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Escherichia coli Heat-Labile Enterotoxin: Comparison of Antitoxin Assays and Serum Antitoxin Levels

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The mouse adrenal tumor cells (Y-1 strain) and the Chinese hamster ovary cells, two routinely used tissue culture assays for *Escherichia coli* heat-labile enterotoxin (LT), were used to detect serum antitoxin responses in culture-positive patients from several well-defined sources. There was no correlation between a significant antitoxin response and isolation of LT-producing *E. coli* in two "domestic" diarrheal outbreaks. Serum samples from a third group of individuals in a rural cholera-endemic area consistently demonstrated significant rises in neutralizing antibody to LT.

The literature contains conflicting reports regarding the significance of a humoral antitoxin response to the heat-labile enterotoxin (LT) elaborated during *Escherichia coli* "cholera-like" diarrhea. Although neutralizing antitoxin (anti-LT) rises in culture-positive cholera are not always demonstrable (with frequency of rises ranging from 60 to 73%) (7), some investigators have suggested that anti-LT rises are necessary for the differentiation of infection versus colonization by *E. coli* and as an indication of infection in culture-negative individuals (2, 13). At least three studies of infections with LT-producing *E. coli* have shown significant rises in serum antitoxin levels from the acute to the convalescent phases of illness (5, 15, 20). At least one other study and two epidemics caused by LT-producing *E. coli* have shown a lack of correlation among illness, the presence of the epidemic strain, and antitoxin response (1, 11, 14).

Because the toxin of *Vibrio cholerae* (CT) and LT are similar immunologically (12) and in that both stimulate adenyl cyclase activity (4, 9), the concepts and technology of cholera research have been directly applied to the study of enterotoxigenic *E. coli* (ETEC) (17). Initial enterotoxicity and neutralization experiments used the rabbit ileal loop (18) and later the rabbit skin permeability test (6), but the advent of tissue culture systems that respond to the toxin stimulation of adenyl cyclase has permitted the processing of large numbers of cultures and serum samples with apparently greater sensitivity (9, 16). The following study examines the neutralizing, humoral immune response to well-documented ETEC infection and compares the Y-1 adrenal cell and the Chinese hamster ovary cell (CHO) tissue culture assays for CT, LT, and their antitoxins.

MATERIALS AND METHODS

Toxins. Purified CT prepared by R. Finkelstein was obtained from the National Institutes of Health. Samples of 500 μg/ml reconstituted in tris(hydroxymethyl)aminomethane buffer were stored at −70°C. *E. coli* strain 10407 was grown in Trypticase soy broth (Difco) with 0.6% yeast extract for 48 h without aeration; the supernatant was then filtered through a 0.22-μm-pore size membrane (Millipore Corp.) and immediately used as a crude LT preparation. Both CT and LT were diluted with phosphate-buffered saline, pH 7.2, containing 0.1% gelatin. In antitoxin studies the optimal toxin concentrations for assays were determined to be 0.1 μg of CT per ml and a 1:4 dilution of crude LT.

Antisera. Swiss Serum and Vaccine Institute (SSVI) cholera antitoxin, lot EC3 (A-2/67)-B (4,470 antitoxin units/ml), obtained from John Feeley, was used as the standard antitoxin and was arbitrarily assigned a value of 1,000 anti-LT units. Paired sera from patients with diarrhea who currently had ETEC in their stools were obtained from the following sources: (i) seven U.S. citizens living in the continental United States who were involved in a Caribbean cruise ship outbreak of diarrhea caused by *E. coli* O25:K98:NM, which produced only LT (1); (ii) six U.S. citizens involved in an outbreak in Crater Lake National Park of diarrhea caused by *E. coli* O6:K15:H16, which produced LT and heat-stable enterotoxin (14); (iii) four Bengalee patients living in rural Bangladesh with isolated episodes of diarrhea caused by *E. coli* that produced LT and heat-stable enterotoxin (15).

ETEC was the predominant aerobic gram-negative organism in each patient's fecal flora. In both the cruise ship and Crater Lake outbreaks, at least four out of five lactose-positive colonies from fecal swabs were ETEC, and in Dacca at least nine out of ten were ETEC. Illness in the Crater Lake patients lasted longer (median length, 8 days) than illness in the cruise ship patients (median length, 2 days) or illness in the Dacca patients (median length, 3 days). All

patients were over 2 years of age, and convalescent serum was collected at least 2 weeks after acute illness (1, 14, 15).

