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Enzyme Immunoassay for Detection of Antibody to Toxins A and B of *Clostridium difficile*

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An enzyme immunoassay (enzyme-linked immunosorbent assay [ELISA]) to detect hamster antibody to toxins A and B of *Clostridium difficile* was developed in which toxin preparations are used to coat the solid phase. The specificity of the assay was supported by blocking tests with the toxin preparations and proteins from a nontoxigenic strain. Sera from immunized and control hamsters were tested by this technique, and results were compared with those from a cytotoxicity neutralization assay. Antibody to toxins A and B assayed by ELISA showed a close quantitative correlation with antibody titers obtained by cytotoxicity neutralization. The ELISA assays described appear to provide a sensitive, specific, and practical method to define the prevalence of antibody to *C. difficile* toxins. These assays could be readily applied to human sera to examine and study the immune response of patients with *C. difficile*-induced disease.

Clostridium difficile has been implicated as the etiological agent of antibiotic-associated diarrhea and colitis in both experimental animals and patients (2). This disease is believed to be toxin mediated, and recent publications from three groups indicate that *C. difficile* produces two distinct toxins (1, 12, 14). The toxins, designated A and B, are antigenically distinct, large-molecular-weight proteins which are separated by anion-exchange chromatography. Both toxins cause actinomorphic changes with fibroblast cells in tissue culture, although titers are substantially different and results with other assays of biological activity show considerable variations. Studies from our laboratory (14) suggest that toxin B is a potent cytotoxin which is responsible for most of the activity in the usual tissue culture assay. Toxin A appears to be more active in assays of enteric disease in experimental animals, suggesting a paramount role in the clinical expression of the disease.

Antibodies to the toxins can be assayed by neutralization of their cytopathic effects; however, tissue culture requires specialized facilities which are not widely available. The enzyme-linked immunosorbent assay (ELISA) is a sensitive and practical assay system which has been used extensively for the detection of bacterial and viral antibodies (5, 11). The purpose of the present study was to develop an enzyme immunoassay to measure antibody to toxins A and B of *C. difficile*.

MATERIALS AND METHODS

Antigen preparation. *C. difficile* strain CTP213, recovered from a patient with antibiotic-associated pseudomembranous colitis, was used for production of toxins A and B. Dialysis bags containing 200 ml of sterile distilled water were suspended in 3 liters of brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.), inoculated with *C. difficile*, and incubated for 5 days as previously described (13). The dialysis bag contents were clarified by centrifugation at 10,000 × *g*. The supernatant fractions were sterilized by filtration, using 0.45-μm (pore size) nitrocellulose membranes (Millipore Corp., Bedford, Mass.), dialyzed against distilled water, and lyophilized. Proteins from a nontoxigenic strain were prepared in a similar manner. A control antigen for the enzyme immunoassay was prepared from the contents of an uninoculated dialysis bag culture.

For ion-exchange chromatography, the concentrated culture filtrate was reconstituted in 0.05 M Tris-hydrochloride buffer, pH 7.5, to a protein concentration of 10 mg/ml, using the Bio-Rad protein assay with bovine gamma globulin as the standard (Bio-Rad Laboratories, Richmond, Calif.). One milliliter of this preparation was applied to a DEAE-Sepharose CL-6B column (1.8 by 20 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.5 (starting buffer), and washed with 2 bed volumes of starting buffer. Toxins A and B were separated by stepwise elution, using 0.25 and 0.45 M NaCl in starting buffer, respectively, and collected in 4.0-ml fractions. Three peak fractions of DEAE-toxin A were combined, dialyzed against 0.01 M glycine–Tris buffer, pH 8.9, and concentrated to 500 μl, using an Amicon YM-30 ultrafiltration membrane in an Amicon 8MC stirred cell (Amicon

Corp., Danvers, Mass.). Toxin B was collected as a single fraction.

