# Secondary *Vibrio cholerae*-Specific Cellular Antibody Responses following Wild-Type Homologous Challenge in People Vaccinated with CVD 103-HgR Live Oral Cholera Vaccine: Changes with Time and Lack of Correlation with Protection

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Peripheral blood immunoglobulin A antibody-secreting-cell (ASC) responses are thought to reflect the mucosal immune response to locally presented antigens. We evaluated the ASC response to cholera toxin (CT) and Inaba lipopolysaccharide (LPS) in 26 North American volunteers following immunization with a single oral dose of live attenuated *Vibrio cholerae* O1 vaccine strain CVD 103-HgR and again upon homologous wild-type challenge with *V. cholerae* classical Inaba 569B. Challenge occurred at either 7, 30, or 180 days after vaccination. The CT and LPS ASC responses of volunteers following vaccination (83 and 55%, respectively) were similar in magnitude and frequency to those of unvaccinated controls following wild-type challenge (80 and 60%, respectively [$0.1 \leq P \leq 0.9$]). The responses were primarily immunoglobulin A. Vaccinated volunteers challenged within 30 days of vaccination had reduced or nondetectable CT and LPS ASC responses. Challenge at 6 months resulted in a heightened ASC response to LPS, confirming the existence of mucosal memory. ASC responses to CT upon challenge at 6 months were detectable but not different from that seen following primary immunization, suggesting that secondary ASC responses to different antigens from a single vaccine operate independently. In spite of these variable ASC responses, the vaccine efficacy was 100% following challenge for all vaccinees. *V. cholerae*-specific ASC responses following antigenic reexposure gave information on the presence of mucosal B memory cells but did not correlate with protective immunity. As such, these ASC assays will have limited usefulness for evaluating vaccine responders in vaccine field trials in cholera-endemic areas where prior *V. cholerae* O1 exposure is unknown.

Specific antibody-secreting cells (ASC) transiently circulate in the peripheral blood after primary oral immunization with live or killed microbial antigens (2, 4, 14). These cells are believed to originate in the mucosal lymphoid tissue, circulating for a short time before homing back to the mucosa for antibody secretion. They are predominantly of the immunoglobulin A (IgA) class, reflecting their mucosal origin. The detection of these cells after primary stimulation, therefore, reflects priming of the mucosal immune system to locally presented antigens.

Information on the activity of these cells after secondary antigenic stimulation in humans is scant. Recently, it has been shown that secondary oral immunization with live oral *Salmonella typhi* Ty21a results in an ASC response that is variably present (5, 6). On the basis of these studies, it has been postulated that vaccines that elicit a more effective local immune response following primary immunization (a process labeled "active immunity") will produce a blunting of a secondary ASC response upon revaccination. The presumed explanation is that B cells primed to produce specific antibody are already in situ and can respond to and contain the antigenic challenge. In addition, it has been postulated that this blunting of the ASC response following secondary antigenic exposure may be indicative of increased mucosal protection (5).

The recent development and testing of a live attenuated

oral *Vibrio cholerae* O1 vaccine, CVD 103-HgR, offered the unique opportunity to evaluate the ASC response following vaccination and challenge and to test this hypothesis. It has been shown that naturally acquired or experimental infection with *V. cholerae* induces long-lasting protective immunity associated with gut mucosal IgA antibacterial and antitoxic antibody (9, 13, 18). A single dose of live attenuated CVD 103-HgR *V. cholerae* O1 vaccine has been shown to be highly protective in North American volunteer challenge studies conducted within 1 month after vaccination (11, 20).

Our studies had several goals. One was to evaluate the ability of CVD 103-HgR live oral cholera vaccine to elicit a primary mucosal immune response as measured by detecting ASC following vaccination. A second aim was to compare the specific ASC response with the one elicited by an experimental wild-type challenge in unvaccinated controls. The third goal was to assess the activity of the cholera-specific ASC response in vaccine-primed individuals upon subsequent exposure to a wild-type challenge. The final goal was to correlate the presence of a secondary ASC response with protection against disease following experimental cholera challenge of vaccinated individuals.

In a series of controlled studies, we evaluated the cholera-specific ASC responses elicited by oral administration of a single dose of the live attenuated *V. cholerae* vaccine, CVD 103-HgR, and again upon challenge at 7, 30, or 180 days after vaccination with wild-type homologous *V. cholerae* O1. In this way, we sought to directly compare the mucosal immu-

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nogenicity of this vaccine with natural infection. In addition, since the time interval between vaccination and challenge varied, we were able to assess the temporal changes in the effector arm of the mucosal immune system as evidenced by the ASC response. This information, coupled with efficacy data obtained from the challenge trials, would expand the current understanding of this mucosal immune response.

