INFECTION AND IMMUNITY, June 1977, p. 781-788
Vol. 16, No. 3
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Humoral Immune Response to the Heat-Labile Enterotoxin of *Escherichia coli* in Naturally Acquired Diarrhea and Antitoxin Determination by Passive Immune Hemolysis

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Received for publication 8 November 1976

Acute- and convalescent-phase sera from 132 students attending a university in rural Mexico were assayed for antibody against the heat-labile enterotoxin (LT) of *Escherichia coli* by neutralization of LT activity in the Y-1 adrenal cell assay and by passive immune hemolysis of LT-sensitized sheep erythrocytes. The two titration methods produced comparable results with respect to antitoxin responses detected. An inverse relationship was found between acute geometric mean antitoxin titer and the occurrence of diarrhea associated with LT-producing *E. coli*, especially in newly arrived students from the U.S.A. A significant correlation ($P < 0.005$) was found between a rise in antitoxin titer detectable by the passive immune hemolysis technique and diarrhea with LT-producing *E. coli* isolated. Thus, humoral antitoxin titers appear to be a useful indicator of immune status with respect to enterotoxigenic (LT) *E. coli* diarrhea.

The heat-labile enterotoxin (LT) of *Escherichia coli* elicits a neutralizing antitoxin response when injected parenterally into animals (4, 5, 13). Antitoxin prepared against LT of one strain of *E. coli* neutralizes LT prepared from other strains irrespective of their source (3, 4, 16). *E. coli* antitoxin also neutralizes the enterotoxin of *Vibrio cholerae* and vice versa (3, 12, 16). It has been demonstrated that exposure to cholera toxoid boosts serum *E. coli* antitoxin titers in man (12). Humoral antitoxin responses to naturally acquired diarrhea associated with enterotoxigenic *E. coli* also have been documented (8, 11, 14, 15). However, the reliability of a humoral antitoxin response as an indicator of enterotoxigenic *E. coli* infection has remained in question. In a recent prospective study of the etiology of acute diarrhea in U.S. and other students attending a university in Mexico (2), we confirmed previous reports (10, 11) indicating that enterotoxigenic strains of *E. coli* are the primary cause of travelers' diarrhea in Mexico. We report here the results of our investigations into the humoral antitoxin response in this study population in relation to the isolation of enterotoxigenic *E. coli* in the presence and absence of diarrhea. Antitoxin titers were determined by the cultured Y-1 adrenal cell technique (1) and by the passive immune hemolysis assay for anti-LT antibody, which has not been reported previously.

MATERIALS AND METHODS

Preparation of *E. coli* enterotoxin and reference antitoxin. LT was obtained from *E. coli* strain H-10407 by the polymyxin release technique (5, 6, 9). The enterotoxin was purified by ammonium sulfate precipitation and back-extraction followed by absorption to and elution from Affi-Gel 202 (Bio-Rad, Richmond, Calif.) as recently described (7). Adult albino rabbits were hyperimmunized with Affi-Gel 202-purified LT as follows. The initial dose consisted of four intramuscular injections of 30 μg of protein each, emulsified in incomplete Freund adjuvant. At 3-day intervals, each rabbit received five additional intramuscular doses of enterotoxin diluted in saline. Doses were increased so that for the sixth injection each rabbit received a total of 400 μg of protein. The animals were bled 14 days after the final injection. Serum was collected, pooled, and stored at −45°C. A single pool of hyperimmune antitoxin was used throughout this study as a reference serum to standardize the passive immune hemolysis assay for antitoxin in the human sera.

Acute- and convalescent-phase sera and the study population. Paired serum samples were obtained from student volunteers participating in an investigation into the etiology of acute diarrhea at the Universidad de las Americas in Cholula, Puebla, Mexico (2). All of the U.S. summer students were newly arrived in Mexico. U.S. full-time students were those who had maintained residence in Mexico for at least 1 year. Latin American students originated from Mexico and from Venezuela. Initial blood samples were obtained when the students developed diarrhea and again 10 to 21 days later.

Paired serum samples were also obtained from a large number of students who did not develop diarrhea; these control blood samples were drawn on the basis of time spent in Mexico.

