Monospecific Equine Antiserum Against Cholera Exo-Enterotoxin

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An antiserum specific for Vibrio cholerae exo-enterotoxin was produced by immunization of a horse with purified cholera-gentoid, a natural cholera toxoid. The serum has a high titer against the toxin antigen in passive hemagglutination tests and a respectable antipermeability factor activity. It also passively protected against cholera-gentoid-induced mouse foot edema. The serum was found to be useful for assaying toxin antigen in crude and refined products by in vitro tests such as radial immunodiffusion, Lf, and quantitative precipitin titrations. Based upon experimental observations, the serum was defined as containing 1,000 flocculating units of anticholera toxin antibody per ml. A flocculating dose, or Lf, of cholera-gentoid approximates 1 μg, and that of cholera-gentoid is 0.625 μg. Formalin toxoids behaved like the parent toxin in these tests. The serum contains approximately 2.2 mg of antibody protein per ml, which appears to be largely, if not entirely, of the Ig(T) type. It is suggested that this serum, which is available in considerable supply, be considered for use as a reference cholera antitoxin. The horse developed symptoms of anaphylactic shock during immunization, suggesting the need for caution in projected studies on toxoid-induced immunity in man.

The cholera exo-enterotoxin, which was designated cholera-gent, has been isolated and purified in this laboratory along with a natural toxoid which we call cholera-gentoid (5, 6). Results of a variety of laboratory studies have given rise to the hope that an antitoxic cholera vaccine may elicit better and longer lasting immunity to cholera than that afforded by conventional, killed cellular vaccines. A field study to test this hypothesis with a Formalin toxoid is currently under active consideration.

If history repeats itself, it may be possible to apply, in cholera research, the classical immunological techniques which have proven so useful in other toxin-mediated diseases, such as diphtheria and tetanus. Accordingly, we undertook the preparation of a large quantity of a monospecific equine antitoxic serum which might serve as an international reference for future immunological studies in cholera. Such a serum could be especially useful as a reagent for analyzing the toxin antigen content of immunizing preparations by radial immunodiffusion (5), Lf, and quantitative precipitin procedures. The results of the present study indicate that this is indeed the case.

MATERIALS AND METHODS

Immunization procedure. After prebleeding, a 1,200-lb (544-kg) gelding was inoculated intramuscularly with 40 mg of purified cholera-gentoid (6) suspended at 1 mg per ml in 50% Freund's complete adjuvant. Cholera-gentoid was used for immunization to avoid untoward reactions which might be expected from administration of large amounts of the toxin itself. The initial injections were made in the four quarters of the horse. After 3 weeks, another 10 mg of cholera-gentoid was inoculated intravenously after the horse had been premedicated with antihistaminics. Eight months after the primary stimulus, the horse was "boosted" with 12 mg of cholera-gentoid; of this, 2 mg was given subcutaneously the day prior to an intravenous dose of 10 mg (again after premedication). The horse was bled periodically via an external jugular vein. Serum was removed from clotted blood and stored frozen. The serum pool which was used most widely consisted of a mixture of sera from four bleedings obtained between the 35th and the 56th day after primary stimulation. A quantity of this serum was furnished to John R. Seal, Cholera Advisory Committee, National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014, for distribution to interested individuals.

