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New Evidence for an Inflammatory Component in Diarrhea Caused by Selected New, Live Attenuated Cholera Vaccines and by El Tor and O139 *Vibrio cholerae*

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Using a lactoferrin latex agglutination assay, we have compared the inflammatory responses to a cholera vaccine candidate, CVD 110, in which all known toxin genes have been deleted or mutated yet still produced significant diarrhea, with a less reactive vaccine strain and wild-type El Tor and O139 *Vibrio cholerae* strains. Data suggest that diarrhea due to attenuated and wild-type El Tor *V. cholerae*, and to a lesser extent O139 *V. cholerae*, involves an inflammatory response. Further study is required to further elucidate the mechanism of the process(es) involved.

Lactoferrin is an iron-binding glycoprotein found in human milk and other epithelial secretions and in the secondary (specific) granules of neutrophils (10). In the evaluation of patients with diarrhea, the lactoferrin latex agglutination assay (LFLA) has repeatedly been demonstrated to be more sensitive in its detection of lactoferrin than is microscopy with methylene blue staining for the detection of fecal leukocytes (11, 14).

The oral cholera vaccine candidate CVD 110 is a derivative of El Tor *Vibrio cholerae* O1 strain E7946 with deletions for the following genes: *ctxA* (cholera toxin A subunit) and the "virulence cassette" which includes *zot* (zonula occludens toxin), *ace* (accessory cholera enterotoxin), and *cep* (core-encoded pilus). Additionally, the *hly* (hemolysin) gene is inactivated by the insertion of a mercury resistance gene. When CVD 110 was administered to 10 healthy volunteers, it produced diarrhea in seven volunteers and fever in an eighth (12). This observation stimulated us to investigate retrospectively the diarrhea associated with this vaccine by testing the stools from CVD 110 volunteers for the presence of lactoferrin as an indicator of inflammation in the intestinal mucosa. The results were compared with the lactoferrin determinations from stool samples of volunteers in three studies. In one study, a classical Inaba vaccine strain, CVD 103HgR2, was used. The CVD 103 HgR2 vaccine, derived from the classical Inaba O1 strain 569B, is also deficient in *ctxA*, does not produce Shiga-like toxin, and contains a mercury resistance gene. In previous trials it has proven to be well tolerated and did not cause diarrhea or fever. In two other dose-response studies, wild-type El Tor *V. cholerae* or wild-type O139 *V. cholerae* was administered.

Study groups and collection of the stools. Stool specimens from four volunteer studies (4, 8, 12, 13) performed at the Center for Vaccine Development, University of Maryland, Baltimore, were examined.

A stool sample collected before the challenge or vaccination was considered the control specimen. In the studies of the CVD 110 and CVD 103HgR2 vaccine strains, formed stools from before and 6 to 7 days after vaccination were assayed for lactoferrin. (Because this study was retrospective, only these stools were available since normally only stool filtrates were saved.) In the wild-type challenge studies of strains 3008 and AI1837, one diarrheal stool per day per volunteer passed during the 96-h observation period was assayed for lactoferrin. Stool samples were sent on ice to our laboratory (University of Virginia) and stored frozen at −70°C until the test for the presence of lactoferrin (Leukotest; Tech Lab, Inc., Blacksburg, Va.) was performed blindly, according to the instructions contained in the manufacturer's manual. Assays of serial twofold dilutions were performed on positive samples to record the highest positive lactoferrin titer. The specimens that did not agglutinate at dilution 1:50 were considered negative. Diarrhea was defined as passage of two or more loose stools within 48 h with at least 200 ml in total volume or a single loose stool of at least 300 ml. In order to estimate the magnitude of inflammation present, the product of lactoferrin geometric mean reciprocal titer and mean diarrhea stool volume or "inflammatory index" was determined in each of the volunteers from all samples submitted and an overall mean was determined for each study group. This calculation was designed to account for the dilution of inflammatory products which could occur in cases of copious diarrhea. Geometric mean reciprocal titers were calculated by using a value of 1:25 for negative results, and data were compared by using an unpaired Student *t* test with the assumption that the variances were unequal. Results were as follows.