Students submitted stool specimens every 3 days, and additional specimens were collected during episodes of diarrhea. These were analyzed for the presence of pathogens including enterotoxigenic *E. coli* and parsites. LT-producing *E. coli* were identified by testing culture filtrates for enterotoxin activity in the cultured Y-1 adrenal cell assay (1). All LT-positive cultures were confirmed by neutralization of the toxic activity with specific rabbit anti-LT serum. All of the adrenal cell-positive culture filtrates in this study were neutralizable by antiserum prepared against the LT of *E. coli* H-10407.

Antitoxin titration by the adrenal cell tissue culture method. Serum samples were heat treated at 56°C for 30 min and assayed for antitoxin by two different methods. The cultured Y-1 adrenal cell assay (1) was used to titrate the sera for neutralizing activity as follows. Sera were diluted in the tissue culture medium (minimum essential medium with Earle base plus 2.0% horse serum), and portions were added to an equal volume of a standard amount of *E. coli* enterotoxin. After preincubation for 30 min at 37°C, the toxin-antitoxin mixtures were added to Microtiter plate wells (Cooke Laboratory Products, Alexandria, Va.) containing cultured Y-1 adrenal cells. The cells were observed for morphological effects of LT after 18 h of incubation. The standard amount of LT was that which produced the typical rounding effect in 80 to 90% of the cells. This culture regularly demonstrated 5 to 10% round cells in the untreated controls. Complete neutralization resulted in cultures showing this background level of rounding; the neutralization titer was taken as the highest dilution of serum demonstrating inhibition of adrenal cell rounding. The same LT preparation was used throughout this study, and positive and negative controls were also performed in duplicate.

Antitoxin titration by the passive immune hemolysis technique. Anti-LT antibody in sera was also quantitated by titration, using the passive immune hemolysis technique. This assay for antitoxin is based on the complement-mediated lysis of LT-sensitized sheep red blood cells (SRBC). Fresh SRBC were sensitized with LT as follows. Five-milliliter portions of sheep blood were washed three times with 12 volumes of phosphate-buffered saline (PBS; 0.1 M, pH 7.2) by resuspension and centrifugation at 1,800 rpm for 8 min. An LT preparation was diluted to 25 to 50 μg of protein per ml in 0.1 M phosphate buffer (pH 6.5), and 1.0 ml of enterotoxin was added per 0.1 ml of packed SRBC. The mixture was then incubated for 30 min at 37°C with intermittent shaking and finally centrifuged as above. The LT-sensitized SRBC (LT-SRBC) were washed three times in PBS and finally suspended in PBS, 1.0 ml per 0.1 ml of packed cells.

Serum antitoxin was titrated by using the Microtiter titration system (Cooke Laboratory Products, Alexandria, Va.) as follows. Twofold dilutions of the sera were prepared in PBS containing 0.02% bovine serum albumin in U-well Microtiter plates, and an equal volume (0.025 ml) of the 1.0% suspension of LT-SRBC was added. After incubation for 30 min at 37°C, 0.025 ml of freshly prepared guinea pig complement (1:10 dilution in PBS-bovine serum albumin) was added, and the plates were incubated at 37°C for 60 min. The lytic reaction was optimal for observation 20 min after returning the plates to room temperature. Each preparation of LT-SRBC was tested for sensitization by using the cells to titrate the standard hyperimmune rabbit antiserum, which consistently demonstrated a 1:128 titer.

Analysis of antitoxin titration data. The major goal of this study was to assess antibody response to LT with respect to the occurrence of diarrhea with or without the isolation of LT-producing *E. coli*. For this purpose, a significant rise in antitoxin antibody was defined as a fourfold or greater rise in hemolytic titer or a twofold rise in hemolytic titer confirmed by a threefold or greater rise in neutralization titer, a definition which allowed maximum utilization of the available data. The validity of this definition was further tested by comparing these results with those obtained with each of the titration methods alone using the more conventional definitions, i.e., that a significant rise in antitoxin could only be represented by a fourfold (two-well) rise in hemolysis titer or by a ninefold (two-well) rise in neutralizing titer. It should be emphasized that data expressed as geometric mean titers (GMTs) are not influenced by the above definitions; GMT data presented in Tables 2, 3, and 4 were obtained with the passive immune hemolysis technique alone.

