Titration of Cholera Antitoxin Levels by Passive Hemagglutination Tests Using Fresh and Formalinized Sheep Erythrocytes[1] (34421)

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(Introduced by M. Pittman)

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In the past, the precise titration of antitoxin against the heat-labile enterotoxin of *Vibrio cholerae* has been made either by the skin capillary permeability factor (SPF) (1–3) or rabbit ileal loop (4) tests. These assays, particularly the latter, are cumbersome, expensive, and not satisfactory for titration of large numbers of sera. The present paper describes a simple passive hemagglutination (HA) test which correlates well with the SPF neutralization test and shows its feasibility for titration of sera for antitoxin.

Materials and Methods. Two toxin preparations were used as antigens in the HA tests. (a) One was a concentrated crude toxin prepared by dialyzing crude cholera exotoxin (3) prepared from *V. cholerae* serotype Inaba, strain 569B against phosphate buffered isotonic saline, pH 7.4, for 24 hr and then concentrated 10-fold by immersion in Carbowax slurry. The final preparations contained 200,000 bluing doses (BD)/ml and 4,000 limit of bluing (Lb) doses/ml as defined by Craig (1, 5). In addition to the toxin, this crude material contained at least 4 other antigens as demonstrated by Ouchterlony double diffusion precipitation. (b) The other toxin preparation was highly purified by sequential dextran–sulfate precipitation, gel filtration and ion-exchange chromatography (6). This material, which contained a single protein-staining band by disc electrophoresis, appeared immunologically pure by disc immunoelectrophoresis and conventional immunodiffusion methods when tested with antiserum against crude toxin. The purified stock toxin contained 20,000,000 BD/ml, 409,000 Lb/ml and 600 μg/ml of Folin protein and was diluted prior to use in 0.85% NaCl.

Sera from rabbits hyperimmunized with a variety of cholera vaccines, toxins, and toxoids were employed. They ranged in antitoxin titers from negative to high. Two sera were absorbed with vibrio cells to remove antibodies against vibrio cellular antigens (7). All sera were stored at —20° without preservatives, and were inactivated by heating at 56° for 30 min before testing. Naturally occurring antibodies against the erythrocyte carrier were absorbed from the sera with packed unsensitized cells.

Fresh sheep blood was collected in Alsever's solution, stored at 4° and used within 2 weeks. Three types of erythrocyte suspensions were employed: (a) fresh untreated, (b) tanned with 1:20,000 tannic acid (8), and (c) formalinized (9). The cells of each preparation after washing were suspended at a 10% concentration in sensitizing toxin containing 4000 Lb/ml (which had been adjusted to pH 6.4–6.6 with 0.1 *N* HCl), incubated at 22–25° for 30 min with occasional shaking, washed 3 times by centrifugation in cold 0.85% NaCl, and adjusted to a concentration of 0.5% for HA tests. Increasing the concentration of pure toxin by 10- or 100-fold or decreasing the concentration by 10-fold did not affect the test sensitivity. However, decreasing the concentration by 100-fold slight-

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ly decreased the sensitivity.

Serial 2-fold dilutions of sera in 0.25-ml volumes were mixed with 0.25 ml of sensitized erythrocytes in 13 × 100-mm tubes. Serum dilutions for tests with untreated or formalinized erythrocytes were prepared in 0.85% NaCl, and for tests with tanned cells, in the same diluent containing 1% normal rabbit serum. Tests were incubated for 2 hr in a 37° water bath and overnight at 4°, and titers were recorded by the pattern of erythrocytes that had settled to the bottom of the tubes. Readings before and after overnight refrigeration were essentially identical; overnight readings are reported.

Vibriocidal antibody titers were determined as previously described (10); antitoxin titers were established by the SPF neutralization test (3) and expressed in terms of the unit defined by Craig (5).

