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### Research Article

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# Intestinal Immune Responses in Humans

## Oral Cholera Vaccination Induces Strong Intestinal Antibody Responses and Interferon- $\gamma$ Production and Evokes Local Immunological Memory

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### Abstract

We have examined secretory antibody and cell-mediated immune responses to oral cholera vaccine in the human gastrointestinal mucosa. Freshly isolated peripheral blood lymphocytes and intestinal lymphocytes obtained by enzymatic dispersion of duodenal biopsies were assayed for numbers of total and vaccine specific immunoglobulin-secreting cells by enzyme-linked immunospot assay (ELISPOT) techniques; the frequency of cells secreting interferon- $\gamma$  (IFN- $\gamma$ ) was also examined by a new modification of the ELISPOT technique.

After booster immunizations with oral cholera vaccine, large numbers of cholera toxin-specific antibody-secreting cells (ASC) appeared in the small intestine. The responses were dominated by IgA ASC. A single immunization, performed 5 mo after the initial vaccinations, gave rise to an ASC response similar to that seen after the first booster immunization, with respect to both magnitude and isotype distribution. Each of the immunizations also evoked an ASC response in blood which was of lower magnitude than that seen in the small intestine, and comprised similar proportions of IgA and IgG ASC. A booster immunization also resulted in increased frequencies of IFN- $\gamma$ -secreting cells, but this increase was confined to the duodenal mucosa.

This study establishes the feasibility of studying, at the single-cell level, intestinal immune reactivity in humans. Furthermore, it indicates that the small intestinal mucosa is an enriched source of IFN- $\gamma$ . It also demonstrates marked differences between intestinal and peripheral blood immune responses after enteric immunization, and confirms the notion that the mucosal immune system in humans displays immunological memory. (*J. Clin. Invest.* 1991. 88:143-148.) **Key words:** antibody • cholera vaccine • interferon • intestine • mucosa

### Introduction

Mucosal surfaces of the alimentary tract represent the largest barrier between internal organ systems and the environment. As such they are frequently exposed to a variety of foreign

matters, including pathogenic microorganisms. Immune responses occurring in mucosa-associated lymphoid tissues are crucial in maintaining the integrity of these surfaces. The concept of a generalized mucosal immune network that links the lymphoid elements of various mucosal tissues with the gut-associated lymphoid tissue (GALT)<sup>1</sup> has been proposed. According to this view, antigen-sensitized B lymphocytes from GALT, and especially from the Peyer's patches, enter into the circulation and preferentially seed in the intestinal mucosa and also in other glandular tissues, such as the salivary and mammary glands. In these new locations, B cells may further differentiate into plasma cells producing secretory immunoglobulins (1, 2).

Secretory immunoglobulins, which in humans are typified by secretory IgA, are thought to play a major role in antibody-mediated protection of mucosal surfaces, although the precise mechanisms involved in this type of protection are incompletely characterized. Oral ingestion of nonreplicating substances induces the concomitant appearance of IgA antibodies in external secretions (3). However, large quantities of antigen are required to induce secretory antibody responses by the mucosal routes, and the responses are generally modest and of short duration (4). A notable exception is cholera toxin (CT) and its B subunit (CTB), which are exceptionally potent immunogens in humans (5). Furthermore, protection against cholera after natural infection (6, 7) or oral vaccination (8) has been shown to last for several years, while coproantibodies are detected only for some months (9).

Knowledge concerning potential regulatory and/or accessory cells in the development of secretory antibody responses is scarce. In this respect, the functional properties and precise anatomic future of T cells activated in GALT are poorly known. Such essential issues are still being addressed mainly in animal systems, especially in mice. However, variations regarding the function and migratory behavior of mucosal lymphocytes in different species have been reported (10). The difficulties in obtaining suitable preparations of lymphocytes from human mucosal biopsy specimens together with the lack of functional assays to characterize mucosal lymphoid cells have impeded the study of secretory immune responses in humans. Such studies performed at the single-cell level have now become possible through the recent developments of T and B

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*1. Abbreviations used in this paper:* AP, alkaline phosphatase; ASC, antibody-secreting cell; B-WC vaccine, cholera toxin B subunit-whole cell vaccine; CD, cluster differentiation; CT, cholera toxin; CTB, cholera toxin B subunit; ELISPOT, enzyme-linked immunospot assay; GALT, gut-associated lymphoid tissues; HRP, horseradish peroxidase; ISC, immunoglobulin-secreting cell; MNC, mononuclear cell; SFC, spot-forming cell.

cell-based enzyme-linked immunospot assays (ELISPOT) (11, 12) together with improved procedures for isolating viable lymphoid cells from small mucosal biopsies (13).

