# Mucosal and Systemic Immune Responses in Humans after Primary and Booster Immunizations with Orally Administered Invasive and Noninvasive Live Attenuated Bacteria

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The mucosal and systemic immune responses after primary and booster immunizations with two attenuated live oral vaccine strains derived from a noninvasive (*Vibrio cholerae*) and an invasive (*Salmonella typhi*) enteric pathogen were comparatively evaluated. Vaccination with *S. typhi* Ty21a elicited antibody-secreting cell (ASC) responses specific for *S. typhi* O9, 12 lipopolysaccharide (LPS), as well as significant increases in levels of immunoglobulin G (IgG) and IgA antibodies to the same antigen in serum. A strong systemic CD4$^+$ T-helper type 1 cell-mediated immune (CMI) response was also induced. In contrast to results with Ty21a, no evidence of a CMI response was obtained after primary immunization with *V. cholerae* CVD 103-HgR in spite of the good immunogenicity of the vaccine. Volunteers who received a single dose of CVD 103-HgR primarily developed an IgM ASC response against whole vaccine cells and purified *V. cholerae* Inaba LPS, and seroconversion of serum vibriocidal antibodies occurred in four of five subjects. Serum IgG anti-cholera toxin antibody titers were of lower magnitude. For both live vaccines, the volunteers still presented significant local immunity 14 months after primary immunization, as revealed by the elevated baseline antibody titers at the time of the booster immunization and the lower ASC, serum IgG, and vibriocidal antibody responses after the booster immunization. These results suggest that local immunity may interfere with colonization of the gut by both vaccine strains at least up to 14 months after basis immunization. Interestingly, despite a low secondary ASC response, Ty21a was able to boost both humoral (anti-LPS systemic IgG and IgA) and CMI responses. Evidence of a CMI response was also observed for one of three volunteers given a cholera vaccine booster dose. The direct comparison of results with two attenuated live oral vaccine strains in human volunteers clearly showed that the capacity of the vaccine strain to colonize specific body compartments conditions the pattern of vaccine-induced immune responses.

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The infectious mechanisms underlying cholera and typhoid fever present important differences and are associated with the induction of distinctive types of immune responses. Current evidence suggests that optimal protection against such diseases is conferred by vaccines that induce a pattern of immune responses matching that induced by natural infection. For instance, it is widely accepted that efficient cholera vaccines must be administered orally in order to optimally stimulate the intestinal immune responses that are critical in mediating protection.

Colonization of the small intestine by *Vibrio cholerae*, a typical noninvasive pathogen, is an important early step in the pathogenesis of cholera. Accordingly, protective immunity against this disease is thought to be essentially humoral and predominantly based on secretory immunoglobulin A (IgA) directed against cell surface components like lipopolysaccharides (LPS). Such antibodies may prevent attachment of the bacteria to gut epithelial cells, contributing to rapid clearing of the vibrios from the intestine. In addition, immunity to cholera toxin (CT), which causes the diarrhea characteristic of cholera, may contribute to protection, although to a lower extent than immunity to LPS (15, 20, 22). The vibriocidal antibody response involves mainly IgM antibodies, which are directed primarily against the O-antigen moiety of *V. cholerae* LPS and promote lysis of vibrio cells in vitro in the presence of guinea

pig complement (2, 10, 18, 29). The level of vibriocidal antibodies in serum seems to be the best measure of induced immunity, since it correlates with the elicitation of a protective intestinal immune response against cholera, as shown in field trials (17, 29). Accordingly, vibriocidal titers in serum are generally considered a correlate of protection, protection being conferred by secretory IgA actively secreted into the intestinal lumen.

In contrast to that of *V. cholerae*, the pathogenesis of *Salmonella typhi* is characterized by mucosal invasion and systemic spreading. This dissemination pattern results from the ability of *Salmonella* spp. to survive within macrophages and leads to the induction of broad-based immunity. For protection against *Salmonella* spp., both antibody and cell-mediated immune (CMI) responses are considered to be important. The O antigen (O9, 12 serotype) is most relevant to protection against typhoid fever; other antigens include the virulence capsule antigen and some outer membrane proteins (for a review, see reference 28). Following oral administration, live attenuated *Salmonella* spp. vaccines can elicit protective immunity associated with the induction of mucosal and serum antibodies as well as a T-cell response (1, 7, 9, 23, 24, 27).