Assays. The CHO and the Y-1 adrenal cell tissue culture assays for the detection of LT and CT have been previously described (4, 9). Both assays have been adapted to miniculture techniques (14, 16) and used to detect the neutralization of enterotoxin activity by antitoxin (5, 8).

Antitoxin activity was determined in the following manner. A 1:2 dilution of serum in phosphate-buffered saline with gelatin was mixed with a 1:4 dilution of LT and incubated at 37°C. After 1 h, 0.05 ml of the toxin-antitoxin mixture was added to the Y-1 monolayer in microtiter plates and to a slide chamber (Lab Tek, eight chambers) into which 0.2 ml of a CHO cell suspension was subsequently added. Both tissue cultures were incubated in humidified 5% CO_2 at 37°C overnight. The Y-1 cells were read after 16 h, and the CHO cells were fixed, stained with fresh 2% Giemsa, and read after 20 h. In the Y-1 cells a rounding response of less than +1 was considered negative, +1 was considered intermediate, and +2 or greater was considered positive. In the CHO assays in a field of 100 cells, a count of 9.2 or fewer elongated cells was negative, 9.3 to 13.5 was intermediate, and 13.6 or more was positive. Although the CHO cells have been evaluated semiquantitatively in microtiter plates (8, 15), we elected to follow the original micromethodology (9). All dose-response studies and antitoxin titrations were run in duplicate.

RESULTS

Toxins. The dose response curves for both CT and LT in both the Y-1 adrenal and CHO assays are presented in Fig. 1. The maximum percentage of elongated cells seen in the CHO assay is 25% of the cell population. The maximum proportion of rounded cells in the Y-1 assay is expressed as +4.

Standard antitoxin. The response of standard concentrations of CT and LT to a series of twofold dilutions of SSVI antitoxin is presented in Table 1 and demonstrates the highly significant correlation of end points between the two tissue culture assay systems. The end point is defined as the smallest amount of serum antitoxin (units) that allows a positive toxin response.

Patient antisera. Epidemiological findings associated with illness in patients included in the present study have been previously described (1, 14, 15).

The results of the antitoxin titrations on sera of patients from the Crater Lake and cruise ship epidemics and the Dacca study are shown in Table 2. A fourfold or greater rise in titer is considered significant; therefore, the significant antitoxin responses occurred in 33% of Crater Lake, 29% of the cruise ship, and 100% of Dacca serum samples. The initial antitoxin units for

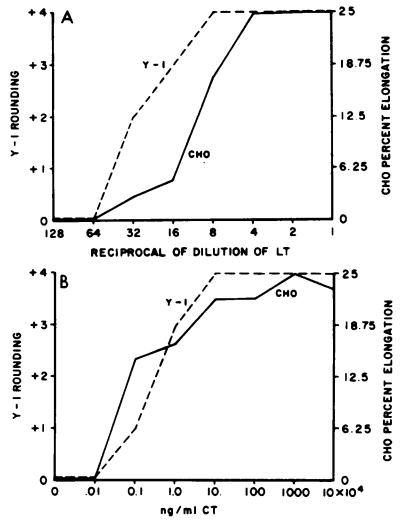

FIG. 1. *Enterotoxin dose response curves in the Y-1 adrenal and CHO tissue culture assay systems. (A) Culture filtrate of E. coli strain 10407 (LT); (B) purified CT.*

TABLE 1. *Tissue culture comparisons in the response of CT and LT to a standardized antitoxin*

Cholera antitoxin[a] (units/ml)	CT (0.1 μg/ml)		LT (culture filtrate)	
	Y-1[b]	CHO[b]	Y-1	CHO
47	−	−	−	−
28.5	−	−	−	−
14.25	−	−	−	−
7.125	−	−	±	−
3.56	−	−	+	+
1.78	+	+	+	+
0.89	+	+	+	+
0.445	+	+	+	+

[a] SSVI cholera antitoxin, originally 4,700 antitoxin units/ml.

[b] Positive and negative interpretations are discussed in Results.

acute-phase serum and the apparently uniform rise in titer are considerably higher in the Dacca patients.

As with the CT and LT observations, the results of Y-1 and CHO tissue culture assays appear to be comparable, with one possible exception, and that is in the case of Crater Lake patient no. 5. A one-tube variation in a series of twofold dilutions is within experimental error.