These developments have prompted us to examine possible changes in B and T cell reactivities within the human intestinal mucosa as a result of local antigenic stimulation, and to which extent these changes would compare to those in the peripheral blood. As prototype immunogen, we used an oral cholera vaccine consisting of killed *Vibrio cholerae* organisms and of CTB (14). This oral vaccine was chosen since it has been shown to be safe and exceptionally immunogenic (5), and to confer long-lasting protection against cholera (15).

## Methods

**Immunizations and collection of specimens.** 10 healthy Swedish volunteers (four males and six females, aged 26–49 y) with no history of cholera vaccination, gave informed consent for participating in this study. Volunteers were immunized twice, 2 wk apart, with oral combined CTB-whole cell (B-WC) cholera vaccine, each dose consisting of  $10^{11}$  killed *V. cholerae* organisms and 1 mg of CTB given together with 150 ml of sodium bicarbonate-citric acid buffer (ACO Pharmaceuticals, Stockholm, Sweden). The vaccine was produced by Institut Merieux, Lyon, France, and was of the same lot as that used in a large field trial (15).

Duodenal biopsies and samples of heparinized venous blood were collected from the volunteers within 24 h before the initial immunization and 7 d after each vaccination at the Department of Gastroenterology, Sahlgrenska Hospital, Göteborg, Sweden. Only five of the volunteers were examined after the first vaccination. Volunteers were sedated with Stesolid, and biopsies were obtained under local anesthesia using a gastrointestinal fibroscope (GIF Q10, Olympus, Stockholm, Sweden) and standard forceps. 12–16 duodenal pinch biopsies, 1–2 mm in diameter, and encompassing the epithelium and lamina propria, were collected on each occasion. In four volunteers randomly selected, a third dose of vaccine was given 5 mo after the initial two immunizations, and blood and duodenal biopsies were collected just before and 7 d after that third immunization.

**Isolation and characterization of cells.** Intestinal lymphocytes were isolated using a novel enzymatic dispersion technique (13). The duodenal biopsy specimens were rinsed thoroughly with 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4 (PBS) (Gibco Europe, Edinburgh, UK) and cut into  $0.1 \times 0.1$ -mm pieces using a semiautomated tissue chopper (McIlwain, Gilford, UK). After two washings with chilled Hepes (25 mM)-buffered Hank's balanced salt solution (Gibco), containing 1 mM  $\text{CaCl}_2$  and 10 mM dithiothreitol, pH 7.4 (extraction buffer), the fragments were dispersed at 4°C in extraction buffer containing 0.5 mg/ml of *Bacillus thermoproteolyticus* thermolysin (Boehringer, Mannheim, Federal Republic of Germany), under continuous stirring. After 30 min, extracted cells were isolated by filtering the suspension through a 150- $\mu\text{m}$  nylon mesh. Remaining fragments were further extracted under continuous stirring at 37°C for 30 min with collagenase/dispase (Boehringer), diluted to 1 mg/ml in Iscove's medium (Gibco) supplemented with 20% fetal calf serum (FCS) (16). Single-cell suspensions, obtained as above by filtration, were pooled with thermolysin extracted cells and pelleted by centrifugation through a cushion of FCS. After two additional washings, the cells were incubated for 20 min at 37°C with 2 mg/ml of deoxyribonuclease (type IV, Sigma Chemical Co., St. Louis, MO) in Iscove's medium with 20% FCS, and single-cell suspensions were finally obtained by filtration through a 50- $\mu\text{m}$  nylon mesh.

Mononuclear cells (MNC) from peripheral blood were isolated by standard isopycnic gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).

Isolated cells were washed, resuspended in Iscove's medium supplemented with 5% FCS and 100  $\mu\text{g}/\text{ml}$  gentamicin (Gibco) (complete medium), and kept on ice until assay.