RESULTS

Composition of the study population. The study population was classified according to national origin, time elapsed since arrival in Mexico, occurrence of diarrheal illness, and isolation of LT-producing *E. coli* from stool at the time of illness (Table 1). Paired acute- and convalescent-phase sera were obtained from 28 newly arrived summer students from the U.S.A. and from 30 full-time U.S. students, all of whom developed diarrhea during the course of the study. Paired sera were obtained on equivalent dates from 22 U.S. summer and 20 U.S. full-time students who did not report diarrhea. Paired sera from 12 Latin American students who presented with illness and from 20 healthy controls of the same national origin were also examined for antitoxin. Isolation of LT-producing *E. coli* from the 70 ill students at the time of illness was significantly greater than isolation of such *E. coli* from the 62 healthy matched controls ($P < 0.005$).

Antitoxin responses of student volunteers. Data pertaining to the number of students demonstrating a rise in antitoxin titer are presented in Table 2. Composite acute- and convalescent-phase titers determined by the passive immune hemolysis titration technique are pre-

TABLE 1. *Student volunteers contributing acute- and convalescent-phase or paired matched control sera during an investigation of diarrhea in Mexico*

Type of student	Occurrence of diarrhea		LT-producing *E. coli*			
			Isolated		Not isolated	
	Ill	Non-ill	Ill	Non-ill	Ill	Non-ill
U.S. summer	28	22	8	2	20	20
U.S. full-time	30	20	12	2	18	18
Latin American	12	20	4	0	8	20
Totals	70	62	24	4	46	58

TABLE 2. *Humoral antibody responses to LT of E. coli in students attending a university in Mexico*

Type of student	No.	No. (%) with rise	GMT (antitoxin)	
			Acute	Convalescent
U.S. summer				
Ill	28	12 (42.8)[a,b]	17.7	29.7 (<0.01)[c]
Not ill	22	2 (9.1)[a]	19.9	23.0 (<0.025)
Total	50	14 (28.0)	18.7	26.7
U.S. full-time				
Ill	30	4 (13.3)[b]	20.6	25.4 (<0.01)
Not ill	20	2 (10.0)	21.1	25.1 (NS)
Total	50	6 (12.0)	20.8	25.3
Latin American				
Ill	12	2 (16.7)	16.0	24.0 (NS)
Not ill	20	1 (5.0)	30.9	32.0 (NS)
Total	32	3 (9.3)	25.3	29.6
Entire study population				
Ill	70	18 (25.7)[d]	19.8	27.2 (NS)
Not ill	62	5 (8.1)[d]	22.6	26.0 (NS)
Total	132	23 (17.4)	21.1	26.6

[a, b, d] Significantly different ($P < 0.025$).
[c] Numbers in parentheses are P values. NS, Not significant.

sented for each of the six subgroups and for the entire study population. Note that the U.S. summer student group had the lowest acute titer (18.7 GMT) and the Latin American students had the highest (25.3 GMT). Of the three types of students, only the U.S. summer students showed a significant correlation between illness and a rise in antibody titer ($P < 0.025$). Also, significantly more ill U.S. summer students showed an antibody response than ill U.S. full-time students ($P < 0.025$). The ill and non-ill U.S. summer student and the ill U.S. full-time student groups showed a significant rise in anti-LT titer (acute versus convalescent, Table 2).

Antitoxin responses in relation to the isolation of LT-producing *E. coli* from students with and without diarrhea. Antitoxin data derived from acute- and convalescent-phase sera of all students with recorded diarrhea are summarized in Table 3. Note that the U.S. summer students who were ill and who also harbored LT-producing *E. coli* had, as a group, a much lower acute antitoxin titer than the U.S. summer students who reported ill but did not harbor such *E. coli* (11.3 GMT versus 21.1 GMT, $P < 0.05$). Ill U.S. summer students from whom LT-producing *E. coli* were isolated also showed a significantly greater number of antitoxin responses than their counterparts who were ill but without LT-producing *E. coli* detected ($P < 0.05$). Significantly more U.S. summer students who were ill with LT-producing *E. coli* present showed antibody responses than U.S. full-time students who were ill with such *E. coli* present ($P < 0.025$). As a group, the U.S. summer students who were ill with LT-producing *E. coli* present showed a highly significant rise in titer ($P < 0.001$), whereas as a group the U.S. full-time students who were ill with such *E. coli* present did not show a significant rise in anti-LT titer. Furthermore, the acute GMT for the entire group of ill students with LT-producing *E. coli* present was less than that of the group without LT-producing *E. coli* isolated (17.6 GMT versus 20.9 GMT), and the convalescent GMT

TABLE 3. *Humoral antitoxin responses and isolation of enterotoxigenic (LT) E. coli in students with diarrhea*