## MATERIALS AND METHODS

**Subjects.** Participants in these vaccination and efficacy studies were healthy adults from the metropolitan Baltimore, Md., area. The methods of medical screening have been previously described, with the modification that all volunteers were tested for human immunodeficiency virus type 1-specific antibody (10). The studies were explained in detail, and informed written consent was obtained. The clinical protocol was approved by the Institutional Review Board of the University of Maryland at Baltimore.

**Vaccine.** *V. cholerae* vaccine strain CVD 103-HgR is a recombinant derivative of pathogenic *V. cholerae* O1 Inaba strain 569B with a deletion of the genes encoding the A subunit of cholera toxin (CT) and introduction of a gene encoding resistance to $Hg^{2+}$ (7). CVD 103-HgR was produced by the Swiss Serum and Vaccine Institute (Berne, Switzerland) as a single-dose formulation containing $3 \times 10^8$ to $5 \times 10^8$ viable lyophilized organisms (with 25 mg of aspartame). The packet containing vaccine was accompanied by another packet containing buffer (2.5 g of sodium bicarbonate and 1.65 g of ascorbic acid). The vaccine was stored at 4°C until use.

**Vaccine administration.** Three cohorts of volunteers received a single oral dose of CVD 103-HgR in separate studies differing in the interval between vaccination and challenge. Each group of volunteers received approximately $3 \times 10^8$ to $5 \times 10^8$ CFU of CVD 103-HgR as outpatients. The vaccine was administered as a suspension made by first mixing the buffer with 100 ml of distilled water and then adding the vaccine packet. Volunteers had nothing to eat or drink for 90 min before and 90 min after vaccination.

**Challenge.** Vaccinees received wild-type classical Inaba *V. cholerae* 569B at either 8 days, 1 month, or 6 months after vaccination with a challenge inoculum of $2 \times 10^6$, $4 \times 10^6$, or $3 \times 10^7$ CFU of *V. cholerae*, respectively. Unvaccinated volunteers served as controls. Challenge studies were carried out in the Research Isolation ward of the Center for Vaccine Development under quarantine for 10 days by the Bureau of Disease Control of the Baltimore City Health Department as previously described (12). Diarrhea was defined as 2 or more loose stools in a 48-h period totalling at least 200 ml in volume or a single loose stool ≥300 ml. Tetracycline (500 mg every 6 h for 5 days) was given for treatment of diarrhea or prior to discharge for all volunteers.

**Immunology.** Heparinized blood was drawn before vaccination and on day 4 (for the 8-day challenge study), 7, or 10 after vaccination and challenge. ASC of the IgG, IgM, and IgA classes against *V. cholerae* O1-specific Inaba lipopolysaccharide (LPS) and CT were measured by ELISPOT as previously described (19). Briefly, peripheral blood mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation (Organon Teknika, Durham, N.C.) and added to specific-antigen-coated or blank microtiter plates at a concentration of $0.25 \times 10^6$ peripheral blood mononuclear cells per well. Wells were run in quadruplicate for each antigen-Ig class. Data were expressed as the number of specific ASC per $10^6$ peripheral blood mononuclear cells for each antigen and Ig class.

**Statistical analysis.** The magnitudes and frequencies of peak geometric mean ASC numbers of vaccinated or control responders following vaccination or challenge were compared by using the Wilcoxon test (paired *t* test). Vaccinee responses were paired with themselves in evaluating the effect of time between vaccination and challenge on the ASC response. The ASC responses of volunteers following vaccination were paired with those of controls following challenge to assess the similarities of natural infection and vaccination with CVD 103-HgR in the elicitation of an ASC response. Frequency data were analyzed by Fisher's exact test.

## RESULTS

**Clinical responses to vaccination and challenge.** The clinical responses to vaccination and challenge are reported elsewhere for the 6-month and 8-day challenges but will be summarized here (20). A total of 36 subjects participated in both vaccination and challenge studies: 11 in the 8-day challenge group, 14 in the 30-day challenge group, and 11 in the 6-month challenge group. No diarrhea occurred after vaccination in any of the groups. A total of 39 volunteers participated in the challenge studies as unvaccinated controls: 11 in the 8-day challenge study, 13 in the 30-day challenge study, and 15 in the 6-month challenge study. Following challenge with *V. cholerae* O1 classical Inaba strain 569B, none of the 36 vaccinated subjects developed diarrhea. In contrast, 25 of 39 (66%) unvaccinated controls developed diarrhea following challenge: 8 of 11 (73%) in the 8-day challenge study, 7 of 13 (54%) in the 30-day challenge study, and 10 of 15 (67%) in the 6-month challenge study ($P < 0.001$ [Fisher's exact test]).