Results. An excellent correlation ($r = 0.98$) between antitoxin and HA titers was obtained with untreated sheep cells sensitized with crude cholera toxin (Fig. 1). Figure 1 also shows that essentially identical HA and antitoxin titers were obtained with two sera rendered monospecific for the toxin by absorption with vibrio cells to remove antibodies against vibrio cellular antigens (7). The HA titers showed scattering in relation to vibriocidal antibody titers (Fig. 2).

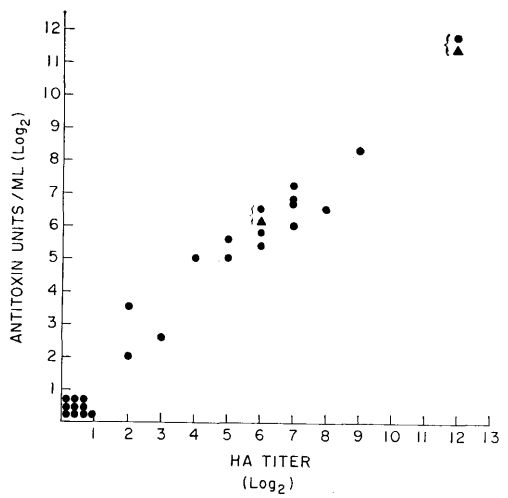

FIG. 1. Relationship of antitoxin levels and hemagglutination titers determined with untreated cells sensitized with crude toxin: (●), unabsorbed serum; (▲), serum absorbed with living washed vibrios to remove vibriocidal antibody; ({), paired unabsorbed and absorbed sera.

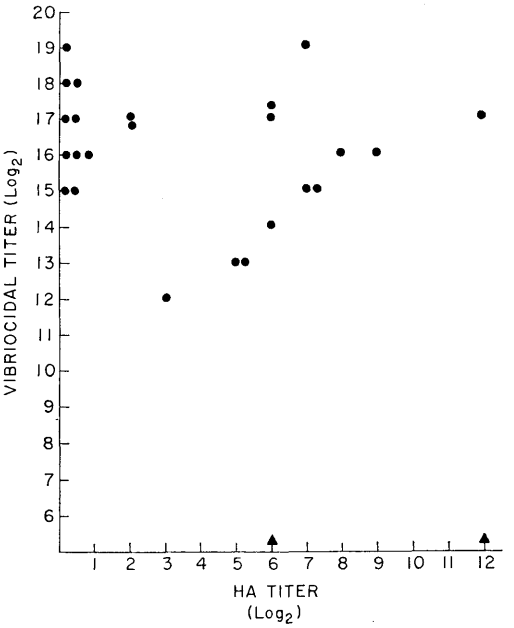

Fig. 2. Relationship of vibriocidal antibody titers and hemagglutination titers determined with untreated cells sensitized with crude toxin: (●), unabsorbed serum; (▲), serum absorbed with living washed vibrios to remove vibriocidal antibody.

Figure 3 depicts a comparison of titers using tanned erythrocytes sensitized with crude toxin. With most sera, a correlation of HA and antitoxin titers was obtained. However, 5 sera with detectable antitoxin by the *in vivo* skin test failed to cause HA. These same sera agglutinated untanned sensitized cells.

Untreated erythrocytes sensitized with purified toxin showed a good correlation ($r = 0.98$) between HA and antitoxin titers (Fig. 4). Three sera containing relatively low antitoxin titers gave negative HA tests. Essentially identical results were obtained with tanned cells. Hemagglutination titers obtained with either tanned or untreated cells sensitized with purified toxin showed no correlation with vibriocidal antibody titers, giving a picture similar to that in Fig. 2.