Polyacrylamide gel electrophoresis of the concentrated DEAE-toxin A was performed with an LKB Multiphor 2117 horizontal electrophoresis unit (LKB Instruments, Rockville, Md.) at a constant setting of 350 V. The slab gels were prepared with 5% total acrylamide (2.6% cross-linking) and 0.1 M glycine–Tris buffer, pH 8.9. Bromophenol blue tracking dye (0.01%) was added to the sample and allowed to migrate 7 cm from the origin. Under these conditions, toxin A banded 0.5 to 0.7 cm from the origin and was electroeluted into 1-cm^2 wells cut from the acrylamide slab 0.2 cm below toxin A. The wells were filled with electrode buffer and 350 V was applied for 15 min. The toxin A which had migrated into the wells was collected, and this process was repeated twice. The electroeluted toxin A samples were combined, dialyzed against 0.001 M Tris-hydrochloride buffer, pH 7.5, and lyophilized. When reapplied to polyacrylamide gel electrophoresis, this preparation produces a single band 0.7 cm from the origin.

Hamster immunization. Fourteen male Golden Syrian hamsters, 6 to 8 weeks old (Charles River Breeding Laboratories, Wilmington, Mass.), were given weekly subcutaneous injections for 12 weeks with 0.1 ml of culture filtrate toxoid (1 mg of protein per ml) mixed 1:1 with Freund incomplete adjuvant. Control hamsters were given 0.1 ml of phosphate-buffered saline (PBS) mixed 1:1 with Freund incomplete adjuvant. Eight animals were bled by retro-orbital puncture before the start of immunization. Four animals from the control group were bled by retro-orbital puncture after 3, 5, 7, and 12 doses of the sham immunogen. Four animals from the immunized group were bled after 3, 5, and 7 doses and six animals were bled after 12 doses of the immunogen. Two and four animals from each group were exsanguinated by cardiac puncture after three and seven doses, respectively. Two hamsters were given weekly subcutaneous injections for 10 weeks with 0.1 ml of a toxin A toxoid (125 to 250 μg of protein per ml) mixed 1:1 with Freund incomplete adjuvant. Both animals were exsanguinated by cardiac puncture 1 week after the last dose of the immunogen. All serum samples were stored at −20°C until tested.

ELISA methods. The ELISA was performed according to a modification of the method of Voller et al. as previously established (15). Duplicate wells of polystyrene, round-bottomed microtiter plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of purified toxin A (0.5 μg of protein per ml in PBS) or partially purified toxin B (1 μg of protein per ml in PBS). Duplicate blank wells were coated with the control antigen (1 μg of protein per ml in PBS). The optimal dilutions of coating reagents were determined by checkerboard titration. After incubation for 14 h at 4°C, the plates were either used immediately or stored at 4°C. Before use the plates were washed five times with PBS containing 0.05% Tween 20 (PBST). A 25-μl sample of serum diluted in PBS was added to the appropriate wells and diluted with 75 μl of PBST–0.5% fetal calf serum. After incubation for 1 h at 37°C, the plates were washed five times with PBST, and 100 μl of peroxidase-labeled rabbit anti-hamster immunoglobulin G (Miles Laboratories, Elkhart, Ind.) diluted 1:800 in PBST-0.5% fetal calf serum was added. The plates were incubated for 1 h at 37°C and then washed five times with PBST, and 100 μl of substrate solution was added. This substrate solution was prepared by adding 40 mg of *O*-phenylenediamine and 40 μl of 30% hydrogen peroxide to 100 ml of 0.01 M citrate buffer (pH 5.0) immediately before use. After incubation for 30 min at room temperature, the absorbance was measured at 450 nm in a microplate colorimeter reader (MR 480; Dynatech Laboratories). Net absorbance was calculated by subtracting the mean absorbance in the wells coated with control antigen from the mean absorbance of the wells coated with toxin A or B.