**ASC responses.** Blood was available for evaluation of the ASC responses in vaccination and challenge studies from 26 of 36 (72%) volunteers and from 17 of 39 (44%) unvaccinated controls. There was no significant difference in the rate of diarrhea for the data of controls available for ASC analysis compared with the data of controls not available for analysis (59 versus 66%; $P = 1.0$ [Fisher's exact test]). The ASC responses to LPS or CT antigens of both vaccinees and unvaccinated controls prior to vaccination or challenge were negligible. None of the vaccinees had any detectable ASC responses to CT and only one vaccinee had a detectable ASC response to LPS prior to vaccination (one cell [IgG class] detected). Of the unvaccinated controls challenged with *V. cholerae*, only one subject had an ASC response specific to CT (four cells) and one other control had an ASC response specific to Inaba LPS (one cell) prior to challenge. All such cells were of the IgG class. The frequencies of response and the peak geometric mean numbers of combined IgA, IgM, and IgG LPS and CT ASC responses following vaccination and challenge are presented in Tables 1 and 2, respectively.

**ASC responses following vaccination compared with those of challenged unvaccinated controls.** The ASC responses to LPS and CT are presented in Tables 1 and 2, respectively. The ASC responses peaked on day 7 in 20 of 26 (77%) vaccinees following vaccination and in 11 of 17 (65%) controls following challenge. The ASC response was for the most part modest, with geometric mean numbers of ASC to both LPS and CT of less than 100 cells per $10^6$ peripheral blood mononuclear cells. The magnitude of the CT or LPS ASC response following vaccination compared with that

TABLE 1. ASC responses to Inaba LPS in vaccinees and controls following vaccination with CVD 103-HgR and/or challenge with wild-type V. cholerae classical Inaba 569B

| Subjects | n | Vaccination | | Challenge | | |
|---|---|---|---|---|---|---|
| | | Peak GMN of ASC[a] (range) | % Responders[b] | Days after immunization | Peak GMN of ASC[a,c] (range) | % Responders[b] |
| Vaccinees | 10 | 20 (0–161) | 50 | 8 | 3* (0–8) | 40 |
| | 10 | 15 (0–120) | 60 | 30 | 4** (0–9) | 30 |
| | 6 | 39 (0–154) | 67 | 180 | 142 (0–318) | 83 |
| Controls[d] | 6 | NA | NA | 8 | 33 (0–595) | 67 |
| | 5 | NA | NA | 30 | 39 (0–70) | 80 |
| | 6 | NA | NA | 180 | 63 (0–100) | 33 |

[a] Peak geometric mean number (GMN) of ASC = the sum of the IgA-, IgG-, and IgM-class ASC/$10^6$ peripheral blood mononuclear cells.
[b] Percent subjects with one or more cells for at least one antibody class.
[c] * and **, significantly different from corresponding values for controls ($P$ = 0.007 and 0.01, respectively [Wilcoxon test]).
[d] Nonvaccinated subjects challenged at the times corresponding to the times of challenges postimmunization for the vaccinees. NA, not applicable.

following wild-type challenge in unimmunized individuals was not significantly different (0.2 ≤ $P$ ≤ 0.9 for LPS ASC response comparisons and 0.1 ≤ $P$ ≤ 0.9 for CT ASC response comparisons [Wilcoxon test]).

The frequency of detection of a CT or LPS ASC response following vaccination ranged from 50 to 90%, while that for the unvaccinated challenged controls was 33 to 100%. Vaccinees responded as frequently to CT and LPS as did the challenged controls ($P$ = 0.4 and 0.6, respectively [Fisher's exact test]). There seemed to be a somewhat greater response rate to CT compared with LPS in both vaccinated subjects and challenged controls, with approximately 60% of vaccinees and challenged controls responding to LPS compared with approximately 80% responding to CT. However, these differences were not statistically significant ($P$ = 0.3 for ASC responses of the controls to LPS and CT and $P$ = 0.1 for ASC responses of the vaccinees to LPS and CT).