Type of student	No.	No. (%) with rise	GMT (antitoxin)	
			Acute	Convalescent
LT *E. coli* isolated				
U.S. summer	8	7 (87.5)[a, b]	11.3[c, d]	32.0[d, e]
U.S. full-time	12	3 (25.0)[a]	25.4	33.9
Latin American	4	2 (52.0)	6.7	19.0
Total	24	12 (50.0)	17.6	30.8[f]
LT *E. coli* not isolated				
U.S. summer	20	5 (25.0)[b]	21.1[c]	28.8[e]
U.S. full-time	18	1 (5.5)	18.0	20.9
Latin American	8	0 (0.0)	26.9	26.9
Total	46	6 (13.0)	20.9	25.4[f]

[a] $P < 0.025$.
[b, c] $P < 0.05$.
[d] $P < 0.001$.
[e, f] $P < 0.025$.

for the entire group of ill students with LT-producing *E. coli* isolated was significantly greater than for the group without such *E. coli* present (30.8 GMT versus 25.4 GMT, $P < 0.025$; Table 3).

Very few (6.0%) of the healthy students harbored LT-producing *E. coli* (Table 4). Only 5 of 58 (8.6%) of the healthy students without LT-producing *E. coli* demonstrated a rise in antitoxin titer. This is in sharp contrast to the fact that 12 of 24 (50%) of those who acquired both diarrhea and LT-producing *E. coli* demonstrated an antitoxin response (Table 3). This difference is further illustrated by the data in Table 2, which show that irrespective of the isolation of LT-producing *E. coli,* 26% of the students who contracted diarrhea showed an antitoxin response, whereas only 8% of the students who remained healthy showed such a response, a significant difference ($P < 0.025$).

Table 5 shows the association of LT-producing *E. coli* with diarrheal illness in the six student subgroups as determined by the isolation of such *E. coli* at the time of illness and by detection of an anti-LT response. By comparing ill students with non-ill matched controls, it can be seen that in all three groups (U.S. summer, U.S. full-time, and Latin American students), both criteria indicate an association between the presence of LT-producing *E. coli* and the occurrence of diarrhea. It is of interest that there was better agreement between isolation of LT-producing *E. coli* and serum response in the ill U.S. summer student group than in the ill U.S. full-time group. The pattern observed with the U.S. full-time students with respect to antitoxin response had a marked resemblance to that observed with the Latin American students.

Relationship between antitoxin neutraliza-

TABLE 4. *Humoral antitoxin responses and isolation of enterotoxigenic (LT) E. coli in students without diarrhea*

Type of student	No.	No. (%) with rise	GMT (antitoxin)	
			Acute	Convalescent
LT *E. coli* isolated				
U.S. summer	2	0 (0.0)	32.0	32.0
U.S. full-time	2	0 (0.0)	32.0	32.0
Latin American	0	0 (0.0)		
Total	4	0 (0.0)	32.0	32.0
LT *E. coli* not isolated				
U.S. summer	20	2 (10.0)	19.0	22.6
U.S. full-time	18	2 (11.1)	20.2	21.7
Latin American	20	1 (5.0)	30.9	32.0
Total	58	5 (8.6)	22.0	25.6

TABLE 5. *Comparison of antitoxin responses and isolation of LT-producing E. coli in students with and without diarrhea*

Type of student	No.	No. (%) with LT-producing *E. coli*	No. (%) with antitoxin response
U.S. summer			
Ill	28	8 (28.6)	12 (42.8)
Not ill	22	2 (9.1)	2 (9.1)
Total	50	10 (20.0)	14 (28.0)
U.S. full-time			
Ill	30	12 (40.0)	4 (13.3)
Not ill	20	2 (10.0)	2 (10.0)
Total	50	14 (28.0)	6 (12.0)
Latin American			
Ill	12	4 (33.3)	2 (16.7)
Not ill	20	0 (0.0)	1 (5.0)
Total	32	4 (12.5)	3 (9.4)
Total for entire study population	132	28 (21.2)	23 (17.4)

tion titer and antitoxin titer as determined by passive immune hemolysis. Previous descriptions of anti-LT responses have been based on detection of neutralizing antibody (8, 11, 14, 15). Because of the potential of the passive immune hemolysis technique as an epidemiological tool, it was considered imperative to assess the relationship between serum passive hemolytic activity and neutralizing activity. Figure 1 shows a direct comparison between antitoxin titers obtained by passive immune hemolysis and by neutralization of LT activity in the Y-1 adrenal cell assay. There is a direct correlation between the antitoxin activity demonstrable by the two methods ($P < 0.001$ for the correlation coefficient).