Immunological techniques. Serum antibody levels were followed by the passive hemagglutination (PHA) microtest (7) with the use of tannic acid-treated chicken erythrocytes sensitized with cholera-gentoid. Results with cholera-gentoid-sensitized erythrocytes were essentially identical. Vibriocidal antibody was titrated by the method of Finkelstein (3). Radial immunodiffusion assays of cholera and cholera-gentoid were performed as before (5), usually in quadruplicate,
with the use of a gel consisting of 1% Noble agar (Difco), 1% sodium azide (J. C. Feeley, personal communication), and 1% of the inactivated horse serum pool. This formula was selected after preliminary tests in a variety of menstrua to determine the optimal level of serum for greatest sensitivity and visibility of the precipitate. Conditions used previously with rabbit serum (5) were not optimal for the horse serum. Gel filtration of serum was performed on a column (2.5 by 100 cm) of Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif.) eluted with tris(hydroxymethyl)-aminomethane-ethylenediaminetetraacetate buffer (5) at a flow rate of 20-ml per h; 2-ml fractions were collected. A fresh column was prepared for use only with serum when it was found that all the antibody activity was removed from serum on passage through a column previously used many times for the purification of choleragen (6). The effluent was concentrated by absorbanse at 280 nm, by the PHA test, and by micro-Ouchterlony tests (5) with choleragenoid (100 μg/ml) in the central wells. Immunoelctrophoresis was performed as described previously (5) except that the rabbit anti-equine sera used for development were purchased from Hyland Laboratories (Los Angeles, Calif.). An antiserum against equine Ig(T) was kindly furnished by Fred Karush (8). It also contained some antibody against equine IgG (Karush, personal communication). Lf determinations and quantitative precipitin reactions were performed by a modification of the procedures recommended by Kabat (10). In the routine procedure, 1 ml of inactivated serum diluted 1:5 in 0.15 M phosphate buffer, pH 7.2, was added to a series of test tubes (13 by 100 mm) containing equal volumes of antigen diluted in the same buffer. Tests were performed in duplicate. The antigens, choleragen and choleragenoid, were prepared as described previously (6). Two preparations of formagen, our designation for Formalin toxoid prepared from pure choleragen (4), were tested. One was the same preparation used previously (4); the other was prepared in a similar manner except that the parent toxin had been dialyzed against 0.1 M sodium borate, pH 8.0, prior to detoxification with Formalin. The serum-antigen mixtures were observed, by transmitted oblique illumination, periodically during 1 hr of incubation at 37°C, to determine the Lf, or most rapidly flocculating mixture. The tubes were then refrigerated for 2 to 4 days, after which they were centrifuged at ca. 1,250 × g for 1 hr in an International refrigerated centrifuge, model PR-1. The supernatants were discarded and the precipitates were washed two times with 2 ml of buffer. The last wash was discarded and the precipitate was dissolved in 1 ml of 10% acetic acid. The absorbance at 280 nm was determined in a Beckman DU spectrophotometer. Values reported are the means of duplicate determinations. On occasion, the concentration or the volume of the reagents, or both, had to be changed. For example, in testing crude filtrates for antigen content, the amount of serum used was 1 ml of a 1:10 dilution and the antigen was tested over a range of 0.5 to 5.0 ml in a 6.0-ml total volume of reaction mixture. The concentrations with weaker or stronger precipitating sera were adjusted accordingly. Lf values appeared to be independent of the volume of reactants within the ranges given, but the total amount of precipitate was reduced when the volume of the reaction mixture was increased. Some of the sera were also titrated by John P. Craig according to his widely used neutralization of skin reactivity procedure (1). Values in "neutralizing units" are those kindly furnished by him. John C. Feeley also titrated some sera in the hemagglutination test of Hochstein et al. (9). Sera designated Wyeth goat 1 and 2 were generously furnished by Ben A. Rubin. Swiss Serum and Vaccine Institute serum, a lyophilized globulin preparation, was provided by John Seal.

RESULTS

Reactions during immunization. Because we had previously observed a type of immediate hypersensitivity reaction to a second antigenic exposure (4), it was of interest to determine the effect of immunization on the health and well-being of the immunized horse. The horse developed rather large, fluctuant sterile abscesses, presumably attributable to the adjuvant, at the sites of four intramuscular injections. Two of these drained spontaneously after several months; one was surgically assisted and yielded approximately 250 ml of straw-colored turbid fluid, and one persisted through the eighth month. At the time of the first intravenous inoculation, the horse experienced a mild respiratory embarrassment, presumably anaphylactic in origin. The last intravenous injection resulted in a severe, life-threatening, anaphylactic reaction requiring adrenalin.

