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Immunological Relationship of Different Preparations of Coliform Enterotoxins

FREDERICK A. KLIPSTEIN* AND RICHARD F. ENGERT

Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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Antisera raised in rabbits to ultrafiltrate toxin preparations containing either the heat-labile (LT) toxin form obtained from whole cell lysates or broth filtrates or the heat-stable (ST) toxin form prepared from broth filtrates from nontoxigenic and toxigenic strains of *Escherichia coli* and *Klebsiella* were examined for their ability to neutralize the secretory effect on water transport of these toxins in the rat jejunum as determined by the in vivo marker perfusion technique. Antisera to the heat-labile toxin derived from whole cell lysate preparations from nontoxigenic strains had no neutralizing effect. Antisera to both types of LT preparation from both toxigenic strains neutralized, with several exceptions, all of the homologous and heterologous LT toxins as well as a heat-labile toxin preparation derived from sequential ultrafiltration of cell-free whole cell lysates which had a defined molecular weight of between 30,000 and 100,000. These antisera also neutralized homologous and heterologous ST preparations obtained from broth filtrates, but they had no neutralizing effect on low-molecular-weight, ST toxin material obtained during the sequential ultrafiltration of cell lysates. Antisera to ST prepared from broth filtrates had no neutralizing capacity against either LT or ST toxin preparations. These observations (i) indicate that the immunological relationship of *E. coli* and *Klebsiella* LT and ST toxins extends to antisera raised against LT prepared by several different methods, (ii) raise the possibility that, based on the response to antisera to LT, there may be several immunologically heterogeneous forms of low-molecular-weight ST toxin, and (c) confirm the lack of immunogenicity of ST.

Transient colonization of the small intestine by strains of *Escherichia coli* that produce a heat-labile (LT) or heat-stable (ST) toxin, either singly or together, is a well recognized cause of episodes of acute diarrhea, particularly among persons living in the tropics (25, 26, 28). Strains of *Klebsiella pneumoniae* are also sometimes isolated from such individuals and, in some, may represent the predominant or only coliform species present (9, 10, 22, 34); when tested, some of these strains have been found to be enterotoxigenic with the capacity to elaborate LT and/or ST forms of toxin as determined by the Chinese hamster ovary (9, 10, 34) or in vivo perfusion assays (17, 19).

The high-molecular-weight LT toxin of *E. coli* is antigenic, and antisera developed against it have been found to afford protection against the action of this toxin in inducing diarrhea in infant rabbits (31), water secretion during perfusion in the rat jejunum (16), fluid accumulation in ligated ileal loops (4, 12, 13, 27, 32) and in stimulating adenyl cyclase as tested by various assay systems (2, 8, 35). *E. coli* LT shares an immunological cross-reactivity with cholera toxin, and antisera against either neutralize the actions of both toxins (13, 23, 31, 33). This immunological relationship appears to extend to the LT toxins of other coliform species. Guerrant and his colleagues found that antiserum to cholera toxin inhibits the effect of the *Klebsiella* LT toxin in the Chinese hamster ovary assay system (10). This laboratory has reported that both equine antiserum to cholera toxin as well as antisera developed in rabbits to preparations containing the LT toxins of *E. coli*, *Klebsiella*, and *Enterobacter cloacae* have a neutralizing effect on the ability of both homologous and heterologous LT toxins to induce water secretion in the rat jejunum. We also observed that the neutralizing effect of these antisera extends, to a lesser extent, to a low-molecular-weight, ST toxin form of several of these species (16).

These observations (16) prompted us to conduct a number of additional studies, the results of which are described in this report. (i) Although we found that normal rabbit serum had no neutralizing effect on the LT and ST toxins

tested, it seemed important to establish the fact that antisera to preparations made in an identical manner to that which we had used for immunization against the LT toxins but derived from nontoxigenic (NTG) strains have no neutralizing capacity. (ii) The material containing LT toxin used for immunization in our previous study consisted of an ultrafiltrate prepared from whole cell lysates. It seemed important to determine whether antisera to preparations of LT toxin obtained from another source, specifically from broth filtrates, would also manifest the same immunological properties. (iii) The antisera were tested in our previous study against the secretory effect of LT toxin preparations which contained all material with a molecular weight of greater than 30,000. It seemed important to determine whether the neutralizing effect of the antisera that we had observed was directed against toxin material, which has a molecular weight within a clearly defined range, rather than possibly against other large-molecular-weight material present in the ultrafiltrate. (iv) Finally, although experience in other laboratories has indicated that crude preparations of *E. coli* ST are not immunogenic (4, 11, 32), it seemed of value to determine whether such is the case when semipurified ultrafiltrate preparations containing this material are used for immunization.