The antibody responses discovered by testing the 132 pairs of acute- and convalescent-phase sera are shown in Table 6. Note that only 1 of 17 paired sera demonstrating a ninefold or greater rise in neutralizing titer failed to demonstrate a rise in hemolytic titer and that only 1 of 13 paired sera demonstrating a fourfold rise in hemolytic titer failed to show a rise in neutralizing titer. Of 132 paired determinations, 127 (96%) fell within the expected range of experimental error for a comparison range of two titration systems dependent on serial dilutions.

The data presented in Table 6 may also be assessed in the light of the results presented in

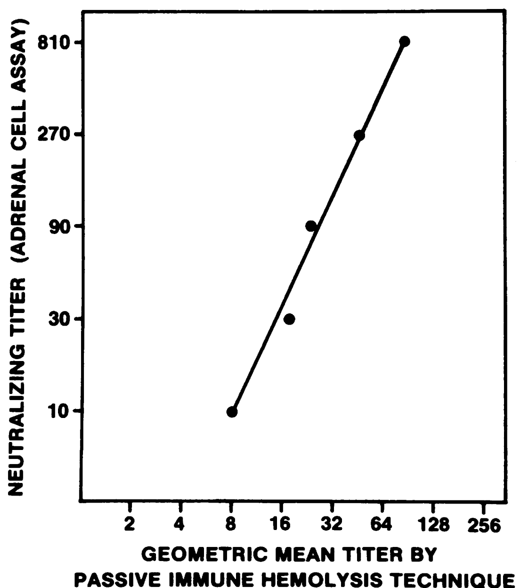

FIG. 1. *Relationship between antitoxin titers obtained by the adrenal cell technique and by the passive immune hemolysis technique. Data are derived from 204 sera with neutralizing titers ≥1:10 and hemolysis titers ≥1:2.*

TABLE 6. *Comparison between antitoxin responses detectable as increased neutralizing activity of serum in the adrenal cell assay and as increased passive immune hemolytic activity*

No. of paired sera demonstrating increased antitoxin titer				
By titration in the passive immune hemolysis assay	By titration of neutralizing activity in the adrenal cell assay[a]			
	0	3×	9×	27×
0	(86)	(13)	1	0
2×	(9)	(3)*	(5)*	2*
4×	1*	(3)*	(4)*	1*
8×	0	(0)	(0)	(1)*
16×	0	0	(0)	(3)*

[a] Parentheses indicate data for 127 acute/convalescent pairs of sera confirming agreement between results of the two assay systems. Asterisks indicate the 23 pairs of sera representing significant antitoxin responses according to the definition: "a fourfold or greater rise in hemolytic titer or a twofold rise in hemolytic titer confirmed by a threefold or greater rise in neutralizing titer."

Tables 2 to 6, i.e., in relation to the source of sera, occurrence of diarrhea, and bacteriological confirmation of the presence of LT-producing *E. coli*. Table 7 shows an analysis of the antibody response data obtained by neutralization alone, by passive immune hemolysis alone, and by both methods. It is clear that both the neutralization data and the passive immune hemolysis data lead to the same conclusions. Approximately 76% of the students who showed an antitoxin response had diarrhea irrespective of the isolation of LT-producing *E. coli*, whereas approximately 50% of those without an antitoxin response were ill, indicating that with respect to the entire study population pathogens other than LT-producing *E. coli* also caused diarrhea. Table 7 shows a significant relationship between antitoxin response and diarrhea accompanied by the presence of LT-producing *E. coli* ($P < 0.025$ by the adrenal cell assay alone, $P < 0.005$ by the passive immune hemolysis assay alone, and $P < 0.01$ by both methods). It is also of interest that of the students showing an antitoxin response, approximately 60% were newly arrived U.S. summer students whereas no more than 30% were U.S. full-time students, although equal numbers of U.S. summer and full-time students (38% each) were enrolled in the study.

