Immunity to Cholera: Relation of Fraction II of Type 2 Cholera Toxin to Vibriocidal Antibody

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Received for publication 2 January 1969

The nontoxic protein component in supernatant fluids of young cultures of the cholera vibrio in peptone dialysate broth contains an antigen identical in specificity to vibrio lipopolysaccharide. This material was heterogeneous after elution from diethylaminoethyl A50 Sephadex, and it contained at least five additional minor antigens. Identity was demonstrated by immunodiffusion methods, by the induction of specific vibriocidal antibody formation, and by specific interference in the vibriocidal reaction. The minor antigens appeared to be unrelated to the vibriocidal reaction. The major antigen was more highly immunogenic than lipopolysaccharide, giving higher and longer-persisting antibody titers in the rabbit, but lipopolysaccharide was the more effective interfering antigen per unit weight in the vibriocidal reaction. The nontoxicity and high immunogenic potency of the protein antigen suggest that it may be useful as an immunizing agent for the production of the antibacterial component of an effective immunity.

The separation of extracellular type 2 toxin, demonstrable in the rabbit ileal loop model, by elution from diethylaminoethyl (DEAE) A50 Sephadex into two components formed in peptone dialysate medium (Difco) has been described by Coleman et al. (1). The toxin-induced water and ion movement from the tissues into the lumen of the bowel is produced by the more strongly positively charged material eluted from the column in deionized water, fraction I. The second peak, fraction II, eluted in electrolyte gradient or by 0.5 M NaCl after washing, had no toxic activity that was demonstrable in the rabbit ileal loop.

Study of the antigenic specificity of these fractions of type 2 toxin showed that they are distinct and that fraction II gave identity reactions in immunodiffusion with the highly purified K-S lipopolysaccharides described by Kaur and Shrivastav (5) which induce the formation of vibriocidal antibody. The relation of fraction II to antibacterial, as distinct from antitoxic, immunity to cholera, measured as vibriocidal antibody, is described in this report.

MATERIALS AND METHODS

Vibrio strains. Two strains of Vibrio cholerae were used in this investigation. One was the toxigenic, rabbit-passed Inaba 569B strain described by Dutta and Habbu (2) and received directly from N. K. Dutta of the Haffkine Institute, Bombay. The other was the American Inaba vaccine strain, NIH 35-a-3; recent culture was supplied by John C. Feeley of the Division of Biologics Standards, National Institutes of Health. Both were stored as freeze-dried stock, and the 569B strain was stored in liquid nitrogen also.

Preparation of fraction II. Peptone dialysate culture supernatant fluids (PSUP) were prepared as described by Coleman et al. (1). Labeled PSUP was prepared by culture of the vibrios in the same medium containing 500 μg per liter of uniformly labeled L-leucine (Tracerlab, Waltham, Mass.). After concentration by flash evaporation at 10 to 12 C and exhaustive dialysis against deionized water, the material was fractionated on DEAE A50 Sephadex (Pharmacia Inc., New Market, N.J.) in columns (2.5 by 45 cm) in a cold room at 4 C. Fraction I, containing the ileal loop reactive toxin, was eluted with deionized water and the column was washed with 0.05 M NaCl; fraction II was eluted with 0.5 M NaCl at a flow rate of 80 ml/hr. Effluents were monitored continuously by ultraviolet (UV) absorption at 280 nm with an 8300A Uvicord II (LKB Produkter AB, Stockholm, Sweden) with a 5-mm (0.3 ml) flow-through cell, by protein analysis by the Folin-Ciocalteau method (7), and, in the case of labeled preparations, by the radioactivity of the fractions as counted in a liquid scintillation counter (Packard Instrument Co., Inc., Maywood, Ill.). Tubes showing high UV absorption, protein, and specific radioactivity were pooled and dialyzed. The dialysand was concentrated by flash evaporation at <20 C, passed through an 0.42-μm membrane filter (Millipore Corp., Bedford, Mass.), and freeze-dried. The dry
preparations were stored in a desiccator in the refrigerator.

Lipopolysaccharide preparations. Lipopolysaccharide (LPS) was prepared from both vibrio strains by coprecipitation from an autolyzed culture with calcium phosphate formed in situ, elution from the precipitate with dilute HCl, and repeated precipitation with ethyl alcohol in the cold as described by Kaur and Shrivastav (5).