Current knowledge about the induction of a local immune response within the human intestinal mucosa, its relationship to systemic immune responses, and the extent to which the local intestinal response displays immunological memory is still slight. In order to further document these issues, we comparatively evaluated mucosal and systemic immune responses after primary and booster immunizations with two live oral vaccine strains, *V. cholerae* CVD 103-HgR (classical Inaba) and

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*S. typhi* Ty21a. The humoral response was determined by (i) the number and kinetics of vaccine-induced antibody-secreting cells (ASC) that circulate in the peripheral blood after mucosal priming, (ii) the levels of vaccine-specific IgG and IgA in serum, and (iii) vibriocidal antibody titers in serum. In addition, the induction of a systemic CMI response was evaluated through the determination of antigen-driven in vitro lymphoproliferative responses and production of cytokines compatible with CD4$^+$ T-helper type 1 (Th1) and Th2 cell responses.

The study population was composed of two groups of five healthy adults with no prior history of infection with or immunization against cholera or typhoid fever. The first group (one female and four males; mean age, 26.6 years; range, 24 to 31 years) received a single oral dose of at least $2 \times 10^8$ CVD 103-HgR live cells (OROCHOL; BERNA), and the second group (three females and two males; mean age, 25.4 years; range, 23 to 28 years) received one dose of at least $1 \times 10^9$ *S. typhi* Ty21a live cells (VIVOTIF Liquid; BERNA) every other day for a total of three doses. Blood samples were collected at baseline and 6, 9, and 22 days after the first vaccine dose. A booster dose was administered by the same vaccine regimen to three subjects of each group 14 months after primary immunization. Blood samples were obtained prior to as well as 9 and 22 days after the booster immunization.

Comparable serological methods were used to evaluate seroconversion for cholera and typhoid vaccines. We quantified serum vibriocidal antibodies in microtiter plates in the presence of guinea pig complement, with CVD 103-HgR as the test strain (3). Titers are defined as the geometric means of the reciprocals of the serum dilutions resulting in at least 50% inhibition of growth. A significant response is defined as a ≥4-fold increase in serum antibody titers compared to baseline titers. Serum IgG antibody responses to CT were measured by enzyme-linked immunosorbent assay (ELISA) (16). Seroconversion is defined as a ≥2-fold increase in antibody titer. Anti-*S. typhi* IgG and IgA Ty2 LPS (serotype O9, 12) antibodies were quantitated by an ELISA as modified previously (5). The serum samples were serially diluted, and their optical densities were determined. Absorbance values within the linear portion of the curve were used to calculate ELISA units (EU). Seroconversion is defined as a ≥2-fold rise in EU over baseline values in experiments in which paired serum dilutions were used and in which the postimmunization sample showed >1 EU/ml.

Vaccine-induced circulating ASC were quantified by an enzyme-linked immunospot assay (6, 11). Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation with Ficoll-Paque (Sigma, St. Louis, Mo.). Isolated cells were washed and resuspended in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. Among total PBMC, numbers of ASC that secreted specific antibodies (IgA, IgG, and IgM isotypes) against *S. typhi* O9, 12 LPS, CVD 103 LPS, formalin-inactivated CT (choleragenoid), or 0.1% formalin-inactivated *V. cholerae* CVD 103-HgR whole cells were determined. After incubation of PBMC in antigen-coated, nitrocellulose-bottomed, 96-well plates (Milliiter HA; Millipore Corporation, Bedford, Mass.) overnight at 37°C, plates were washed and incubated overnight at 4°C in the presence of either goat anti-human IgG, IgA, or IgM alkaline phosphatase conjugates (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Plates were developed by the addition of the phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP)–nitroblue tetrazolium (KPL). Colored spots were enumerated in quadruplicate wells, and data were adjusted to numbers of spot-forming cells per $10^6$ PBMC.