The morphology of isolated cells was determined by May-Grünwald staining of cytocentrifuged preparations. On average, the enzymatic dispersion procedure yielded  $2.1 \times 10^6$  nucleated cells (range 0.95–4.3  $\times 10^6$ ) per pool of 15 duodenal biopsies, comprising 30–40% MNC and 60–70% epithelial cells. Cell viability was > 90% for MNC and 60–80% for epithelial cells as determined by trypan blue dye exclusion. Cytoplasmic immunoglobulin-containing immunocytes accounted for 25–35% ( $n = 6$ ) of the total duodenal MNC population; among immunoglobulin-containing immunocytes, 71% were accounted for by IgA-, 11% by IgG-, and 18% by IgM-containing cells, as determined by indirect immunofluorescence on ethanol/acetic acid (95:5, vol/vol)-fixed cytocentrifuged suspensions. Phenotypic characterization of isolated MNC was performed by flow cytometry using a fluorescence-activated cell sorter (Becton, Dickinson & Co., San Jose, CA) and phycoerythrin- or fluorescein-conjugated monoclonal antibodies specifying the cluster differentiation (CD) antigens listed below. Preliminary studies revealed that gating out epithelial cells also removed large lymphoblastoid cells (including presumably plasma cells). Therefore, these flow cytometric analyses underestimate the proportion of MNC present in duodenal cell suspensions. Among membrane stained cells gated arbitrarily as MNC,  $80 \pm 13\%$  were T cells ( $\text{CD}3^+$ ),  $2 \pm 3\%$  were B cells ( $\text{CD}19^+$ ),  $2 \pm 3\%$  were of the monocyte/macrophage lineage ( $\text{CD}14^+$ ), and  $2 \pm 4\%$  were natural killer (NK) cells ( $\text{CD}16^+$ ,  $57^+$ ).

**Detection of total and specific immunoglobulin-secreting cells (ISC).** Peripheral blood and duodenal cell suspensions were assayed for numbers of total and specific antibody-secreting cells (ASC) by a two-color micromodification (12) of the original ELISPOT (17, 18). Individual wells of nitrocellulose-bottomed 96-well Millititer HA plates (Millipore Corp., Bedford, MA) were coated at 4°C with a 3  $\mu\text{M}$  solution of GM1 ganglioside (Sigma Chemical Co.) in PBS overnight, washed three times with PBS, and further exposed for 3 h at ambient temperature to 0.1 ml of PBS containing 2.5  $\mu\text{g}/\text{ml}$  of purified CT (List Biological Laboratories, Inc., Campbell, CA) or 1  $\mu\text{g}/\text{ml}$  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 complete medium, and incubated at 37°C for 30 min. The contents of the wells were replaced with 0.1 ml of complete medium containing various numbers of MNC. For each determination, duplicates of three different cell concentrations were assayed. Plates were then incubated undisturbed for 4 h at 37°C in air with 7.5%  $\text{CO}_2$ . Plates were then rinsed three times with PBS and four times with PBS containing 0.05% Tween 20. Next, 0.1 ml of PBS-Tween containing 1% FCS and either a mixture of affinity-purified goat antibodies to human IgA and IgG conjugated with alkaline phosphatase (AP) and horseradish peroxidase (HRP), respectively, or a mixture of affinity-purified, HRP-conjugated goat antibody to human IgA and AP-conjugated goat antibody to human IgM, was added to the wells. All enzyme-conjugated antiglobulin reagents were purchased from Southern Biotechnology Associates, Birmingham, AL, and their specificity was confirmed by enzyme-linked immunosorbent assay (ELISA) block titrations against a panel of human IgA and IgM paraproteins and purified human polyclonal IgG. Plates were incubated with enzyme-conjugated antiglobulins at 4°C overnight and washed three times with PBS-Tween, twice 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 AP chromogen substrate for 10–20 min, washed with PBS, and exposed to 0.1 ml of HRP chromogen substrate for 5–10 min (12). The AP chromogen substrate consisted of 0.15 mg/ml of 5-bromo-4-chloro-3-indolylphosphate toluidine (Bio-Rad Laboratories, Richmond, CA) and 0.3 mg/ml of *p*-nitroblue tetrazolium chloride (Bio-Rad Laboratories) in 0.1 M  $\text{NaHCO}_3$ , 5 mM  $\text{MgCl}_2$ , pH 9.8. The HRP chromogen substrate consisted of 0.3 mg/ml of 3-amino-9-ethylcarbazole (Sigma Chemical Co.) and 0.015% (vol/vol)  $\text{H}_2\text{O}_2$ , in 0.1 M sodium acetate, pH 5.0. Dishes were thoroughly rinsed with tap water, dried, and examined for the presence of blue (AP) and red (HRP) spots. These reactions were enumerated under low magnification ( $\times 40$ ).

