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Microtiter Enzyme-Linked Immunosorbent Assay for Immunoglobulin G Cholera Antitoxin in Humans: Sensitivity and Specificity

ROY M. ROBINS-BROWNE,[1] CHARLES R. YOUNG,[1] MYRON M. LEVINE,[1]* AND JOHN P. CRAIG[2]

The Center for Vaccine Development, Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] and the Department of Microbiology and Immunology, Downstate Medical Center, State University of New York, Brooklyn, New York 11203[2]

Serum samples were obtained from 92 informed, community volunteers before and 10, 21, and 28 days after they ingested 10^3 to 10^6 *Vibrio cholerae* of Inaba or Ogawa serotype and classical or El Tor biotype as part of a cholera vaccine development program. Pre- and postchallenge sera were examined for neutralizing antibody to cholera toxin by the rabbit skin permeability factor and adrenal cell techniques. Immunoglobulin G-binding antibodies to cholera toxin were quantitated by enzyme-linked immunosorbent assay (ELISA) in serum diluted 1:200. The results obtained in these cholera volunteers were compared with a negative control population comprising 30 people who ingested enteropathogenic *Escherichia coli* or *E. coli* which produced heat-stable but not heat-labile enterotoxin. Although all three antitoxin assays correlated closely with each other in both groups of volunteers, ELISA was more sensitive than either neutralization assay in detecting both subclinical and overt cholera infections. Seroconversion was demonstrated by ELISA in 58 of 66 (88%) volunteers who excreted *V. cholerae*, including 50 of 54 (93%) with clinical cholera, compared with 47 of 66 (71%) and 52 of 66 (79%) by the rabbit skin permeability factor and adrenal cell techniques, respectively. Although ELISA does not measure the toxin-neutralizing activity of antibodies directly, it provides a practical alternative to the rabbit skin permeability factor and adrenal cell assays.

Measurement of cholera antitoxin is useful in the serological detection of clinical and subclinical cholera, in epidemiological investigations, and in studies of immunity to *Vibrio cholerae* (1, 2, 7, 8). We have recently reported a microtiter enzyme-linked immunosorbent assay (ELISA) capable of measuring immunoglobulin G cholera antitoxin in serum at a single dilution (9). ELISA values correlated closely with those obtained in the rabbit skin vascular permeability factor (PF) assay. It was concluded that ELISA, which detects antibodies that bind to cholera toxin, provides a reliable in vitro correlate of in vivo neutralizing activity.

In this paper we compare the sensitivity and specificity of microtiter ELISA with PF and Y-1 adrenal cell techniques, standard tests which measure cholera toxin-neutralizing antibodies. In addition, criteria are laid down which define a significant rise in ELISA titer when pre- and postinfection sera are examined at a single dilution.

MATERIALS AND METHODS

Sera. Sera were obtained from volunteers admitted to the 22-bed Isolation Ward of the Center for Vaccine Development during studies to evaluate cholera vaccines and to investigate the fundamental mechanisms of cholera immunity. Test sera were collected from 92 volunteers before and 10, 21, and 28 days after ingesting 10^3 to 10^6 *V. cholerae* of Ogawa or Inaba serotype and classical or El Tor biotype. Pre- and postchallenge sera were also obtained from a negative control population comprising 30 volunteers who ingested enteropathogenic *Escherichia coli* or *E. coli* that produced heat-stable but not heat-labile enterotoxin. Sera were stored at −20°C, and all specimens from an individual were tested simultaneously.

Antitoxin assays. Microtiter ELISA for immunoglobulin G cholera antitoxin was performed on sera diluted 1:200 in phosphate-buffered saline–Tween 20 buffer (pH 7.2) as previously described (9). Neutralizing antibody was assayed by the rabbit skin PF and Y-1 adrenal cell techniques as reported elsewhere (3, 4, 6).

In the PF test a significant rise in titer was denoted by a maximum postchallenge value at least twice that of the prechallenge value, provided the difference amounted to more than 2 antitoxin units per ml. In the adrenal cell test, seroconversion was signified by a fourfold or greater difference between pre- and maximum postchallenge antitoxin titers.

Classification of illness. Cholera infections were classified bacteriologically and clinically as follows: class 0 signified that all stool or rectal swab cultures

performed at least once daily after ingestion were negative for *V. cholerae* and that no diarrhea occurred; class 1 denoted that at least one stool or rectal swab yielded *V. cholerae* but that no diarrhea occurred; class 2 indicated that at least one stool culture was positive and that there were, within a 48-h period, at least two diarrheal stools with a combined volume of not less than 250 ml; and class 3 signified diarrhea in excess of 5 liters.