Tests with formalinized erythrocytes sensitized with crude (Fig. 5) or purified (Fig. 6)

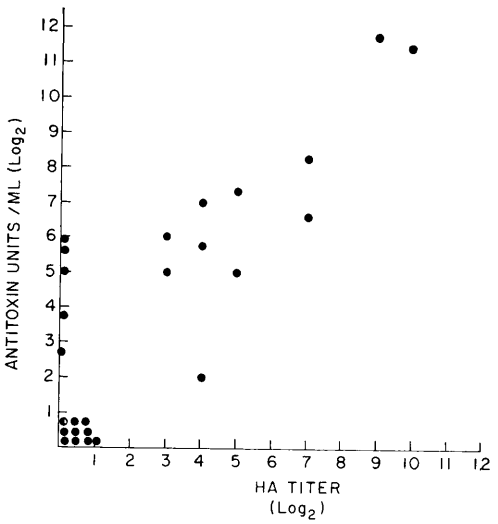

FIG. 3. Relationship of antitoxin levels and hemagglutination titers determined with tanned cells sensitized with crude toxin: (●), unabsorbed serum.

toxins displayed high degrees of correlation ($r = 0.99$ and 0.98, respectively) between HA and antitoxin titers. Only one serum having a low antitoxin titer failed to show HA with formalinized cells sensitized with purified antigen. Tanning was unnecessary. No correlation was obtained with vibriocidal antibody titers. Stability and sensitivity of sensitized formalinized erythrocytes stored as a 0.5% suspension containing 1:10,000 thimerosal were maintained for at least 60 days at 4° and 20–25° (the longest time tested), and up to 1 week at 37°.

Discussion. The present study shows clearly that results of HA tests with sheep erythrocytes sensitized with either crude or purified cholera toxin preparations correlate well with antitoxin levels in rabbit sera measured by the SPF neutralization tests (3). It is of considerable interest that the toxin antigen, thought to be a protein (6, 11, 12), was capable of sensitizing untreated erythrocytes. Tanning is generally required with protein antigens (8, 13). Elimination of this rather troublesome procedure is advantageous. In the present study, tanned cells gave

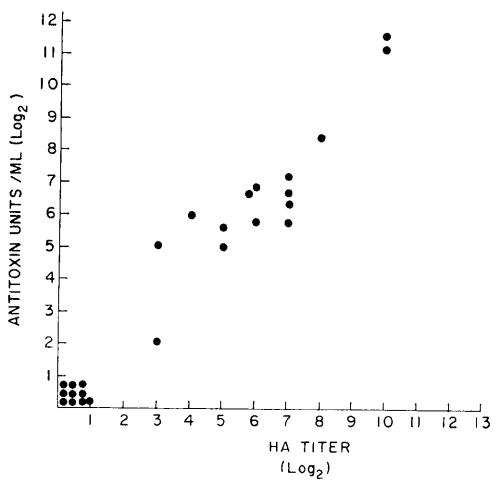

Fig. 4. Relationship of antitoxin levels and hemagglutination titers determined with untreated cells sensitized with purified toxin: (●), unabsorbed serum.

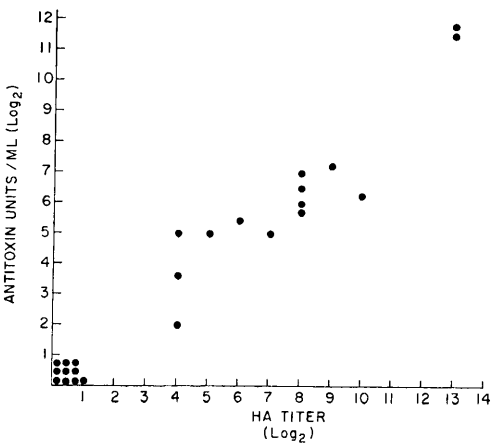

Fig. 5. Relationship of antitoxin levels and hemagglutination titers determined with formalinized cells sensitized with crude toxin: (●), unabsorbed serum.

false negative results with certain low titer sera.

