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Immunological Cross-Reactivity Between a Heat-Labile Enterotoxin(s) of *Escherichia coli* and Subunits of *Vibrio cholerae* Enterotoxin

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Heat-labile enterotoxin from *Escherichia coli* is immunologically related to both subunits, A and B, of cholera enterotoxin as demonstrated by neutralization and immunodiffusion.

An immunological interrelationship between the heat-labile enterotoxin(s) of *Escherichia coli* (LT) and the enterotoxin (choleragen) of *Vibrio cholerae* is firmly established. Gyles and Barnum (14) first reported neutralization of a porcine *E. coli* LT by antisera to cholera enterotoxin. Subsequently, Smith and Sack (26) and Evans et al. (5) demonstrated neutralization of LT rabbit ileal loop response and permeability factor activity, respectively, by antiserum to purified choleragen. Partial neutralization of the effects of several crude preparations of toxigenic *E. coli* on adrenal cells by antisera to cholera toxin was also reported (3). Gyles (13) observed that the neutralizing effect of equine anticholeragenoid (anti-B region of cholera enterotoxin [11]) against both porcine and human LTs was removed by adsorption of the serum with choleragenoid. Immunodiffusion studies subsequently revealed that a precipitin line common to enterotoxin preparations from human and porcine strains of enterotoxigenic *E. coli* gave a reaction of partial identity with the precipitin line between anticholeragenoid and cholera toxin (12). Similar results were recently observed by Richardson et al. (23).

Individuals who respond to immunization with cholera toxoid with significant increases in serum cholera antitoxin titers also show increases in serum *E. coli* enterotoxin neutralizing activity (20).

More recently, Dafni and Robbins (2) claimed purification of heat-labile enterotoxin from *E. coli* by affinity chromatography with antiserum to *V. cholerae* toxin, although not all enterotoxic activity was removed from their crude concentrate even after repeated passes through an immunoabsorbent column, and the specific biological activity of the eluted product was not documented.

The cholera enterotoxin is an 84,000-dalton polymeric protein composed of two major, immunologically distinct regions or domains ("cholera-A" and "cholera-B") (9). Of these, the 56,000-dalton B region, or choleragenoid (11), is responsible for binding of the toxin to the G_{M1} ganglioside receptor on the intact host-cell membrane (9). The A region (28,000 daltons) is responsible for the diverse biological effects of the toxin, which are dependent upon activation of host-cell membrane-associated adenylate cyclase leading to the production of excessive amounts of cyclic AMP. To date, immunological relationships have been shown between *E. coli* LT and choleragen in its B region, only. It should be noted that these observations have been based on crude and partially purified preparations of LT that vary in physicochemical properties from laboratory to laboratory (4, 7, 10, 15, 17-19, 22, 25, 27).

The present study establishes that the *E. coli* LT and choleragen are immunologically related in both the cholera-A and cholera-B regions, as demonstrated by both neutralization of biological activity and immunodiffusion studies.

The strains used were *E. coli* 711(F1LT) (*phe trp pro his* Nx_R *lac*), which is a derivative of strain P307, and its isogenic *tox* parent strain, 711 (kindly provided by S. Falkow). The organisms were cultured at 37°C in Evans medium (6) in three 10-liter fermentor jars (VirTis, Gardiner, N.Y.) with vigorous aeration and agitation for 14 to 16 h after inoculation with 10^6 viable bacteria per ml. The bacteria were removed by continuous-flow centrifugation (40,000 × *g*) at 4°C. The cell-free supernatant was concentrated 50-fold by ultrafiltration on Amicon PM-10 membranes (Amicon Corp., Lexington, Mass.). LT was precipitated with 60% saturated $(NH_4)_2SO_4$, and the precipitate was harvested and dialyzed against TEAN buffer [0.05 M tris(hydroxymethyl)aminomethane, 0.001 M ethylenediaminetetraacetic acid disodium salt, 0.003 M NaN_3, 0.2 M NaCl]. This product, after

concentration by ultrafiltration to 8 ml, is designated "CFS." The bacterial cells were also collected, resuspended, and extracted in 500 ml of 1 M NaCl in phosphate buffer. After centrifugation, the supernatant was dialyzed against TEAN, precipitated with $(NH_4)_2SO_4$, redialyzed against TEAN, and concentrated to 8 ml by ultrafiltration on Amicon PM-10 membranes. This product is designated "EXT."

