Antitoxic Immunity in Experimental Cholera: Observations with Purified Antigens and the Rat Foot Edema Model

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The recently introduced choleragen-induced rat foot edema model has been employed as a bioassay for evaluating the immunogenicity of three purified preparations containing cholera exo-enterotoxin antigen, choleragen, choleragenoid, and Formalin-treated choleragen (formagen). The results indicated that choleragen evoked antitoxic immunity. Both the degree of resistance to challenge and the serum antibody levels of immunized animals were found to be related to the immunizing dose. Responses to the natural toxoid, choleragenoid, were erratic: some animals responded well and some failed to respond with either serum antibody or resistance to challenge. On the other hand, the artificially prepared toxoid, formagen, was found to be superior to the parent toxin in immunogenicity. Resistance to the choleragen-induced rat foot edema could be transferred passively by means of antibody-containing serum from previously immunized animals. Each of the antigens induced a state of hypersensitivity manifested by an immediate edematous response to challenge with either choleragen or choleragenoid. This condition, which was also passively transferable, suggests that untoward reactions should be anticipated in people receiving multiple doses of immunogens containing the cholera exo-enterotoxin antigen. Some of these observations were repeated, in a preliminary fashion, in an apparently equally suitable mouse foot edema model.

It is now quite clear that the diarrhea of cholera is mediated by an exo-enterotoxin elaborated by cholera vibrios. In the previous paper (7), it was shown that parenteral immunization with purified toxin antigen elicited an effective immunity against live vibrio intestinal challenge under conditions in which antitoxic immunity was the only logical resistance mechanism. It has also been observed that cholera patients develop antibodies capable of neutralizing the enterotoxin, as assayed in the ligated rabbit ileal loop model (12), as well as antibodies capable of neutralizing a skin reactive factor (2, 4) found in crude (4) and partially purified (10) culture filtrates. The relevance of these observations to each other has recently been established by the finding (9; R. A. Finkelstein and J. J. LoSpalluto, J. Infec. Dis., in press) that the pure exo-enterotoxin is an extremely potent permeability factor. Furthermore, the serum antitoxin levels of cholera convalescents, as determined in a passive hemagglutination test with the pure antigen, were directly correlated with their neu-

tralizing activity in both skin and ileal loop neutralization tests (11).

These observations have given rise to the hope that antitoxic immunity in man, and particularly in children in endemic areas, may be more effective and longer lasting than the antibacterial immunity provided by current cellular or lipopolysaccharide somatic antigen vaccines (13, 14). A field study to test this hypothesis in man is under active consideration.

However, at present, there are no entirely satisfactory methods of evaluating or comparing the immunogenic activity of proposed antitoxic vaccines in the laboratory. The method of Kasai and Burrows (12), involving immunization of groups of rabbits with two doses of antigen and then titration of their serum antibody by neutralization of ileal loop activity of toxin in other groups of rabbits, is too cumbersome for most laboratories. Active immunization of dogs, as proposed by Curlin et al. (5), is likewise not feasible as a vaccine bioassay for most laboratories. Methods suggested by Feeley and Roberts

(6) appear most promising but, again, involve immunization of groups of animals, bleeding, and then titration of serum antibody levels in other animals; in this case, the measure of activity is neutralization of skin reactivity. The active mouse protection test, of some value in assaying antibacterial vaccines, is entirely useless as a measure of antitoxic immunogenicity, as antitoxic immunity plays no role in protection against the artificial, experimental septicemic infection (7).

We have recently introduced an additional experimental cholera model, the rat foot edema test, which was proposed as a useful technique for screening anticholera drugs (8). In the present study, we undertook to evaluate the rat foot edema test as a means of assaying the immunogenicity of preparations of purified cholera exoenterotoxin (choleragen), natural cholera toxoid (choleragenoid) (9; R. A. Finkelstein and J. J. LoSpalluto, *in press*), and Formalin-treated choleragen ("formagen"). In the course of the investigation, it was observed that the antitoxic immunity could be passively transferred. It was also noted that rats which had had previous experience with choleragen antigen developed a type of immediate hypersensitivity response to a second dose. The latter effect was also passively transferable. Some observations were repeated, in a preliminary fashion, in the apparently equally suitable mouse foot edema model.