A further comparison of the antibody response data obtained via the two titration techniques is shown in Table 8. It is again clear that the results obtained with both techniques lead to the same conclusions. As a group and irrespective of the presence of LT-producing *E. coli*,

TABLE 7. *Comparison of antitoxin response data obtained with paired sera by titration of neutralizing activity in the adrenal cell assay and by titration of passive immune hemolytic activity*

Students grouped according to antibody response[a]	No. in group	Percent ill	Percent ill plus LT *E. coli* isolated	Type of student as % USS:USFT:LA[b]
(+) by AC	17	76.5	41.2[c]	65:17:18
(−) by AC	115	49.6	14.8[c]	34:41:25
(+) by PIH	13	76.9	61.5[d]	54:31:15
(−) by PIH	119	50.4	13.4[d]	36:38:26
(+) by AC/PIH[e]	23	78.2[f]	52.2[g]	61:26:13
(−) by AC/PIH	109	47.7[f]	22.0[g]	33:40:27
Total group:	132	53.0	18.2	38:38:24 = 100%

[a] Using the adrenal cell assay (AC) alone, an antitoxin response was defined as a ninefold (two-well) or greater rise in neutralizing titer; in the passive immune hemolysis assay (PIH), an antitoxin response was defined as a fourfold (two-well) or greater rise in hemolytic activity.
[b] USS:USFT:LA = U.S. summer:U.S. full-time:Latin American students, relative percentage.
[c] $P < 0.025$.
[d] $P < 0.005$.
[e] AC/PIH refers to the definition of antitoxin response used in this study (see Table 6).
[f] $P < 0.025$.
[g] $P < 0.010$.

TABLE 8. *Correlation between diarrheal illness, isolation of LT-producing E. coli, and isolation of Shigella (control group) and antitoxin response as detected by titration of neutralizing activity in the adrenal cell assay and by titration of passive immune hemolytic activity*

Students grouped according to three independent criteria	No. in group	Percent of students in group demonstrating antibody response according to: AC	PIH	AC/PIH[a]
All with diarrhea	70	18.6	14.3	25.7[b]
All without diarrhea	62	6.4	4.8	8.1[b]
All with LT *E. coli* isolated	28	25.0	28.6[c]	42.9[d]
All without LT *E. coli*	104	9.6	4.8[c]	10.6[d]
All with *Shigella* isolated	13	0.0	7.7	7.7
Total group	132	12.9	9.8	17.4

[a] Using the adrenal cell assay (AC) alone, an antitoxin response was defined as a ninefold (two-well) or greater rise in neutralizing titer; in the passive immune hemolysis assay (PIH), an antitoxin response was defined as a fourfold (two-well) or greater rise in hemolytic activity. AC/PIH refers to the definition of antitoxin response used in this study (see Table 6).
[b] $P < 0.025$.
[c] $P < 0.005$.
[d] $P < 0.05$.

those participants who reported ill with diarrhea exhibited a threefold-greater number of antitoxin responses than their non-ill counterparts ($P < 0.025$, using data obtained with both techniques). Also, students from whom LT-producing *E. coli* were isolated, irrespective of illness, exhibited as a group a fourfold-greater number of antitoxin responses than their counterparts from whom such *E. coli* were not isolated ($P < 0.005$, using data obtained by passive immune hemolysis). For comparison, similar data are presented for those participants from whom *Shigella* was isolated, representing 10% of the study population. Only 1 of 13 students with *Shigella* demonstrated an antitoxin response.

DISCUSSION

We recently reported the results of an investigation in Mexico in which the occurrence and etiology of acute diarrhea in newly arrived U.S. students (a tourist-like group), in full-time U.S. students in residence in Mexico for 1 year or longer, and in Latin American students were studied (2). We reported that pathogens, primarily enterotoxigenic *E. coli* and *Shigella*, were found in 57% of students with diarrhea and in 18% of those who did not develop diarrheal illness. The occurrence of diarrhea not associated with detectable pathogens and the high incidence of multiple pathogens (12% in ill students, 1% in controls) precluded any clear definition of the etiology of diarrhea in the study population. However, there was a clear association between the presence of LT-producing *E. coli* and diarrhea in newly arrived students. This result confirmed previous observations which associated LT-producing *E. coli* with travelers' diarrhea in Mexico (10, 11).

