

## Isolation of Special Antibodies Which React Only With Homologous Enterotoxins From *Vibrio cholerae* and Enterotoxigenic *Escherichia coli*

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Cholera enterotoxin (CT) and heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* share a common antigenic determinant. In addition, CT and LT each have a unique antigenic determinant. Antisera were prepared by immunoaffinity chromatography against these unique antigenic determinants, that is, antiserum that reacted with CT but not with LT and antiserum that reacted with LT but not with CT. Antiserum against the common antigenic determinant to CT and LT was also prepared by immunoaffinity chromatography. The specificities of these antisera were demonstrated by Ouchterlony double-gel diffusion tests and by neutralization of the activities of CT and LT to cause morphological changes in Chinese hamster ovary cells.

Immunological and biological similarities between cholera enterotoxin (CT) and heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* have been demonstrated (1, 2, 6, 7, 9-11, 18, 19). Gyles and Barnum (10) first suggested that CT and LT were immunologically related by demonstrating the neutralization of porcine *E. coli* LT activity by anti-LT antiserum. Subsequently, neutralization of LT activities by either anti-CT or anti-cholerae antigen antiserum in the rabbit ileal loop test (19), the rabbit skin vascular permeability test (7), and the Y-1 adrenal cell assay (1, 6, 11) has been reported by several workers. More directly, the immunological partial identity of CT and LT was demonstrated by the double-gel diffusion test (1, 9, 17). Recently, Clements and Finkelstein (2) clearly showed that LT is immunologically related to subunits A and B of CT by neutralization of activities and by the double-gel diffusion test. This paper reports the preparation of antiserum which reacts with CT, but not with LT, and of antiserum which reacts with LT, but not with CT. Isolation of a common antibody against both CT and LT is also reported.

### MATERIALS AND METHODS

**Preparation of purified CT and LT.** CT purified from the culture filtrate of *Vibrio cholerae* 569B by the method of Ohtomo et al. (17) was purchased from Sanko Junyaku Co., Tokyo, Japan.

LT was purified essentially as described by Clements and Finkelstein (3). In brief, *E. coli* 536-5, a strain producing LT only, isolated from a patient with traveler's diarrhea, was cultured with vigorous shaking at 37°C for 24 h in Casamino Acids-yeast extract medium

(16, 21) containing 90 µg of lincomycin hydrochloride per ml (Japan Upjohn Ltd., Tokyo), which enhances the synthesis of LT (13, 15). The bacterial cells were collected by centrifugation and suspended in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.6) containing 0.9% NaCl. The suspension was sonicated with a Sonifier model 185 (Branson Sonic Power Co., Danbury, Conn.), since a whole-cell lysate is reported to be the richest source of LT (3). The supernatant containing LT was obtained by centrifugation and fractionated with 60% saturation of ammonium sulfate. Then LT was purified by successive column chromatographies on Bio-Gel A5m (Bio-Rad Laboratories, Richmond, Calif.) and Sephacryl S-200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). LT was eluted from the Bio-Gel A5m column with 0.3 M D-galactose (Wako Fine Chemicals Co., Osaka, Japan) as described by Clements and Finkelstein (3). The purified LT thus obtained gave a single band on polyacrylamide gel disc electrophoresis.

**Preparation of antisera against purified CT and LT.** Antisera against purified CT and LT were prepared essentially as described previously (13). A 25-µg amount of purified CT or purified LT in 1 ml of phosphate-buffered saline (pH 7.0) was emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). The emulsion was inoculated intramuscularly into young rabbits weighing about 2 kg each. Two booster injections were given on days 25 and 50 with 25 µg of toxin emulsified with Freund complete and incomplete adjuvant, respectively. The highest dilution of the antisera obtained which gave a precipitin line against 3 µg of homologous toxin in an Ouchterlony gel diffusion plate was 1:16. The gel diffusion test was performed as described elsewhere (14) in a 0.9% Noble agar (Difco) plate with phosphate-buffered saline (pH 7.0) containing 0.3% sodium azide as a preservative.

**Immunoaffinity column chromatography.** Im-

munoaffinity column chromatography was carried out as described previously (4, 13). A 1-g amount of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) was coupled with 7 mg of purified CT or LT. A 1-ml amount of either anti-CT or anti-LT antiserum was applied to a column of cyanogen bromide-activated Sepharose 4B coupled with either CT or LT, and the column was washed with phosphate-buffered saline (pH 7.0) until the optical density of the eluate at 280 nm reached a basal level. The immunoglobulin bound to the column was then eluted with 0.2 M glycine-hydrochloride buffer (pH 2.7) containing 0.5 M NaCl.

