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# Protection Against Experimental Cholera by Oral or Parenteral Immunization

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Comparisons were made between the antigenic potency and protective capacity of several cholera toxin derivatives. Rabbits were immunized parenterally with 50 μg of cholera toxin, A subunit, B subunit, procholeragenoid, or Wyeth glutaraldehyde toxoid 20101. Examination of the antibody response curves revealed that cholera toxin elicited serum antitoxin responses that rose more quickly than in the subunit-immunized animals; however, antitoxin levels were of the same magnitude after 10 weeks. Parenteral immunization with procholeragenoid evoked antibody titers that were similar to the toxin, whereas Wyeth toxoid yielded only one-tenth the level of antitoxin. Oral immunization with procholeragenoid as well as Wyeth toxoid resulted in lower serum antitoxin titers than that achieved with parenteral immunization, despite the oral administration of 10 times the parenteral dose. Analysis of protection against live-cell challenge revealed that parenteral administration of procholeragenoid provided the best protection against fluid accumulation. Oral immunization with procholeragenoid also was very effective, whereas oral immunization with B subunit or Wyeth toxoid resulted in minimal protection. Also, the A subunit provided surprisingly more protection than did cholera toxin.

The objective of this study was to compare the antigenic potency of cholera toxin with that of several of its derivatives, including its A and B subunits, heat-aggregated toxin (procholeragenoid) and glutaraldehyde toxoid. The toxin subunits were isolated and characterized by Kurosky et al. (11) and were shown to be of high purity. Hejtmancik et al. (8) and Markel et al. (12) have shown that immunization of animals with the B subunit preparation evokes antisera containing antibodies specific only for the B subunit, whereas antisera to the A subunit contain both antibodies to the A subunit (or α chain) and the B subunit. Using the same subunit preparations, Peterson et al. (15) observed that antisera to both subunits could effectively neutralize cholera toxin, but the toxin-neutralizing capacity of each type of serum could be attributed to antibodies reacting only with the B subunit. Use of cholera toxoid preparations containing high concentrations of B subunit as proposed by Holmgren et al. (9) should provide the best possible protection against cholera.

Studies designed to optimize antitoxic immunity are particularly pertinent since initial field trials with glutaraldehyde and formaldehyde toxoids have not provided significant protection against cholera (2, 13). Although some investigators interpret the poor protection observed in field trials of these toxoids as evidence that antitoxic immunity can not be effective against cholera, the field trial studies were performed only with 100-μg doses of glutaraldehyde or formaldehyde toxoids. The dose level was not sufficient to elicit the maximum antitoxin responses in humans (17), and these chemical toxoiding procedures have been shown to be quite damaging to the antigenic integrity of cholera toxin (19). Furthermore, animal experimentation has shown that antitoxic immunity against cholera is as effective as antisomatic immunity, but the ratio of toxoid dose per body weight used in animal studies (14) was at least 15 times greater than that employed in the human trials (2, 13).

Finkelstein et al. (4) described a heat-aggregated form of cholera toxin which he referred to as procholeragenoid. Fujita and Finkelstein (6) found that procholeragenoid was effective in protecting mice against challenge with toxin or live vibrios when given by the parenteral or oral routes. Since it could have had some side effects, Germanier et al. (7) reported that formaldehyde could be used to detoxify procholeragenoid without affecting its antigenicity. Therefore, procholeragenoid appeared to be a viable alternative to toxoids currently being tested. The current study indicates that procholeragenoid administered by the parenteral or oral routes provided rabbits with protection that was superior

to glutaraldehyde toxoid and other toxin derivatives against experimental cholera using the intestinal loop model initially described by De (3).

## MATERIALS AND METHODS

**Antigens.** Cholera toxin was purified and analyzed as previously described (8, 10). Cholera toxin A subunit and B subunit were prepared and characterized by the procedure of Kurosky et al. (11). Procholeragenoid was prepared by heating purified cholera toxin (5 mg/ml) at 60°C for a period of 5 min. Nonaggregated cholera toxin and free subunits were removed by chromatography on a column (2.5 by 100 cm) of agarose (A 0.5 M; Bio Rad Laboratories). The void volume peak containing the high molecular weight procholeragenoid was pooled and stored at 4°C before use. Wyeth glutaraldehyde toxoid 20101 (without adjuvant) was an experimental toxoid prepared by Rappaport et al. (18) and acquired from Carl E. Miller at the National Institutes of Health (NIH) in Bethesda, Md. It was prepared in response to an NIH contract for the purpose of eventual field trial.