RESULTS

Results of the PF and adrenal cell assays corresponded with the class of illness (Table 1). Of 54 volunteers who developed overt illness (class 2 or 3) after ingesting *V. cholerae*, 46 (85%) showed a rise in antitoxin titer by the adrenal cell technique and 42 (78%) showed a similar rise by the rabbit skin PF assay. Of 12 people with subclinical (class 1) infection, 6 and 5 were positive by these assays, respectively. In contrast, only 4 of 26 (15%) individuals who ingested *V. cholerae* and who failed to develop clinical or subclinical cholera (class 0) exhibited a rise by either assay. Overall, results of the rabbit skin PF and adrenal cell assays concurred in 79 of 92 sets of sera, whereas in 4 only the PF assay, and in 9 only the adrenal cell assay, showed a rise. Both assays were negative in all 30 volunteers who ingested *E. coli* and who comprised the negative control population.

TABLE 1. *Correlation between class of illness of induced cholera and antitoxin levels measured by the adrenal cell and rabbit skin PF techniques*

Class of illness[a]	No. of volunteers	Significant rise in antitoxin titer[a] by:		Both assays positive	Both assays negative
		Adrenal cell test	PF assay		
0	26	4 (15)[b]	4 (15)	3 (12)	21 (81)
1	12	6 (50)	5 (42)	4 (33)	5 (42)
2	44	38 (86)	33 (75)	32 (73)	5 (11)
3	10	8 (80)	9 (90)	8 (80)	1 (10)
Total	92	56 (61)	51 (55)	47 (51)	32 (35)

[a] See the text for definitions.
[b] Parentheses indicate percentages.

Matched sera from 47 individuals in whom antitoxin rises were detected by both PF and adrenal cell assays were deemed a positive reference sample for comparison with ELISA. All 47 paired sera also showed rises by ELISA, with values (expressed as net optical density) from 0.26 to 1.15 (mean ± standard deviation = 0.72 ± 0.22) higher in postchallenge than in prechallenge specimens (Fig. 1). All but 10 of the 47 had rises of 0.60 or greater, and all but 5 showed rises of 0.40 or greater. In contrast, paired sera from the 30 persons who ingested *E. coli*, all of which were negative in both the PF and adrenal cell assays, showed a rise of between −0.09 and 0.12 (mean ± standard deviation = 0.03 ± 0.06). Fifteen persons had no rise or rises of less than 0.05, whereas 29 of 30 had rises of 0.10 or less. These results are displayed in Fig. 1, which also

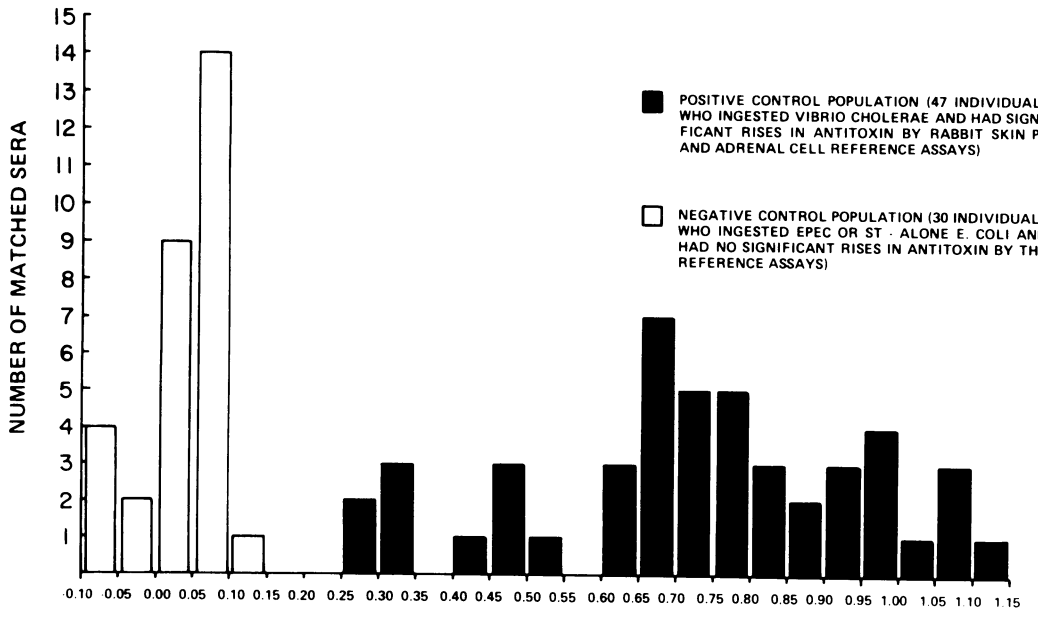

FIG. 1. *Distribution of changes in net optical density values between pre- and postchallenge sera of negative and positive control populations. EPEC, Enteropathogenic E. coli; ST, heat-stable enterotoxin.*