MATERIALS AND METHODS

The rat foot edema test was conducted precisely as described in an earlier publication (8). Rats were immunized in groups of four by subcutaneous inoculation of measured amounts of antigen in the fleshy region of the neck. Choleragen and choleragenoid were prepared as described elsewhere (R. A. Finkelstein and J. J. LoSpalluto, J. Infec. Dis., *in press*). Challenge doses of choleragen (usually 0.8 μg in 0.1 ml) were diluted immediately before use from a single lot which had been maintained in the refrigerator as a 2 mg/ml solution in 0.4 M ammonium formate with 0.02% sodium azide (*p*H 6.06 to 6.09). Its activity (choleragenicity and edema-promoting) was stable over the 7-month duration of this work. To prepare the formagen, the ammonium formate menstruum was replaced with *p*H 7.2 phosphate buffer by dialysis on a UM-10 membrane (Amicon Corp., Lexington, Mass.). Formalin was then added to a 1 mg/ml solution of choleragen to a final concentration of 0.4%. The mixture was kept at room temperature for 30 min and then stored in a stoppered tube in the refrigerator. A control preparation was handled similarly except for the addition of the Formalin. Three weeks later, the control preparation caused fatal choleraic diarrhea in infant rabbits administered 10 μg by mouth in 5 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.0 (8, 9), whereas the treated preparation

caused only slight to moderate fluid outpouring and no diarrhea or deaths in rabbits administered 100 μg in the same manner. It was then considered to be sufficiently detoxified for use. Earlier or later toxicity tests were not performed. Rats were bled, either by cardiac puncture or from the retro-orbital sinus, and the serum was titrated in the passive hemagglutination test described previously (11). Choleragenoid was usually used as the erythrocyte-sensitizing antigen in these assays, but identical results were obtained when randomly selected sera were tested with choleragen-sensitized erythrocytes. Sera from immunized rats as well as immunized rabbits (11) and an immunized horse (*unpublished data*) were tested for their ability to protect against the rat foot edema response by intravenous administration of 0.5 ml of undiluted serum, via a tail vein before challenge. Results, unless otherwise stated, are based on mean values of groups of four animals. Control animals were included in every assay, and, in every instance, the contralateral foot of control and experimental animals, inoculated with the diluent as an additional control, demonstrated no edematous response.

RESULTS

Immunization with choleragen. Prior immunization with choleragen was found to have a marked protective effect against the choleragen-induced rat foot edema. As illustrated in Fig. 1, when rats had been inoculated with 0.4 μg of choleragen 2 weeks prior to challenge (with 0.8 μg of choleragen), the edematous response was approximately half that of control animals. An immunizing dose of 2.0 μg gave somewhat greater protection, whereas doses of 10, 25, or 50 μg 2 weeks previously were completely protective. The rats which had previously received choleragen did, however, exhibit an immediate response to the challenge dose which was not evident in the control group. This was striking in the 0.5-hr readings and had practically subsided by 5 hr postchallenge (Fig. 1). We regard this effect as a manifestation of hypersensitivity of the immediate type.

Choleragen-immunized rats were also found to be hypersensitive to choleragenoid injected in the foot. Choleragenoid ordinarily elicits no edematous response. It should be noted, from the inset in Fig. 1, that the immunized rats responded with circulating antibody demonstrable in the passive hemagglutination test. Furthermore, the geometric mean titers of sera from the groups of immunized rats were directly related to doses of immunizing antigen employed.

Additional experiments, in groups of rats immunized with 50 μg of choleragen and challenged at intervals, indicated that partial immunity was evident 1 week after immunization. Immunity against the 0.8-μg challenge was complete at 2 weeks and persisted for at least 8

weeks. The transient, immediate response to challenge occurred in all of these groups of immunized rats. It should be mentioned that several rats succumbed within 2 to 3 days after the immunizing dose of 50 μg of choleragen. Smaller immunizing doses resulted in no fatalities, although at the higher doses approaching that level the site of inoculation was grossly edematous.