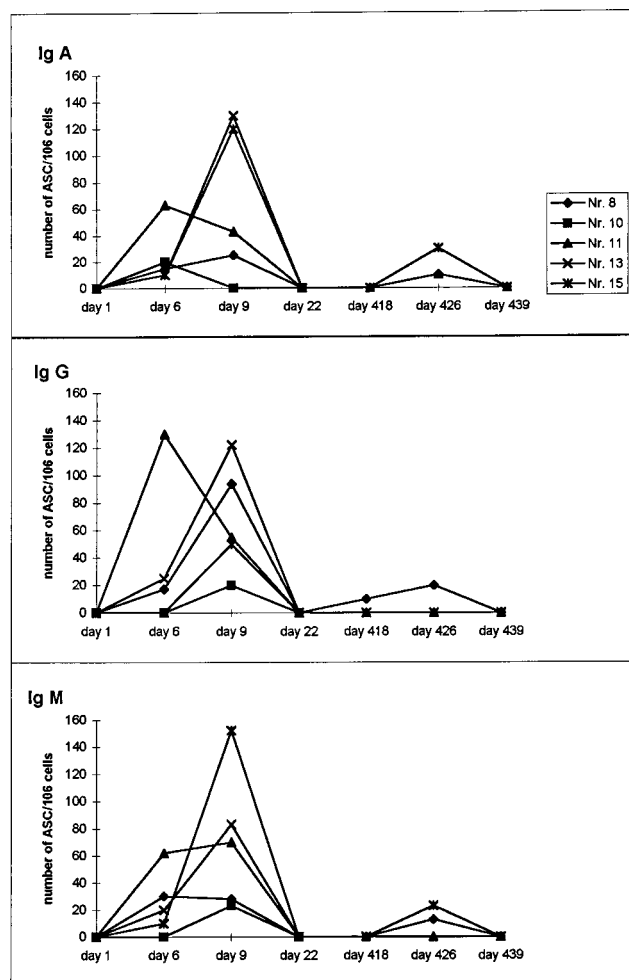For the measurement of T-cell proliferation, PBMC isolated



FIG. 1. Specific ASC responses to *S. typhi* Ty2 LPS after primary (day 1) and booster (day 418) immunizations. Results are expressed as mean numbers of IgA, IgG, and IgM ASC per $10^6$ PBMC. Nr., volunteer number.

as described above were resuspended in RPMI 1640 medium (Sigma) containing 10% heat-inactivated, dialyzed human AB serum (Sigma). PBMC were cultured in round-bottom, 96-well microtiter plates (Costar, Badhoevedorp, The Nederlands) at an initial cell density of $7.5 \times 10^5$ cells/ml in the presence or absence of the separate test antigens. Antigens consisted of 0.1% formalin-inactivated *S. typhi* Ty21a or *V. cholerae* CVD 103-HgR whole cells added at concentrations of $0.1 \times 10^6$ to $5 \times 10^6$ cells/ml. Six days after initiation of the assay, 1.0 μCi of [*methyl*-$^3$H]thymidine (37 MBq/ml; Amersham International plc, Little Chalfont, Buckinghamshire, United Kingdom) was added to each well. After further incubation for 18 h, the cells were harvested onto glass-fiber filter mats with a cell harvester (Skatron Instruments Inc., Sterling, Va.). Antigen-induced [$^3$H]thymidine uptake by cultured PBMC was measured as the numbers of counts per minute of triplicate cultures and expressed as a proliferation index (antigen-induced levels divided by control levels of [$^3$H]thymidine uptake). For cytokine excretion studies, isolated PBMC were cultivated for 96 h in duplicate with or without stimulation with the same antigens as those used for the lymphoproliferative assay (see above). Supernatant fluids were collected after centrifugation at $300 \times g$ for 5 min and frozen at $-70°C$. Levels of interleukin-4 (IL-4) and gamma interferon (IFN-γ) were measured by enzyme im-
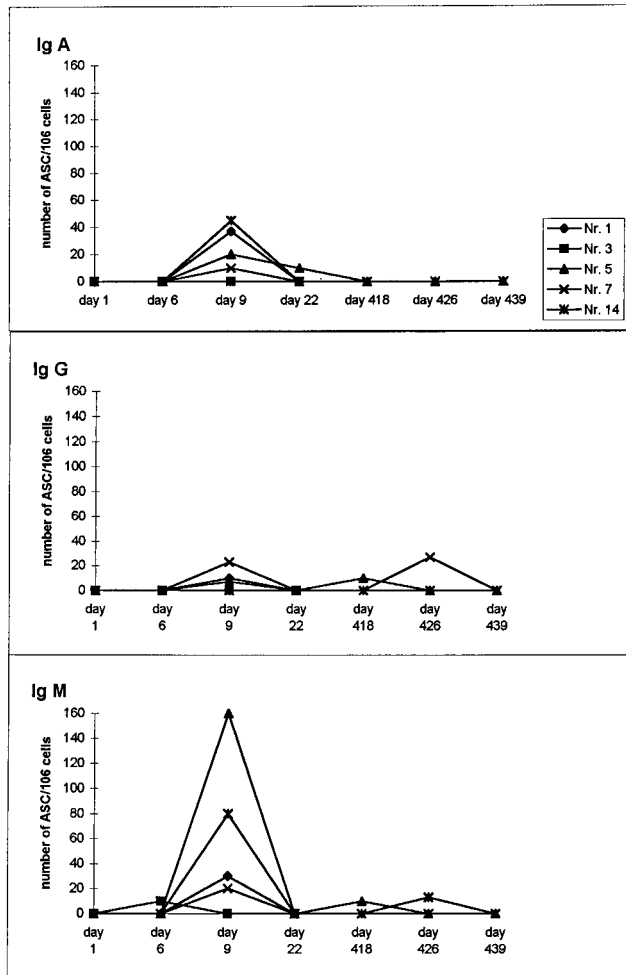
FIG. 2. Specific ASC responses to *V. cholerae* CVD 103-HgR after primary (day 1) and booster (day 418) immunizations. Results are expressed as mean numbers of IgA, IgG, and IgM ASC per $10^6$ PBMC. Nr., volunteer number.

