

NOTES

Neutralization of *Salmonella* Toxin-Induced Elongation of Chinese Hamster Ovary Cells by Cholera Antitoxin

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A partially purified preparation of the delayed skin permeability factor from *Salmonella typhimurium* caused Chinese hamster ovary cells to elongate. The elongation effect and the skin test activity were blocked by monospecific rabbit antisera against cholera toxin and against the B fragment of cholera toxin.

Enterotoxins have been described for a number of genera of enteric bacilli. Two of these organisms, *Vibrio cholerae* and toxigenic *Escherichia coli*, produce enterotoxins that have several fundamental properties in common. The heat-stable and heat-labile (LT) enterotoxins of *E. coli*, as well as cholera enterotoxin (which is heat labile), cause hypersecretion of fluid and electrolytes from the intestinal mucosa (5, 8, 14). *E. coli* LT and cholera enterotoxin also elicit vascular permeability changes in rabbit skin, which are best observed 18 to 24 h after intradermal injection (1, 4). Elongation of Chinese hamster ovary (CHO) cells and steroidogenesis of adrenal cells have been demonstrated with both *E. coli* LT and cholera toxin (2, 7). The common mechanism of action determined by these in vivo and in vitro assay systems involves the stimulation of adenyl cyclase (7, 12, 13), which results in increased levels of cyclic adenosine 5'-monophosphate. Moreover, the B fragment of cholera toxin (6, 10) and *E. coli* LT share antigenic determinants (W. F. Osborne and S. H. Richardson, Abstr. Annu. Meet. Am. Soc. Microbiol., 1976, B73, p. 23), indicating a degree of antigenic relatedness between the two protein toxins (15). This relationship is particularly significant with regard to the biological activity of the two toxins. Cholera antitoxin will neutralize *E. coli* LT, and antisera to *E. coli* LT will neutralize cholera toxin (3).

Recent studies on the pathogenesis of *Salmonella* infections have suggested the potential involvement of an exotoxin(s). Koupal and Deibel (9) described a cell wall-associated enterotoxic factor in *Salmonella enteritidis* that caused intestinal fluid loss when administered orally to infant mice. Sandefur and Peterson (11) have demonstrated two skin permeability factors (P.F.) in culture filtrates of *Salmonella*

typhimurium. One factor is heat stable and elicits an erythematous response within 1 to 2 h after intradermal injection into rabbit skin. The other factor, which requires chromatography on Sephadex G-100 for demonstration of activity, results in a delayed erythematous and edematous response within 18 to 24 h.

In the present study, we have demonstrated that chromatographed crude culture filtrates of *S. typhimurium* elicit changes in CHO cells that are morphologically indistinguishable from those produced by cholera toxin. As in the skin permeability assay for *Salmonella* delayed PF, toxic activity for CHO cells is demonstrable only after chromatography of crude culture filtrates to remove an inhibitor-like substance. Furthermore, we are able to demonstrate that the CHO cell elongation factor produced by *Salmonella* can be neutralized by monospecific cholera antitoxin and by monospecific antisera against the B fragment of cholera toxin.

Both rapid and delayed PFs elute together close to the void volume on a Sephadex G-100 column (1 by 90 cm), and both have an approximate molecular weight of 90,000 as reported by Sandefur and Peterson (11). More recently, we have achieved some degree of separation of the rapid and delayed PFs by modification of the Sephadex G-100 chromatography. By decreasing the pump speed, peak resolution was improved sufficiently such that several fractions that trailed the first peak (comprised of both rapid and delayed PF) contained only the delayed PF activity (Fig. 1). In this instance, the rapid PF activity was contained in elution volumes of 36 to 60 ml, whereas the delayed PF activity was demonstrable in elution volumes of 36 to 116 ml. The reasons for the broadness of the delayed PF activity peak are unknown; however, it is possible that the *Salmonella* factor(s) may exist in active aggregate forms.