Immunization with choleragenoid. Responses to immunization with choleragenoid differed from those given above with choleragen, even though the two have been shown to be antigenically identical. As indicated in the right side of Fig. 2,
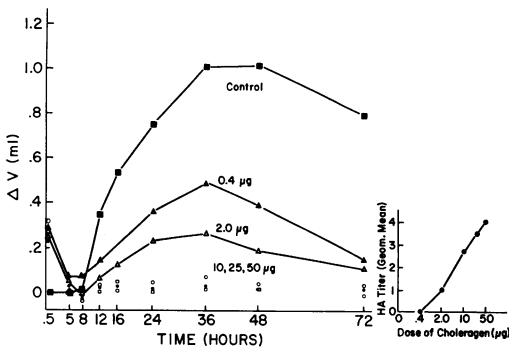

FIG. 1. *Effect of immunization with various doses of choleragen on response to challenge with 0.8 μg of choleragen 2 weeks later. Results are expressed as the mean change in volume (ΔV), relative to initial values, of four animals per group. Inset: relationship of mean serum hemagglutination (HA) titers of the same groups of animals to immunizing dose of choleragen employed. HA titers: 0, undetected at 1:10; 1, 1:10; 2, 1:20; 3, 1:40, etc.*
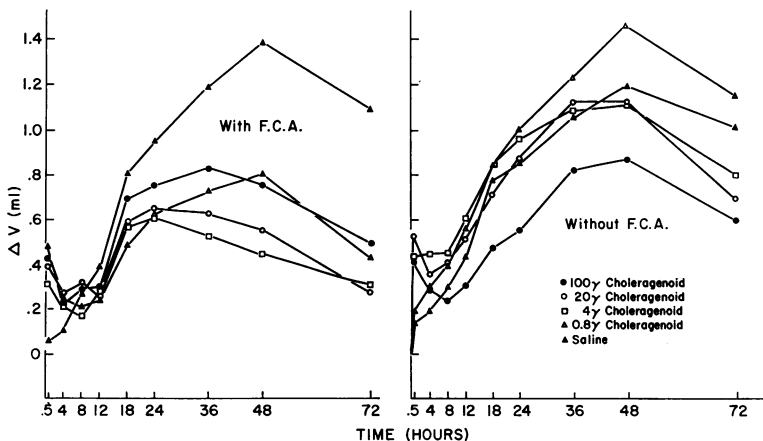
only a partial immunity could be attained in groups of choleragenoid-immunized rats even when doses as high as 100 μg were used. Furthermore, when choleragenoid was the immunizing antigen, the dose-response relationship, so apparent in Fig. 1, was not clear-cut. This was also the case when Freund's complete adjuvant was included with the choleragenoid immunizing doses (Fig. 2, left side). However, the animals which had received choleragenoid, either with or without adjuvant, did exhibit the immediate type of hypersensitivity response on challenge with choleragen.

Giving the choleragenoid in two 10-μg doses, spaced 1 week apart, did not exert any material influence on the response to challenge. Increasing the interval between immunization and challenge, up to 8 weeks, was likewise without beneficial effect. However, a more careful scrutiny of the individual animals within the choleragenoid-immunized groups did shed some light on the mechanism of the "partial immunity" observed. As illustrated in Fig. 3, which depicts responses to challenge of choleragen- and choleragenoid-immunized animals at 8 weeks, complete protection was again observed in the choleragen-treated group and only partial resistance was observed in the choleragenoid group. But, it was noted that, within the choleragenoid group, two of the animals were practically completely resistant to the choleragen challenge and two were completely susceptible. Examination of the sera from the resistant animals by the hemagglutination test revealed substantial titers (1:40), whereas the susceptible animals had no detectable serum antibody. Thus, it appears that the partial