Rabbits were immunized with ultrafiltrates containing material made as if to contain the LT toxin from NTG strains of *E. coli* and *Klebsiella* and with ultrafiltrates of LT toxin, prepared by various techniques, and ST toxin from toxigenic (TOX) strains of these species. The neutralizing capacity of the antisera thus raised was determined by ascertaining their ability to inhibit the secretory effect of these various toxin preparations, as well as toxin materials with clearly defined molecular weights which were derived from sequential ultrafiltration, of the TOX strains as measured by the in vivo marker perfusion technique in the rat jejunum.

MATERIALS AND METHODS

Bacterial strains. (i) TOX strains. *E. coli* strain 334 (API profile biotype 5044572; serotype O15:H11) was isolated from an Indian in Calcutta with acute undifferentiated diarrhea (7); it has been used in a number of previous immunological investigations (4, 8). *K. pneumoniae* strain TS-19 (API profile biotype 5205573; serotype 19) was isolated from the jejunum of a Puerto Rican with untreated tropical sprue (18).

(ii) NTG strains. *E. coli* GU-1191 (API profile biotype 5144552) and *Klebsiella* GU-558 (API profile biotype 5205773) were both isolated from the urinary tracts of healthy individuals. The results of various assays for enterotoxigenicity that were performed on these strains are shown in Table 1. The Chinese hamster ovary (8) and suckling mouse (1) assays were performed by Richard L. Guerrant, Charlottesville, Va.

TABLE 1. *Enterotoxigenicity of strains examined*

Strain	Assay			
	CHO[a]	SM[b]	Rat perfusion MEC[c]	
			LT	ST
TOX *E. coli* 334	16	0.1187	1 ng	0.1 ng
NTG *E. coli* GU-1191	3	0.0681	NTG	NTG
TOX *Klebsiella* 19	24	0.0935	1 ng	0.1 ng
NTG *Klebsiella* GU-558	4	0.0709	NTG	NTG

[a] Percent of Chinese hamster ovary (CHO) cells elongated. Values greater than 13.5% are considered positive for LT toxigenicity (8).

[b] Suckling mouse assay. Values greater than 0.0875 are considered positive for ST toxigenicity (1).

[c] MEC, Minimum effective concentration required to induce water secretion. Values of 10 ng per ml or less are considered positive for toxigenicity (17). NTG indicates that the toxin fraction was inactive at the highest concentration tested.

Preparation of toxin fractions. The methods used for preparing toxin fractions have been described in detail previously (17). Briefly, LT fractions from whole cell lysates (LT-WCL) were prepared by treating the harvested confluent growth of aerobic agar cultures on a Sonifier cell disrupter (Branson Sonic Power Co., Plainview, N.J.) for 10 min; the suspension thus obtained was centrifuged at 4°C for 45 min at 30,000 × *g*, and the supernatant was passed through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). The cell-free material was passed through an Amicon PM-30 ultrafiltration membrane (Amicon Corp., Lexington, Mass.), which has a molecular weight cutoff of 30,000; the retentate fractions were washed twice with 2× the original volume, using 0.02 M ammonium bicarbonate (pH 7.8). LT from broth filtrates (LT-BF) was obtained after agitated aerobic growth in Trypticase soy broth (BBL, Cockeysville, Md.). The cultures were centrifuged, the supernatant was passed through a 0.45-μm membrane, and the filtrate was precipitated with 90% saturated ammonium sulfate. The precipitate was redissolved in 0.02 M ammonium bicarbonate and then fractionated by ultrafiltration as described for WCL-LT. The ultrafiltration retentates were lyophilized and stored at −20°C until assay.