The majority of the ASC responses were of the IgA class. Following vaccination with CVD 103-HgR, IgA accounted for 84% of the CT-specific ASC responses and 83% of the LPS-specific ASC responses. Similarly, 80% of the CT ASC and 76% of the LPS ASC responses of the control groups challenged with V. cholerae 569B were of the IgA class. There was only one individual who did not have a predominantly IgA ASC response. This was an unvaccinated control who had a marked IgM LPS ASC response (595 cells) following challenge, constituting 90% of his ASC response.

The effect of the time interval between vaccination and challenge on the ASC response. As shown in Tables 1 and 2, subjects challenged with homologous wild-type V. cholerae O1 within 30 days of vaccination had meager mean peak ASC LPS and CT responses (3 or 4 cells). The magnitudes of these ASC responses to both CT and LPS in the challenged vaccinees were markedly decreased from those generated in the challenged controls (all $P$ values were <0.05 except for the day 30 challenge CT ASC value, which approached significance [$P$ = 0.06]). In contrast, when vaccinees were given wild-type V. cholerae 569B 6 months after vaccination, prominent LPS and CT ASC responses were detected. Although 3 of 5 vaccinated individuals had at least a threefold increase in actual numbers of ASC to CT and LPS following challenge, suggesting a booster response, the differences between ASC responses after vaccination and challenge in studies with small subject numbers did not achieve statistical significance ($P$ = 0.19 and 0.9 for LPS ASC differences and CT ASC differences, respectively [Wilcoxon signed-rank test]).

This gradual recovery of the antigen-specific ASC response as the interval between vaccination and challenge increases is further suggested by ASC data obtained from three additional volunteers challenged 4 months after vaccination. These volunteers were challenged in the same study as the vaccinees challenged at 6 months. Upon challenge, these vaccinees had a peak LPS ASC response of 18 (range, 6 to 24) cells and no detectable CT ASC response.

TABLE 2. ASC responses to CT in vaccinees and controls following vaccination with CVD 103-HgR and/or challenge with wild-type V. cholerae classical Inaba 569B

| Subjects | n | Vaccination | | Challenge | | |
|---|---|---|---|---|---|---|
| | | Peak GMN of ASC[a] (range) | % Responders[b] | Days after immunization | Peak GMN of ASC[a,c] (range) | % Responders[b] |
| Vaccinees | 10 | 46 (0–572) | 70 | 8 | 3* (0–14) | 60 |
| | 10 | 30 (0–110) | 90 | 30 | 4** (0–6) | 40 |
| | 6 | 92 (0–248) | 90 | 180 | 42 (12–319) | 100 |
| Controls[d] | 6 | NA | NA | 8 | 57 (0–73) | 50 |
| | 5 | NA | NA | 30 | 34 (0–66) | 90 |
| | 6 | NA | NA | 180 | 125 (11–166) | 100 |

[a] Peak geometric mean number (GMN) of ASC = the sum of the IgA-, IgG-, and IgM-class ASC/$10^6$ peripheral blood mononuclear cells.
[b] Percent subjects with one or more cells for at least one antibody class.
[c] * and **, significantly different from corresponding values for controls ($P$ = 0.007 and 0.01, respectively [Wilcoxon test]).
[d] Nonvaccinated subjects challenged at the times corresponding to the times of challenges postimmunization for the vaccines. NA, not applicable.

## DISCUSSION

The development of an effective cholera vaccine has never been as imperative as at present in the face of the cholera pandemic spreading from Asia and Africa to the shores of Latin America (1). Our studies show that a single oral dose of the live attenuated *V. cholerae* Inaba CVD 103-HgR vaccine produces ASC responses to CT and Inaba LPS that are very similar to the responses produced by the wild-type *V. cholerae* challenge. The responses are primarily IgA, suggesting that the mucosal priming of this vaccine is similar to that seen in our experimental *V. cholerae* challenge model.

Kantele et al. evaluated the ASC responses following primary and booster immunizations with live oral typhoid vaccine Ty21a (5, 6). We have extended these studies by describing primary ASC responses after vaccination with the live oral cholera vaccine, CVD 103-HgR, followed by wild-type cholera infection. Antigenic reexposure of vaccinated individuals to wild-type *V. cholerae* infection results in a variable secondary ASC response. Blunted ASC responses to *V. cholerae* Inaba LPS and CT were observed up to 4 months following vaccination with CVD 103-HgR. The time interval for this reduced ASC response upon secondary exposure to antigen probably depends on the quality of the original immunizing event. It has been shown, for example, that primary vaccination with a liquid suspension of *S. typhi* Ty21a followed by a secondary vaccination 1 year later with the same vaccine formulation results in a decreased ASC response. In contrast, vaccination and boosting with an enteric coated capsule formulation of Ty21a gives an elevated secondary ASC response within the same time interval (5, 6). Secondary challenge with wild-type *Shigella sonnei* 1 month after primary immunization with 5076-1C, a hybrid vaccine consisting of Ty21a expressing *S. sonnei* LPS for both *S. typhi* and *S. sonnei*, results in a marked increased specific ASC response (21).