The cholera enterotoxin subunits, cholera-A and cholera-B, were isolated from pure choleragen (11) by gel filtration under dissociating conditions as described previously (16) and, after sequential dialysis against diminishing concentrations of urea in TEAN buffer, were used as antigens for each of two goats (anti-A and anti-B), which were immunized over a 1-year period. Monospecific equine antiserum to choleragenoid was described previously (8). Specificity of the subunit antisera was demonstrated by radioimmunoassay by a procedure to be described (manuscript in preparation) and by immunodiffusion. Some sera directed against cholera-A were adsorbed with cholera-B to remove any potential contaminating anti-B activity.

The antisera prepared against purified cholera-A or against purified cholera-B completely neutralize the biological activity of LT to a high titer (Table 1).

TABLE 1. *Neutralization of adrenal cell activity[a] of cholera and E. coli enterotoxins[b]*

Antisera	Cholera-gen (8 pg)[c]	*E. coli*	
		CFS (1.13 μg)[c]	EXT (3.39 μg)[c]
Goat anti-cholera-A[d]	1,280[e]	320	320
Goat anti-cholera-B	6,400	12,800	6,400
Equine anticholeragenoid	12,800	12,800	6,400

[a] The adrenal cell assay was conducted with mouse Y-1 adrenal cells in miniculture (24).

[b] Approximately 4 "minimal rounding doses" of toxin were used.

[c] Protein determinations were made according to the method of Bradford (1).

[d] Antiserum was adsorbed with cholera-B to remove any potential anti-B activity.

[e] Reciprocal of highest serum dilution showing complete neutralization of biological activity. Base line sera had no effect.

Immunodiffusion experiments to determine the cross-relationship between *E. coli* LT and the isolated subunits of cholera enterotoxin were performed in 1% Noble agar (Difco), with 1% sodium azide added as a preservative. Immunodiffusion reactions were allowed to develop overnight at room temperature. Wells contained 50 μl and were 9 mm apart, center to center.

Antiserum to cholera-A reacted with concentrated *E. coli* supernatant preparations (CFS) and formed a precipitin line that showed partial identity with cholera-A (Fig. 1A.1, A.6). Antiserum to cholera-B forms two closely spaced precipitin lines with CFS (Fig. 1A.2, A.5), both of which merge with the cholera-B anti-cholera-B band (Fig. 1A.2, A.3). Of these, one line merges with the line given by antiserum to cholera-A and CFS (Fig. 1A.1). Control wells show no precipitation of cholera-B by antiserum to A (Fig. 1A.3, A.4) or of cholera-A by antiserum to B (Fig. 1A.5, A.6).

Isolated specific anticholeragenoid antibody (EAG), obtained by immunoaffinity chromatography of monospecific equine anticholeragenoid (21), produces two precipitin bands with the concentrated salt extract (EXT) (Fig. 1B.4). Of these, one merges with the band recognized by the antiserum to A (Fig. 1B.3, B.4) and the antiserum to B (Fig. 1B.4, B.5); the other is continuous with the second precipitin line recognized by antiserum to B only (Fig. 1B.4, B.5). CFS does not show this second line with EAG (Fig. 1B.1).

Preadsorption of antiserum to A with cholera-A and of antiserum to B with cholera-B prevents the formation of a precipitin line between either antiserum and its respective antigen or with CFS (Fig. 1C) or EXT (Fig. 1D).

Treatment of the *E. coli* antigen preparations with rabbit antiserum prepared against concentrated CFS from the isogenic *tox* parent strain had no effect on the recognition by antiserum to B (Fig. 1E) or by antiserum to A of either *E. coli* preparation. The antiserum against CFS of the *tox* parent strain gave multiple precipitin bands against both CFS and EXT from the tox^+, but none of these bands corresponded with the bands which resulted with either cholera antiserum (data not shown).