ST fractions were prepared by stationary anaerobic growth in Trypticase soy broth, followed by centrifugation and passage of the supernatant through a 0.45-μm membrane; the filtrate was precipitated with 8 volumes of acetone, and the precipitate was then solubilized in distilled water. This material was passed sequentially through UM-10 (molecular weight cutoff, 10,000) and UM-05 (molecular weight cutoff, 500) ultrafiltration membranes. The UM-05 retentate was washed twice with 0.02 M ammonium bicarbonate and the material was then lyophilized and frozen until assay.

Immunization procedure. Each toxin preparation

was administered to two New Zealand white rabbits. A 5-mg portion of toxin, as measured by protein content (determined by the Lowry technique [20]), was made up to 1 ml in saline and mixed with 1.0 ml of complete Freund adjuvant; 0.25 ml of this material was injected into each footpad. After 2, 4, and 6 weeks, 2 mg (by protein) of the toxin preparation in 0.5 ml sterile saline was injected intravenously. Serum was obtained for study 9 weeks after the initial immunization procedure.

Perfusion technique. The procedures used for the evaluation of water transport by means of the in vivo marker perfusion technique in rats have been described in detail (19, 24). Single 20-cm jejunal segments of anesthetized, tracheostomized Charles River Sprague-Dawley rats were perfused with the toxin fractions contained in isosmolar balanced electrolyte solution (24) at a rate of 0.5 ml/min using a peristaltic pump (model 1201, Harvard Apparatus Co., Millis, Mass.). The osmolalities of all test perfusates were directly determined by measuring the freezing point depression, and each was shown to be isosmotic with rat plasma at 317 mosmol/kg (19). Six 30-min test fractions were collected after a 30-min steady-state period. Net transport of water, expressed in microliters per centimeter per 30 min, was calculated by using the usual marker technique formula from changes in polyethylene glycol 4000 concentration, which were determined by the method described by Malawer and Powell (21). Values reported are for the mean of the six perfusion periods in each animal. Net lumen-to-blood transport is termed absorption and is signified by a plus sign, whereas a minus sign refers to net blood-to-lumen transport or secretion.

Water transport in 30 rats perfused with the electrolyte solution alone ranged from absorption of +21 to 78 μl with a mean ± standard error of the mean of +46 ± 3 μl/cm per 30 min. Values reported for the secretory action of the toxin preparations alone represent the mean for perfusion assays in five or more individual rats and those for these preparations after exposure to either heat or each of the antisera are the average for perfusion in two rats. Toxin dosages perfused were selected on the basis that they were the minimum ones that consistently yielded a mean value of net secretion during the six perfusion periods; this was achieved by concentrations of 10 ng/ml for LT-WCL and ST preparations, and 1 μg/ml for the LT-BF preparations of the TOX strains. The effect of exposure of the toxins to 100°C for 30 min is shown in Table 2.

Neutralization. The neutralizing capacity of the various antisera was evaluated by determining their ability to inhibit the capacity of the various toxin preparations to induce water secretion in the jejunum. Either 1 or 3 ml of antiserum was incubated at 37°C with the toxin preparations for 30 min before perfusion. Complete neutralization of toxin activity was considered indicated by reversal from secretion to absorption values within the normal range (>+21 μl/cm per 30 min), and partial neutralization was indicated by absorption values of between 0 and +21 μl/cm per 30 min (16). The statistical difference between different groups was determined by Student's *t* test for independent means.

We have previously reported the absence of neutralizing effect in this test system of normal rabbit serum and the positive neutralizing effect of equine cholera antiserum against the secretory action of the LT-WCL and ST toxin fractions of the two TOX strains used in this study (16).

RESULTS

Neutralizing capacity of antisera to preparations from NTG strains. The addition of either 1 or 3 ml of antisera to the large-molecular-weight retentates of whole cell lysates (made identically as the LT-WCL preparations from TOX strains) prepared from the NTG strains of *E. coli* and *Klebsiella* had no neutralizing effect on the secretory activity of either the LT or ST toxin preparations of the TOX strains of these species (Table 2).