Total IgA-, IgG-, and IgM-secreting cells were similarly enumerated

in wells coated with affinity-purified goat antibodies to the F(ab)<sub>2</sub> fragment of human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Exposure of cell suspensions to 2 mM of cycloheximide (Sigma Chemical Co.) for 3 h at 37°C before and during cell plating, abrogated specific as well as total Ig-mediated spot formation, thus demonstrating that this assay detects cells synthesizing and actively secreting immunoglobulins. Incubation with 10 µg/ml of CT during plating of cells inhibited anti-CTB-mediated spot formation by ≥ 60%, but did not appreciably affect the total number of immunoglobulin producing cells detected. Moreover, when peripheral blood lymphocytes (PBL) were submitted to the enzymatic dispersion treatment used to isolate duodenal cells, the number and the isotype distribution of detectable Ig-producing PBL were not affected by this treatment (data not shown).

**Enumeration of interferon-γ (IFN-γ)-secreting cells.** The number of peripheral blood and duodenal cells secreting IFN-γ was determined by a two-site reverse ELISPOT technique using two distinct epitope specific mouse monoclonal anti-IFN-γ antibodies as capture and developing reagents, respectively (11). Cells were incubated for 20 h at 37°C in anti-IFN-γ-coated wells in air supplemented with 7.5% CO<sub>2</sub>. Plates were developed by stepwise exposure of the wells to biotinylated mouse monoclonal antibody to human IFN-γ, diluted to 2 µg/ml, 4 µg/ml HRP-labeled egg avidin (Extravidin, Sigma Chemical Co.) and HRP chromogen substrate. Exposure of the cells to cycloheximide (2 mM) (Sigma Chemical Co.) before and during cell plating inhibited by up to 90% IFN-γ-mediated spot formation. Incubation of phytohemagglutinin-stimulated PBL with thermolysin/collagenase under conditions identical to those employed for isolating duodenal lymphocytes decreased by 30% the frequency of IFN-γ-secreting cells detectable by this assay.

**Quantitation of secreted IFN-γ.** Aliquots of cell suspensions containing 8 × 10<sup>4</sup> duodenal MNC or 10<sup>6</sup> peripheral blood MNC were cultured for 24 h at 37°C in 200 µl of complete medium in flat-bottom 96-well culture plates (Nunc, Roskilde, Denmark). Thereafter, cell suspensions were centrifuged, and the culture supernatants were harvested and kept frozen (-20°C) before being assayed. Secreted IFN-γ in cell culture supernatants was measured by an ELISA (19) that employed the same capture and developing mouse monoclonal antibodies to human IFN-γ as the reverse ELISPOT described above. The assay, when calibrated against known amounts of purified recombinant human IFN-γ (National Institutes of Health standard) can detect as little as 0.05 IU of IFN-γ per ml (19).

## Results

**Baseline frequencies of ISC and IFN-γ-secreting cells in duodenal mucosa and in peripheral blood.** We first examined the numbers of ISC and that of IFN-γ-secreting cells in suspensions of MNC obtained from peripheral blood and from enzymatically dispersed human duodenal biopsies before vaccination. On a per MNC basis, cell suspensions from duodenal biopsies displayed considerably higher frequencies of ISC than corresponding peripheral blood MNC (Fig. 1). In keeping with previous studies (20) and with our immunocytological data obtained on enzymatically dispersed duodenal cell suspensions, the majority (mean 72%) of the ISC in the duodenum were accounted for by IgA ISC. IgG and IgM ISC were also detected, but in considerably lower frequencies. This pattern contrasted with that seen in peripheral blood, where IgA and IgG ISC were detected in comparable frequencies and predominated over IgM ISC (Fig. 1).