Preparation of antisera. Antisera were prepared in rabbits by hyperimmunization and by the inoculation of single graded doses of antigen. For the former, ultrasonic lysate (WCL) of agar-grown vibrios was prepared as described by Kasai and Burrows (4) and given on alternate days in doses of 1, 2, and 4 mg intraperitoneally (ip) followed by three doses in the same amounts given intravenously (iv). Animals were similarly immunized with fraction II and LPS on the same dose schedule. Rabbits were also immunized with conventional cholera vaccines, one from the Central Research Institute, Kasauli, India, and the other that produced by Eli Lilly & Co., Indianapolis, Ind., in the same way in doses of 0.5, 1.0, and 2.0 ml by the ip and iv routes. The animals were bled 4 days after the last inoculation.

For the preparation of antisera to single doses of antigen, animals were given a single ip inoculation. Fraction II and LPS were used as the immunizing antigens. The doses of fraction II were 10, 25, 50, 100, 200, 250, and 400 μg. Those of LPS were 50, 60, 80, 100, 200, 250, and 400 μg. Each antigen dose group was made up of six animals. The groups which received the 250-μg doses were bled at weekly intervals, beginning with preimmunization specimens, and the remainder were bled 3 weeks after inoculation. Sera from the indicated groups of animals were pooled as equal amounts from each animal for the titration of antibody.

Immunodiffusion. Specific precipitation by double diffusion in agar was carried out according to Ouchterlony (8). Commercially available immunodiffusion plates (Hyland Laboratories, Los Angeles, Calif.) and plates prepared in the laboratory were used for both the semimicro test and the macro test. Agarose (1%) containing thimerosal (1:10,000) was poured to a depth of 5 mm in small petri dishes. For the semimicro test, 2-mm wells were cut with an LKB cutter, and for the macro test with a Feinberg agar gel cutter (Shandon, Consolidated Laboratories, Chicago, Ill.), the latter giving a center well 10 mm in diameter and outer wells 7 mm in diameter. The reactions were allowed to develop for 24 hr to several days at room temperature in a humid chamber.

Vibriocidal antibody titration. Vibriocidal antibody was titrated by the plate culture method used at the Central Research Institute, Kasauli, India, and by the dilution-subculture method of counting viable bacteria described by Freeman et al. (3), with NIH 35-a-3 as the test organism. In the former method, serum samples were inactivated at 56 C for 30 min and then diluted by 10-fold steps in a 1:20 dilution of reconstituted freeze-dried guinea pig complement (Markham Laboratories, Chicago, Ill.) in 0.1% peptone water. The vibrios were grown in 3% peptone water for 16 hr, and then diluted to contain 200 to 500 colonies per standard 4-mm loop. A 1-ml amount of each of the serum dilutions and 1 loopful of the vibrio suspension were incubated in a water bath at 37 C for 40 min and then plated on 3% peptone agar plates. Plates were observed after 24 hr of incubation, and the highest serum dilution which killed 50% of the vibrios was considered to be the vibriocidal titer.

In the dilution-subculture method used here, the reaction mixture contained 2 × 10⁸ vibrios per ml as measured photometrically, complement in 1:25 dilution, and serial dilutions of inactivated antiserum in a total volume of 5 ml. The mixture was incubated in a water bath at 37 C for 40 min, and then subcultured in trypticase soy broth for assay of numbers of viable bacteria. The number of vibrios given by the complement control tubes was taken as 100%. The plot of percentage of survival against log serum concentration was linear and allowed the interpolation of a 50% end point. The antibody titer of the serum could then be expressed as vibriocidal units, i.e., the interpolated amount of serum required to kill 50% of the vibrios per ml. Although this titration is laborious, it gives results of the order of precision required for quantitation of interfering antigen; in sixfold replicates, the maximal difference in observed titers has been <20 per cent.

Interference test. The concentration of antibody was found to be critical in the titration of interfering antigen. This concentration was determined by using varied serum dilutions against several constant amounts of antigen. Efficiency may be calculated as interfering units of antigen per microgram, i.e., the amount of antigen effectively combining with a vibriocidal unit of antibody. In such titrations, it was found that this increases to a peak with increasing serum dilutions and then falls off, presumably a reflection of the equivalence zone. Over the range of usable antigen concentrations, the critical dilution of serum used contained about 10 vibriocidal units per ml. In the interference test, the maximal serum dilution was held constant and that of the interfering antigen varied.

As the test was carried out, graded amounts of the interfering antigen in 1 ml of saline were added to 1 ml of diluted serum and incubated at 37 C for 30 min. After chilling, 1 ml of this mixture was added to 1 ml of 1:5 complement. 1 ml of vibrio suspension (8 × 10⁸ cells per ml), and 2 ml of saline for titration of residual vibriocidal antibody as described above.