**Animals.** New Zealand white rabbits initially weighing 4 to 5 points (ca. 1.8 to 2.3 kg) were obtained and housed under the same conditions for the duration of this study. A total of six rabbits were immunized with each antigen.

**Immunization of rabbits.** Each of the preparations described above was administered without adjuvants, either by parenteral injection or orally. Parenteral administration consisted of a single, intramuscular injection into the hind leg. Oral immunization was accomplished by feeding the antigen solutions, contained in 5 ml of an antacid (Mylanta; Stuart Pharmaceuticals), into the mouth of the rabbit through a syringe and catheter. All rabbits were given three consecutive doses of antigen at 4-week intervals. Each parenteral dose of antigen was 50 μg, whereas each oral dose was 500 μg.

**Serological titrations.** Cholera antitoxin titers of sera were determined by the passive hemagglutination test (5). All titers were expressed as antitoxin units per milliliter, based on the Swiss Serum and Vaccine Institute reference serum (1).

**Intestinal loop challenge.** Rabbits were challenged by a procedure described in detail previously (14). Briefly, rabbits were anesthetized by intramuscular injection of 3 ml of Ketaset (Bristol Laboratories) before ligation of the small intestine into a series of eight loops, each about 10 cm in length. Injections (1 ml each) of live vibrios suspended in phosphate-buffered saline with gelatin and ranging from $10^3$ to $10^8$ cells per ml, were injected via 2-cm interspaces between the loops. The sequence of injections was reversed in alternate animals. Proximal and distal loops always received 1 ml of phosphate-buffered saline with gelatin as a control. Rabbits were sacrificed 18 h after surgery, and the loop responses were measured by recording the ratio of fluid accumulation (milliliters) to the length of the loop (centimeters). The number of bacterial cells causing accumulation of 1 ml of fluid per cm of intestinal loop was established as the effective dose evoking 50% maximal response ($ED_{50}$). Protection was expressed as the ratio of the $ED_{50}$ of immunized animals to nonimmunized controls.

## RESULTS

**Serological titrations.** Figure 1 shows a comparison of the geometric mean cholera antitoxin responses of rabbits immunized parenterally with cholera toxin or its A and B subunits. It appeared initially that intact cholera toxin was more antigenic than either of its two components on a weight basis; however, after 8 to 10 weeks the antitoxin titers of the A and B subunit immunized rabbits rose to approximately the same level. Vibriocidal assays (21) performed on the rabbits immunized with cholera toxin revealed no rise in serum vibriocidal antibody titers at any time after immunization.

Rabbits immunized parenterally with procholeragenoid responded with antitoxin titers similar to those immunized with cholera toxin, (Fig. 2). Oral administration with 10 times the parenteral dose (500 μg) of procholeragenoid resulted in significantly lower serum antitoxin responses for 8 weeks after immunization. By 10 weeks, the serum antitoxin response of the orally immunized rabbits was approximately one-half that of rabbits given 50 μg of procholeragenoid by parenteral injection.

Immunization of rabbits parenterally with Wyeth glutaraldehyde toxoid resulted in approx-

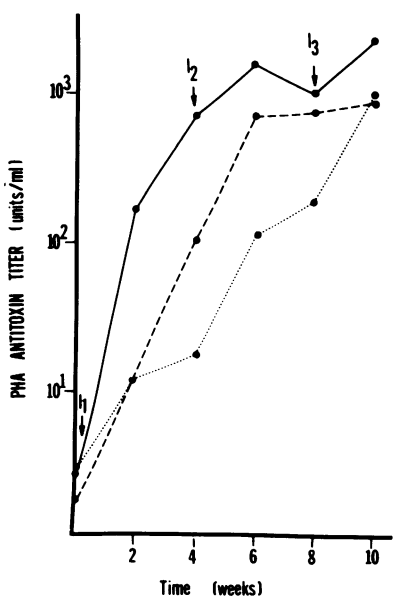


FIG. 1. *Geometric mean cholera antitoxin responses as determined by passive hemagglutination of sera from rabbits immunized with cholera toxin* (——), *cholera toxin A subunit* (-----), *and cholera toxin B subunit* (·····) *by the parenteral route.*