Reverse ELISPOT analyses, performed on cell suspensions from enzymatically dispersed duodenal tissues revealed that the human duodenal mucosa is a major source of cells producing IFN-γ. When compared to peripheral blood MNC, duo-

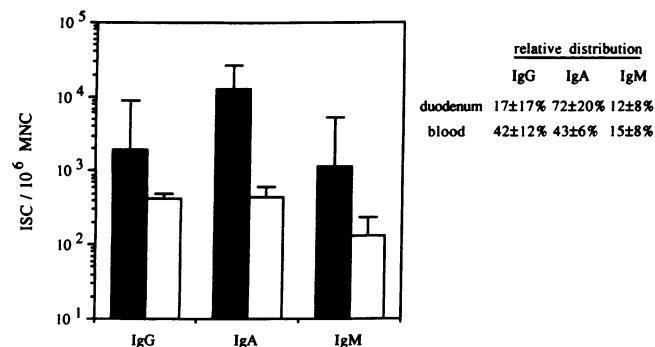


Figure 1. Frequency of intestinal and peripheral blood ISC before any intentional immunization. Data are expressed as geometric mean numbers of ISC±SD per 10<sup>6</sup> intestinal (solid bars) and peripheral blood (open bars) MNC. The relative distribution (mean±SD) of IgG-, IgA-, and IgM-ISC is indicated at right.

denal MNC contained eight times as many IFN-γ-secreting cells (Fig. 2). In addition, the amount of IFN-γ produced in supernatants from short-term (24 h) cultures of duodenal cells was particularly impressive, since immunoassayable quantities (≥ 0.05 IU IFN-γ/ml) of IFN-γ could be detected in cultures containing as few as 8 × 10<sup>4</sup> duodenal MNC in 200 µl of medium. In contrast, IFN-γ production by high-density (10<sup>6</sup> MNC per 200 µl) cultures could not be detected with PBL, even after prolonged (96 h) incubation period (data not shown).

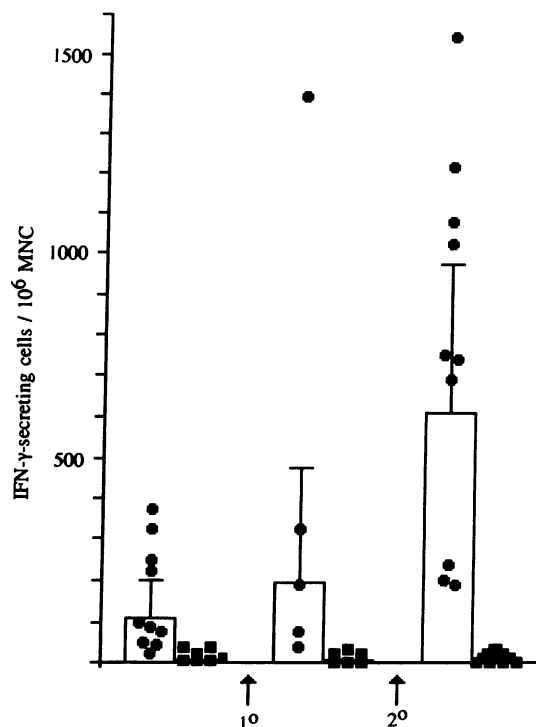


Figure 2. Frequencies of duodenal and peripheral blood INF-γ-secreting cells before and after B-WC vaccinations. Results are expressed as geometric mean numbers of INF-γ-secreting cells±SD per 10<sup>6</sup> MNC. Cell suspensions were assayed before and 7 d after each of the vaccinations (arrows). The individual frequencies of INF-γ-secreting cells in peripheral blood (■) and duodenal (●) cell suspensions are indicated.

*Vaccine-induced specific ASC in the duodenal mucosa.* We then examined whether single or repeated oral immunizations with B-WC vaccine would elicit an antitoxin ASC response in the duodenal mucosa. In 10 individuals examined before immunization, no duodenal anti-CTB activity could be demonstrated, except for two individuals who displayed low numbers of CTB-reactive IgA ASC. In contrast, 1 wk after a primary oral immunization, duodenal cell suspensions from four out of five volunteers examined displayed increased numbers of ASC to the CTB test antigen (Fig. 3 A). Among CTB-specific ASC, IgM ASC predominated in two of the volunteers and IgA ASC in the other two. Small numbers of IgG anti-CTB ASC were detected in two individuals.

A second oral immunization with B-WC vaccine gave rise to a more pronounced increase in the frequencies of CTB-specific ASC in 8 out of the 10 vaccinees examined. This secondary anti-CTB response was dominated by IgA ASC, but an increase in CTB-specific IgG ASC was also observed (Fig. 3 A). Small numbers of CTB-specific IgM ASC were detected at this time (Fig. 3 A).