We have presented conclusive evidence that the LT preparations described here have antigenic determinants common to each of the isolated subunits of cholera enterotoxin. The biological activity of both preparations, CFS and EXT, is completely neutralized by specific antiserum to each of the cholera subunits (A and B). By immunodiffusion, there appears to be one molecular species possessing determinants immunologically related to both (A and B) subunits of cholera toxin, possibly representing intact *E. coli* enterotoxin, and a second molecular species with determinants related to the B subunit of cholera toxin only. The precipitin lines establishing these immunological interrelationships are (i) abolished by preadsorption of the antisera to cholera subunits by their homologous antigens, (ii) not affected by preadsorption of

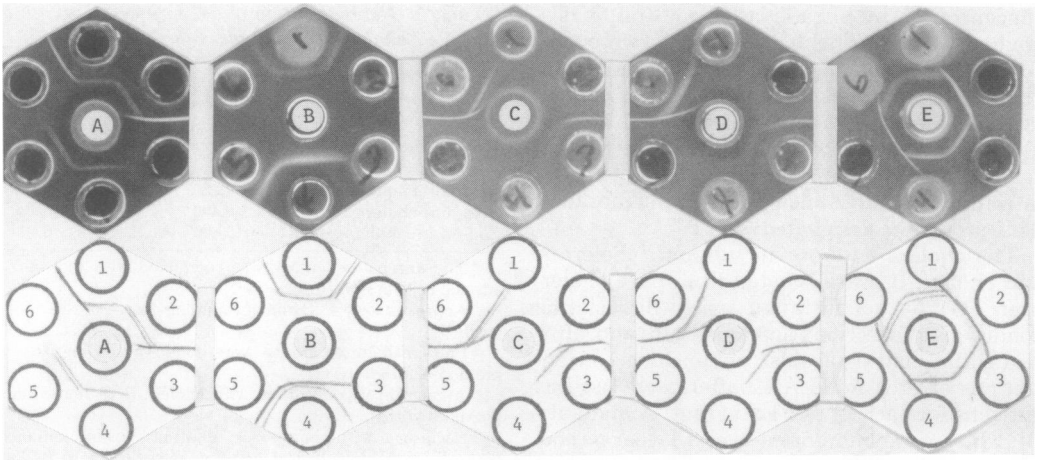

FIG. 1. *(A) Reactions of antiserum to cholera-A (wells 1 and 4) and antiserum to cholera-B (wells 2 and 5) with E. coli-concentrated supernatant (CFS) (center well). Wells 3 and 6 contain 5 μg of isolated cholera subunits B and A, respectively. One precipitin line given by both antisera shows a reaction of partial identity with both cholera subunits. A second line given by antiserum to B is cross-reactive with B only. Antisera do not recognize their heterologous antigens. (B) Reaction of antiserum to cholera-A (wells 3 and 6), antiserum to cholera-B (wells 2 and 5), and purified equine anticholeragenoid antibodies (EAG) (center well) with E. coli-concentrated supernatant (CFS) (well 1, 800 μg) and salt extract (EXT) (well 4, 1,600 μg). EAG produces two precipitin bands with EXT: one identical with the band recognized by anti-A and anti-B and one identical with the band recognized by anti-B only. Only one band, the anti-A and anti-B band, is given between EAG and CFS. (C and D) Preadsorption of antiserum to A with cholera-A (well 1) and of antiserum to B with cholera-B (well 4) prevents the formation of a precipitin line between either antiserum and its respective antigen (as shown by cholera-A/anti-A [wells 2/3] and by cholera-B/anti-B [wells 5/6]) or with CFS (800 μg) (C, center well) or EXT (1,600 μg; D, center well). (E) Pretreatment of E. coli CFS (well 1) or EXT (well 4) with rabbit antiserum to CFS of the isogenic tox parent strain has no effect on the recognition of CFS (well 6) or EXT (well 3) by antiserum to cholera-B (center well). Wells 2 and 5 contain cholera-B.*

either *E. coli* preparation by antiserum to the isogenic *tox* parent *E. coli* strain, and (iii) also recognized by purified equine antibodies to choleragenoid. Lack of complete identity between *V. cholerae* and *E. coli* products is shown by spurring reactions in immunodiffusion (e.g., Fig. 1A.2, A.3, A.6). Although we are dealing with cross-reactive antigens and therefore are limited in making valid comparisons, our results also suggest that the amounts of cholera-related antigens produced by *E. coli* are very much less than those produced by *V. cholerae,* and, furthermore, their distribution may differ in CFS and EXT.

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