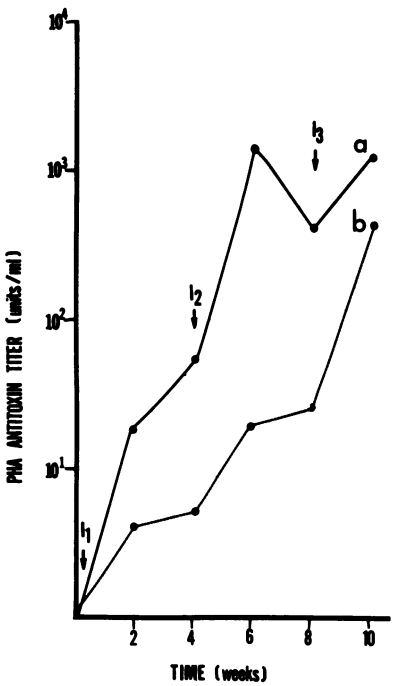


FIG. 2. *Geometric mean cholera antitoxin responses as determined by passive hemagglutination of sera from rabbits immunized with procholeragenoid by either the parenteral (a) or oral (b) route.*

imately 10-fold lower titers than the level obtained parenterally with cholera toxin or procholeragenoid (Fig. 3). In addition, serum antitoxin responses rose after oral immunization with the glutaraldehyde toxoid, but the amount was less. After 10 weeks, the serum titers were about one-half those observed after parenteral immunization.

**Live-cell challenge.** Table 1 summarizes the relative degree of protection against experimental cholera observed after immunization with the various cholera toxin derivatives. The best protection observed against live-cell challenge was obtained by parenteral immunization with procholeragenoid (6,216-fold). Oral immunization with procholeragenoid also produced highly significant protection (2,973-fold). Parenteral immunization with A subunit or the glutaraldehyde toxoid conferred protection of the same magnitude. Parenteral immunization with cholera toxin was protective, but less so than parenteral immunization with the A subunit, procholeragenoid, or the Wyeth toxoid. Interestingly, oral immunization with the Wyeth toxoid was significantly less effective than oral immunization with procholeragenoid. Protection appeared to be related to the general level of serum antitoxin but was difficult to predict based upon the serum antitoxin titer at the time of challenge.

## DISCUSSION

Procholeragenoid administered by the parenteral route evoked a serum antitoxin response which was equivalent to that of cholera toxin. The A and B subunits elicited lower antitoxin responses several weeks after immunization; however, after 10 weeks the antitoxin levels in the A and B subunit sera approached the same magnitude as that in sera of rabbits immunized with cholera toxin or procholeragenoid. Apparently, the A and B subunits are less potent immunogens, but this can be minimized if sufficient time is allowed for antigen processing, lymphocyte proliferation, and antibody formation. In contrast, glutaraldehyde toxoid (Wyeth 20101), which was employed in a previous field trial, appeared to be a less potent antigen by as much as 10-fold. This lower antitoxic response observed with glutaraldehyde toxoid could be explained by structural damage occurring during the toxoiding procedure. Oral immunization with either procholeragenoid or glutaraldehyde

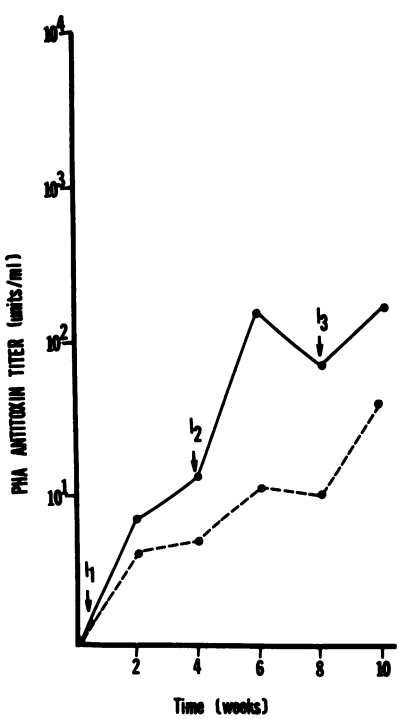


FIG. 3. *Geometric mean cholera antitoxin responses as determined by passive hemagglutination of sera from rabbits immunized with plain Wyeth toxoid 20101 by either the parenteral* (——) *or oral* (-----) *route.*