ELISA blocking test. An ELISA blocking test was performed by the method of Yolken and Stopa (17). A dilution of the test serum in PBST-0.5% fetal calf serum was selected which gave an absorbance of 0.400 to 0.500 in wells coated with toxin A or B. The serum was mixed with increasing concentrations of toxin A, toxin B, or proteins derived from a nontoxigenic strain of *C. difficile*, incubated for 45 min at 37°C, and then reacted in the ELISA system in wells coated with toxin A or B.

Cytotoxicity neutralization assay. The cytotoxicity of toxins A and B was determined with WI-38 human lung fibroblasts grown to a confluent monolayer in a microtiter tissue culture system as previously described (3). Serial 10-fold dilutions of partially purified toxin B or purified toxin A preparations were tested. The greatest dilution which caused typical morphological changes in 100% of the cell population was defined as the 100% cytopathic dose (TCD_{100}). The TCD_{100} of the toxin B preparation used for the neutralization assay was 27 pg of protein (540 pg/ml). The TCD_{100} of the toxin A preparation was 28.3 ng of protein (566 ng/ml). The neutralization assay was performed as follows. Serial twofold dilutions of test serum were prepared in PBS in a final volume of 50 μl and were mixed with an equivalent volume of toxin A or B at a concentration 20-fold greater than the respective TCD_{100}. After incubation at room temperature for 20 min, 50 μl of each mixture was added to microtiter wells containing WI-38 cells in 50 μl of tissue culture medium. Thus, the concentration of toxin in the well was 10 TCD_{100}. The antitoxin titer was the greatest dilution of serum which completely neutralized 10 TCD_{100}.

RESULTS

Use of serum at a single dilution. Preliminary studies with a high-titer positive hamster serum revealed a linear relationship between serum dilution and net absorbance (Fig. 1). Net absorbance of serial twofold dilutions of pooled sera from five unimmunized hamsters showed no significant ELISA activity for dilutions from 1:128 to 1:1,024 (net absorbance, ≤0.053). The linearity of the positive control serum suggested that antibody could be quantified by testing serum at a single dilution; therefore, all serum samples were tested in duplicate at a dilution of 1:128 in PBS (10, 18). The positive control serum at a dilution of 1:128 was included on every plate. To control for day-to-day and plate-to-plate variation, the results for all samples

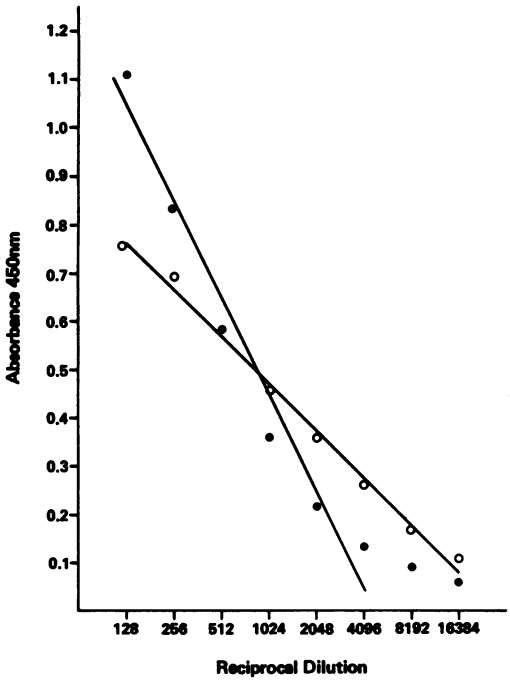

FIG. 1. Relationship between ELISA values (net absorbance) for anti-A (○) and anti-B (●) antibody and serial twofold serum dilutions of a positive control serum. The regression equation for anti-A antibody is $y = -0.10 \log_2 x + 1.44$, and $r = -0.997$. The regression equation for anti-B antibody is $y = -0.20 \log_2 x + 2.43$, and $r = -0.983$.

were expressed as corrected absorbance values: $\text{absorbance}_{\text{corrected}} = \text{absorbance}_{\text{sample}} \times [\text{absorbance}_{\text{positive control (reference day)}}/\text{absorbance}_{\text{positive control (test day)}}]$.