Neutralizing capacity of antisera to LT toxin preparations from TOX strains. The addition of either 1 or 3 ml of antisera to the LT-

TABLE 2. *Neutralizing effect of antisera to LT-WCL preparations from NTG strains*[a]

Perfused		Alone	Heated[c]	Antiserum added					
				E. coli		*Klebsiella*		Mean[d]	*P*[e]
Strain	Toxin[b]			1 ml	3 ml	1 ml	3 ml		
TOX *E. coli*	LT-WCL	−22 ± 6	+25	−5	−11	−14	−6	−9 ± 2	NS
TOX *E. coli*	LT-BF	−10 ± 2	+28	−6	−3	−11	−9	−7 ± 2	NS
TOX *E. coli*	ST	−17 ± 4	−20	−6	−6	−7	−9	−7 ± 1	NS
TOX *Klebsiella*	LT-WCL	−16 ± 4	+27	−6	−5	−8	−5	−6 ± 1	NS
TOX *Klebsiella*	LT-BF	−11 ± 2	+13	−13	−3	−4	−4	−6 ± 2	NS
TOX *Klebsiella*	ST	−28 ± 4	−19	−8	−6	−4	−12	−8 ± 2	NS

[a] Values represent water transport in microliters per centimeter per 30 min.

[b] LT-WCL and ST preparations were perfused at a concentration of 10 ng per ml; LT-BF was perfused at 1 μg per ml.

[c] After exposure to 100°C for 30 min.

[d] Mean value plus standard error of the mean for all perfusions where antiserum was added.

[e] The *P* value indicates whether there was a statistically significant difference between these values and those for perfusion with the toxin alone.

WCL of the TOX strains of *E. coli* and *Klebsiella* resulted in neutralization in every instance of the secretory activity of both the homologous and heterologous LT toxins (prepared either from whole cell lysates or broth filtrates) and ST toxin preparations (Table 3). All preparations were completely neutralized except for the following where neutralization was partial: the homologous and heterologous LT-BF by antiserum to *Klebsiella* LT-WCL, the *Klebsiella* LT-BF preparation by antiserum to *E. coli* LT-WCL, and *E. coli* ST antiserum to *E. coli* LT-WCL.

Antisera to the LT-BF of *E. coli* and *Klebsiella* produced complete neutralization in one instance: 3 ml of antiserum to *Klebsiella* LT-BF completely neutralized the secretory activity of the same toxin preparation (Table 3). These antisera produced partial neutralization of the secretory effect of all of the other preparations with these exceptions: they neutralized the homologous but not the heterologous preparations of WCL-LT, and antiserum to *E. coli* LT-BF did not neutralize the ST preparation of *Klebsiella*.

Neutralization of toxin fractions separated on the basis of molecular weight. To determine whether the neutralizing effect of antisera in the variously prepared LT toxin preparations was directed against an LT toxin fraction with a specific molecular weight, cell-free preparations derived from whole cell lysates were sequentially passed through a series of four ultrafiltration membranes. The activity of the washed retentate of each membrane was then tested by perfusing this material, in serial 10-fold dilutions starting at a concentration of 100 μg/ml, through the rat jejunum to determine the minimum effective concentration at which it induced water secretion. All ultrafiltrate fractions of the NTG strains were inactive. The XM-300 (molecular weight, >300,000) and the XM-100 (molecular weight, 100,000 to 300,000) retentates of the TOX *E. coli* and *Klebsiella* strains were either inactive or evoked secretion only at a concentration of 100 μg/ml. The PM-30 retentates (molecular weight, 30,000 to 100,000) of these strains yielded highly potent material that was found to be heat labile (Table 4). The addition of antisera to either type of LT preparation (LT-WCL or LT-BF) of either *E. coli* or *Klebsiella* neutralized the secretory activity of each of the homologous and heterologous LT toxin preparations in this molecular weight range. The addition of 3 ml of each of the antisera yielded partial neutralization in the case of the *E. coli* toxin ultrafiltrates and complete neutralization in the case of the *Klebsiella* ultrafiltrates so tested.

The UM-05 retentate (molecular weight 500 to 30,000) of both TOX strains also yielded potent secretory material which proved to be heat stable (Table 4). The addition of either 1 or 3 ml of antisera to the various *E. coli* and *Klebsiella* LT preparations had no neutralizing effect on the secretory action of this material.

Neutralizing capacity of antisera to the ST toxin preparations from TOX strains. Antisera produced against the ST toxin derived from broth filtrate of TOX strains of *E. coli* and *Klebsiella* had no neutralizing effect against any of the LT or ST toxin preparations of these strains (Table 5).

Neutralizing capacity of the antisera based on Δ water. The criterion used in Tables 2–5 for a positive neutralizing effect of the various antisera was reversal of the absolute value of water transport from secretion to absorption. By this criterion, none of the individual antisera evaluated in Tables 2 and 5 were active, and there was no statistically significant difference between values for absolute water transport in rats perfused with the different toxin preparations alone and those to which these antisera had been added. Although water transport was not reversed to absorption, in some instances the addition of antisera appeared to diminish the amount of secretion, i.e., the values moved in the direction of absorption although not

TABLE 3. *Neutralizing effect of antisera to different LT preparations from TOX strains*[a]

Perfused			Antiserum added							
			E. coli				*Klebsiella*			
Strain	Toxin	Alone	LT-WCL		LT-BF		LT-WCL		LT-BF	
			1 ml	3 ml	1 ml	3 ml	1 ml	3 ml	1 ml	3 ml
TOX *E. coli*	LT-WCL	−22 ± 6	+25		−6	+4	+23		−6	−6
TOX *E. coli*	LT-BF	−10 ± 2	+6	+27	−5	+6	−5	+5	−4	+12
TOX *E. coli*	ST	−17 ± 4	+4	+13	−7	+2	+33		−2	+14
TOX *Klebsiella*	LT-WCL	−16 ± 4	+21		−9	−2	+21		−8	+5
TOX *Klebsiella*	LT-BF	−11 ± 2	−13	+2	−10	+4	−7	+8	+3	+27
TOX *Klebsiella*	ST	−28 ± 4	+47		−7	−4	+24		+1	+12

[a] Values represent water transport in microliters per centimeter per 30 min.

TABLE 4. *Neutralizing effect of antisera to LT preparations from TOX strains on sequentially separated whole cell lysate preparations*[a]

Strain	Mol wt of prepn	Alone[b]	Heated[c]	Antiserum added								Mean[d]	P[e]
				E. coli				*Klebsiella*					
				LT-WCL		LT-BF		LT-WCL		LT-BF			
				1 ml	3 ml	1 ml	3 ml	1 ml	3 ml	1 ml	3 ml		
TOX *E. coli*	30–100	−11 ± 4	+16	+3	+6	+5	+3	+3	+10	−2	+9	+5 ± 1	<0.01
TOX *E. coli*	0.5–30	−6 ± 2	−4	−2	−1	−5	−5	−12	−9	−9	−13	−7 ± 2	NS
TOX *Klebsiella*	30–100	−14 ± 4	+30	+10	+33	+12	+26	+8	+25	+20		+19 ± 4	<0.001
TOX *Klebsiella*	0.5–30	−10 ± 3	−5	−11	−15	−15	−16	−3	−6	−7	−8	−10 ± 2	NS

[a] Values represent water transport in microliters per centimeter per 30 min.
[b] The *E. coli* 0.5 to 30 molecular weight fraction was perfused at a concentration of 100 ng per ml; all other fractions were perfused at 10 ng per ml.
[c] Exposed to 100°C for 30 min.
[d] Mean value plus standard error of the mean for all perfusions where antiserum was added.
[e] The *P* value indicates whether there was a statistically significant difference between these values and those for perfusion with the toxin alone. NS, No significant difference.

TABLE 5. *Neutralizing effect of antisera to ST toxin preparations from TOX strains*[a]

Perfused		Alone	Antiserum added				Mean[b]	P[c]
			E. coli ST		*Klebsiella* ST			
Strain	Toxin		1 ml	3 ml	1 ml	3 ml		
TOX *E. coli*	LT-WCL	−22 ± 6	−6	−3	−4	−11	−6 ± 3	NS
TOX *E. coli*	LT-BF	−10 ± 2	−8	−9	−9	−3	−7 ± 1	NS
TOX *E. coli*	ST	−17 ± 4	−12	−6	−20	−7	−11 ± 3	NS
TOX *Klebsiella*	LT-WCL	−16 ± 4	−11	−4	−4	−5	−6 ± 2	NS
TOX *Klebsiella*	LT-BF	−11 ± 2	−5	−11	−5	−7	−7 ± 1	NS
TOX *Klebsiella*	ST	−28 ± 4	−14	−4	−15	−8	−10 ± 3	NS

[a] Values represent water transport in microliters per centimeter per 30 min.
[b] Mean value plus standard error of the mean for all perfusions where antiserum was added.
[c] The *P* value indicates whether there was a statistically significant difference between these values and those for perfusion with the toxin alone. NS, No significant difference.

reaching values in the absorption range. In addition, certain of antisera which were called positive based on this criterion yielded only partial neutralization with absorption values of between 0 and +21 μl/cm per 30 min (Tables 3 and 4). These observations led us to consider whether there was a significant difference between values called inactive or partial neutralization when considered in terms of Δ water transport. Δ water is defined as the change in water transport, in microliters per centimeter per 30 min, between values when the toxin is perfused alone and then with antisera.

To answer this question, we analyzed the data in Table 3 from several points of view. The first question we asked was whether those values in this table, which are regarded as positive for neutralization on the basis of absolute values, differ significantly from those considered inactive when compared in terms of Δ water. The answer is yes. Values of Δ water (mean ± standard error of the mean in microliters per centimeter per 30 min) for antisera as yielding either complete (Δ 46 ± 4) or partial (Δ 22 ± 2) protection were significantly greater ($P < 0.001$) than those called inactive (Δ 10 ± 2). The second question was whether those values which were considered positive in terms of absolute water transport differ significantly, when expressed as Δ water, from values given in Tables 2 and 5, all of which were considered inactive in terms of absolute water transport. The answer again is yes. Values or Δ water were statistically significantly greater for all groups of antisera to LT

preparations from TOX strains than for antisera to either the LT preparations from NTG strains or to ST (Table 6).

DISCUSSION

The major purpose for conducting the studies described in this report was to provide supplementary information, principally of a control nature, to our previously reported observations which indicated that antisera developed to LT toxin preparations of either *E. coli* or *Klebsiella* have an immunological relationship (16). Current evidence indicates that there is an antigenic similarity among the LT toxins produced by various different strains of *E. coli* (28, 29); however, in view of the fact that recent studies concerned with purification have shown that the molecular weight and certain other properties of LT depend on the method of toxin production (3, 5, 6, 30), it remains uncertain whether these different forms of *E. coli* LT share the same exact antigenic properties. Since we used rather impure preparations of LT toxin consisting of all material with a molecular weight of >30,000 derived from whole cell lysates in our previous study, it seemed important to ascertain the immunogenicity of preparations made in this manner from NTG strains as well as that of LT toxins prepared by other techniques from the TOX strains, and to evaluate the neutralizing capacity of these various antisera against LT toxin preparations which have a clearly defined molecular weight such that large-molecular-weight material unrelated to the toxins had been excluded.

We found that antisera to the LT preparations from NTG strains were inactive; absolute values for water transport were not significantly increased above those for perfusion with the toxin alone, and values for Δ water transport following the addition of these antisera were significantly less than those following the addition of antisera to the same preparations from TOX strains. Antisera to the LT preparations made from broth filtrates of TOX strains were active in neutralizing the homologous and heterologous LT and ST toxins, except in the case of both heterologous LT-WCL toxin preparations and the heterologous LT toxin preparation in one instance. In general, antisera to LT-WCL preparations were less active against the LT-BF toxin preparations, usually requiring 3 ml of antiserum to neutralize the secretory activity, and the same was true of the neutralizing capacity of the antisera to the LT-BF preparations against all of the LT toxin preparations. We suspect, but cannot prove, that this was related to the fact that the potency of the LT in LT-BF preparations was 100-fold less than that of the other preparations; thus the antigen used for immunization was weaker and the total amount of this toxin required for perfusion to test the neutralizing capacity of the various antisera was greater. Antisera to both types of LT preparation neutralized homologous and heterologous LT toxin fractions that were contained in material within a defined range of molecular weights; we do not know why the neutralization of the *Klebsiella* ultrafiltrate was more complete than that of the *E. coli* toxin ultrafiltrate, but we suspect that this was related to the fact that a larger amount of toxin ultrafiltrate had to be used for perfusion in the case of the *E. coli* preparation.

The evidence available from the present and our previous study (16) which suggests that an immunological cross-reactivity exists between the LT toxins of *E. coli* and *Klebsiella* may now be summarized as follows. (i) Control preparations consisting of either normal, nonimmunized rabbit serum or antisera to preparations made similarly to those containing LT toxin but obtained from NTG strains are inactive. (ii) Anti-

TABLE 6. *Effect of inactive versus active neutralizing antisera in terms of Δ water transport*[a]

Toxin	Antiserum added[b]					
	NTG LT	TOX ST	TOX LT-WCL[c]		TOX LT-BF[c]	
			Mean	*P*	Mean	*P*
LT	8 ± 1		30 ± 5	<0.001	22 ± 3	<0.001
ST	15 ± 2		46 ± 9	<0.01	30 ± 4	<0.02
LT		7 ± 1	30 ± 5	<0.001	22 ± 3	<0.001
ST		12 ± 3	46 ± 9	<0.01	30 ± 4	<0.01

[a] Values are the mean ± standard error of the mean for Δ water (in microliters per centimeter per 30 min) between values obtained during perfusion with the toxin alone and after incubation with specific antiserum.

[b] Values for NTG LT (Table 2) and TOX ST (Table 5) were considered inactive and those for TOX LT-WCL and TOX LT-BF (Table 3) were considered to yield neutralization based on absolute values for water transport.

[c] *P* values indicate the statistical difference between values in this column and those obtained with antisera to either NTG LT or ST.

sera to LT preparations prepared either from whole cell lysates or broth filtrates exhibit, in nearly all instances, cross neutralization to the heterologous toxin preparations. (iii) These active antisera also neutralize the secretory activity of potent LT toxin ultrafiltrates whose molecular weight falls within a well-defined range. (iv) Adsorption of antisera to the LT-WCL toxin preparations of either *E. coli* or *Klebsiella* by incubation with a heat-inactivated LT toxin preparation of either the homologous or heterologous strain completely abolishes their neutralizing capacity.

Our observations also continue to suggest that an immunological relationship exists between the LT and ST toxins of both *E. coli* and *Klebsiella*. Antisera to both the LT-WCL and LT-BF toxin preparations of *E. coli* and *Klebsiella* neutralized, with one exception, both the homologous and heterologous ST toxin preparations; antisera to similarly made LT preparations from NTG strains did not. We have previously shown that in the case of antiserum to the LT-WCL toxin preparation, this neutralization can be abolished by adsorption of the antiserum by a heat-inactivated preparation of either the homologous or heterologous LT toxin (16).

In the course of attempting to obtain a better defined LT toxin preparation by means of sequential ultrafiltration of whole cell lysates, we found that ultrafiltrates of both *E. coli* and *Klebsiella* which had an apparent molecular weight in the range of between 500 and 30,000 contained potent material that induced water secretion and, further, that this activity was heat stable. We had been unaware that an ST toxin could be obtained from whole cell lysates, and we have no idea as to the nature of this material other than the fact that its molecular weight and heat lability characteristics are similar to those described in the literature for the ST toxins of *E. coli* (14) and *Klebsiella* (15). We had presumed that this ST material was similar to, or identical with, that contained in the ultrafiltrate fractions of broth filtrates that were used elsewhere in both the present and previous studies, since both have the same apparent molecular weight. Immunological studies, however, suggest that such is not the case: whereas the secretory activity of preparations derived from broth filtrates was neutralized by antisera to LT, that of the preparations obtained from whole cell lysates was completely unaffected by any of these antisera. We can only conclude from these observations that there appear to be different, immunologically heterogeneous forms of low-molecular-weight, ST toxin material. It should be emphasized that although we have identified two apparently dissimilar low-molecular-weight, ST materials that are capable of inducing water secretion, we do not know whether either of these is identical with the ST toxin that is usually identified by a positive response in the suckling mouse assay and which has been shown to be a causative agent of acute diarrhea (26). In the final analysis, the definitive assessment of the immunological relationship between LT and ST toxins awaits evaluation of the immunological response between antiserum to completely purified LT and totally pure ST.

Finally, we found no evidence that the ST toxin preparation derived from broth filtrates is itself immunogenic. Such would not be anticipated to be the case in view of the small apparent molecular weight of this material (14, 15), but since only crude preparations of ST were used as the antigen and the immunological response was assayed by the relatively insensitive ligated rabbit ileal loop model in previous studies which have considered this (4, 11, 32), we thought it would be worthwhile to see if, by using a more purified ultrafiltrate preparation of ST for the antigen and the more sensitive perfusion technique as the assay for the neutralizing response, an immunogenic response could be detected; no immunogenic response was detected.

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