munoassays according to the specifications of the manufacturer (PharMingen, San Diego, Calif.).

In order to compare the patterns of immune responses induced by live oral vaccines and to evaluate their capacity to induce local immunological memory, the responses to primary and booster immunizations with vaccines against cholera and

typhoid fever were determined. None of the 10 volunteers examined before primary immunization had detectable levels of circulating ASC against anti-*S. typhi* O9, 12 LPS or *V. cholerae* CVD 103-HgR whole cells (Fig. 1 and 2). Except for that of one volunteer vaccinated with Ty21a (peak on day 6), ASC responses peaked on day 9 and returned to baseline by day 22 postvaccination, irrespective of the number of vaccine doses administered (CVD 103-HgR, one dose; Ty21a, three doses). After primary oral vaccination with Ty21a, all five volunteers displayed high levels of O9, 12 LPS-specific ASC. The response was dominated by IgG ASC (mean, 83 ASC/$10^6$ PBMC; range, 20 to 130) and IgA ASC (mean, 74 ASC/$10^6$ PBMC; range, 20 to 130), followed by IgM ASC (mean, 57 ASC/$10^6$ PBMC; range, 23 to 152) (Fig. 1).

After primary immunization with the cholera vaccine, the ASC response to CVD 103-HgR whole cells was dominated by IgM ASC (mean, 150 ASC/$10^6$ PBMC; range, 10 to 608), followed by IgA ASC (mean, 28 ASC/$10^6$ PBMC; range, 10 to 45) and IgG ASC (mean, 13 ASC/$10^6$ PBMC; range, 7 to 23) in five of five, four of five, and three of five volunteers, respectively (Fig. 2). Interestingly, we observed qualitatively and quantitatively similar ASC responses to purified Inaba LPS (IgM ASC [mean, 177 ASC/$10^6$ PBMC; range, 25 to 368], IgA ASC [mean, 52 ASC/$10^6$ PBMC; range, 10 to 110], and IgG ASC [mean, 18 ASC/$10^6$ PBMC; range, 10 to 30] in three of five, three of five, and four of five subjects). The vaccine also elicited in three of five subjects anti-CT responses dominated by IgA ASC (mean, 75 ASC/$10^6$ PBMC; range, 35 to 107) and IgG ASC (mean, 48 ASC/$10^6$ PBMC; range, 10 to 83), followed by IgM ASC (mean, 9 ASC/$10^6$ PBMC; range, 7 to 10).

Vaccination with Ty21a elicited significant increases in IgG and IgA antibodies to O9, 12 LPS in the sera of four of five volunteers (Table 1). After a single dose of CVD 103-HgR, significant seroconversion of vibriocidal antibodies occurred in four of five immunized subjects (Table 2). In addition, a >2-fold rise in CT-specific IgG titers was observed in the sera of three of five volunteers (Table 2).

