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Titration of Cholera Antitoxin in Human Sera by Microhemagglutination with Formalinized Erythrocytes[1]

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Microtiter hemagglutination tests employing formalinized sheep erythrocytes sensitized with either crude or purified cholera toxin were used to assay the cholera antitoxin content of human sera. Comparable results were obtained with either crude or purified toxin-sensitized cells with the exception of two sera that gave unusually high hemagglutination titers with the crude toxin. Sera from 13 convalescent cholera patients showed a high degree of correlation between antitoxin levels as determined in vitro by the hemagglutination test and in vivo by the skin permeability factor neutralization test. Fourfold or greater rises in antitoxin levels between acute and convalescent sera were detected in 9 of 15 patients with bacteriologically proven cholera. No significant increases in titer were observed in 14 cases of noncholera diarrhea. Cholera antitoxin was detected by hemagglutination in only 1 of 33 sera, obtained from eight countries, containing vibriocidal antibodies. Formalinized sheep erythrocytes sensitized with toxin and stored at 4 C in the presence of 1:10,000 thimerosal were stable and sensitive for at least 6 months (the longest time tested).

Hochstein et al. (7) reported the successful titration of antitoxin in the sera of rabbits, immunized against *Vibrio cholerae* heat-labile enterotoxin, by means of passive hemagglutination (HA) tests with either fresh or Formalin-preserved sheep erythrocytes. Titration of cholera antitoxin by HA tests has been demonstrated by Ghosh et al. (6) by use of unmodified sheep erythrocytes and by Finkelstein and Peterson (5) who used tanned chicken cells.

The present paper extends our study to include the examination of sera from human cholera and noncholera patients by use of the microtiter technique and stable formalinized sheep erythrocytes.

MATERIALS AND METHODS

Sera. Seventy-one human sera were provided by W. H. Mosley, Pakistan-SEATO Cholera Research Laboratory, Dacca; 13 sera collected 10 days after hospital admission with cholera, Inaba serotype; 30 paired acute and convalescent sera from 15 cholera patients, Inaba and Ogawa serotypes; and 28 paired acute and convalescent sera from 14 noncholera diarrhea patients. Thirty-three sera with demonstrable vibriocidal antibody titers collected during surveys in six countries were obtained from the National Communicable Disease Center (NCDC) serum bank: 1 from American Samoa, 2 from Honduras, 4 from Ghana, 6 from Vietnam, and 10 each from Panama and the Philippines. All sera were stored at −20 C and inactivated by heating at 56 C for 30 min before testing.

Sensitization of erythrocytes. Formalinized sheep erythrocytes (3) were sensitized as described previously (7). Briefly, formalinized cells were washed three times in cold 0.85% NaCl and a 10% concentration of cells was suspended in either crude (7) or purified (S. H. Richardson, Bacteriol. Proc., p. 89, 1969) toxin [each toxin was diluted in 0.85% NaCl to 4,000 Lb/ml (2) and then adjusted to *p*H 6.4 to 6.6 with 0.1 N HCl]. The toxin-cell suspension was incubated for 30 min at 22 to 25 C with occasional shaking and washed three times in cold 0.85% NaCl; the cell concentration was adjusted to 0.5% for HA tests. Treatment of the cells with tannic acid was unnecessary (7).

Titration of sera. Naturally occurring antibodies

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against the erythrocyte carrier were absorbed from the sera with fresh, washed packed sheep cells. Serial two-fold dilutions of sera in 0.025-ml volume were mixed with 0.025 ml of formalinized sensitized cells in microtiter "U" plates (Cooke Engineering Co., Alexandria, Va). Cell controls, as well as a titration of positive and negative reference sera, were included with each titration. Plates were agitated gently, sealed with plate sealers, incubated at 37 C for 2 hr, refrigerated overnight at 4 C, and read by the pattern method over a microtiter mirror. No further change in titer was observed after overnight incubation. Titers were recorded as the highest primary serum dilution showing a positive HA pattern. The antitoxin content of each serum was calculated by comparison of the titer of serum under test to that of the simultaneously titrated reference rabbit antitoxin (lot 18) known to contain 160 units of antitoxin per ml as defined by Craig (2). Results are based on the geometric mean titer of three tests.

In vivo antitoxin titers were established by the skin permeability factor (SPF) neutralization test (4) and expressed as antitoxin units per milliliter (2). Vibriocidal antibody titers were determined as described by McIntyre and Feeley (10).