CVD 110. Ten specimens from the 10 volunteers given CVD 110 were tested for lactoferrin. Over 1 to 5 days after ingestion of CVD 110, seven volunteers developed diarrhea, all with high titers of lactoferrin ranging from 1:400 to 1:3,200. Of the three volunteers who did not develop diarrhea after ingesting CVD 110, one developed general malaise, headache, and fever (38.5°C) and had a titer of lactoferrin of 1:800. The other two individuals were found to have lactoferrin titers of 1:800 (with one loose stool of 115 ml which did not satisfy the definition of diarrhea) and 1:200.

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TABLE 1. Diarrheal numbers and volumes with reciprocal fecal lactoferrin titers and inflammatory indices after ingestion of *V. cholerae* vaccines and strains

Inoculum	Dose (CFU)	No. of volunteers	MDSV (ml)[a]	Avg no. of loose stools	Post-LFLA GMRT[b]	Inflammatory index ($\times 10^5$)[b]
CVD 110	10^8	10	603	4.3	857a	8.5e
CVD 103HgR2	10^8	9	81	0.6	27b	<0.1f
Wild type						
El Tor 3008	10^6	8	2,871	11.5	54c	1.6g
O139 AI1837	10^6	24	3,139	14.1	32d	1.0h

[a] MDSV, mean diarrheal stool volume.
[b] GMRT, postinoculation lactoferrin geometric mean reciprocal titer. Inflammatory index = (MDSV) ×-LFLA (post GMRT), for each individual and averaged for the study group. Significance by unpaired *t* test: a versus b: $P = 0.006$; c versus d: $P = 0.005$; e versus f: $P = 0.03$; e versus g: not significant; e versus h: $P = 0.003$; f versus g: $P = 0.002$; f versus h: $P < 0.02$; g versus h: not significant.

CVD 103 HgR2. CVD 103 HgR2 caused diarrhea in 1 of the 10 volunteers. Ten postchallenge specimens were tested for LFLA. Lactoferrin was detected in only two patients (titers of 1:50 and 1:400). The specimen with diarrhea was lactoferrin negative. The specimen with the lactoferrin titer of 1:400 did not meet the criteria for diarrhea (241 ml over 3 days); curiously, the prechallenge stool of this patient had a lactoferrin titer of 1:100 before the challenge. Because the elevated lactoferrin titer in this preinnoculation sample indicated the probability of some previous inflammatory process, the samples from this individual were not used in statistical analysis. All other nondiarrheal samples were lactoferrin negative (titer <1:50).

***V. cholerae* El Tor.** *V. cholerae* El Tor caused diarrhea in seven of the eight volunteers; 18 fecal specimens were tested for fecal lactoferrin. Six of the seven volunteers with diarrhea had lactoferrin detected (≥1:50) in their stools. The geometric mean reciprocal titer of lactoferrin was calculated to be 54. One volunteer who did not meet criteria for diarrhea had fecal lactoferrin present at titer of 1:100.

***V. cholerae* O139.** The O139 cholera strain caused diarrhea in all but one of 24 volunteers. One half of the volunteers (12 of 24) had lactoferrin detected in at least one stool sample. One volunteer had no loose stools, and no lactoferrin was detected in samples from this individual. There was no significant difference in the diarrheal volumes for individuals who did (mean volume = 3,039 ml) and did not (mean volume = 3,239 ml) have a LFLA result of ≥1:50 in one or more of their stool samples.

Table 1 summarizes the results of these four trials, and Fig. 1 graphically presents the data for reciprocal LFLA titer (postchallenge).

In this study, the mean titer of lactoferrin detected in stools of volunteers ingesting vaccine candidate CVD 110 was significantly higher than the mean titer found for CVD 103HgR2 vaccine volunteers (857 versus 27; $P = 0.006$). This supports a direct correlation between lactoferrin titers and the degree of adverse symptoms seen with these vaccine strains. It is worth noting that some volunteers, especially in the CVD 110 cohort, had highly elevated lactoferrin titers without diarrhea. Furthermore, recent preliminary findings document increased fecal interleukin-8 (as determined by Quantikine enzyme-linked immunoassay; R&D Systems, Minneapolis, Minn.) in the stools of seven of nine volunteers with diarrhea and elevated fecal lactoferrin after CVD 110 challenge (mean IL-8 = 238 ± 203 (standard error of the mean) pg/ml versus 16.5 ± 13.7 for prechallenge controls, $n = 9$ pairs; $P < 0.05$ by Wilcoxon signed rank test).