RESULTS

The resolution of eluates from column fractionation of the admixture of substances present in PSUP was least by UV absorption at 280 nm, although location of main protein peaks has been useful. Considerably better resolution was obtained by analysis of the fractions for protein. The elution of fraction II in 0.5 M NaCl, after removal of fraction I and washing, as monitored by protein analysis is illustrated in Fig. 1. Frac-
tion II, as used here, was collected as a pool of the fractions eluted between 80 and 160 ml. Although the validity of monitoring by such protein analysis is open to question because of the paucity of aromatic amino acids as noted by Coleman et al. (1) and because there is no assurance that the substances of interest are necessarily protein, it is apparent that fraction II as so prepared is not homogeneous. When the PSUP preparation had been labeled with tritium to give maximal resolution of the eluates, the heterogeneity became more marked (Fig. 2) and was separable into three pieces designated A, B, C, and D, with D presumably representing the trailing edge of C.

Immunodiffusion. As noted above, this study was initiated by the observation that the heavy, or main, precipitin line shown by fraction II when tested against anti-WCL hyperimmune serum gave an identity reaction with the single precipitin line produced by 569B LPS against the same antiserum. This identity reaction is shown in Fig. 3 in which alternate antigen wells contain fraction II and LPS, the former in the odd-numbered wells. The reaction was produced in the semimicro test by the use of Hyland plates and was read 24 hr before the secondary precipitin lines given by fraction II had developed.

This identity reaction was confirmed by the mutual dilution method described by Preer (9), in which the antigens are mixed in varying proportions. The fraction II to LPS ratios were 7:1, 3:1, 1:1, 1:3, and 1:7 in antigen wells 2 through 6, and well 1 contains undiluted fraction II (Fig. 4). The plate was developed for 24 hr as shown here, and the secondary precipitin lines of fraction II are not yet apparent. Clearly, the reaction of the antigens in mixture in varying proportions shows identity in all proportions and with the unmixed
fraction II. This kind of test was also carried out in Oudin tubes (9), in which the secondary precipitin bands were developed. The main precipitin line given by the mixtures was single and in the same position as those of fraction II and LPS alone, whereas the secondary lines given by fraction II, nearer the antibody side, were also in the same position as those produced by fraction II alone.

The heterogeneity of fraction II observed in the elution pattern of tritiated PSUP was also reflected in its antigenic specificity. It was found to give at least six precipitin lines with anti-WCL serum, but the number of demonstrable lines tended to vary from one preparation to another. The maximal number of lines found is illustrated in Fig. 5 in which fraction II is present in well 3. The greater antigenic complexity of WCL is shown by the greater number of precipitin lines of well 1. Well 1 contains LPS, and the identity reaction with fraction II and the presence of LPS antigenic specificity in WCL are apparent.

The antigens of LPS and fraction II prepared from the NIH 35-a-3 strain are compared with those prepared from 569B (Fig. 6). The identity reaction between the two LPS preparations is shown between wells 1 and 6, and it is apparent that the main precipitin lines show both homologous and heterologous identity. Well 2 contains NIH 35-a-3 fraction II, and it appears to
lack one precipitin line given by 569B fraction II in well 4; it is uncertain whether this is a concentration effect, analogous to differences in preparations of 569B fraction II, or an indication that an antigen present in 569B is lacking in NIH 35-a-3. Since the main precipitin line showing identity between LPS and fraction II was present in all preparations, this point was considered peripheral to the present study and was not pursued further.

The heat stability of LPS antigenicity has long been established, and, because of the identity reaction given by LPS and fraction II, the heat stability of the latter was investigated. A preparation of fraction II showing four precipitin bands was heated at 56°C and in a boiling water bath. These were compared with one another, with unheated fraction II, and with LPS (Fig. 7). It is apparent that the main precipitin line was stable to boiling for 1 hr and continued to give the identity reaction, whereas the secondary lines of fraction II were destroyed at 56°C after 10 to 30 min.

The heterogeneity of tritium-labeled fraction II as indicated by the elution pattern shown in Fig. 2 was investigated with respect to antigenic specificities. As shown there, four subfractions were separated. These were examined by immuno-diffusion and compared with intact fraction II (Fig. 8). Each subfraction is adjacent to fraction II. It is apparent that the main precipitin line showing identity with LPS is contained in subfraction B, in this instance represented by a single collection tube containing 8 ml, but it is contaminated with a secondary line shared with subfractions A and C; only two secondary lines are found in subfraction C, and a single secondary line is given by A. D is apparently no more than the trailing edge of C. More detailed study of the separation of the component antigens of fraction II, and their biological activity, will be reported elsewhere.