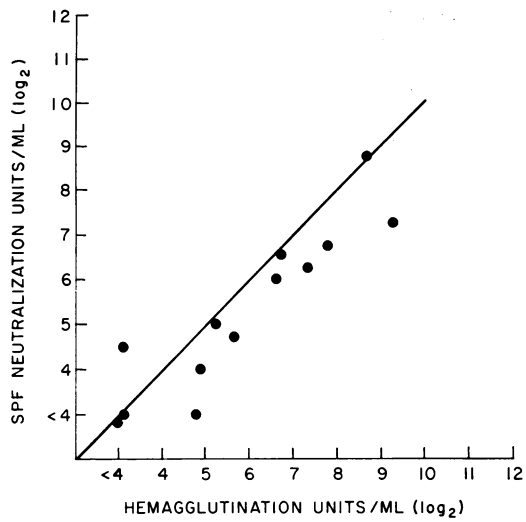

FIG. 1. *Correlation of antitoxin units in convalescent human sera as determined by SPF and HA tests with purified toxin* (r = 0.98). *Diagonal line represents equal titers.*

RESULTS

Convalescent cholera patient sera. Table 1 shows an excellent agreement between in vitro HA titers obtained by use of purified and crude sensitizing antigens. The only exceptions are with sera 4067 and 4393, which gave inordinately high titers with the crude toxin-sensitized cells. This was perhaps due to HA by some other antigen-antibody system. It should be pointed out that the close agreement of "titer" (expressed in serum dilutions) and calculated "units" is fortuitous, since the dilution end point in this system was usually very close to the antitoxin level in Craig units (2).

TABLE 1. *Comparison of antitoxin levels in cholera convalescent sera as determined by hemagglutination (HA) and skin permeability factor (SPF) neutralization tests*

Serum no.	HA titer, erythrocytes sensitized with				SPF neutralization titer (units/ml)
	Purified toxin		Crude toxin		
	Titer[a]	Units/ml	Titer[a]	Units/ml	
4794	<16	<17	<16	<17	<16
4859	<16	<17	<16	<17	<16
4067	24	26	79	86	<16
3903	26	28	<16	<17	16
4410	<16	<17	<16	<17	23
4393	49	53	388	422	24
4024	32	35	39	42	32
3598	91	99	49	53	64
4053	128	139	97	106	79
3957	91	99	79	86	91
4512	208	226	256	278	111
3814	512	557	388	422	158
3579	362	394	512	557	446
Reference antitoxin[b]	147	(160)	147	(160)	(160)

[a] Geometric mean titer of three tests.
[b] Amount was 160 units/ml.

Table 1 also shows that the HA antitoxin units determined with cells sensitized with purified toxin were in good agreement with the in vivo units determined by the SPF neutralization test, except perhaps for serum 3814 which was 3.5-fold higher by the HA test. A graphic display of antitoxin levels determined by HA with purified toxin and by the SPF neutralization test is given in Fig. 1. Antitoxin unitage determined by HA tended to be slightly higher, but the methods show excellent correlation (r = 0.98). A correlation of r = 0.96 was obtained when crude toxin-sensitized cells were used for HA.

HA titrations (with cells sensitized with purified toxin) of paired acute and convalescent cholera patient sera are recorded in Table 2. Nine of the 15 patients developed a fourfold or greater increase in antitoxin level and 3 other patients developed an almost two-fold increase. The antitoxin level of one serum (9117) dropped from 80 to 20 units/ml in 42 days. The serotype of the causative vibrio seemed to have no effect on the antitoxin response. It is of interest to note that most of the patients had detectable antitoxin on admission.

TABLE 2. *Hemagglutination titration of paired sera from cholera patients*

Case no.	Acute serum[a]		Convalescent serum		Four-fold or greater increase in titer
	Vibrio cholerae serotype isolated	Antitoxin titer (units/ml)[b]	Time after admission (days)	Antitoxin titer (units/ml)[b]	
9396	Ogawa	<16	5	<16	−
9342	Inaba	160	6	197	−
9015	Inaba	20	7	160	+
9023	Inaba	50	7	638	+
9353	Ogawa	160	7	259	−
9513	Inaba	32	7	80	−
9408	Inaba	<16	8	318	+
9437	Ogawa	98	9	1037	+
9524	Inaba	16	9	98	+
9539	Inaba	24	10	40	−
9538	Inaba	65	10	1037	+
9061	Inaba	98	10	1568	+
9419	Inaba	<16	10	318	+
9698	Inaba	65	37	518	+
9117	Ogawa	80	42	20	−

[a] Serum drawn on day of hospital admission.
[b] Geometric mean titer of three tests.

TABLE 3. *Hemagglutination titrations of paired sera from noncholera diarrhea patients*

Case no.	Acute serum[a] (antitoxin titer in units/ml)[b]	Convalescent serum	
		Time after admission (days)	Antitoxin titer (units/ml)[b]
7188	<16	6	<16
7169	<16	6	<16
7238	259	6	40
6970	40	7	65
6994	<16	7	<16
7167	<16	7	<16
7240	<16	7	<16
7008	<16	8	<16
7220	<16	9	<16
7208	<16	9	<16
6978	<16	9	<16
6984	<16	9	<16
6942	<16	11	<16
6949	<16	11	<16

[a] Serum drawn on day of hospital admission.
[b] Geometric mean titer of 3 tests.