Paired acute- and convalescent-phase sera collected prospectively from 132 of the 189 participants in the above study were used to test

the hypothesis that humoral anti-LT antibody (antitoxin) might serve as a useful indicator of immune status or at least provide a tool for the retrospective diagnosis of enterotoxigenic *E. coli* diarrheal illness. This hypothesis was based on firm evidence of the antigenicity of LT (3, 5, 13) and reports by others concerning antitoxin responses in man (11, 14, 15). The results reported here were obtained with paired sera from students with diarrhea and from carefully matched controls, using two different and independent methods to detect humoral antitoxin responses. These were the Y-1 adrenal cell assay (1) for the titration of neutralizing antibody and the in vitro passive immune hemolysis assay recently developed in our laboratory. A preliminary analysis of the data obtained by the two techniques clearly showed a correlation between neutralizing antitoxin and passive immune hemolytic activity in the sera. Thus, the analysis reported here used data obtained with both titration systems, although the use of data derived from either technique alone would have led to the same conclusions. However, the passive immune hemolysis assay for anti-LT antibody proved to be superior to the adrenal cell technique in many respects, particularly in that the Microtiter procedure is rapid, easy to perform, relatively economical, and free of extraneous serum effects that complicate the tissue culture assay. Titers determined by the passive immune hemolytic assay proved to be more reproducible since hemolytic end points were more readily determined and therefore less subjective than end points determined by toxin neutralization.

One of the questions addressed by this study was: Could humoral antitoxin serve as a useful indicator of immune status with respect to LT-producing *E. coli* diarrhea? There are numerous instances in this study in which an inverse relationship between acute, or base-line, GMTs and either illness or isolation of LT-producing *E. coli*, or both, are documented. For example, the select group of eight U.S. summer students ill with LT-producing *E. coli* present had an acute GMT of 11.3, the entire group of U.S. full-time students had an acute GMT of 20.8, and the entire group of Latin American students had an acute GMT of 25.3. The group of 20 Latin American students who exhibited neither diarrhea nor enterotoxigenic *E. coli* had an acute GMT of 30.9. Thus, the answer to the above question appears to be affirmative. Of course, this does not indicate whether humoral antitoxin itself mediates immunity, because an antitoxin response may be only one of a myriad of immune responses resulting from exposure to LT-producing *E. coli*.

The second question addressed by this study was: Could an antitoxin response detectable with paired sera serve as a useful indicator of exposure to LT-producing *E. coli*? The answer to this question also appears to be affirmative. Groups of students who contracted diarrhea and particularly those from whom LT-producing *E. coli* were isolated demonstrated the highest percentage of antitoxin responses and were also the most reactive with regard to the magnitude of the humoral response as a group. Of 28 students who harbored LT-producing *E. coli,* irrespective of illness, 12 (43%) demonstrated an antitoxin response, but by the same criteria only 1 of 13 (7.7%) of those who harbored *Shigella* demonstrated a response.

In the group of 28 U.S. summer students who contracted diarrhea, 29% harbored LT-producing *E. coli* whereas 43% demonstrated an antitoxin response, indicating that the isolation of toxigenic *E. coli* failed in somewhat more than 10% of these cases. This result is not entirely unexpected. However, in the group of 30 full-time U.S. students with documented illness, 40% harbored LT-producing *E. coli* but only 13% demonstrated an antitoxin response. Thus the pattern of humoral antitoxin responses exhibited by the U.S. full-time students showed a marked similarity to that exhibited by the Latin American students. The reason for this is not immediately evident, since with respect to percent isolation of enterotoxigenic *E. coli* the full-time U.S. student group more closely resembled the U.S. summer student group. It would be of interest to perform a more comprehensive prospective study of the etiology of acute diarrhea in an equivalent full-time U.S. student group over a longer period of time, beginning with their arrival in Mexico and including periodic monitoring of their humoral antitoxin status. This type of population might prove to be a prime target for testing the efficacy of immunoprophylactic measures when appropriate products become available.

In summary, the results reported here show that the passive immune hemolysis assay for serum anti-LT antibody effectively detects humoral antitoxin responses when this titration system is used to titrate paired acute- and convalescent-phase sera. Furthermore, humoral antitoxin responses were found to correlate well with the occurrence of diarrhea associated with the presence of LT-producing *E. coli*. This was particularly obvious in the case of the tourist-like group, the newly arrived U.S. summer students, who participated in this study.

ACKNOWLEDGMENTS

This investigation was supported by contract no. 200-75-

0536 from the Center for Disease Control, Atlanta, Ga., and by Public Health Service grants AI-12699 and AI-13385 from the National Institute of Allergy and Infectious Diseases.

We wish to acknowledge Jeff Lewis, D'Lynn Satterwhite, and Darshan Singh for technical assistance and K. Boyle for typing this manuscript.

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