As indicated in Table 1, the inflammatory index for CVD 103HgR2 was significantly different from the products for CVD 110, wild-type 3008, and wild-type O139 ($P = 0.03$, $P = 0.002$, and $P < 0.02$, respectively). Although higher in the CVD 110 volunteers, as opposed to the wild-type 3008 volunteers, the inflammatory indices were not significantly different in these two groups. However, the diarrhea caused by wild-type O139 had a significantly lower inflammatory index than did CVD 110 diarrhea ($P = 0.003$).

While the inflammatory index measurement is an imperfect estimate of the inflammatory activity of a diarrheal process, it provides the best attempt available to include an assessment of overall diarrhea with lactoferrin titer in this study. The data presented here are limited by the inability to measure lactoferrin in all stools from all volunteers in all studies and the differences in collection times for those stools which were available for assay. Future, prospective work may clarify these concerns. Nonetheless, the index provides an approximation for overall inflammation and can aid in the interpretation of the data presented herein, especially since there is a correlation between postchallenge LFLA and inflammatory index across all four cohorts.

A number of interpretations of these results are possible. CVD 103HgR2, produced from classical Inaba strain 569B may produce different hemagglutinins or other colonization factors which allow for epithelial colonization but do not produce a robust inflammatory response. Furthermore, the greater inoculum of CVD 110 might have triggered the lactoferrin responses; however, this was not seen with the comparable inoculum of CVD 103 HgR. Colonization, which has been linked to effective immunogenicity in vaccines, must occur to some extent (9). The large flux of fluids and "washing out" of the intestine associated with the diarrhea from El Tor and O139 cholera may limit colonization and inflammation induced by wild-type vibrios. In the CVD 110 trial, a highly inflammatory process was present. Lacking functional toxins (*ctxA*, *ace*, *zot*), CVD 110 may produce a mechanistically different diarrheal process than that seen with wild-type *V. cholerae* infection. As shown by Madara et al., polymorphonuclear leukocytes themselves may induce chloride secretion similar to that seen in toxigenic secretory diarrheal illnesses, through the stimulus-induced release of 5′ AMP (6) after chemotaxis into the lamina propria of colonized epithelium. This is perhaps due to actions of chemotactic cytokines, including interleukin-8 and interleukin-6, which have been shown to be released by epithelial cells in response to *E. coli* colonization or *Salmonella* infection in vitro (2, 3, 5, 7). The gene deletions in CVD 110

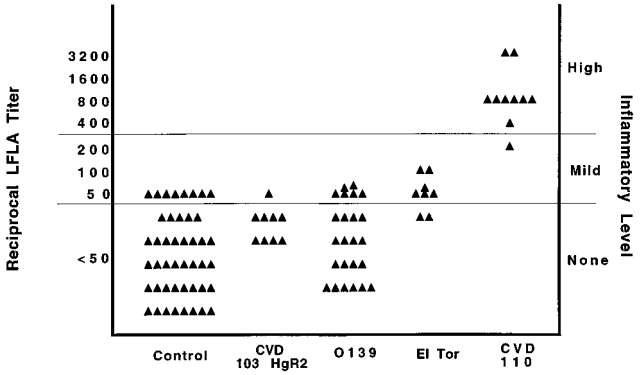

FIG. 1. Reciprocal lactoferrin titers in stools of prechallenge controls and volunteers fed cholera vaccine CVD 103HgR2, O139 cholera, El Tor cholera, and cholera vaccine candidate CVD 110.

may reduce the activity of some not yet characterized inhibitory mechanism which normally minimized the inflammatory response to colonizing *V. cholerae*. Indeed, cholera toxin has been shown to reduce leukocyte chemotaxis in vitro (1). Preliminary studies of a classical Ogawa strain lacking the A subunit of cholera toxin (CVD 101) show that it too causes diarrhea with increased fecal lactoferrin (all five volunteers tested having fecal lactoferrin reaching 1:100 to 1:800). Finally, however, it is possible that the inflammatory diarrhea produced by CVD 110 occurred via a mechanism unique to it or to its parent strain, E7946. Hence, these results cannot be extrapolated to other vaccine constructs or wild-type strains. Further studies will need to address in more detail the inflammatory responses to E7946, CVD 110, and other strains to explore these possibilities.

Taken together, our findings raise an important new concept of a potential role of neutrophils or neutrophil products in the diarrhea seen with a newly engineered live attenuated cholera vaccine as well as with El Tor and O139 cholera. Further, prospective studies, perhaps with fecal cytokines, will be necessary to clarify the potential mechanisms involved in these important findings.

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