FIG. 2. *Effect of immunization with various doses of choleragenoid, with or without Freund's complete adjuvant (FCA), on response to challenge with 0.8 μg of choleragen 2 weeks later. Results expressed as in Fig. 1.*

resistance observed in groups of choleragenoid-immunized animals was due to heterogeneity in response of the individual animals comprising the groups, some responding well insofar as both resistance and serum antibody were concerned and some failing to respond. Reexamination of earlier data and serological tests of
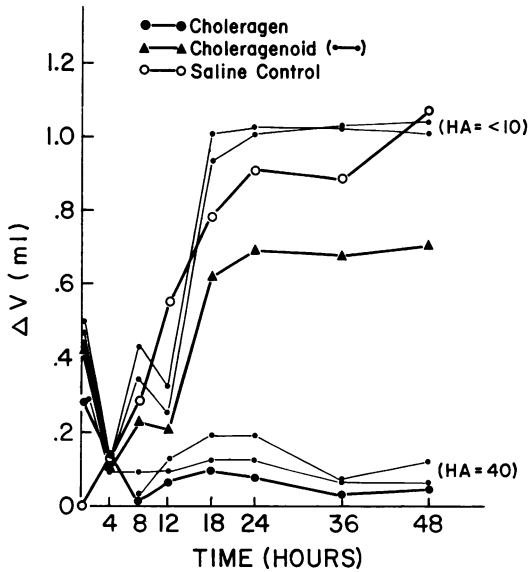

FIG. 3. *Effect of immunization with choleragen or choleragenoid (50 µg) on response to challenge with 0.8 µg of choleragen 8 weeks later. Heavy line, open circles, control group; heavy line, filled circles, choleragen immunized group; heavy line, triangles, choleragenoid immunized group, mean values; lighter lines, small filled circles, individual rats of choleragenoid-immunized group.*
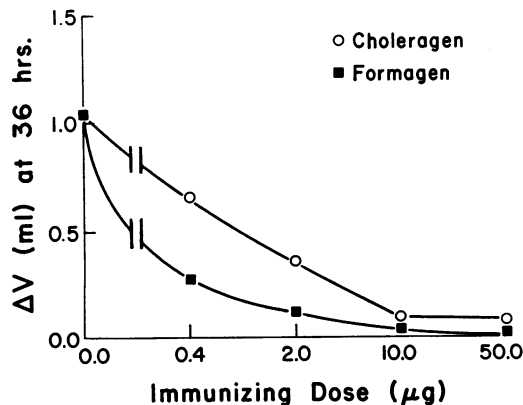

FIG. 4. *Comparison of immunogenic potency of choleragen and formagen against 0.8 µg of choleragen challenge 2 weeks after immunization with indicated antigen dose. ΔV values 36 hr after challenge.*
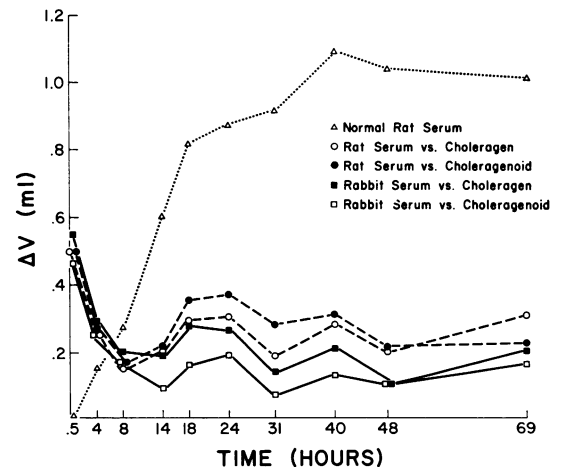
additional sera from choleragenoid-immunized rats substantiated these observations.

Immunization with formagen. It was therefore of considerable interest to examine the responses of rats to immunization with formagen, an artificially prepared "toxoid" (as opposed to the natural toxoid, choleragenoid). The results (Fig. 4) indicated that formagen was somewhat more active than choleragen in stimulating antitoxic immunity in the rat foot edema model. Formagen also stimulated the immediate hypersensitive response.

Passive transfer of antitoxic immunity and immediate hypersensitivity. To determine whether the observed antitoxic immunity and immediate hypersensitivity were passively transferable by serum, groups of rats were passively immunized by intravenous inoculation of 0.5-ml samples of pools of sera from rats or rabbits immunized with choleragen or choleragenoid. The control group received normal rat serum in lieu of the antibody-containing sera. The results (Fig. 5) were consistent with the hypothesis that both antitoxic immunity and immediate hypersensitivity were mediated by serum antibody. Serum from a choleragenoid-immunized horse (*in preparation*) was also found to protect rats against the edematous response when given at the 0.5-ml level but not when only 0.5 ml of a 1:10 dilution was administered. The larger dose elicited the hypersensitive response but the smaller dose did not.