Unfortunately, a limited quantity of serum prevented determination of antitoxin by the SPF neutralization test.

HA titers of paired sera from noncholera diarrhea patients are recorded in Table 3. None showed a significant increase in titer. The antitoxin level of 1 (6970) of the 14 patients increased insignificantly, whereas another (7238) showed a fourfold decrease. In contrast to the patients with cholera, nearly all of the sera were lacking in detectable antitoxin.

Results of vibriocidal and HA titrations of the 33 sera from the NCDC serum bank given in Table 4 show that there was no relation between vibriocidal and antitoxin properties. Vibriocidal titers ranged from 1:320 to 1:5,120. Thirty-two of the sera had less than 5 antitoxin units/ml by the HA titration. One serum (from Samoa) had 40 antitoxin units/ml.

DISCUSSION

In the titration of sera from convalescent cases of cholera, HA levels were found to be essentially the same, regardless of whether erythrocytes were sensitized with crude or purified cholera toxin. Although the results were comparable, the use of purified toxins would be preferable, at least on theoretical grounds, since the crude toxin preparation employed contained at least four antigens other than toxin by the Ouchterlony gel diffusion test (7). In fact, with crude toxin, two cholera convalescent sera had unusually high HA titers in relation to SPF antitoxin titration levels. For this reason, only erythrocytes sensitized with purified toxin were employed for tests with paired cholera and noncholera sera.

The present study shows clearly that HA levels correlated well with cholera antitoxin levels as measured in vivo by the SPF neutralization test. These observations confirm and extend our previous study (7) with rabbit sera and show in addition the feasibility of employing the microtiter system. Finkelstein and Peterson (5) have reported a similar correlation based on data derived from more laborious HA tests employing fresh chicken erythrocytes treated with tannic acid.

In HA titrations (purified toxin-sensitized cells) on paired acute and convalescent sera, most (9 of 15) of the bacteriologically proven cholera cases showed a significant rise in antitoxin levels, al-

TABLE 4. *Vibriocidal and hemagglutination (HA) titers of 33 sera from serological surveys in six countries*

Country	No. of sera	Vibriocidal titer[a] (range)	HA (antitoxin units/ml)
American Samoa	1	1,280	40
Honduras	2	320–640	<5[b]
Ghana	4	320–2,560	<5[b]
Vietnam	6	640–1,280	<5[b]
Panama	10	320–5,120	<5[b]
Philippines	10	320–2,560	<5[b]

[a] Reciprocal end point dilution.
[b] Each serum had <5 antitoxin units/ml.

though great variation between patients was observed. In contrast, significant increases were not detected with paired sera from 14 diarrhea cases thought not to be cholera based on bacteriological findings. The presence of antitoxin in the acute-phase sera of most noncholera as compared to cholera cases is striking, but at this time it is unexplained. Benenson et al. (1) and Martin et al. (9) noted the presence of cholera antitoxin, as judged by the SPF neutralization test, in many "acute-phase" cholera patient sera.

The use of formalinized erythrocytes (3) for the HA test had the advantage that a large number of tests can be performed over an extended period of time with the same lot of red cells. An even greater advantage is that the formalinized sheep cells sensitized with cholera toxin, if stored at 4 C in the presence of 1:10,000 thimerosal, can be employed for at least 6 months (longest time tested to date) without loss of stability or sensitivity. This avoids the time-consuming process of daily cell preparations such as those employed by Finkelstein and Peterson (5).

A disadvantage of employing sheep erythrocytes is the fact that human sera must be absorbed with unsensitized cells to avoid false-positive reactions. To eliminate the absorption procedure, we have attempted to sensitize and employ both fresh and formalinized human "O" erythrocytes. The same technique used for sheep cells has consistently failed to sensitize fresh or formalinized (tanned and untanned) human "O" cells. Some success has been achieved in sensitizing human "O" cells with very high toxin concentrations, but the limited supply of purified toxin available renders this approach impractical.

It should be emphasized that, although only antitoxin determined by the SPF neutralization test was used as the in vivo method in this paper, Mosley et al. (11), using the same sera, have shown an excellent correlation of results obtained by the SPF test with those obtained by the ileal loop neutralization test (8).

The use of stable formalinized sheep erythrocytes and the microtiter system should facilitate studies on the evaluation of the potential role of "antitoxic" immunity and "toxoid" vaccines in immunization against cholera. With this system, it should be possible to titrate the antitoxin unitage of a large number of sera available only in small volumes.

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