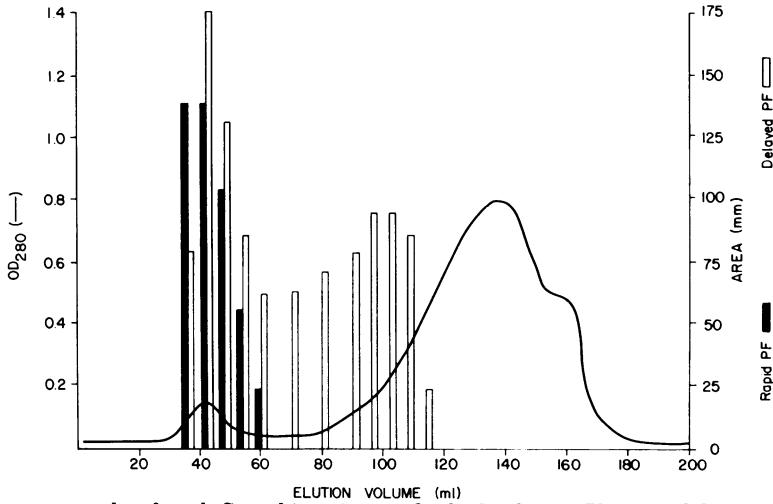


FIG. 1. Chromatography of crude *S. typhimurium* on Sephadex G-100. The optical density (OD) at 280 nm is represented by a solid line. The skin test reactivity of representative fractions is given in square millimeters; the closed bars represent rapid PF activity; the open bars depict delayed PF activity.

Stock cultures of CHO cells (designated by S. C. Barranco as CHO-LA) were grown at 37°C with 4% CO₂ in F-12 medium (GIBCO) supplemented with 10% fetal calf serum and polymyxin B. For the CHO cell elongation tests, cells were suspended in F-12 medium containing 1% fetal calf serum and polymyxin (7). A 0.1-ml amount of the suspension containing approximately 500 cells was delivered to each well, and the plates were incubated at 37°C with 4% CO₂ for 18 to 24 h. The normally trapezoid-shaped CHO cells elongated to a spindle form within 18 to 24 h after exposure to the toxins, as illustrated in Fig. 2. At least 100 cells were counted in each well, and the effectiveness of the test preparations was expressed as percent elongation. The appearance of CHO-LA cells after exposure to *Salmonella* or cholera toxins is somewhat different than that described by Guerrant et al. (7) for CHO-K1 cells (American Type Culture Collection, Rockville, Md.). The CHO-LA cells appear to be more sensitive to the toxins, and the morphological change observed is more exaggerated. These cells often exhibit a "spider-like" appearance rather than a simple elongation effect, making them more easily distinguished from nonelongated cells.

Neutralization experiments were performed using monospecific rabbit antisera, prepared in this laboratory, against highly purified cholera toxin and antisera against the B fragment of cholera toxin (10). In all cases, the respective preimmunization serum was included as a control. The control serum had a cholera antitoxin titer of less than 8.7 units/ml, whereas the hyperimmune serum had a titer of 4,618 units/ml,

as determined by the passive hemagglutination test for cholera antitoxin. *Salmonella* toxin preparations containing only the delayed PF activity were incubated with equal volumes of 10-fold dilutions of the above-mentioned rabbit sera at 37°C for 1 h. A 10- μ l amount of each preparation was added per microtiter well containing CHO cells. The plates were incubated for approximately 18 h in a 37°C incubator with 4% CO₂. Cells were either fixed in methanol and stained with Giemsa stain before counting, or the unfixed, unstained cells were counted immediately after incubation.

A rabbit antiserum to cholera toxin provided substantial protection to CHO cells from the *Salmonella* CHO cell elongation factor (Fig. 3). The preimmunization serum offered no protection, showing that the neutralization effect was specific and directly associated with the presence of antibodies to cholera toxin. A similar neutralization effect, not illustrated here, was obtained using antisera to the B fragment of cholera toxin.

The neutralization of the *Salmonella* toxin effect was also demonstrable using the rabbit skin permeability test. Dilutions of the rabbit sera described above were preincubated for 1 h at 37°C with equal volumes of chromatographed *Salmonella* crude culture filtrates containing both rapid and delayed PFs. A 0.1-ml amount of each sample was injected intradermally in rabbits whose hair had been removed with clippers and a depilatory cream. Pontamine Sky Blue dye was injected intravenously 1 h after skin testing as described by Sandefur and Peterson (11). Neutralization of the delayed bluing and induration factor was detectable 18 to 24 h after