The level and type of CMI response induced by each vaccine were evaluated by determining lymphoproliferative responses to specific vaccine antigens and cytokine production patterns. Sensitized lymphocytes were found only after immunization with *S. typhi* Ty21a (Fig. 3). All five volunteers vaccinated with this strain exhibited significantly increased proliferative responses and IFN-$\gamma$ production (mean, 160 pg/ml; range, 52 to 233 pg/ml) after Ty21a whole-cell stimulation of PBMC, collected 21 days after primary vaccination, whereas their IL-4 levels remained low (the levels of four subjects were <6 pg/ml,

TABLE 1. Anti-*S. typhi* Ty2 LPS responses in serum following primary and booster oral immunizations with live attenuated typhoid vaccine in healthy adults

| Immunization (day) | Volunteer | Serum IgG anti-Ty2 LPS | | | Serum IgA anti-Ty2 LPS | | |
|---|---|---|---|---|---|---|---|
| | | Baseline titer | Peak titer | Response[a] | Baseline titer | Peak titer | Response[a] |
| Primary (1) | 8 | 4.69 | 11.17 | + | 0.53 | 2.53 | + |
| | 10 | 1.57 | 2.20 | − | 0.47 | 0.73 | − |
| | 11 | 10.76 | 44.41 | + | 2.66 | 7.66 | + |
| | 13 | 2.20 | 59.14 | + | 1.51 | 10.88 | + |
| | 15 | 0.23 | 17.33 | + | 0.19 | 5.74 | + |
| Booster (418) | 8 | 8.55 | 13.28 | − | 0.54 | 1.01 | − |
| | 11 | 10.88 | 16.05 | − | 1.99 | 3.95 | − |
| | 15 | 0.11 | 1.04 | + | 0.09 | 0.55 | + |

[a] A significant response (+) is defined as a ≥2-fold increase in antibody titers. −, no response.

TABLE 2. Systemic anti-cholera responses following primary and booster oral immunization with live recombinant
cholera vaccine in healthy adults

| Immunization (day) | Volunteer | Serum vibriocidal antibody[a] | | | Serum IgG anti-CT[b] | | |
|---|---|---|---|---|---|---|---|
| | | Baseline titer | Peak titer | Response | Baseline titer | Peak titer | Response |
| Primary (1) | 1 | <10 | 1,280 | + | 63 | 263 | + |
| | 3 | <10 | <10 | − | 80 | 86 | − |
| | 5 | <10 | 10,280 | + | 75 | 538 | + |
| | 7 | <10 | 640 | + | 219 | 199 | − |
| | 14 | <10 | 10,280 | + | 67 | 161 | + |
| Booster (418) | 5 | 20 | 40 | − | 483 | 545 | − |
| | 7 | 40 | 80 | − | 426 | 403 | − |
| | 14 | <10 | 1,280 | + | 474 | 376 | − |

[a] We quantified vibriocidal antibodies in microtiter plates in the presence of guinea pig complement, with CVD 103-HgR as the test strain. A significant response (+) is defined as a ≥4-fold increase in serum antibody titers compared to baseline titers. −, no response.
[b] A significant response (+) is defined as a ≥2-fold increase in antibody titers. −, no response.

and that of one subject was 25 pg/ml). In contrast, no single volunteer vaccinated with CVD 103-HgR presented any evidence of a CMI response after primary immunization (Fig. 4).

Three volunteers of each group were given booster doses after 14 months by the same immunization protocol used for primary immunization. At the time of the booster, two of three Ty21a vaccinees still presented elevated O9, 12 LPS-specific IgG titers in their sera whereas their IgA titers had returned to baseline levels (Table 1). Booster vaccination resulted in the seroconversion of only one subject for both O9, 12 LPS-specific IgG and IgA antibody titers in serum but did not stimulate a serum antibody response in the volunteers with elevated baseline titers (Table 1). Only a modest or no ASC response was
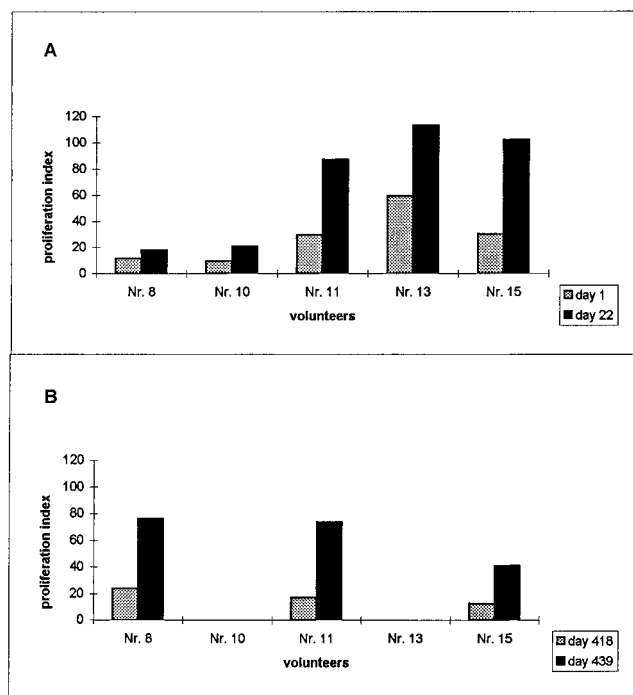