Immunological response. Serum antibody levels during the first 56 days after immunization, as determined by the PHA microtest, are illustrated in Fig. 1. (Subsequent values are given later in Table 1.) The titer rose steadily from the 7th day and tended to plateau after the 35th day, with only slight rises to the 56th-day bleeding. The serum pool, from the last four bleedings, had a geometric mean titer of 17,060 based upon 11 independent titrations (95% confidence limits, 17,060 ± 8,130). In the early days of the experiment, we had used the titer against two unrelated antigens, choleragen and cholera toxin. As has been subsequently observed, the PHA titer to choleragen was higher than that to cholera toxin by a factor of ten.

Fig. 1. Serum antibody levels, determined by PHA (7), during the first 8 weeks after immunization.
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9,905 to 31,020). In the hemagglutination test of Hochstein et al. (9), it was found to have a geometric mean titer (log2) of 13.0 (i.e., approximately 8,200) in four tests, corresponding to 7,250 units (Craig) per ml (Feeley, personal communication). As indicated later in Table 1, the titer declined somewhat by 25 weeks and rose following the booster inoculation.

Although the horse initially had a vibriocidal antibody titer between $10^2$ and $10^3$ in a highly sensitive test (3), the titer did not rise on immunization with choleragenoid.

Characterization of equine anticholera toxin antibody. To determine the nature of the equine antitoxic antibody, 2.5 ml of the serum was subjected to gel filtration on Bio-Gel A-5m. The results of that chromatographic procedure are depicted in Fig. 2. Only fractions in the region characteristic of 7S immunoglobulins contained antibody demonstrable by Ouchterlony micro-precipitin tests. When the antibody-containing region, pool V, was concentrated to the original serum volume by pressure ultrafiltration, its titer was equivalent to that of the original serum in the PHA test. Selected fractions from other areas were devoid of detectable hemagglutinating antibody. Thus, apparently all of the antibody was contained in this region.

The results of immunoelectrophoresis of pool V and of the parent serum are shown in Fig. 3. The relationship between the precipitin patterns obtained in Fig. 3C and 3D and those published previously (8, 11) suggests that the equine anticholera toxin antibody is largely, if not entirely, of the Ig(T) type. Additional studies, still in progress (Peterson and Finkelstein, unpublished data), in which the antibody has been dissociated from specific immune precipitates and has been purified, support this contention.

The serum, in a 0.01-ml amount inoculated intravenously, was found to afford about 50% protection against mouse foot edema induced by 0.4 µg of choleragen (4).

The specificity of the serum with regard to products found in crude Vibrio cholerae culture filtrates and the pure antigens, choleragen and choleragenoid, is illustrated in Fig. 4. The horse serum gave a single reaction of identity with crude concentrated culture filtrate, choleragen, and choleragenoid, whereas an antiserum against crude filtrate exhibited multiple bands with the crude filtrate and single bands with the purified antigens.

Radial diffusion immunoassays of cholera gen and choleragenoid. The equine antiserum was in-
corporated in agar for the assay of choleragen and choleragenoid by the radial immunodiffusion procedure (5). The results are illustrated in Fig. 5. Over the ranges illustrated, the precipitin ring diameter was linearly related to the antigen concentration plotted logarithmically. At lower concentrations, with this serum, the precipitin rings became somewhat fuzzy and deviated from linearity. As was shown previously (5), choleragenoid, being the smaller molecular species, gave larger precipitin zones than choleragen per microgram of antigen. In fact, the ratio of the concentrations of choleragen to choleragenoid required to produce the same precipitin ring diameter, approximately 1.5, approximates very closely the ratio of their molecular weights (choleragen, 90,000; choleragenoid, 60,000; LoSpalluto and Finkelstein, to be published). The formagen prepared after dialysis against borate buffer gave results identical to the control and to the parent toxin in radial immunodiffusion tests. In addition, ultracentrifugation studies revealed it to be the same molecular size as choleragen.

**Fig. 4.** Ouchterlony test (macro) of horse serum (well 1) and antiserum against crude culture filtrate (well 4) against XM-100 membrane concentrate (reference 6; in central well) and isolated choleragen and choleragenoid, as follows: wells 2 and 5, choleragen; wells 3 and 6, choleragenoid.