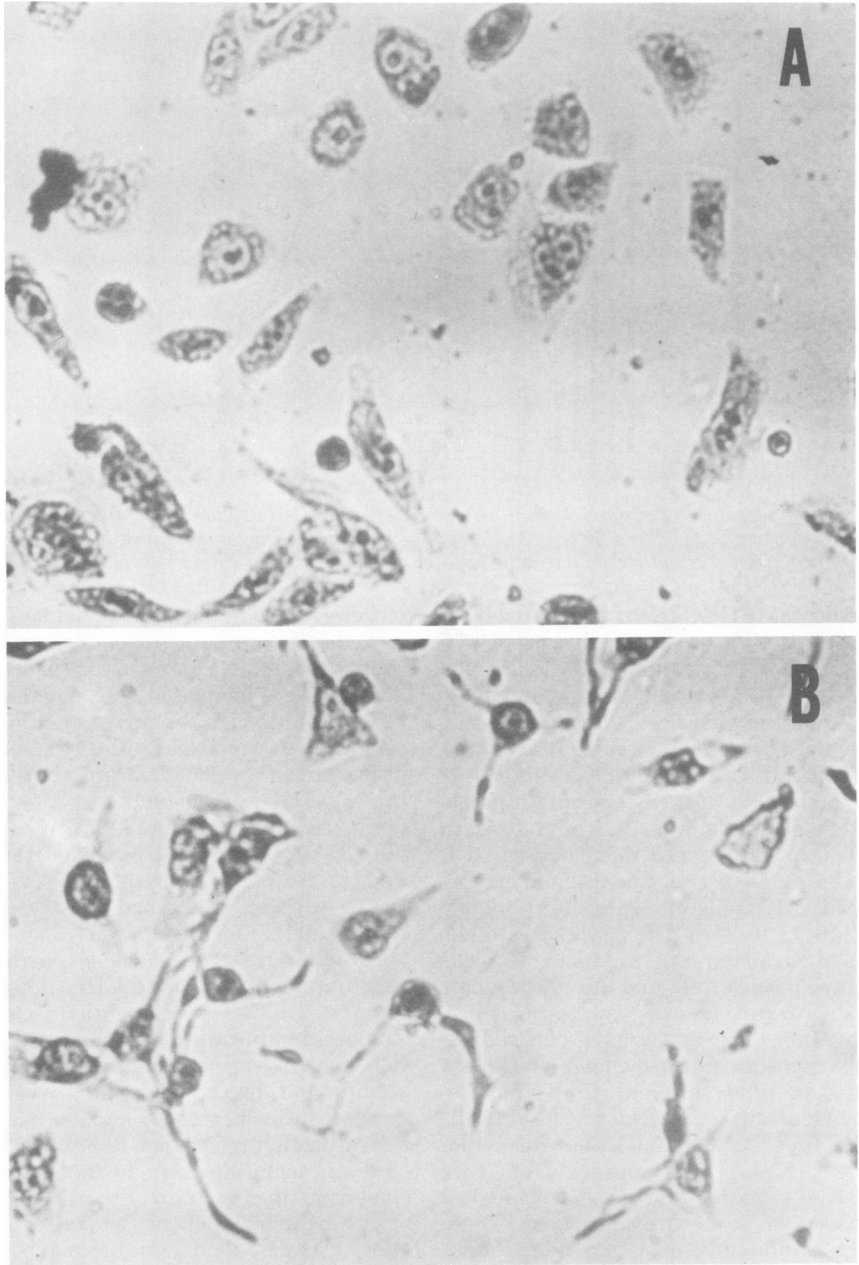


FIG. 2. Effect of chromatographed *S. typhimurium* crude culture filtrates on the morphology of CHO cells. The normal trapezoid-shaped cells are shown in (A), and elongated cells treated with a *Salmonella* toxic preparation are shown in (B).

skin testing. There was no neutralization of the delayed PF by the preimmunization sera, whereas antisera against cholera toxin and against the B fragment of cholera toxin significantly neutralized the delayed bluing and induction effect (Fig. 4). The rapid-bluing PF, also

present in the preparation, was not neutralized by either serum.

Although the actual pathogenic role of the substances elaborated by *Salmonella* is unknown, the biological properties of skin permeability alteration and CHO cell elongation are

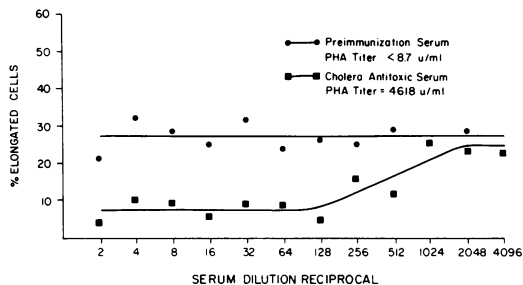


FIG. 3. Neutralization of the CHO cell elongation factor from *S. typhimurium* by preincubation with cholera antitoxic serum. Control cells were exposed to the delayed *Salmonella* factor preincubated with the preimmunization serum. Each control and test dilution was run in triplicate. PHA refers to the cholera antitoxin titers as determined by the passive hemagglutination test.

