PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 1187–1191 (1974)

Radiolabeled Toxin for Studying Binding of Cholera Toxin and Toxoids to Intestinal Mucosal Receptor Sites[1] (37978)

JOHNNY W. PETERSON AND W. F. VERWEY

Department of Microbiology, University of Texas Medical Branch, Galveston, Texas 77550

The massive loss of fluid and electrolytes during cholera is initiated by a heat-labile protein exo-enterotoxin elaborated by *Vibrio cholerae* (1–4). The toxin, designated choleragen by Finkelstein, and its spontaneously formed toxoid, choleragenoid, have been isolated from culture supernatants in highly purified form (5, 6). Cholera toxoids, prepared by treatment of purified toxin with formaldehyde or glutaraldehyde, have now been produced by Wyeth Laboratories. The availability of purified toxin and toxoids has stimulated increasing interest in antitoxic immunity and the elucidation of the mode of action of the toxin. Recent studies suggest that the cholera toxin exerts its effect on the intestinal epithelium by first binding to a receptor on the plasma membrane of the mucosal epithelial cells and then stimulating the activity of the membrane-associated enzyme, adenyl cyclase, thereby increasing the cellular concentration of cyclic AMP (3, 4, 7, 8). One effect of overproduction of cyclic AMP on intestinal epithelial cells is the hypersecretion of fluid and electrolytes from the cells.

Cholera toxin has been detected on the brush border membranes of mouse intestinal epithelial cells, employing fluorescein and horseradish peroxidase-labeled antibodies and tritium-labeled choleragen (9). Evidence was presented that choleragen and choleragenoid, but not formalin toxoids, were adsorbed uniformly to the mucosal surfaces of villi from ligated intestinal loops of adult mice after intralumenal inoculation of the purified proteins.

Information relating to the nature of cholera toxin receptor(s) is largely unknown; however, some studies suggest that the receptor site may be a ganglioside, more specifically a sialidase-resistant monosialosylganglioside (10, 11). The conclusion was drawn from studies that showed cholera toxin was deactivated by the addition of gangliosides. The resulting mixture failed to elicit both the characteristic permeability reaction in rabbit skin and fluid accumulation in ileal loops of adult rabbits. More recently, it has been found that monosialosylgangliosides (GM_1) are effective in inhibiting the binding of radiolabeled choleragen to fat cell membranes at concentrations as low as 1 ng/ml (12).

The present study was designed to investigate further the binding of choleragen, choleragenoid, and artificial cholera toxoids to intestinal epithelial receptor sites. An *in vitro* binding technique employing ^{125}I-labeled choleragen was developed and shown to be specific for both choleragen and choleragenoid. The procedure appears to have considerable potential usefulness in the study of toxin binding.

Materials and Methods. Highly purified choleragen (Lot #0572, prepared for the N.I.H. by Dr. R. A. Finkelstein) was used throughout this study. Iodination was accomplished by mixing 500 μg of choleragen in 100 μl of 0.05 *M* tris buffer (pH 7.5) with 2.5 mCi of carrier-free Na ^{125}I (IMS .30, Amersham/Searle) in 25 μl of NaOH solution and 50 μl of 0.5 *M* potassium phosphate buffer (pH 7.2). One-hundred microliters of chloramine T (500 μg/ml)

[1] This work was supported by Public Health Service research grant AI-08518 from the National Institute of Allergy and Infectious Diseases.

in 0.05 *M* phosphate buffer was added and the mixture incubated for 2 min at 4°. The reaction was stopped by the addition of 100 μl of sodium metabisulfite (500 μg/ml) in 0.05 *M* phosphate buffer. Seconds later, 100 μl of potassium iodide (10 mg/ml) in 0.05 *M* phosphate buffer was added. After iodination, the labeled toxin was separated from the unbound radiolabel by chromatography on a 1 × 60 cm column of Sephadex G-25 equilibrated with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (Cohn fraction V, Nutritional Biochemicals Corp.) at a pH of 7.2. Quantitation of the radiolabeled toxin protein was accomplished by the radial immune diffusion assay of Finkelstein (5), using 4 μl of Finkelstein equine antitoxin (available from the NIH) per ml of 0.5% Noble agar in PBS. Radioactivity was measured in a Nuclear-Chicago gamma scintillation counter (Model 1185 A) with a counting efficiency of 80%. This procedure routinely gave labeled toxin preparations having an average specific activity of 13 μCi/μg of toxin antigen. The iodinated toxin was stored at 4° and used for periods as long as one month.