DISCUSSION

Both antitoxin assays were performed concurrently in one laboratory with samples of single reagent preparations in an attempt to reduce the number of variables present in earlier comparisons of toxin activity (12). Although evi-

TABLE 2. *Tissue culture comparisons of antitoxin levels[a] on acute- and convalescent-phase sera from culture-positive (ETEC) patients*

Patient no. and source	Y-1		CHO	
	Acute	Convalescent	Acute	Convalescent
Cruise ship				
1	1.25	2.5	2.5	2.5
2[b]	5	20	5	20
3	10	5	5	5
4	<1.25	<1.25	2.5	2.5
5	5	2.5	2.5	5
6	5	2.5	5	2.5
7[b]	1.25	5	1.25	5
GMT[c]	2.7	3.3	3.0	4.5
Crater Lake				
1	<0.6	<0.6	1.2	1.2
2[b]	1.2	20	2.5	29
3[b]	<0.6	5	2.5	10
4	1.2	10	2.5	5
5	2.5	5	10	5
6	40	20	40	20
GMT	1.5	5.6	4.4	7.0
Dacca				
1[b]	20	>160	20	>80
2[b]	5	>160	5	>80
3[b]	10	40	10	40
4[b]	10	>160	5	>80
GMT	10	133.7	8.4	133.7

[a] Results are expressed as antitoxin units based on SSVI cholera antitoxin standard as 1,000 units/ml.

[b] Fourfold or greater rise in titer.

[c] GMT, Geometric mean titer. When the end point fell below the first serum dilution, a number half the distance between that number and zero was chosen. When the end point was beyond the last dilution, 200 units was selected as the upper limit.

dence for binding sites on the Y-1 cells was thought to make these cells dissimilar to the CHO cells (2, 8), the dose response curves for both assays to LT and CT are strikingly similar (Fig. 1). Furthermore, the neutralization of LT and CT by a standard antitoxin was detected at identical titers in the two systems (Table 1).

Antitoxin levels in U.S. residents with diarrhea positive for LT-producing ETEC confirm reports of poor or insignificant humoral response to natural infection with this organism in other U.S. populations (11, 19). This study also confirms the significant titer rise in culture-positive patients from a rural area of Bangladesh, where continued exposure to both *V. cholerae* and ETEC is highly probable. The distinction between the two types of populations may be seen in the laboratory as (i) individual variation, (ii) the result of a larger antigenic dose to the Bengalee patients, (iii) the stimulation of a secondary, anamnestic immune response in the Bengalee patients to the previously encountered toxin (LT) or related antigen (CT), or (iv) a difference in the severity of illness in each group.

Although the Crater Lake patients had the illness of longest duration (median, 8 days), the significant differences in response to LT in this group seem to reflect individual immune conditions. The Bengalee patients were ill for a shorter period of time (median, 3 days), but the four individuals uniformly manifested both initially high titers and significant rises in convalescent serum. These data suggest previous exposure either to LT or the immunologically related CT in an area where ETEC and *V. cholerae* are causes of endemic diarrhea (15).

A study sometimes cited when antitoxin titers are sought as a prerequisite to differentiate colonization and infection (3, 13) is that in which volunteers were experimentally infected (5). Although the number of volunteers who achieved significant titer rises was extremely high (83%, or six of seven volunteers) and the experiment was conducted in this country, the method and amount of inoculation was unnatural, and the volunteers showing the greatest response were veterans who had served in North Korea, New Guinea, and North Africa (5). Other indirect evidence against the importance of a humoral response to a local challenge with LT and CT is presented in recent studies using dog Thiry-Vella loops. Although repeated challenge seemed to elicit local immunity, serum antitoxin levels were not affected (21).

The requirement of a significant fourfold rise in the serum antitoxin level in diagnosing first infections seems impractical. Although the two assays tested were reproducible and are available, the neutralization experiments are time consuming and often given equivocal answers. For these reasons, we question the appropriateness of demands for a rise in antitoxin titer to confirm infection (3, 13) and emphasize a need to develop a more refined and less cumbersome serological assay, using the newly available purified reagents.

On reviewing the epidemiological data pertaining to all three sets of patients included in the present study, we found that 8 out of 17 (47%) diarrheal patients with ETEC and 6 out of 17 (35%) diarrheal patients with no ETEC demonstrated a fourfold rise in titer. In the culture-negative, antitoxin-positive patients with diarrhea, the humoral response was interpreted as an indication of infection by an enterotoxigenic organism and was used in the epidemiological investigations (1, 14). Obviously, there is a need for increased sensitivity for both the

current bacteriological and serological diagnoses of ETEC diarrhea.

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