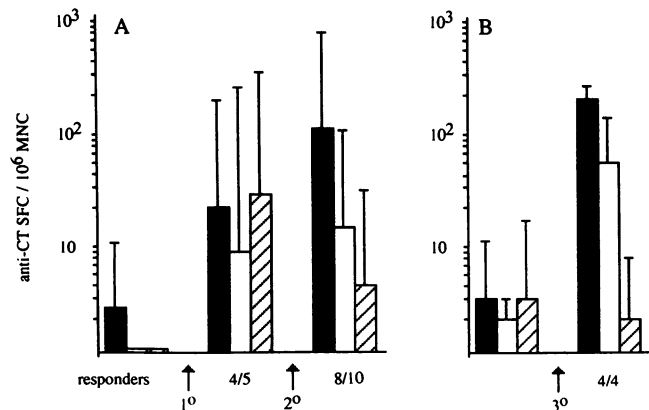
Four of these volunteers were reexamined 5 mo later, before immunization with a third dose of B-WC vaccine. Whereas negligible numbers of CTB-reactive duodenal ASC were detected before immunization, the third dose of vaccine induced a strong duodenal antibody response in all four volunteers examined (Fig. 3 B). This response was comparable to that seen after the first booster immunization with respect to both magnitude and isotype distribution (Fig. 3 A).

Overall, the frequency and isotype distribution of duodenal ISC were not significantly influenced by the immunization regimen. However, in 3 out of the 10 volunteers examined 7 d after the second oral immunization with B-WC vaccine, the frequency of duodenal IgA ISC was increased by 3.3- to 8.5-fold from preimmune values (data not shown).

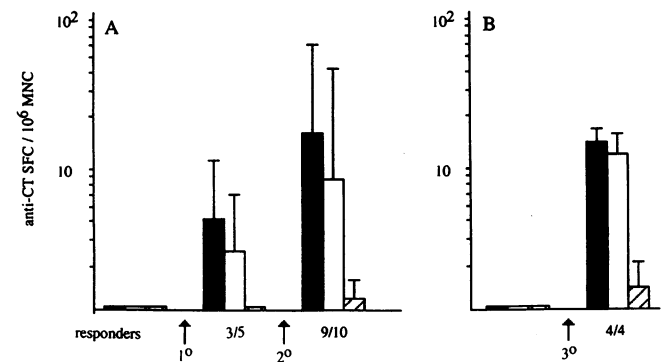
*Vaccine-induced specific ASC in peripheral blood.* We also examined the extent to which the intestinal ASC response to B-WC vaccine was associated with an ASC response in peripheral blood. None of the 10 volunteers examined before immuni-

zation had detectable anti-CTB ASC in the peripheral circulation (Fig. 4 A). However, 1 wk after the primary oral cholera immunization, three of the five volunteers examined had detectable circulating anti-CTB ASC (Fig. 4 A). These individuals also had duodenal anti-CTB ASC at that time. The second oral immunization markedly increased both the magnitude of the blood response and the percentage of volunteers responding with specific anti-CTB ASC 1 wk later (9 out of 10) (Fig. 4 A). These responses were highly transient and CTB-reactive blood ASC had essentially disappeared within the next few days (data not shown). The third dose of vaccine given 5 mo later induced a circulating anti-CTB ASC response of the same magnitude and isotype distribution as that seen after the second immunization (Fig. 4 B). Although comparable in kinetics, the ASC response in blood differed markedly from that recorded in the intestine, with respect to both magnitude and isotype distribution. Thus, ASC responses in blood were generally of lower magnitude and comprised comparable numbers of specific IgA and IgG ASC, while specific IgA ASC predominated the duodenal responses. Also at variance with the duodenal ASC responses, specific IgM ASC were rarely detected in the circulation, even in individuals whose primary duodenal response was dominated by IgM ASC. In one individual, we observed a fivefold increase in the numbers of IgA and IgM ISC, but in the other individuals there were no notable changes in the total number of circulating ISC after primary and booster immunizations, irrespective of isotype (data not shown).