shows that there was no overlap in the frequency distribution of the negative reference population and that of the population that had significant rises in both neutralization assays. Based on this observation, a rise in net optical density of 0.20 or greater between matched pre- and postchallenge sera was taken as evidence of a significant rise in antitoxin titer. This value, which was approximately midway between the lowest rise in net optical density in the positive control population (0.26) and the highest in the negative control population (0.12), exceeded the mean of the negative control population by 3 standard deviations (Fig. 1).

By this criterion, 61 of 92 (66%) volunteers who ingested *V. cholerae*, including 50 of 54 (93%) who developed overt clinical illness, showed a rise in circulating antitoxin titer, i.e., a difference in net optical density between pre- and postchallenge sera of 0.2 or greater by ELISA (Table 2). Rises in binding antibody measured by ELISA corresponded closely with rises in neutralizing antibody. ELISA values, expressed as the maximum positive difference in net optical density between matched pre- and postchallenge sera, also correlated with the extent of illness (Table 2). For all classes of illness, except class 0, ELISA demonstrated significant rises in antitoxin more frequently than either neutralization assay (Tables 1 and 2). The superiority of ELISA in detecting antibody rises was consistent in sera from all volunteers regardless of their history of prior exposure to cholera toxin or toxoid or of the challenge strain biotype (Table 3).

TABLE 2. *Correlation between class of illness of induced cholera and antitoxin levels measured by ELISA*

Class of illness[a]	No. of volunteers	Significant rise in antitoxin by ELISA[a] (%)	ELISA values[b]
0	26	3 (12)	0.09 ± 0.22
1	12	8 (67)	0.39 ± 0.29
2	44	40 (91)	0.63 ± 0.31
3	10	10 (100)	0.62 ± 0.25
Total	92	61 (66)	

[a] See the text for definitions.

[b] Mean ± standard deviation of maximum positive difference in net optical density between matched pre- and postchallenge sera.

TABLE 3. *Comparison of ELISA, adrenal cell, and rabbit skin PF assays in matched sera from volunteers challenged with V. cholerae of classical or El Tor biotype*

Challenge	Biotype	No. of volunteers	No. of volunteers with significant rise in antitoxin titer by:		
			ELISA	Adrenal cell assay	PF assay
Initial	Classical	24	17	16	14
	El Tor	36	32	29	26
Rechallenge or vaccinee	Classical	25	12	11	11
	El Tor	7	0	0	0

DISCUSSION

We previously showed that results of ELISA for serum immunoglobulin G cholera antitoxin correlate closely with those obtained in the rabbit skin PF assay (9). It was argued that, because cholera toxin-neutralizing activity is found chiefly in the immunoglobulin G class (5), titers of immunoglobulin G-binding antibodies would be expected to correspond with those of neutralizing antibodies. The present study provides additional evidence for this suggestion. Thus, all of 47 volunteers who ingested *V. cholerae* and who showed a significant rise in antitoxin titer by both the adrenal cell and PF methods were also positive by ELISA (Fig. 1). On the other hand, ELISA revealed rises in 2 of 12 subclinical infections and 4 of 54 overt infections that were not detected by the adrenal cell assay, and in 3 of 12 and 8 of 54 not detected by the PF technique. In all, seroconversion was demonstrated by ELISA in 50 of 54 (93%) overt and 8 of 12 (67%) subclinical infections (Table 2). Comparable figures for the adrenal cell and PF assays were 46 of 54 (85%) and 6 of 12 (50%), and 42 of 54 (78%) and 5 of 12 (42%), respectively (Table 1). It has recently been shown, however, that the sensitivity of the PF method can be increased by using smaller doses of toxin (J. P. Craig, unpublished data).

Increased sensitivity of ELISA over the neutralizing antibody assays was achieved without any loss of specificity. Thus ELISA was positive no more frequently than either neutralization assay in people with class 0 cholera (no diarrhea, negative cultures) or in volunteers who ingested enteropathogenic *E. coli* or heat-stable enterotoxin-producing *E. coli*.

Microtiter ELISA permits quantification of circulating antitoxin at a single dilution of serum, is readily adapted to dealing with large numbers of specimens, and is comparatively easy to perform. Although it does not measure the toxin-neutralizing capacity of antibodies, ELISA provides a practical alternative to the adrenal cell and rabbit skin PF assays.

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