Definition of a positive sample. The pooled negative control serum was assayed in duplicate on 10 plates. The means and standard deviations of the corrected absorbance values for anti-A and anti-B antibody were 0.040 ± 0.015 and 0.038 ± 0.013, respectively. Any sample with a corrected absorbance of >2 standard deviations above the mean of the negative control was considered positive for antitoxin.

Reproducibility of ELISA. The reproducibility of the ELISA was studied by testing three positive sera in quadruplicate on five plates over 4 days. The coefficients of variation for the three samples, determined from the mean and standard deviation of the corrected absorbance, were 9.2, 16.6, and 9.2% for anti-A antibody and 11.1, 9.8, and 18.6% for anti-B antibody.

Response of immunized hamsters. Antibody to toxin B as measured by the cytotoxicity neutralization assay was detected in parenterally immunized hamsters after 5 weeks, and titers continued to rise with additional booster doses of the immunogen. Antibody to toxin A as measured by the cytotoxicity neutralization assay was also detected after 5 weeks, but titers either remained stable or dropped with additional booster doses. All of the control and preimmunization sera failed to neutralize either toxin at the lowest dilution tested (1:16). The positive control serum obtained from a hamster sacrificed after 14 doses of the immunogen had an anti-B antibody titer of 1:512 by the cytotoxicity neutralization assay and an anti-A antibody titer of 1:256. This same serum also had a neutralizing antibody titer of 1:1,024 for 10 mouse lethal doses of partially purified toxin A. Sera obtained from the two hamsters immunized with toxin A had an anti-A antibody titer of 1:400 in the cytotoxicity neutralization assay. Both sera failed to neutralize the cytopathic effect of toxin B at a 1:2 dilution. Antibody to both toxins as measured by ELISA was detected in immunized hamsters after 3 weeks and continued to rise with subsequent doses of the immunogen. The sham-immunized hamster failed to develop a detectable antibody response to either toxin (Table 1). The titration curves in the ELISA assays of the sera from the two hamsters immunized with a toxin A toxoid are shown in Fig. 2. Both animals had high-titer antibody to toxin A

TABLE 1. ELISA values for anti-A and anti-B antibody in parenterally immunized and control hamsters

No. of doses	Mean net absorbance (corrected)[a]			
	Anti-toxin A		Anti-toxin B	
	Immunized	Control	Immunized	Control
Pre-bleed	0.028 ± 0.013		0.034 ± 0.011	
3	0.102 ± 0.026	0.024 ± 0.029	0.180 ± 0.083	0.025 ± 0.030
5	0.305 ± 0.060	0.013 ± 0.009	0.703 ± 0.074	0.027 ± 0.010
7	0.294 ± 0.058	0.022 ± 0.029	0.814 ± 0.180	0.020 ± 0.022
12	0.501 ± 0.042	0.012 ± 0.024	0.892 ± 0.046	0.011 ± 0.022
14	0.735[b]		1.110[b]	

[a] All values are means ± 1 standard deviation of four to eight serum samples.
[b] Represents a single serum determination.

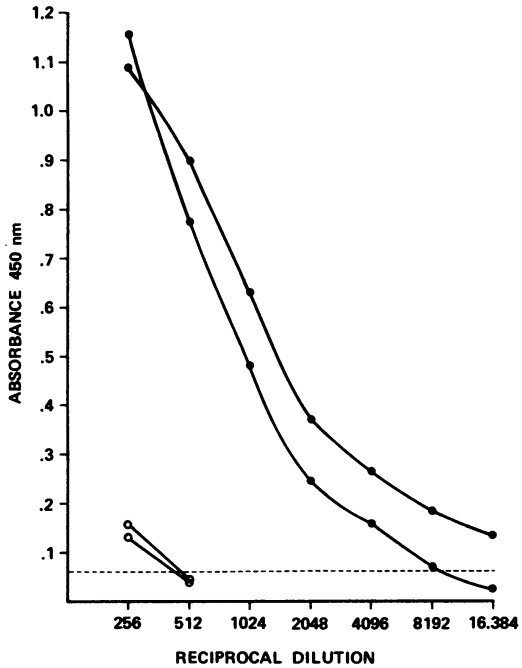

FIG. 2. Relationship between ELISA values (net absorbance) for anti-A (●) and anti-B (○) antibody and serial twofold serum dilutions of sera from two hamsters immunized with a toxin A toxoid. The dotted horizontal line represents the absorbance corresponding to 2 standard deviations above the mean of the negative control serum.