FIG. 3. Lymphoproliferative responses of subjects orally immunized with *S. typhi* Ty21a after primary (A) and booster (B) immunizations. PBMC obtained before and 21 days after immunization were tested for proliferative responses to Ty21a. Subjects 10 and 13 were not given booster immunizations. Nr., volunteer number.

observed for the three volunteers available for booster immunization (Fig. 1). However, specific peripheral blood T-cell proliferative responses to whole Ty21a cells were induced in all three subjects given booster doses (Fig. 3).

Before booster immunization with the cholera vaccine, two of three volunteers still presented measurable vibriocidal antibody titers, the third one having a titer below the detection limit (Table 2). After the booster, the titers of the two volunteers with measurable vibiocidal antibody titers did not rise significantly, in contrast to the titer of the third volunteer, who showed a >4-fold rise. Interestingly, only this volunteer showed a significant (fivefold) increase in the lymphoproliferative response to CVD 103-HgR whole cells after booster immunization (Fig. 4). Prebooster anti-CT IgG titers were comparable to or even higher than peak values observed 22 days after primary immunization for all three volunteers; however, the titers did not significantly rise after the booster immunization (Table 2). As for Ty21a, only low (IgG and IgM) or undetectable (IgA) levels of ASC were observed after the booster immunization (Fig. 2).

This study demonstrates that oral immunization with a single oral dose of the recombinant cholera vaccine strain CVD 103-HgR or with three oral doses of a liquid formulation of the attenuated *S. typhi* vaccine strain Ty21a elicits strong mucosal and systemic secretory immune responses in human volunteers. This observation is of particular interest since the rates of seroconversion of vibriocidal antibodies in cholera challenge studies and anti-O9, 12 LPS IgG antibodies were shown to correlate with protective efficacy in the field. A significant rise in serum vibriocidal antibodies (CVD 103-HgR) always correlated with a positive ASC response to the corresponding antigen.

Primary oral immunization with vaccine strain CVD 103-HgR induced a systemic humoral response to CT in three of five volunteers and persistently high baseline IgG titers 14 months later in the sera of three volunteers available for booster immunization, including two responders and one nonresponder to the primary immunization. No boosting effect was observed after the secondary immunization. These data are consistent with published information showing that CT induces long-term immunological memory (19, 26). Current evidence points to a greater significance of antibacterial over antitoxin immunity in protection against cholera (14, 15). CT may, however, play a potentiating role, since it is a potent mucosal immunogen that induces strong secretory and systemic immune responses after oral immunization in experimental
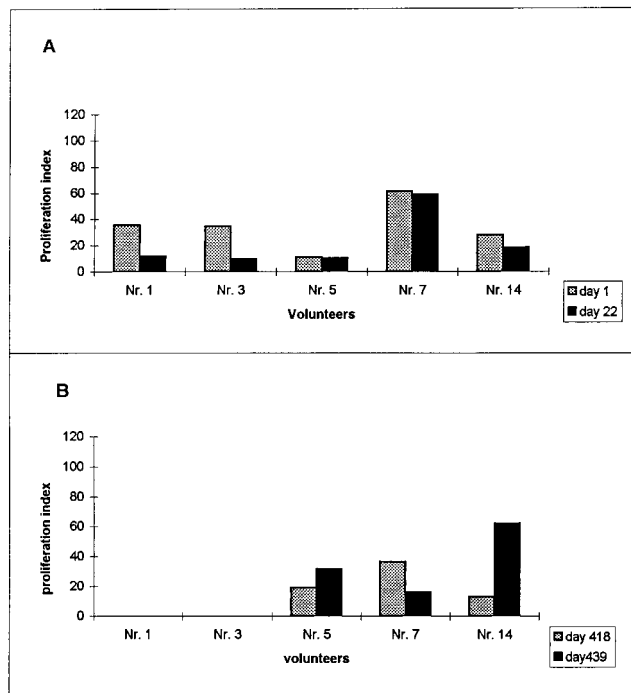
FIG. 4. Lymphoproliferative responses of subjects orally immunized with *V. cholerae* CVD 103-HgR after primary (A) and booster (B) immunizations. PBMC obtained before and 21 days after immunization were tested for proliferative responses to CVD 103-HgR. Volunteers 1 and 3 were not given booster immunizations. Nr., volunteer number.