*Vaccine-induced duodenal and peripheral blood IFN- $\gamma$ -producing cells.* Even though the prevaccination numbers of IFN- $\gamma$ -secreting cells were high in the duodenal mucosa, the primary oral cholera immunization resulted in increased numbers of IFN- $\gamma$ -secreting cells in two out of five volunteers examined. A second immunization given 2 wk later resulted in an increased frequency of IFN- $\gamma$ -producing cells in all but one of the 10 volunteers examined (Fig. 2). As compared with the prevaccination values, the increase in IFN- $\gamma$ -secreting cell frequency after the second dose of vaccine ranged from 2- to 37-fold (mean increase sixfold). In contrast, no appreciable change in the frequency of circulating IFN- $\gamma$ -producing PBL was de-



**Figure 3.** Intestinal anti-CTB ASC responses before and after B-WC vaccinations. Results are expressed as geometric mean numbers of IgA (closed bars), IgG (open bars), and IgM (cross hatched bars) anti-CTB spot-forming cells (SFC)  $\pm$  SD per  $10^6$  MNC. Cell suspensions were assayed before and 7 d after each of two immunizations performed 2 wk apart (A), and immediately before and 7 d after a third immunization performed 5 mo later (B). The proportion of volunteers responding to each vaccination (arrows), and in any isotype, is indicated.



**Figure 4.** Anti-CTB ASC responses in peripheral blood before and after B-WC vaccinations. Results are expressed as geometric mean numbers of IgA (closed bars), IgG (open bars), and IgM (cross hatched bars) anti-CTB SFC  $\pm$  SD per  $10^6$  MNC. Cell suspensions were assayed before and 7 d after each of two immunizations performed 2 wk apart (A) and immediately before and 7 d after a third immunization, performed 5 mo after the initial two vaccinations (B). The number of volunteers responding to each vaccination (arrows), and in any isotype, is indicated below the horizontal axis.

tected at the times examined after either the primary or the secondary vaccination (Fig. 2).

## Discussion

In the present study, we have examined whether single or repeated oral immunizations would elicit an immune response in the intestinal mucosa, and also the extent to which this response could be reflected in the peripheral blood. We monitored the frequencies of total as well as specific ISC of different isotypes and of IFN- $\gamma$ -secreting cells in these compartments before and after immunizations with a combined B-WC cholera vaccine. Recent micromodifications of the ELISPOT technique, which allow analyses of small numbers of cells (11, 12), together with the development of an improved enzymatic tissue dispersion technique for isolating mucosal cells (13), have enabled us to address these questions. With the same methodology we also evaluated to which extent an immunological memory would develop.

With respect to local duodenal immunoglobulin production, IgA-secreting cells predominated. This is in keeping with the many studies, and our own immunocytological data, showing that IgA is the dominating immunoglobulin as well as specific antibody isotype present in human mucosal glandular tissues and external secretions (20, 21). On the other hand, our findings are at variance with those recently reported by Peters et al. (22) who, using an AP-based ELISPOT technique, reported similar numbers of cells secreting IgA, IgG, and IgM among small intestinal MNC. This discrepancy may be explained in part by the use of different tissue dispersion techniques to extract intestinal lymphocytes. Alternatively, the presence of substantial numbers of cells with strong endogenous phosphatase activity (e.g., epithelial cells) in the duodenal cell suspensions can generate artifacts (falsely positive spots) when AP chromogen substrates are used in the ELISPOT (Czerkinsky, C., unpublished observations). In the present study, this drawback was avoided by using a bichromatic ELISPOT technique (12).

IgA was also the predominant isotype found among duodenal cells secreting specific antibodies to CTB after oral immunizations with B-WC vaccine. This vaccine, which is known to stimulate an intestinal antibody response associated with protective immunity against cholera disease (15), induced a duodenal ASC response which was already manifest after a single immunization. Thus, CTB-specific intestinal IgM and IgA ASC appeared within 1 wk after a primary immunization; a second vaccination 2 wk later gave rise to a sharp rise in the numbers of anti-CTB duodenal ASC, the vast majority being IgA ASC. The frequency of CTB-specific intestinal IgG ASC also increased after a second immunization, although to a lesser extent than that of IgA ASC. By that time, CTB-specific intestinal IgM ASC were detected in a few volunteers and only in small numbers.

This study also confirms the notion that the intestinal IgA system in humans displays immunological memory. Intestinal immunologic memory to CTB after natural or experimental infection (6, 23) or after vaccination (8, 24, 25) has been reported. In this regard, we found a persisting capacity of duodenal lymphocytes to mount an impressive ASC response after a single booster dose of B-WC vaccine given 5 mo after the initial immunizations. This response was comparable to that seen after the second of two initial immunizations, regarding magnitude and isotype distribution. Our results clearly indi-

cate that the first two doses of B-WC vaccine were efficient at inducing local intestinal immunologic memory.