Crude cholera toxin, which contained at least four antigens in addition to the toxin by gel diffusion gave the most satisfactory results. The somewhat surprising failure of cell-wall lipopolysaccharide antigens present in the crude toxin (14) to sensitize the cells and cause false positive reactions with sera having high vibriocidal titers might be caused by the requirements for heat or alkali treatment of lipopolysaccharides for effective erythrocyte sensitization. Purified toxin theoretically would offer a greater assurance against false positive reations. However, false negative HA reactions using purified toxin were obtained

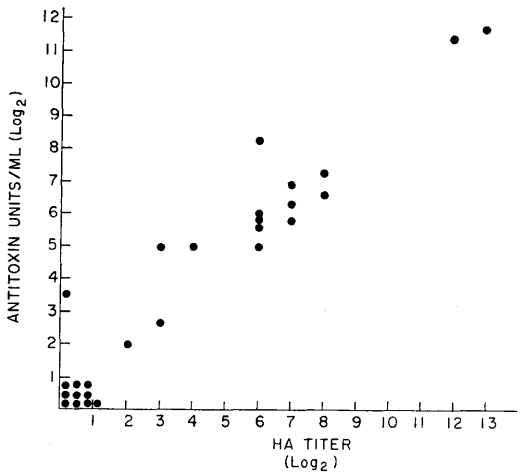

FIG. 6. Relationship of antitoxin levels and hemagglutination titers determined with formalinized cells sensitized with purified toxin; (●), unabsorbed serum.

with several sera having relatively low antitoxin titers by the *in vivo* test. The significance of this, if any, awaits further studies with additional sera.

The use of formalinized erythrocytes for the HA test has the advantage that a very large number of tests could be performed over an extended period of time with the same lot of red cells. In addition, our experience indicates that sensitized formalinized cells can be prepared and used for at least 60 days, the longest time tested to date, thereby avoiding the time-consuming process of daily washing and sensitization of cells.

At present, there is great interest in the development of toxoid-containing cholera vaccines, since most presently available vaccines lack the ability to stimulate antitoxin (3). The HA technique should prove useful in conjunction with serologic surveys during field trials of toxoid vaccines, especially since this method should be readily adaptable to studies on large numbers of small serum samples by the microtiter technique. Even the rabbit skin test procedure, while much simpler than the ileal loop test, would be most difficult to apply to large numbers of sera, especially in field laboratories. Another possible use of the HA test would be for diagnosis. Benenson *et al.* (2), using the skin test, found that the measurement of antitoxin was of diagnostic value in cholera.

After our present study was completed, Ghosh *et al.* (15) using untreated sheep erythrocytes and different antigens (whole-cell lysates, peptone water supernatants, and purified toxin fractions), reported a relationship of HA titers with antitoxin levels measured in the rabbit ileal loop (4).

Since antitoxin levels as measured by the SPF and ileal loop neutralization test show close correlation (16), it is indicated that the two studies are in agreement and that each shows the feasibility of using the HA test for titration of cholera antitoxin. Our study provides additional information on the suitability of different toxin and erythrocyte preparations and the lack of interference of somatic antigens in the HA test. Ghosh *et al.* (15) did encounter some evidence of false positive reactions due to antibodies other than antitoxin when very crude antigens (*e.g.*, whole-cell lysates) were used for sensitization. Additional studies are in progress to evaluate the use of microtiter procedures and to apply HA tests for titration of antitoxin in sera from cholera patients and from persons immunized with cholera toxoids.

Summary. Hemagglutination titers of cholera antitoxin (rabbit sera) determined with sheep erythrocytes sensitized with crude or purified cholera toxin correlated with the antitoxin levels as determined by the rabbit skin permeability factor neutralization test. Tannic acid treatment of erythrocytes was unnecessary. Both fresh and formalinized erythrocytes were satisfactory, and the latter, sensitized with toxin, could be stored without loss of sensitivity for at least 60 days. The presence of "somatic" antigen in crude toxin did not interfere with the specificity of the test.

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