**Fig. 5.** Radial immunodiffusion assays of choleragen and choleragenoid with equine antiserum, 1%, in gel. Inset: photograph, of precipitin rings observed.

**Fig. 6.** Lf determinations and quantitative precipitin curves of five preparations, each, of choleragen and choleragenoid and of an equal protein concentration mixture of the two. Lf mixtures indicated by arrows. The choleragen curve which reaches base line on antigen excess side was from an assay refrigerated for only 2 days; the remainder were held for 4 days.

Lf determinations and quantitative precipitin reactions of choleragen and choleragenoid with equine antiserum. Results of Lf determinations and quantitative precipitin reactions with five separate lots each of choleragen and choleragenoid, as well as an equal protein concentration mixture of the two, are illustrated in Fig. 6. It can be seen that the results were quite reproducible. The precipitin curves are of the flocculation type with sharp zones of inhibition in both antigen and antibody excess. In the case of choleragen, the Lf values with 0.2 ml of serum ranged from 175 to 200 μg in the five preparations. With choleragenoid, the most rapidly flocculating mixture was obtained within a range from 112 to 125 μg. The mixture of choleragen and choleragenoid gave a smooth flocculation curve with an Lf, at 150 μg, intermediate between the Lf values of the two components. Lf values ranged from approximately 9 to 21 min. Choleragenoid usually
showed slightly faster flocculation. PHA and passive hemagglutination-inhibition tests (7) for antibody and antigen in the supernatant fluids removed after sedimentation of the immune precipitates indicated the neutrality of the mixtures surrounding the peak of the precipitin curve. Free antibody was detectable only in the lower portions of the ascending limb of antibody excess, and free antigen could be recognized only in the descending portion of antigen excess. These results were similar with both antigens.

The flocculating antibody content of the serum was estimated from the absorbance of the specific precipitate at the equivalence point (assumed to be the Lf mixture) by deducting the antigen contribution \( [E_{280} \text{nm} \approx 10.0 \text{ (5)}] \), and using a value for equine Ig(T) of \( E_{280} \text{nm} = 14.8 \text{ (8)} \). The average value was calculated to be approximately 445 \( \mu \text{g} \) of antibody protein per 0.2 ml or 2.225 mg per ml. Individual values determined with choleraen and choleraenoid did not differ significantly and were included in the calculations.

Similar tests were conducted with two preparations of formalin. In each instance, the Lf values for the Formalin-treated product were identical within experimental error to those of the control preparation. However, in the one case in which the preparations had been stored in phosphate buffer under refrigeration for approximately 6 months, both the Formalin-treated and control preparations were significantly less active than the parent lot. The precipitin curve was also much broader, suggesting that some aggregation may have occurred. Unfortunately, ultracentrifugal studies could not be performed on the phosphate-dialyzed preparation.

Additional studies were made of the flocculating activity of five crude fermenter supernatants (5, 6). In these instances, the tests were performed with 1.0 ml of 1:10 serum and an increased volume of antigen. The Lf value was not precisely determined because flocculation was delayed. However, the amount of crude supernatant giving maximal precipitation, determined from the precipitin curve, ranged from 2.0 to 4.0 ml in the five preparations. After purification and recovery of the individual antigens, it was possible to calculate the flocculating doses recovered and to compare these values with the flocculating doses originally present in the fermenter culture. Results of these calculations indicated recovery of antigen ranging from 18 to 57%—in reasonable accord with earlier estimates by other methods (6).