Each of the three oral immunizations with B-WC vaccine also gave rise to the transient appearance of specific ASC in the peripheral blood. However, conspicuous differences were noted between duodenal and peripheral blood anti-CTB ASC responses. First, the frequency of anti-CTB antibody-producing cells in peripheral blood was considerably lower than that in the duodenal mucosa. Secondly, peripheral blood ASC responses to oral B-WC vaccine comprised comparable numbers of specific IgG and IgA ASC and practically no specific IgM ASC. These specific ASC probably represent B cells en route to various mucosal tissues including the intestinal lamina propria (26, 27). Consistent with this view are our recent findings demonstrating the sequential appearance of such cells in peripheral blood and in minor salivary glands from oral cholera vaccinees (28). Animal studies have shown that a proportion of B cells, once activated by luminal antigens, appear in the circulation, and preferentially home to the intestinal mucosa (29). The large numbers of vaccine specific ASC found in the duodenal mucosa suggest that the same migratory pathway occurs in humans.

Another interesting observation in our studies was the high frequency of duodenal cells spontaneously secreting IFN- $\gamma$ , in the absence of any intentional vaccination. After vaccination increased numbers of duodenal IFN- $\gamma$ -producing cells were detected in a few vaccinees after the first and in all but one vaccinee after the second immunization. This finding suggests that a mucosal cell-mediated immune response associated with immunological memory was also induced after cholera vaccination. In contrast, no changes in the frequency of circulating IFN- $\gamma$ -secreting cells could be detected, at least at the times examined. The high numbers of duodenal IFN- $\gamma$ -secreting cells detected after enteric immunization are particularly intriguing in view of several recent reports. IFN- $\gamma$ , beside its known antiviral and immunoregulatory properties (30), increases expression of secretory component, a receptor for locally produced polymeric immunoglobulins on epithelial cells (31). Therefore, IFN- $\gamma$  is likely to influence the transepithelial transport of potentially protective secretory immunoglobulins. Other *in vitro* studies suggest that IFN- $\gamma$  may directly modulate such fundamental physiological properties of intestinal epitheliae as permeability and barrier function (32, 33). Our data support the contention that local IFN- $\gamma$  production in the intestinal wall plays an important role in maintaining a normal balance between absorptive and secretory properties of this organ.

The expansion of duodenal IFN- $\gamma$ -secreting cell populations, mainly observed after the second B-WC vaccination, might be the result of local activation of primed T cells by vaccinal antigens. This interpretation is supported by recent studies, both in murine (34) and in human systems (35), indicating that memory T cells rather than naive T cells, are the major producers of IFN- $\gamma$  upon activation. The apparently adaptive character of the intestinal IFN- $\gamma$  response does not rule out the possibility that natural killer cells partly contributed to the duodenal IFN- $\gamma$  production.

The magnitude of duodenal ASC and IFN- $\gamma$ -secreting cell responses to B-WC vaccine is impressive, especially when compared with corresponding blood responses. Given (a) the multitude of antigens present in this vaccine and that we measured only the ASC response to CTB, (b) the fact that the time for cell isolation and analyses had to be chosen considering available

information (36), and (c) the observation that treatment of mitogen stimulated PBL with thermolysin/collagenase lowers the frequency of detectable IFN- $\gamma$ -secreting cells, it is likely that the duodenal ASC and IFN- $\gamma$ -secreting cell responses after cholera vaccinations were of even higher magnitude than those recorded.

In conclusion, this study demonstrates the feasibility of studying, at the single-cell level, humoral and cell-mediated immune responses in the human intestinal tract. Appropriate tissue dispersion procedures and sensitive ELISPOT techniques have enabled us to evaluate the magnitude, localization, and dissemination of an immune response induced at enteromucosal sites. Furthermore, a mucosal immunological memory could be demonstrated. The above methodological developments should now make it possible to study anatomically segmented immune responses in the human gastrointestinal tract, and to evaluate the immunogenicity of other candidate vaccines. They should also serve the purpose of studies aiming at the possible disclosure of functional abnormalities of the enteromucosal immune system in a number of pathological conditions.

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