**CHO cell assay.** The Chinese hamster ovary (CHO) cell assay was carried out as described previously (12). Eagle minimal essential medium was used in place of the F12 medium originally described by Guerrant et al. (8), since this modification decreased the extent of elongation of CHO cells in the absence of enterotoxins.

## RESULTS AND DISCUSSION

Antiserum against purified CT was applied to an immunoaffinity column in which purified LT was coupled to cyanogen bromide-activated Sepharose 4B. Chromatography was carried out as described in Materials and Methods. A typical elution profile from the immunoaffinity column is shown in Fig. 1. The two major peaks in Fig.

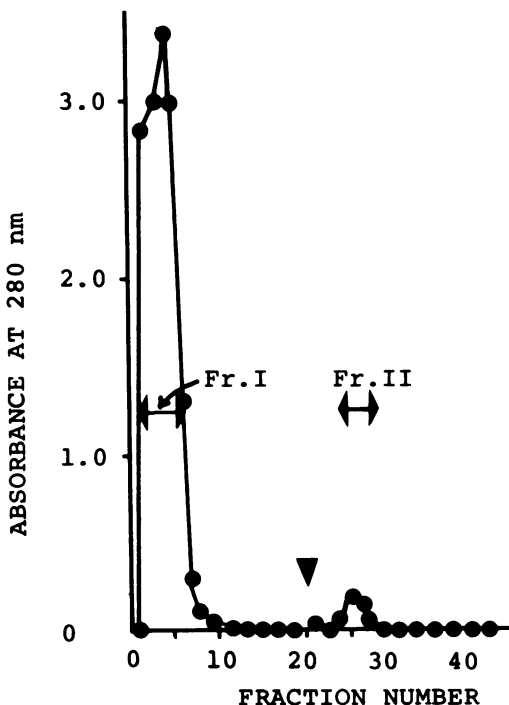


FIG. 1. Immunoaffinity column chromatography of anti-CT on LT-coupled Sepharose 4B. Immunoaffinity column chromatography was carried out as described in the text. Elution with 0.2 M glycine-hydrochloride buffer (pH 2.7) was started at the time indicated by the arrow. Fr., Fraction.

1 were designated fractions I and II, respectively, and each of them was concentrated by ultrafiltration on an Amicon PM-10 membrane to a volume of 1 ml. Similarly, antiserum against purified LT was chromatographed on an immunoaffinity column in which purified CT was coupled to cyanogen bromide-activated Sepharose 4B. A typical elution profile is shown in Fig. 2. The two major peaks, designated fractions III and IV, were each concentrated to 1 ml as described above.

The immunological specificities of fractions I through IV were examined in Ouchterlony double-gel diffusion tests (Fig. 3 and 4). Fraction I gave a precipitin line with CT, but not with LT, whereas fraction III gave a precipitin line with LT, but not with CT (Fig. 3). This observation indicated that a monospecific antiserum to CT was obtained by LT-coupled immunoaffinity column chromatography of anti-CT antiserum, in which antibody against the common antigen to LT was adsorbed to the column and antibody which is not related to LT was not adsorbed to the column. Similarly, a monospecific antiserum to LT was obtained by a CT-coupled immunoaffinity column of anti-LT antiserum. The results in the upper half of Fig. 3 and 4 confirm the previously reported ideas that LT and CT share a common antigen (1, 9, 11) but that each

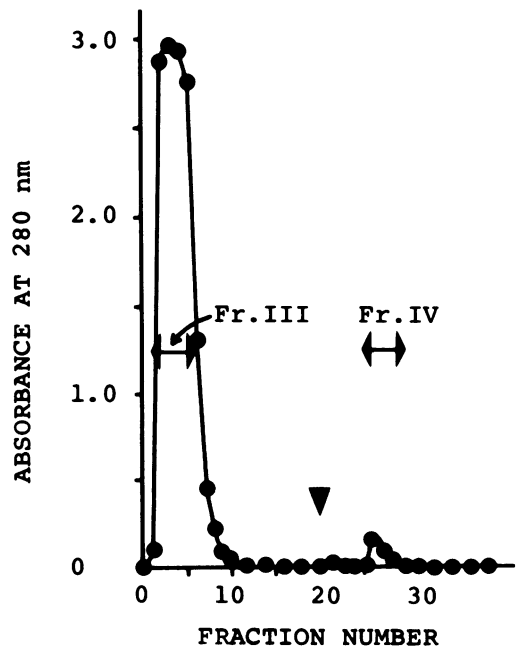


FIG. 2. Immunoaffinity column chromatography of anti-LT on CT-coupled Sepharose 4B. Immunoaffinity column chromatography was carried out as described in the text. Elution with 0.2 M glycine-hydrochloride buffer (pH 2.7) was started at the time indicated by the arrow. Fr., Fraction.