Vibriocidal antibody. Since vibriocidal antibody is produced in response to immunization with LPS (6, 10), the presence in fraction II of an antigen apparently identical in specificity with that of LPS raises the question of whether fraction II was similarly related to vibriocidal antibody. This was considered in two ways, that fraction II would function as a complete antigen and stimulate the production of vibriocidal antibody and that the combining power demonstrated in the immunodiffusion reaction represented combination with vibriocidal antibody to produce a functional inhibition of its activity.

As regards the former, fraction II, together with WCL and LPS and conventional cholera vaccines, was used to hyperimmunize rabbits.
Two preparations of each of the soluble antigens and two lots of cholera vaccine were used. Vibriocidal antibody was titrated by the plate culture method to give approximate, but sufficiently accurate, titers for this purpose (Table 1). These data indicate that the immunogenicity of fraction II with respect to vibriocidal antibody roughly equals that of the other immunizing antigens in the amounts used.

The relative immunogenicity of fraction II and LPS may be assayed by immunization with graded doses of antigen. Preliminary experiments had indicated that the amount of antigen given in a single IP dose was linearly related to the log of the antibody titer over the appropriate dose range. Groups of animals were, therefore, so immunized with the two antigens, and serum pools were titrated for vibriocidal antibody by the plate method. The results are shown in Fig. 9 as the log of the geometric mean titers plotted against micrograms of antigen. The superior immunogenic potency of fraction II is clearly indicated. The maximal titer within the linear range, 1:320,000, produced by LPS required a dose of 100 μg of antigen, whereas the same titer produced by fraction II was in response to 41 μg of antigen. Similarly, the maximal titer, 1:1,320,000, given by fraction II antigen in response to a dose of 50 μg of antigen was higher than the maximal titer elicited by LPS, i.e., 50% of the amount of fraction II antigen gave a threefold greater antibody response.

Animals receiving doses of 250 μg of the antigens, an amount well beyond the range of linear antibody response, and bled at weekly intervals also showed the superior antibody response to fraction II (Fig. 10). The initial response to fraction II was more rapid than that to LPS, as indicated by the antibody titers of 1:8,000 and 1:102, respectively, at 1 week postinoculation, although, thereafter, the titer to fraction II rose more slowly. The peak titer to fraction II was higher than that to LPS, 1:1,325,000 and 1:1,000,000, respectively. It may be noted that this peak titer was the same as that produced by 50 μg of fraction II; i.e., the additional 200 μg, or fivefold the antigen dose, was not required to reach peak titer. In addition, vibriocidal antibody produced by fraction II declined more slowly in titer than that produced by LPS, the titers persisting at 7 weeks postinoculation being 1:100,000 and 1:102, respectively.

![Fig. 9. Assay of the relative immunogenicity of 569B fraction II and LPS as shown by the antibody response to graded doses of the antigens. Log of vibriocidal antibody titer plotted against micrograms of antigen.](http://jb.asm.org/)

![Fig. 10. Time course of the vibriocidal antibody response to single doses of 250 μg of 569B fraction II and LPS antigens.](http://jb.asm.org/)

**Table 1. Vibriocidal antibody titers produced by hyperimmunization**

<table>
<thead>
<tr>
<th>Immunizing antigen</th>
<th>Antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCL (A)</td>
<td>$10^4$ to $10^8$</td>
</tr>
<tr>
<td>WCL (B)</td>
<td>$10^4$ to $10^8$</td>
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<tr>
<td>Fraction II (A)</td>
<td>$10^7$</td>
</tr>
<tr>
<td>Fraction II (B)</td>
<td>$10^7$</td>
</tr>
<tr>
<td>LPS (A)</td>
<td>$10^7$</td>
</tr>
<tr>
<td>LPS (B)</td>
<td>$10^6$ to $10^7$</td>
</tr>
<tr>
<td>Kasauli vaccine</td>
<td>$10^4$ to $10^7$</td>
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<tr>
<td>Lilly vaccine</td>
<td>$10^7$ to $10^8$</td>
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In the titration of the combining power of fraction II and LPS in the vibriocidal reaction, the relation between log per cent survival, as determined by the dilution-subculture method, and log antigen concentration was linear over a usable range, and could be used to interpolate a 50% end point. The results of a series of titrations with various amounts of antigen in the presence of antifraction II serum diluted to contain 12 vibriocidal units of antibody per ml are shown in Fig. 11. It is evident that fraction II, in contrast to its greater immunogenic potency, is a less effective interfering antigen than is LPS. The marked difference at 10 µg of antigen is to be disregarded perhaps as beyond the usable concentration range of fraction II antigen, but the lesser efficiency of this antigen is evident at the 50% survival point. The amount of antigen required to inactivate all but one vibriocidal unit of antibody, i.e., the one remaining to be titrated, is 75 µg in the case of fraction II, but less than 50% of that amount, 35 µg, in that of LPS. If 1 combining unit of antigen is considered that amount required to inactivate 1 unit of vibriocidal antibody under these conditions, LPS contains about 0.32 combining units per µg, whereas fraction II contains about 0.15 units per µg. These numbers are less exact than might be inferred, but the approximate twofold difference is clearly significant with the observed maximum variation of $< \pm 10\%$ in replicate titrations.