It is probable that the quality of the secondary antigenic stimulation also influences the presence or absence of a secondary ASC response. In our studies, we used a potent secondary antigenic stimulus, namely, a wild-type challenge with an inoculum that leads to a high attack rate in controls. This challenge is clearly different from the booster immunizations with attenuated vaccines used in other studies.

Our data do not support the hypothesis that the absence of a secondary specific ASC response following antigenic rechallenge correlates with protective efficacy. We show that subjects who did not have secondary ASC responses to *V. cholerae*-specific LPS and CT at 7 or 30 days after vaccination were no less protected than subjects who had marked ASC responses to these antigens upon challenge at 6 months. These antigens were selected because of previous studies suggesting that they are important immunoprotective antigens of *V. cholerae* (9, 13, 18). Our conclusions may be limited by the fact that we may not be measuring the ASC responses to all protective antigens of *V. cholerae* such as surface proteins that mediate virulence through adherence and colonization (3, 15, 17).

The presence of a specific ASC response after primary immunization also was not correlated with protection. Only approximately 50% of subjects had a primary ASC response to LPS, and 70 to 90% had a CT ASC response, yet protection was 100%. Part of the reason for this may be the nature of the assay itself. The assay is performed to detect mature B cells already producing antibody. These cells probably constitute only a small fraction of the specific

B-cell population circulating after immunization. It has been shown that there are many immature B-cell forms recognizing a specific antigen but not producing antibody present in the blood after parenteral immunization (8). This possibility will be the subject of future investigation.

The circulation of specific ASC after secondary antigenic exposure is probably dependent on the number of specific-memory B cells present in the lamina propria. Antigen would be processed at lymphoid sites to induce a secondary ASC response only if there were not sufficient numbers of primed antibody-producing B cells to prevent antigen absorption and processing in intestinal lymphoid tissue. Mice orally vaccinated and rechallenged with CT develop secondary antitoxin plasma cells responses in the lamina propria both from antigen-driven proliferation of memory cells within the lamina propria and also predominantly from systemically migrating antitoxin-containing plasmablasts (16). In contrast, our data would suggest that the majority of the mucosal antibody response within 6 months after vaccination with CVD 103-HgR is due to memory cells contained within the lamina propria and not to recirculating lymphoblasts.

The magnitude of the secondary ASC response also seems to be antigen specific. For example, the secondary ASC response to *V. cholerae* LPS was detectable at the 4-month challenge but a CT-specific ASC response was not. The LPS response increased markedly at 6 months. These results suggest that the CT may be a more potent mucosal priming antigen than LPS. The fact that there are variable secondary ASC responses to different antigens in the same vaccine suggest that a reduced secondary ASC response is not simply due to active mucosal immunity reducing bacterial load. If that were the case, specific secondary responses would be the same for all antigens contained in a given vaccine. However, this hypothesis is valid only if antigenic expression of the pathogen is not changed in the immune gut. It is possible that an active mucosal immune response inhibits the production of CT by the invading organisms and thus limits the subsequent specific CT immune response.

The fact that vaccinated volunteers were all uniformly protected against challenge regardless of the time interval before challenge suggests that specific mucosal immunity was not impaired. The uniform protection of vaccinees against challenge also makes it unlikely that suppressor T-cell activity was responsible for the absent secondary ASC response. Detection of a secondary ASC response most likely is dependent on the numbers of specific activated memory B cells present in situ in the lamina propria.

The practical implications of these findings are apparent. The primary ASC responses to *V. cholerae* LPS and CT are of limited value in predicting vaccine efficacy, although they do give vital information on the potency of mucosal priming. The secondary ASC responses to specific LPS and CT are of even more-limited use in predicting the efficacy of a vaccine, since they are dependent on the strength of the secondary antigenic stimulus and the time interval from primary vaccination. In field conditions, in which the prior exposure history of a given vaccinee is usually unknown, an absent or reduced ASC response following vaccination would be difficult to interpret.

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