Comparison of flocculating, hemagglutinating, and neutralizing activities of various cholera antitoxic sera. Results of flocculation tests, PHA titrations, and neutralization tests on a variety of sera are summarized in Table 1. In Table 1, the flocculating unitage is derived from the number of micrograms of choleraen and choleraenoid which would react with 1 ml of serum at equivalence. It becomes apparent that certain of the activities are not directly proportional to each other. For example, the Wyeth goat serum 1, which is approximately twice as active as our equine serum (Texas horse pool) in flocculation tests, is approximately three times as active in hemagglutination, and seven to nine times as active in Craig's neutralization test. It is also interesting that the Swiss Serum and Vaccine Institute serum, also a horse serum but prepared

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<th>Table 1. Comparison of flocculating, hemagglutinating (HA), and neutralizing activities of various cholera antitoxic sera</th>
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\( ^a \) Procedure of Finkelstein and Peterson (7).
\( ^b \) Tests performed by J. P. Craig. ND = not determined.
\( ^c \) Tests performed with earlier preparations of choleraen and choleraenoid.
\( ^d \) Two weeks after booster.
\( ^e \) Could not be determined because of insufficient flocculating activity.
against a crude filtrate and having multiple serological reactivities, had respectable hemagglutinating activity and almost twice the neutralizing activity of our serum in Craig's test, but could not be made to flocculate in test tubes. Actually, it was possible to demonstrate flocculation with this serum in capillary tests with 25 μl of serum and 25 μl of 2.5 μg of cholera-oid antigen, but a unitage for comparison could not be established. Finally, serum from rabbit "H," which had almost four times the neutralizing activity of our horse serum, had a slightly lower PHA titer, and less than half of the flocculating activity. Although the data are not given here, it should be mentioned that the Wyeth goat serum 1 gave a much broader precipitin curve, in both antigen and antibody excess, than did our horse serum. This was less marked with Wyeth goat serum 2, whereas the rabbit sera gave nearly classical flocculation-type curves, with a sharp rise in the antibody excess side, although the descent on the antigen excess side was somewhat more gradual than with the horse serum.

**DISCUSSION**

Although the knowledge that cholera is a toxin-mediated disease was somewhat late in coming in comparison with the classical toxin diseases, in a sense it is fortunate in that we can apply to cholera the experience gained previously and, at the same time, avoid some of the problems faced by the pioneer workers in diphtheria and tetanus. In those earlier studies, which need not be reviewed here, sera were, of necessity, prepared against impure antigens. Undoubtedly, much, but not all, of the confusion of the early in vitro serological observations and their comparisons with neutralization tests resulted from this factor. In the present study, we were fortunate to be able to start with an antigen of relative purity (5, 6), and the resultant antiserum is monospecific, at least insofar as its capacity to react visibly with other components of cholera filtrate is concerned. Additional evidence was the failure of the horse to demonstrate a rise in vibriocidal antibody, presumably a sensitive indication of the absence of somatic antigen in the immunizing preparation.

The present antiserum has many attributes which suggest its suitability for use as an international reference preparation. It has respectable neutralizing activity, it is available in relatively large amount, it provides sharp and reproducible precipitin curves, and it can be used in Ouchterlony and radial immunodiffusion tests without the confusion which might result from use of a serum with multiple reactivities against other cholera antigens.