Similar titrations were carried out with anti-LPS serum and the two antigens and gave closely similar results. LPS was again the more efficient interfering antigen, and the difference between the two antigens was about twofold in this system also.

**DISCUSSION**

Our data show clearly that the nontoxic material present in young culture supernatant fluids from peptone dialysate broth and eluted from DEAE A50 Sephadex in 0.5 M NaCl is a mixture of substances. Earlier studies (1) showed it to have an average molecular weight of 30,000 to 35,000, and to contain about 90% protein, 3% carbohydrate, and 6 to 7% extractable lipid. Whereas after elution this fraction appeared, on the basis of UV monitoring, to be relatively homogeneous, monitoring by protein analysis suggested the heterogeneity confirmed here. The antigenic homogeneity implied earlier was based on the results of immunoelectrophoresis and immunodiffusion carried out on slides. In our hands, these methods have been less sensitive, perhaps because of dilution effects, than the semimicro and macro methods.

We were able to define more precisely the heterogeneity of the material designated fraction II by the use of macro immunodiffusion methods and of tritium-labeled preparations. By the former, at least six antigenic components differentiable by immunodiffusion were demonstrated. Of these, the major component, so designated because it gave the most intense precipitin line, occurred nearest the antigen well and was of primary interest because it gave identity reactions with K-S lipopolysaccharides. This antigen was found to be contained entirely in a subtraction, B, of fraction II, making up $< 5\%$ of the total eluate volume, but it remained contaminated with a single minor antigen. Its separation as an antigenically homogeneous substance is a prerequisite to elucidation of the nature of the common antigenicity with LPS; such studies will be reported elsewhere.

To our knowledge, the antigens related to vibriocidal antibody formation remain obscure. Although it is established that LPS is such an antigen, it is by no means clear whether other antigenic specificities may participate also. The additional antigens present in fraction II might play such a part, or they may have other biological activity. We doubt that the minor antigens are concerned with the induction of, and reaction
with, vibriocidal antibody. If they were, it might be anticipated that fraction II would be superior in combining power, manifested in the interference reaction, to LPS when tested against anti-fraction II serum. Not only was it not superior, but rather was only 50% as efficient on a weight basis. This lesser efficiency may be attributable to its appreciable contamination with antigens unrelated to vibriocidal antibody. The observed difference can hardly be accounted for on the basis of differences in molecular weight; on the contrary, given the same number of combining sites per molecule, the much larger LPS molecule would be less efficient on a weight basis, and the titration curves would be characterized by differences in slope. The close similarity, if not identity, of the slopes (Fig. 11) suggests that the number of combining sites is relatively constant per unit weight, i.e., that the larger LPS molecule contains additional combining sites to counteract an effect of differences in molecular weight that would otherwise be apparent.

The major interest here was the possible functioning of fraction II as an antigen giving rise to the antibacterial component of an effective immunity to cholera. Assuming that the vibriocidal antibody response is a reflection of antibacterial immunity, the observations reported here suggest that it does contribute to immunity, and may, therefore, have some practical utility as an immunizing antigen. It appears to be nontoxic in the general sense in that it has not been possible to determine a mouse LD₅₀ for fraction II; as much as 20 mg ip has failed to kill mice, in contrast to the mouse LD₅₀ of LPS of perhaps 2 mg. Such apparent lack of toxicity, coupled with the marked immunogenic potency and persistence of antibody formed to it, would support the potential applicability of this kind of preparation as an immunizing antigen.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI 07624 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED