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# Immunological Properties of Purified *Klebsiella pneumoniae* Heat-Stable Enterotoxin

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*Klebsiella pneumoniae* heat-stable enterotoxin was purified to apparent homogenicity by the same techniques used to purify *Escherichia coli* heat-stable enterotoxin. The two toxins had the same potency in the suckling mouse assay and showed immunological cross-reactivity in enzyme-linked immunosorbent assay, neutralization of secretory activity by specific hyperimmune antisera, and protection against active challenge in rats immunized with a vaccine containing synthetically produced *E. coli* heat-stable enterotoxin.

Diarrheal epidemics among nursery children in which *Klebsiella pneumoniae* was the principal enteric bacteria isolated from stool cultures were first described by Olarte and his colleagues in Mexico more than two decades ago (22). The epidemic nature of the diarrhea and the consistent isolation of a single serotype (type 19) of *Klebsiella* led to their suggestion at that time that this organism might be the causal agent, but assays for enterotoxigenicity were not then available. Subsequently, *Klebsiella* sp. has been isolated from the stool of infants during nursery epidemics of acute diarrhea in the United States (8) and India (3); among children with sporadic episodes of diarrhea in Brazil (9), Ethiopia (26), and South Africa (24); and among Swedish expatriates who developed diarrhea while living in Cyprus (2). The enterotoxigenicity of these *Klebsiella* strains was established by assay of cell-free culture filtrates in rabbit ileal loops (3, 8, 9, 26), in Y1 adrenal cell or Chinese hamster ovary tissue culture assays for heat-labile toxin (LT) (2, 8, 9, 24, 26), and in the suckling mouse assay for heat-stable toxin (ST) (8, 24). The *Klebsiella* LT and ST enterotoxins identified by these assays have not been purified, and their relationship to similar toxins produced by *Escherichia coli* is unknown. In the present study, we purified the *Klebsiella* ST toxin to apparent homogeneity and determined its immunological relationship to *E. coli* ST.

ST was obtained from (i) *Klebsiella* strain TS 9 (serotype 19), an isolate from the small bowel of a Puerto Rican with tropical sprue (17) whose culture filtrate has previously been shown to evoke a positive response in the suckling mouse assay (16); and (ii) *E. coli* strain 18 D (O42:H47), a strain producing only ST and isolated from the stool of a child with acute diarrhea (25). The ST toxins were purified by the methods described by Staples et al. for the purification of human ST from *E. coli* strain 18 D (25). ST activity in elution fractions obtained during each of the purification procedures was monitored by the suckling mouse assay in which a positive response, referred to as 1 mouse unit (MU), is that amount of toxin, based on its protein concentration (20), which yields an intestinal/carcass ratio by weight of >0.083 (6). In each of the three consecutive chromatographic separative procedures used for purification, the *Klebsiella* ST eluted in the same region and had the same potency in the suckling mouse assay as *E. coli* ST. The initial broth filtrate contained $4.4 \times 10^6$ MU, and the final product had $8.78 \times 10^5$ MU, representing a 20% recovery; expressed on a unit basis, the potency of the ST in broth filtrate was 1.14 MU/μg and that of the pure toxin was 169 MU/μg, representing a 148-fold purification. Thin-layer chromatography of the pure toxin showed a single band with an $R_f$ of between 0.09 and 0.19.

Pure *Klebsiella* ST was as equally potent as pure *E. coli* ST: the minimum effective dosage in the suckling mouse assay of *Klebsiella* ST was 5.9 ng and that of *E. coli* ST was 4.0 ng; this difference is within the range of experimental variation for this assay. Exposure to 100°C for 30 min or to trypsin on a 1:1 weight basis for 60 min at 37°C did not affect the potency of either ST toxin. Destruction of the disulfide bridges of *E. coli* ST by treatment with reducing reagents, such as 2-mercaptoethanol or dithiothreitol, abolishes its biological activity (25); exposure of the *Klebsiella* ST to $5 \times 10^{-4}$ M dithiothreitol for 60 min also abolished its secretory activity in the suckling mouse assay.

The antigenic relationship between *Klebsiella*

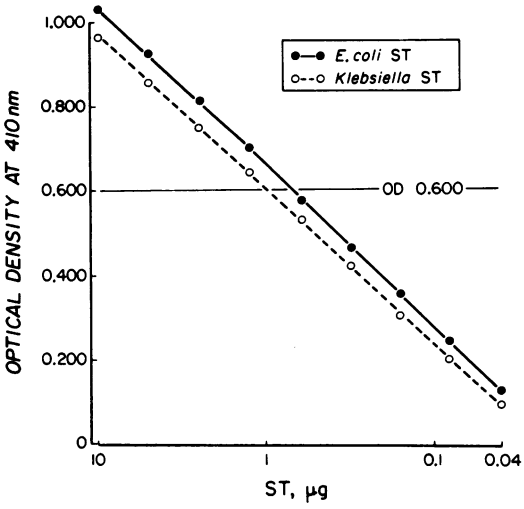


FIG. 1. Comparison of the antigenicity of *Klebsiella* ST and *E. coli* ST as determined by a double-sandwich enzyme-linked immunosorbent assay technique with hyperimmune antisera to *E. coli* ST.

and *E. coli* ST toxins was examined by means of a double-sandwich enzyme-linked immunosorbent assay by using hyperimmune antisera to *E. coli* ST raised in rabbits and goats as described previously (14). When those concentrations at which each ST toxin yielded an optical density of 0.600 at 430 nm were compared, the antigenicity of *Klebsiella* ST was 69% that of *E. coli* ST (Fig. 1).

To determine whether it was haptenic, hyperimmune antiserum was raised to *Klebsiella* ST conjugated to a protein carrier by the same methods used previously for *E. coli* ST (14). *Klebsiella* ST was mixed with porcine immunoglobulin G (IgG) at a ratio by weight of 1.33 to 1 (this was considered to approximate a molar ratio of 100 to 1), and 1-ethyl-3-(dimethylaminopropyl)carbodiimide was added at a ratio by weight of 2:1 to total protein for 18 h. The conjugate was then exhaustively dialyzed against water at 4°C for 72 h. Based on the incremental rise in Lowry protein, the conjugate contained 49% *Klebsiella* ST by weight; 1 mg was given intramuscularly to rabbits, with Freund complete adjuvant for primary immunization and Freund incomplete adjuvant for two booster immunizations given at monthly intervals.

The neutralizing effect of serial dilutions of hyperimmune rabbit antiserum to each of the ST toxins on the secretory effect of these toxins was tested in the suckling mouse assay. For each datum point three mice were given 100 μl intraintestinally containing 2 MU (twice the minimum effective dosage) of each ST toxin that had been incubated with the designated antiserum dilution for 3 h at 37°C. The number of MU neutralized by 1 ml of antiserum was derived by multiplying the projected antiserum dilution required to neutralize the secretory effect (i.e., yield of the gut/carcass ratio of >0.083) times the 10-fold dilution factor times a factor of two

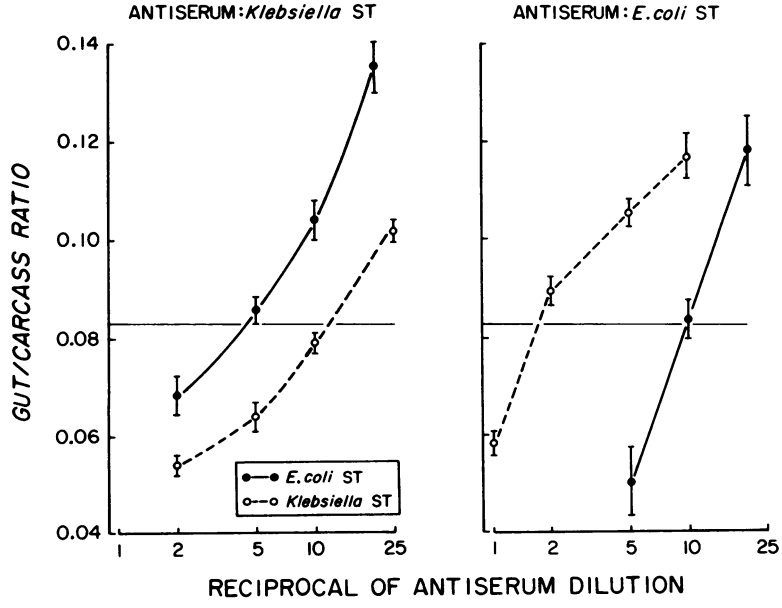


FIG. 2. Neutralizing effect of specific rabbit hyperimmune antisera to either *Klebsiella* or *E. coli* ST on the secretory effect of these toxins in the suckling mouse assay.

to adjust for the 2 MU used. In each instance, the neutralizing capacity of the specific hyperimmune antiserum was greater for the homologous than for the heterologous toxin (Fig. 2). A total of 1 ml of hyperimmune antiserum to *E. coli* ST neutralized the secretory effect of 200 MU of *E. coli* ST and of 34 MU of *Klebsiella* ST, whereas 1 ml of hyperimmune antiserum to *Klebsiella* ST seroneutralized the secretory effect of 240 MU of *Klebsiella* ST and of 90 MU of *E. coli* ST.

A synthetically prepared peptide with the same structure, potency, and immunological properties as native human *E. coli* has been produced (14) and incorporated into a vaccine by cross-linking it with the B subunit of LT toxin (12). Peroral immunization of experimental animals with this vaccine raises dose-dependent specific mucosal IgA antitoxin titers to each of the toxin components thereby providing protection against challenge in ligated ileal loops with either the ST or LT toxins or with viable bacterial strains which produce these toxins either singly or together (13, 15). To determine whether the synthetic *E. coli* ST component of this vaccine would also provide protection against *Klebsiella* ST, rats were immunized with it, and the results of challenge with *Klebsiella* ST were compared with those previously observed (15) for challenge with *E. coli* ST in rats immunized with the same dosage of this vaccine.

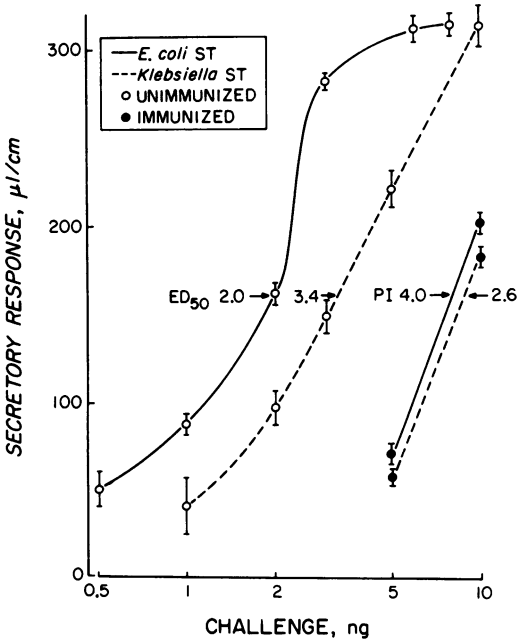


FIG. 3. Protection in rats immunized with a vaccine consisting of synthetically produced *E. coli* ST cross-linked to the LT toxin B subunit. $ED_{50}$, That dosage which produces 50% maximum secretion in unimmunized animals; PI, protection index. Data for challenge with *E. coli* ST has been published previously (15).

Sprague-Dawley rats, weighing between 150 and 175 g, were given primary immunization with vaccine, containing 200 antigen units of both ST and the LT B subunit, by the intraperitoneal route with Freund complete adjuvant; this was followed at 4-day intervals by two peroral booster immunizations of 1,000 antigen units each that were given 2 h after the peroral administration of cimetidine (Tagament; Smith Kline & French Laboratories, Carolina, Puerto Rico) at a dosage of 50 mg per kg of body weight to ablate gastric secretion. Unimmunized control rats and immunized rats were challenged 4 to 6 days after the final booster immunization by the instillation of graded dosages of the ST toxin into ligated ileal loops for 18 h as described previously (15). Each datum point for fluid secretion (presented as the mean ± standard error of the mean) was derived from challenge in four or five immunized and five control rats. The protection index was determined by dividing that dosage of toxin in immunized animals which yielded the same secretion as the 50% effective dose in unimmunized animals by the value for unimmunized animals. The protection index against challenge with *E. coli* ST was 4.0 and that against challenge with *Klebsiella* ST was 2.6 (Fig. 3).

It is now apparent that, although minor variations exist, the amino acid composition and sequence of human and animal *E. coli* STs are remarkably similar (1, 19) and that these different STs are closely interrelated immunologically (4, 7, 13). *Yersinia enterocolitica* also produces a low-molecular-weight ST that resembles *E. coli* ST in that it also causes secretion by means of activating cyclic GMP (23) and is immunologically cross-reactive with *E. coli* ST (21). The results of the present study show that such is the case for some strains of *Klebsiella*. *Klebsiella* ST, purified by the same techniques used for *E. coli* ST, had the same secretory potency in the suckling mouse assay. Although not examined in the present study, Gentile et al. have shown that a partially purified *Klebsiella* ST both stimulates cyclic GMP and affects ion transport in the rabbit ileum (5). An immunological relationship between *Klebsiella* and *E. coli* STs was identified by enzyme-linked immunosorbent assay, by cross-neutralization with specific hyperimmune antiserum in suckling mice, and by protection against active challenge with either ST toxin in rats immunized with a vaccine containing synthetically produced human *E. coli* ST.

The strain of *Klebsiella* used to produce ST in the present study resembles other enterotoxigenic strains of *Klebsiella* isolated from the stools of persons with acute diarrhea in that the

toxins elaborated by these strains yield a positive response in assays stimulated by the *E. coli* toxins, namely, the Y1 adrenal cell or Chinese hamster ovary tissue culture assays for LT and/or the suckling mouse assay for ST. Other strains of *Klebsiella*, principally those isolated from the small intestine of persons with tropical sprue, produce toxins which are inactive in these assays but which can be identified by the findings that (i) cell-free culture supernatants cause fluid secretion in rabbit ligated ileal loops (10, 17); (ii) in vivo perfusion of nanogram concentrations of semipurified ultrafiltration fractions of broth filtrates, containing either large-molecular-weight heat-labile or low-molecular-weight heat-stable forms, stimulates water and electrolyte secretion in the rat jejunum (16, 18); and (iii) the peroral administration of strains which elaborate these toxins causes acute diarrhea in neonatal piglets (27). The low-molecular-weight heat-stable form of these toxins has been purified (11), but its relationship, if any, to the suckling mouse positive *Klebsiella* ST toxin described in the present report is unknown.

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