TABLE 1. *Protection against experimental cholera after immunization*[a]

| Group | Inaba V86 Challenge $ED_{50}$ Value | Protection Factor | Serum Antitoxin at Challenge |
|---|---|---|---|
| Nonimmunized Controls | $3.7 \times 10^3$ | 1 | 0.99 |
| Cholera Toxin (50 μg) (Parenteral) | $1.0 \times 10^6$ | 270 | 2235 |
| A Subunit (50 μg) (Parenteral) | $1.0 \times 10^7$ | 2700 | 996 |
| B Subunit (50 μg) (Parenteral) | $1.0 \times 10^5$ | 27 | 847 |
| Procholeragenoid (50 μg) (Parenteral) | $2.3 \times 10^7$ | 6216 | 1255 |
| Procholeragenoid (500 μg) (per os) | $1.1 \times 10^7$ | 2973 | 443 |
| Wyeth Toxoid 20101 (50 μg) (Parenteral) | $7 \times 10^6$ | 1892 | 160 |
| Wyeth Toxoid 20101 (500 μg) (per os) | $1 \times 10^5$ | 27 | 40 |

[a] Analysis of protection conferred to rabbits against intestinal loop challenge with live *V. cholerae* Inaba V86 by immunization with cholera toxin, A subunit, B subunit, procholeragenoid, and glutaraldehyde toxoid. The protection factor is the ratio of the $ED_{50}$ of immunized rabbits to the nonimmunized controls. Cholera antitoxin titers were determined by passive hemagglutination and are expressed in units per milliliter, based on the Swiss Serum and Vaccine Institute reference serum.

toxoid revealed that serum antitoxin developed, but was of lower magnitude than that after parenteral injection. Furthermore, the mean serum response after oral immunization with the glutaraldehyde toxoid was 10-fold lower than that obtained with animals immunized orally with procholeragenoid. This could be explained by the lack of tissue-binding capacity of the glutaraldehyde toxoid (16), which would allow it to pass readily through the gastrointestinal tract without being retained on epithelial cell surfaces.

Correlation of antitoxin titers with protection against fluid loss from live-cell challenge was not predictable, but they appeared to be generally related. For example, rabbits immunized with cholera toxin developed a geometric mean antitoxin titer of 2,235 U/ml and exhibited only 270-fold protection against intestinal loop challenge, whereas rabbits immunized with A subunit developed a geometric mean titer of 996 U/ml and expressed a protection factor of 2,700-fold. In contrast, rabbits immunized with B subunit developed a similar geometric mean antitoxin titer of 847 U/ml but only allowed 27-fold protection. By far the best protection was observed in rabbits immunized with procholeragenoid by the parenteral route (6,216-fold). About one-half that amount of protection was conferred by 10 times as much procholeragenoid given orally. The superior protection observed with procholeragenoid may be attributed to the residual toxicity of the aggregated cholera toxin complex. It may tend to modulate the immune response to itself via stimulation of adenylate cyclase. Despite the low antitoxin response to the glutaraldehyde toxoid (160 U/ml), it provided 1,892-fold protection against live-cell challenge. Glutaraldehyde toxoid administered by the oral route evoked a poor serum antitoxin response (40 U/ml) and little protection (27-fold). These data suggest that there might be differences between animals in the amount of antibody that can pass from the blood to the intestinal surface.

The possibility that vibriocidal antibody responses to somatic antigen contamination in the toxin or toxoid preparations might affect the results reported here was diminished after examination of the serum titers from rabbits immunized with cholera toxin and Wyeth toxoid 20101. We have previously reported that Wyeth

toxoid 20101 was relatively free of somatic antigen, since it gave no appreciable rise in vibriocidal antibody titers in rabbits at doses up to 200 g (17). Similarly, no detectable rise in vibriocidal antibody titer was observed in the sera from the cholera toxin immunized rabbits tested in this study. Since the toxin subunits and the procholeragenoid preparations were derived from the same lot of cholera toxin, no further vibriocidal titrations were performed.

Based upon the antibody responses and protective capacity of procholeragenoid administered by the parenteral or oral routes, it should be concluded that this toxin derivative deserves further consideration as a potential immunogen. Together with the observations of Germanier et al. on detoxification of procholeragenoid (7) and the initial studies of Fujita and Finkelstein on oral and parenteral immunization with procholeragenoid (6), there is sound basis to anticipate that procholeragenoid would increase protection against cholera. Incorporation of a superior immunogen such as procholeragenoid or formalinized procholeragenoid in whole-cell vaccines might enhance the impressive synergistic protection observed against experimental cholera (14, 20) and subsequently provide improved protection for humans against cholera.

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