Mucosal homogenates were prepared from the small intestines of normal adult guinea pigs. Segments of entire small intestine were excised and cut lengthwise prior to extensive washing in cold PBS. The mucosal layer was removed from the serosa by gentle scraping with the edge of a microscope slide and homogenized at low speed in a Virtis tissue homogenizer for 1 min, after which the insoluble material was collected and washed with a total of 500 ml PBS. The mucosal homogenate was subsequently quantitated on the basis of dry-weight determinations using a Mettler M5 microbalance. Weights for buffer salts were subtracted from all total solids determinations.

The basic toxin-binding technique described here consisted of combining 1–1.5 × 10^5 cpm of ^{125}I-choleragen with 5–10 mg of the insoluble mucosal homogenate material, suspended in 0.1% BSA-PBS in 12 × 75 mm test tubes. After 1-hr incubation at 37°, the mixture was centrifuged and washed twice with 3-ml vol of PBS, after which the tubes containing the mucosal pellets were counted in the gamma scintillation counter. Some of these experiments measure the ability of various substances, including unlabeled choleragen, choleragenoid, formaldehyde toxoid (Wyeth lot No. 00101), and glutaraldehyde toxoid (Wyeth lot Nos. 11101 and 11201), to inhibit the binding of ^{125}I-choleragen to the mucosal homogenate. This was accomplished by preincubation of the mucosal homogenate with the inhibitor at 37° for 1 hr prior to the addition of the labeled choleragen. The amount of ^{125}I-choleragen that became bound to the homogenate was determined as described. Inhibition of ^{125}I-choleragen binding to the mucosal homogenate by specific equine antitoxin was accomplished by a similar procedure except that ^{125}I-choleragen and antitoxin dilutions (1 ml) were mixed and incubated for 1 hr at 37° prior to a similar incubation with the mucosal homogenate. Then the amount of radiolabeled toxin bound to the homogenate after washing with PBS was determined. All determinations were expressed as the average of duplicate determinations.

Results. Figure 1 illustrates the adsorption of ^{125}I-choleragen to the mucosal homogenate. Three concentrations of radiolabeled choleragen were incubated with a series of tubes containing increasing amounts of mucosal homogenate. The increasing levels of radioactivity in the washed homogenate indicate an increase in toxin adsorption as the mucosal homogenate concentration was increased. However, 100% binding of the labeled toxin was not observed, even when lower concentrations of radiolabeled toxin were added (as illustrated in the lower two curves). At the highest concentration of homogenate tested (16 mg of homogenate), approximately 50% of the total radiolabel was bound for each of the three concentrations of ^{125}I-choleragen added. The remaining unbound radioactivity may be an indication of damage from the iodination process.

In order to determine if the binding of this radiolabeled toxin was specific, and not due to mechanical trapping or nonspecific

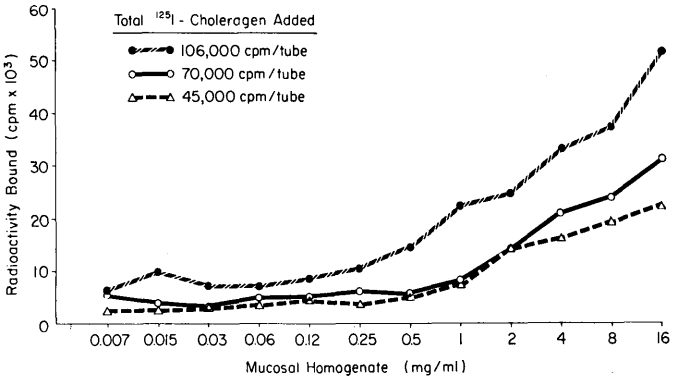

FIG. 1. Binding of ^{125}I-choleragen to intestinal mucosal homogenates.

adsorption, experiments were designed to determine if the binding of ^{125}I-choleragen could be specifically inhibited by preexposure of the mucosal homogenate to unlabeled choleragen or choleragenoid. In addition, normal rabbit serum was used as a source of heterologous proteins as a control for nonspecific protein adsorption. The results of these experiments are presented in Fig. 2. The solid horizontal bar indicates the average amount of binding of ^{125}I-choleragen in tubes containing 10 mg of mucosal homogenate. It can be observed that this binding was inhibited by nanogram levels of both choleragen and choleragenoid, as illustrated by the center two curves. Choleragen and choleragenoid, at concentrations as low as 50–100 ng/ml, appear to be equally effective in inhibiting the binding of ^{125}I-choleragen to the mucosal homogenate. Normal rabbit serum proteins, at comparable and even higher concentrations not illustrated here (e.g., 1:5 dilution of serum), failed to inhibit the binding of ^{125}I-choleragen to the mucosal homogenate, as depicted by the open circled curve. The relative failure of goat immunoglobulin, iodinated by an identical procedure, to bind to the mucosal homogenate (bottom curve) further supports the specificity of ^{125}I-choleragen binding.