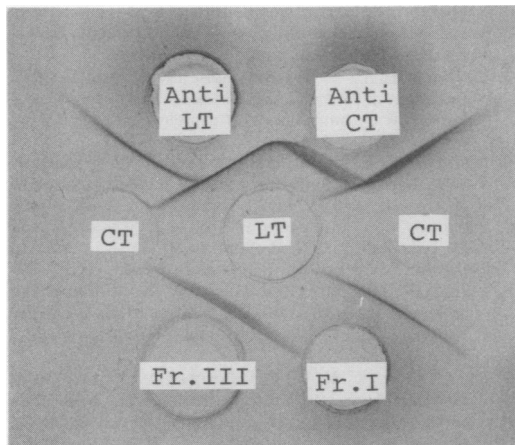


FIG. 3. Ouchterlony double-gel diffusion test with fractions (Fr.) I and III.

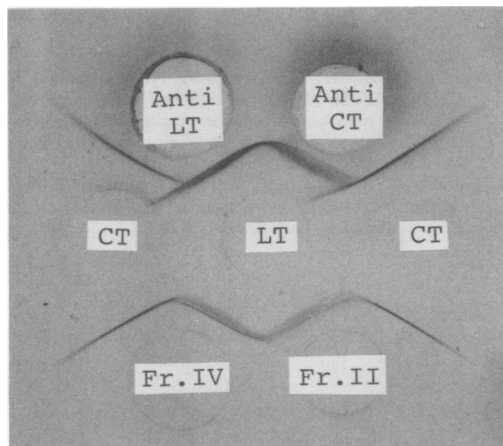


FIG. 4. Ouchterlony double-gel diffusion test with fractions (Fr.) II and IV.

also has a unique antigen (2). The above results showed that antibodies against these unique antigens were isolated by immunoaffinity column chromatographies.

Antibody against the common antigen of CT and LT was adsorbed to an immunoaffinity column coupled with either CT or LT and was eluted with glycine-hydrochloride buffer (pH 2.7). This occurrence was demonstrated by the Ouchterlony double-gel diffusion test (Fig. 4). Fractions II and IV both gave a precipitin line against both LT and CT, and these lines completely fused. The monospecific antisera against CT and LT were designated as anti-CTs and anti-LTs, respectively, and the antisera against the common antigens of CT and LT, derived from anti-CT and anti-LT, were designated as anti-CTc and anti-LTc, respectively.

The immunological specificities of anti-CTs,

TABLE 1. Neutralization of the activities of CT and LT to cause morphological changes in CHO cells by various preparations of antisera

Antiserum	% CHO cells elongated by: <sup>a</sup>	
	CT	LT
Anti-CT	2.2	2.8
Anti-LT	1.7	2.1
Anti-CTs	3.0	49.5
Anti-LTs	52.7	1.4
Anti-CTc	2.1	1.5
Anti-LTc	1.4	2.3
None	44.7	41.5
No toxin	2.2	2.2

<sup>a</sup> A 10- $\mu$ l amount of each antiserum was mixed with either 1 ng of CT or 4 ng of LT and incubated at room temperature for 20 min, and then the activity of the preparation to cause morphological changes in CHO cells was examined as described in the text.

anti-LTs, anti-CTc, and anti-LTc were further demonstrated by neutralizing the activities of CT and LT to cause morphological changes in CHO cells (Table 1). Elongation of CHO cells caused by CT was neutralized by anti-CTs as well as by anti-CT, anti-LT, anti-CTc, and anti-LTc, but not by anti-LTs. Likewise, elongation of CHO cells caused by LT was neutralized by anti-LTs as well as by anti-CT, anti-LT, anti-CTc, and anti-LTc, but not by anti-CTs.

The present data clearly showed the existence of common and unique antigens in CT and LT, as proposed by Clements and Finkelstein (2). The preparations of anti-CTs and anti-LTs may be useful in analyzing the molecular structures of CT and LT. Quite recently, Dallas and Falkow (5) and Spicer et al. (20) reported similarities in the amino acid sequences of the B and A subunits of CT and LT. It will be interesting to determine which parts of the sequence can be recognized by anti-CTs, anti-LTs, anti-CTc, and anti-LTc. Experiments along these lines are now in progress.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by a grant from the Yamada Science Foundation.

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