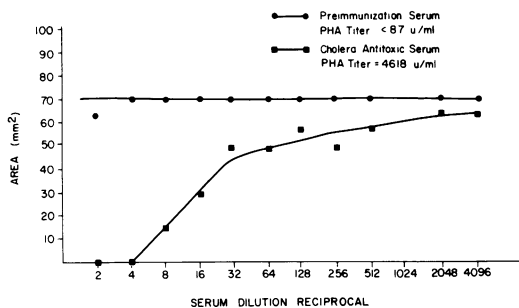


FIG. 4. Neutralization of delayed PF by preincubation with cholera antitoxic serum. Control spots were injected with *Salmonella* factor preincubated with preimmunization sera. Each control and test dilution was tested in duplicate, and observations were recorded at 18 to 24 h. PHA refers to the cholera antitoxin titers as determined by the passive hemagglutination test.

shared by cholera toxin and *E. coli* LT, both of which are responsible for fluid and electrolyte loss during intestinal infection with the respective organisms. Since CHO cells respond to *Salmonella* toxin preparations containing delayed but not rapid PF activity, and since crude filtrates expressing rapid but not delayed PF activity do not elongate CHO cells, it is probable that the CHO cell elongation factor is the same as the delayed PF.

In the present study we have shown that cholera antitoxin and antisera to the B fragment of cholera toxin can neutralize the biological activity of *Salmonella* delayed PF and the CHO cell elongation factor. Thus, an interesting antigenic relationship exists between the toxins of *V. cholerae*, *E. coli*, and *S. typhimurium* in which the *E. coli* LT and *Salmonella* delayed PF possess antigenic determinants

present on the B fragment of cholera toxin. Whether or not the toxins of *Salmonella* and *E. coli* are antigenically related remains to be determined. The antigenic similarities of the factors elaborated by these three enteric pathogens, as well as an apparent common mode of action, are suggestive of some common genetic origin for the information leading to their synthesis. Finally, experiments are in progress to determine if cholera toxoid-immunized rabbits are protected against intestinal loop challenge with live *Salmonella*, as might be expected based on the data presented here.

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LITERATURE CITED

- Craig, J. P. 1965. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. *Nature* (London) 207:614-616.
- Donta, S. T., M. King, and K. Sloper. 1973. Cholera enterotoxin: induction of steroidogenesis in tissue culture. *Nature* (London) 243:246-247.
- Evans, D. G., D. J. Evans, Jr., and S. L. Gorbach. 1973. Identification of enterotoxigenic *Escherichia coli* and serum antitoxin activity by the vascular permeability factor assay. *Infect. Immun.* 8:731-735.
- Evans, D. J., Jr., D. G. Evans, and S. L. Gorbach. 1973. Production of vascular permeability factor by enterotoxigenic *Escherichia coli* isolated from man. *Infect. Immun.* 8:725-730.
- Finkelstein, R. A. 1973. Cholera. *Crit. Rev. Microbiol.* 2:553-623.
- Finkelstein, R. A., M. Boesman, S. H. Neoh, M. K. LaRue, and R. Delaney. 1974. Dissociation and recombination of the subunits of the cholera enterotoxin (choleragen). *J. Immunol.* 113:145-150.
- Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman. 1974. Cyclic adenosine monophosphate and alteration of chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for enterotoxins of *Vibrio cholerae* and *Escherichia coli*. *Infect. Immun.* 10:320-327.
- Gyles, C. L. 1971. Heat-labile and heat-stable forms of the enterotoxins from *Escherichia coli* strains enteropathogenic for pigs. *Ann. N.Y. Acad. Sci.* 176:314-322.
- Koupal, L. R., and R. H. Deibel. 1975. Assay, characterization, and localization of an enterotoxin produced by *Salmonella*. *Infect. Immun.* 11:14-22.
- Kuroskey, S., D. E. Markel, B. Touchstone, and J. W. Peterson. 1976. Chemical characterization of the structure of cholera toxin and its natural toxoid. *J. Infect. Dis.* 133:514-522.
- Sandefur, P. D., and J. W. Peterson. 1976. Isolation of

- skin permeability factors from culture filtrates of *Salmonella typhimurium*. *Infect. Immun.* 14:671-679.
12. Schafer, D. E., W. D. Lust, B. Sircar, and N. D. Goldberg. 1970. Elevated concentration of Adenosine 3':5'-cyclic monophosphate in intestinal mucosa after treatment with cholera toxin. *Proc. Natl. Acad. Sci. U.S.A.* 67:851-856.
 13. Sharp, G. W. G., and S. Hynie. 1971. Stimulation of intestinal adenyl cyclase by cholera toxin. *Nature (London)* 229:266-269.
 14. Smith, H. W., and C. L. Gyles. 1970. The effect of cell free fluids prepared from cultures of human and animal enteropathogenic strains of *Escherichia coli* on ligated intestinal segments of rabbits and pigs. *J. Med. Microbiol.* 3:403-409.
 15. Smith, N. W., and R. B. Sack. 1973. Immunologic cross-reactions of enterotoxin from *Escherichia coli* and *Vibrio cholerae*. *J. Infect. Dis.* 127:164-170.