Figure 3 shows the results of additional efforts to establish the specificity of the binding of ^{125}I-choleragen to the intestinal mucosa preparations. The solid horizontal bar represents the average level of binding of ^{125}I-choleragen to the intestinal mucosal homogenate. When dilutions of equine antitoxin were incubated with ^{125}I-choleragen prior to the addition of the mucosal homogenate, the amount of radiolabeled toxin bound decreased to a low level as

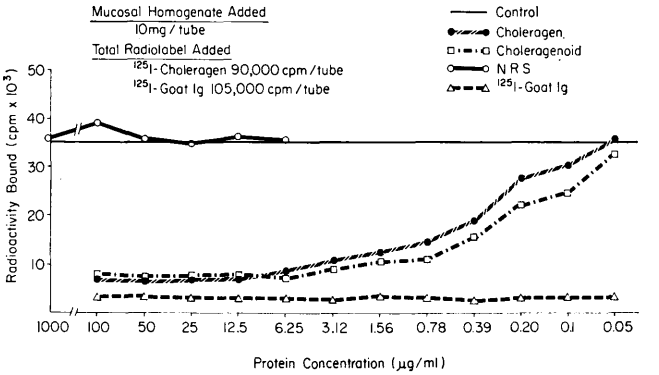

FIG. 2. Inhibition of ^{125}I-choleragen binding to intestinal mucosal homogenates by unlabeled choleragen and choleragenoid.

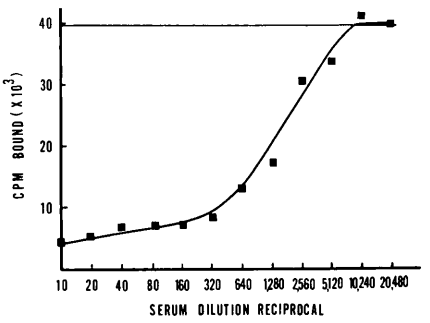

FIG. 3. Inhibition of ^{125}I-choleragen binding to guinea pig mucosal homogenates by equine antitoxin.

the concentration of antitoxin was increased, as seen in the bottom curve.

Previous immunohistochemical studies (9) indicated that unlike choleragen and choleragenoid, formaldehyde toxoids did not bind to the mucosal surface when inoculated into mouse intestinal loops *in vivo*. Confirmation of this observation was achieved in these studies when formaldehyde and glutaraldehyde toxoids were tested for their ability to inhibit mucosal binding of ^{125}I-choleragen. The lower curve shown in Fig. 4 again shows inhibition of ^{125}I-toxin binding by unlabeled choleragen. In contrast, the binding of ^{125}I-choleragen to the mucosal homogenate was not inhibited by either formaldehyde or glutaraldehyde toxoids at concentrations ranging from 0.05 to 100 μg/ml, depicted by the dashed and solid curves, respectively.

Discussion. A sensitive *in vitro* technique for studying the binding of cholera toxin and toxoids to intestinal mucosal tissue has been described. The data indicate that considerable amounts of labeled choleragen retain the ability to bind to the intestinal mucosa after iodination. As indicated in Fig. 1, some of the radiolabeled material did not bind to the mucosal homogenates with the same efficiency, which suggests some protein damage from the iodination process. More specific analysis of the non-tissue-binding portion of the iodinated toxin preparation is currently under investigation.

The ^{125}I-choleragen which became bound appears to be highly specific, since it could be inhibited equally well by preincubation of mucosal homogenates with nanogram concentrations of unlabeled choleragen or choleragenoid and not by heterologous proteins. Furthermore, the bound radiolabel must represent antigenically intact cholera toxin since equine antitoxin was capable of inhibiting ^{125}I-choleragen binding to the mucosal homogenates.

The inhibition of ^{125}I-choleragen binding by antitoxin also suggests a possible mechanism by which antitoxic immunity *in vivo* may be explained. Whether inhibition of the tissue-binding property is the sole means of toxin neutralization is at present unknown, since no toxin molecule has been found which possesses the adenyl cyclase activation moiety without a tissue-binding site.