With such a reagent, historical precedent and current practicality suggest that an antitoxin unit should be defined so that results in various laboratories may be compared more readily. In this regard, it should be mentioned that a cholera antitoxin unit has already been defined by Craig, based upon neutralization of skin reactivity of toxin. This bioassay system has indeed already found rather wide and practical usage among some cholera workers, even though the methodology and definitions have not yet been presented formally in the scientific literature. (Craig's procedure and provisional standard were actually defined at a symposium on cholera, sponsored by the U.S.-Japan Cooperative Medical Science Program, in Palo Alto, Calif., in 1967. Although use of the printed abstracts from that meeting is stated to be restricted, details of his procedure have generously been made available by Dr. Craig to interested workers.) In fact, the unitage established by Craig with the present serum (Table 1) could be adopted per se. However, to do so would carry the implication that values derived from tests in which this serum might find most wide usage—in vitro serological tests of the type described herein—would be related to those obtained in the system from which the unitage was derived, the neutralization of permeability activity. The present study, in accord with previous experience with diphtheria and tetanus, indicates that this is not the case. Although the reasons for the present discrepancies remain obscure as in the past, they could depend on the presence of varying amounts of nonprecipitating antibody, differences in avidity, and reactivity with different sites on the antigen molecule. It is apparent that in vitro measurements offer a number of advantages, as they have particularly with diphtheria and tetanus, although they should not be expected to supersede neutralization-type bioassays. Therefore, not wishing to detract in any way from the established merit of Craig's system and its continued use, we propose that the present cholera antitoxic serum be defined, independently, as containing 1,000 flocculating units of cholera toxin antibody per ml. This definition, although arbitrary, is based on the observation that 1 ml of the serum reacts at equivalence, as determined by the LF mixture, with very close to 1,000 μg of toxin antigen which appears to be, for practical purposes, pure (5, 6). Extending this definition, a flocculating dose of choleragen, at least of the preparations tested, approximates 1.0 μg (i.e., 0.962 ± 0.056 μg). If purer toxin preparations can be obtained and demonstrated to be free from choleraoid, one would expect that the flocculating dose may become slightly smaller, although, judging from the
criteria of purity already applied to these preparations, this is unlikely. In any case, establishment of a unitage enables precise in vitro comparisons. According to the same definitions, a flocculating dose of choleragenoid is approximately 0.613 ± 0.028 µg. The ratio between these two values, as well as the behavior of the two antigenically identical moieties in radial immunodiffusion tests, has been found to be quite consistent with their molecular size differences. Furthermore, this ratio would define the range of error that might be expected in assaying an unknown mixture containing both moieties. In this regard, it is interesting to note that, despite the large number of laboratories currently engaged in this kind of work, there has, as yet, been no independent confirmation of the existence of choleragenoid, although we have recently been able to demonstrate the conversion of choleragen to choleragenoid in vitro (Finkelstein et al., submitted for publication). Although choleragenoid is indeed a natural cholera toxoid in the classic sense, it may well be an anomaly resulting from our method of preparing high-potency toxic filtrates in bulk. Unless and until it is encountered elsewhere, consideration of its complicating effects (in other laboratories than ours) may be minimized. In our experience, two Formalin toxoids behaved identically with their control preparations in the in vitro tests described and, in contrast to choleragenoid, a Formalin toxoid was found to be identical in size to choleragen. Previous studies (2, 4) have indicated the Formalin toxoids to be equivalent or somewhat superior to the parent toxins as immunogens. Choleragenoid, on the other hand, was somewhat erratic as an antigen or immunogen in rodents (4), although it has served quite well in the present study.

The present serum has also been used to determine the flocculating activity of crude fermenter supernatants, and the results, compared with the flocculating activity of the isolated flocculating antigens, have compared favorably with previous estimates of recovery (6). The present antisera contains approximately 2.225 mg of antibody per ml, and the antibody seems to be of the Ig(T) type. Additional studies on the nature of the antibody are in progress.

A final consideration relates to the untoward reactions of the horse during immunization and their pertinence to proposed immunization of human beings. In the present study, the amounts of antigen used for immunization were enormous compared to what may be considered to be a potential immunizing dose for man. In our previous study (4), which demonstrated an immediately type hypersensitivity on second exposure to antigen, the system for observation was extremely sensitive and probably revealed reactions which would otherwise be unnoticed. The only conclusion which can be drawn is that investigators concerned with this aspect should proceed with due caution.

ACKNOWLEDGMENTS

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I am grateful for the assistance of Beverly Brock, Russell C. Hollingsworth, Udorn Lecomboon, and Johnny W. Peterson during portions of this study. Discussions with my colleague Joseph J. LoSpalluto were always stimulatory and rewarding. He also performed the ultra centrifugal studies on the formagen preparation. Immunization and bleeding of the horse was accomplished with the assistance of Franklin J. Stein.

ADDENDUM IN PROOF

After acceptance of the manuscript, the horse serum pool was tested and found to react in "multiple proportions" in the quantitative precipitin test over the 20-fold range of serum concentrations from 0.05 to 1.0 ml. Tests with 4H-labeled cholera indicated that close to 90% of the radioactivity was precipitated at equivalence.

LITERATURE CITED