Observations in mice. In preliminary studies in 20-g Swiss albino mice (Euer's Farm, Austin,

FIG. 5. *Passive protection against choleragen-induced rat foot edema by serum from choleragen- or choleragenoid-immunized rabbits or rats. In the case of the choleragenoid-immunized rat serum, the pool was made from serological responders.*

Tex.), challenged in a similar manner but with 0.4 μg of choleragen in 0.01 ml of diluent using a microsyringe, similar results were obtained. Responses to choleragenoid were only partial and not dose-related. On the other hand, the responses to choleragen and to formagen were more regularly related to the immunizing dose, but complete protection was not obtained with 10 μg of antigen, the highest dose tested in this preliminary work. The immediate hypersensitivity reaction was observed in animals with previous experience with each of the antigens.

DISCUSSION

The present study demonstrates the potential usefulness of the rat foot edema model in the bioassay of immunogens directed against the cholera exo-enterotoxin. The test requires only minimal experimental manipulations and equipment. It has the decided advantages that reproducible data are obtained in small experimental groups, the data are quantitative rather than of the attribute type, only single doses of antigen are required to induce immunity which is related to the antigen dose employed, and it is a direct type test in that the immunized animal is the one which is challenged, rather than an indirect type test in which sera from the immunized animals are subsequently laboriously titrated for toxin neutralization activity in other groups of animals. If it is considered desirable, however, sera from immunized rats could be titrated in the hemagglutination test which provides in vitro results which correlate with in vivo neutralization tests (11) and with the observed resistance to challenge. The rat foot edema immunoassay does have one potentially significant disadvantage in that the immunological responses to choleragenoid, the natural cholera toxoid, are erratic. Some animals respond quite well with both antibody rises and resistance to subsequent challenge, whereas others respond with neither. All of the animals, however, apparently "recognize" choleragenoid in that they do respond with the immediate type of hypersensitivity found in animals with previous antigenic experience. With the information at hand, it would be useless to speculate about the possible reasons for this heterogeneity of response among the rats we have been using. In our experience, rabbits apparently respond quite uniformly to choleragenoid with high-titer neutralizing sera as well as resistance to challenge (7), and we experienced no difficulty in producing a high-titer protective antiserum in a horse immunized with choleragenoid. Preliminary work suggests that mice respond similarly to rats. It would be of interest to extend these observations in other inbred strains of rats and mice and also, more significantly, to determine whether human beings respond uniformly to immunization with choleragenoid.

Our results clearly indicate that resistance is passively transferable by serum from immunized animals. Most likely, antibody of the $7S$ variety is involved (11). This suggests the possibility of passive immunization for prophylaxis of people at high risk of exposure to cholera, although, from the nature of the distribution of the disease in endemic and neoepidemic areas, this is not likely to be a widely practiced procedure. It is of significance, in this regard, to point out that a recent study (G. T. Curtin and C. C. J. Carpenter, J. Infec. Dis., in press) strongly suggests that circulating antibody is indeed protective against (experimental) cholera.

In accord with Feeley and Roberts (6), the present study also demonstrates the superior protective potency of the Formalin-treated toxoid as compared with the parent enterotoxin. These observations are in direct contradiction to those of Burrows (3), who reported that toxin inactivated by formaldehyde loses 90% or more of its immunogenic potency. The differences probably lie in the source and purity of the toxin preparations employed as well as the conditions of formalinization. In the present study, we used purified toxin derived from syncase medium and Feeley and Roberts used a syncase medium filtrate, whereas Burrows was apparently using a peptone medium-derived product. These considerations are important because a detoxified antigen will probably be necessary for projected studies on antitoxic immunity in man.

In this regard, it is well to issue a word of caution suggested by the results of the present study, as they concern the immediate hypersensitivity observed in animals with previous antigenic experience. If these observations can be extrapolated to man, one might expect a high proportion of reactions to a second injection of a cholera toxin antigen-containing vaccine. Certainly, investigators concerned in this area should proceed with due prudence.

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