These data confirm and extend previous observations that choleragen and choleragenoid, but not artificial toxoids, bind to the intestinal mucosa. The lack of inhibition by

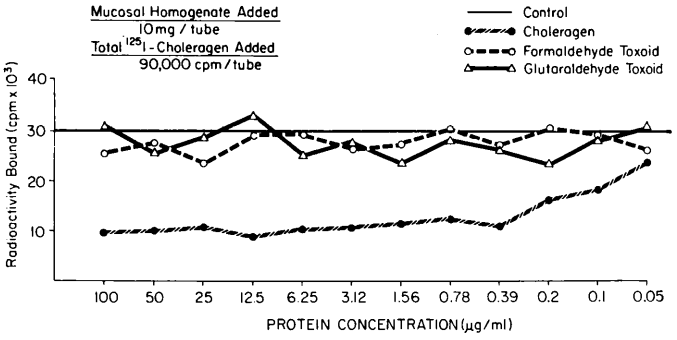

FIG. 4. A comparison of the efficiency of choleragen, formaldehyde toxoid, and glutaraldehyde toxoids to inhibit ^{125}I-choleragen binding to guinea pig mucosal homogenate.

glutaraldehyde and formaldehyde toxoids suggests that the tissue-binding sites on the toxin molecule are masked or destroyed by the toxoiding process, which possibly is one means by which the loss of toxicity is achieved.

In contrast, these studies indicate that the toxin-binding sites are retained in the case of choleragenoid and suggest that another region of the toxin molecule, also contributing to the toxicity, is damaged or lost during conversion of choleragen to choleragenoid (13). This region presumably is responsible for adenyl cyclase activation, since previous studies have shown that choleragenoid is relatively nontoxic (1).

One limitation of earlier studies concerned with localization of the cholera toxin (9) has been the need for a more sensitive means of toxin detection. This work has demonstrated that binding of cholera toxin to mucosal tissue can be demonstrated with nanogram concentrations, while previous studies required the use of microgram concentrations of toxin. It is hoped that the sensitive nature of this basic procedure will allow further investigation into the mechanism of action of the cholera toxin.

Summary. Recent evidence suggests that the mode of action of the cholera toxin involves at least two steps, which include binding to a membrane receptor and stimulation of the membrane-bound enzyme adenyl cyclase. A sensitive *in vitro* technique for studying toxin binding to tissue receptor sites is described and appears to have considerable potential usefulness in the study of toxin interaction with mucosal tissue. Highly purified ^{125}I-labeled cholera toxin was found to bind specifically to insoluble mucosal homogenates from the small intestines of normal adult guinea pigs. Binding of labeled toxin could be inhibited by nanogram levels of both cholera toxin and choleragenoid (a spontaneously formed toxoid), but not by heterologous rabbit serum proteins. Artificial cholera toxoids prepared by treatment of purified toxin with formaldehyde or glutaraldehyde failed to inhibit radiolabeled toxin binding to mucosal homogenates. The data indicate that cholera toxin binding is highly specific for the toxin and is not inhibited by heterologous proteins. In contrast, artificial toxoids, currently under investigation as potential human vaccines, appear to have lost their affinity for the tissue receptor.

The authors would like to express their appreciation to Mr. Ronald E. Roberts and Mrs. Arabella Black for excellent technical assistance.

1. Finkelstein, R. A., Critical Reviews in Microbiology **2**, 553 (1973).

2. Finkelstein, R. A., Texas Reports on Biology and Medicine **27**, 181 (1969).

3. Carpenter, C. C. J., Amer. J. Med. **50**, 1 (1971).

4. Pierce, N. F., Greenough, W. B., and Carpenter, C. C. J., Bact. Rev. **35**, 1 (1971).

5. Finkelstein, R. A., and LoSpalluto, J. J., J. Exp. Med. **130**, 185 (1969).

6. Finkelstein, R. A., and LoSpalluto, J. J., J. Infect. Dis. **121**, S63 (1970).

7. Field, M., N. Eng. J. Med. **284**, 1137 (1971).

8. Carpenter, C. C. J., J. Infect. Dis. **126**, 551 (1972).

9. Peterson, J. W., LoSpalluto, J. J., Finkelstein, R. A., J. Infect. Dis. **126**, 617 (1972).

10. Van Heyningen, W. E., Carpenter, C. C. J., Pierce, N. F., and Greenough, W. B., J. Infect. Dis. **124**, 415 (1971).

11. King, C. A., and Van Heyningen, W. E., J. Infect. Dis. **127**, 639 (1973).

12. Cuatrecasas, P., Biochemistry **12**, 3547 (1973).

13. Finkelstein, R. A., Peterson, J. W., and LoSpalluto, J. J., J. Immunol. **106**, 868 (1971).

Received Nov. 12, 1973. P.S.E.B.M., 1974, Vol. 145.