animals (8). The mucosal immune response to CT is T cell dependent and major histocompatibility complex class II restricted. CT is also characterized by a strong ability to enhance mucosal immune responses to unrelated antigens. However, since CVD 103-HgR expresses only the CT B subunit, it may not elicit such a response. Indeed, while adjuvant activity was clearly shown for the cholera holotoxin, some debate exists as to whether the nontoxic CT B subunit alone can serve as an adjuvant in the absence of the enzymatically active A subunit (21).

Serum vibriocidal antibodies induced by the cholera vaccine still persisted in two of three vaccinees 14 months after the primary immunization, and their presence correlated with the prevention of a boosting effect. Prior kinetic studies have shown that after oral immunization with CVD103-HgR, vibriocidal antibody titers generally peak around day 10 (29). In a long-term kinetic study, about 64% of vaccinees that seroconverted after primary immunization had returned to baseline levels of vibriocidal antibodies after 15 or 24 months (4). Losonsky et al. (18) also found significant vibriocidal titers 1, 4, and 6 months after primary vaccination of volunteers with CVD 103-HgR but not 33 to 36 months after clinical infection of the same volunteers with a wild-type strain of *V. cholerae*. The maximal duration of protection elicited by CVD 103-HgR is not yet known, but in one challenge study the vaccine was shown to be 100% protective against diarrhea caused by wild-type *V. cholerae* up to 6 months after vaccination (25).

In contrast to immunization with CVD 103-HgR, which did not provide evidence of a CMI response except in a single volunteer after booster immunization, both primary and booster oral immunizations with Ty21a induced strong systemic CMI responses, characterized by the production of IFN-γ in the absence of IL-4, a cytokine pattern compatible with a

Th1-like response. The CD4+ Th1 cell subset is preferentially involved in CMI responses and is thereby a major component of the host defense against intracellular pathogens. Therefore, our results fully confirm previous findings that intracellular pathogens elicit CMI responses and establish that a noninvasive pathogen such as *V. cholerae* is unlikely to do so.

In addition, both vaccines are able to induce long-term immunological memory. Indeed, lower ASC and vibriocidal antibody responses after the booster indicate that the vaccinees can still rely on a significant local immunity that may interfere with colonization of the gut by the vaccine strain for at least 14 months after basis immunization. Prior studies by Kantele et al. (12, 13) have shown that the level of the immune response to a booster immunization with Ty21a inversely relates to the level of residual active immunity conferred by primary vaccination. Interestingly, despite the low secondary ASC response, Ty21a was able to significantly boost both humoral and CMI responses.

In conclusion, the direct comparison, in human volunteers, of results of oral vaccination with two attenuated live strains derived from a noninvasive (*V. cholerae*) and an invasive (*S. typhi*) enteric pathogen shows that the patterns of vaccine-induced immune responses clearly reflect differences in the capacities of the vaccine strains to colonize specific body compartments, although the fact that the typhoid vaccine is administered in three successive doses may also favor the induction of a CMI response. Therefore, in addition to being useful in the prevention of infectious diseases, well-characterized and safe live attenuated vaccines allow us in a manner better than that of any animal model to gain direct insight into human immune responses to pathogens. Based on this preliminary study, the duration of the immune response induced by primary oral immunization and the effect of long-term immunological memory on subsequent booster immunization deserve to be more carefully examined in a study involving more volunteers.

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