and showed only minimal reactivity to toxin B.

ELISA blocking assay. Figures 3 and 4 show the results of the ELISA blocking assay. Activity of the positive control serum for toxin A-coated plates was 93% blocked by toxin A at a concentration of 6.4 μg/ml, whereas similar concentrations of toxin B and proteins from a nontoxigenic strain resulted in a 3% increase and an 8% decrease in activity, respectively. Activity of the serum for toxin B-coated plates was 67% blocked by toxin B at a concentration of 1.6 μg/ml, whereas similar concentrations of toxin A and proteins from a nontoxigenic strain resulted in 10 and 46% decreases in the activity, respectively.

Correlation between ELISA and tissue culture neutralization assay. In serum samples from immunized hamsters, levels of toxin B-binding immunoglobulin G antibody measured by ELISA correlated closely with titers of toxin-neutralizing antibody determined by the cytotoxicity neutralization assay (Fig. 5). The Spearman rank correlation coefficient, $r^s = 0.98$, indicates that the relationship between the two assays was statistically significant ($P < 0.001$). Levels of toxin A-binding immunoglobulin G measured by ELISA also correlated closely with titers by the cytotoxicity neutralization assay (Fig. 6) (Spearman rank correlation coefficient, $r^s = 0.97$; $P < 0.001$).

DISCUSSION

The data presented indicate that antibody to toxins A and B of *C. difficile* can be measured by an enzyme immunoassay. The specificity of the assay for anti-A antibodies is supported by several lines of evidence. The toxin A preparation used for the initial coating step showed a single protein band by polyacrylamide gel electrophoresis. Hamsters immunized with this protein developed antibodies which completely neutralized the cytopathic effect of toxin A and failed to neutralize that of toxin B. This observation, previously reported by other investigators, demonstrates that the toxins are biochemically separable and immunologically distinct (8). In the ELISA, sera from hamsters immunized with toxin A toxoid reacted very strongly with toxin A-coated wells and only minimally with toxin B-coated wells. The ELISA blocking test, which showed that anti-A activity could not be inhibited by toxin B or proteins from a nontoxigenic strain, also confirms the antigenic distinctness of

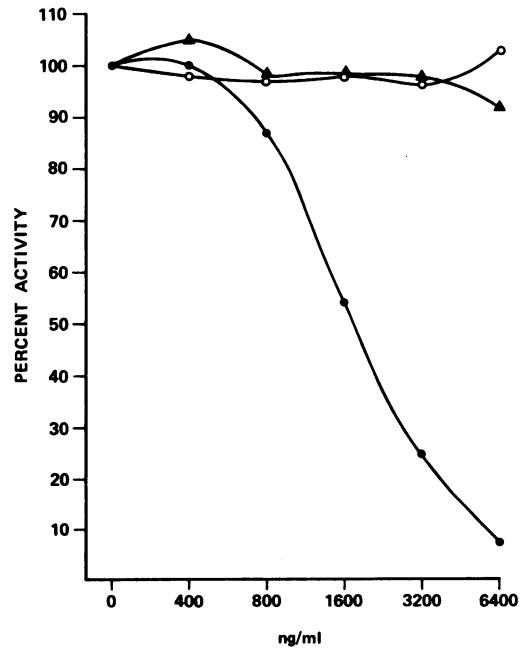

FIG. 3. Percent activity for toxin A-coated plates of the positive control serum preincubated with increasing concentrations of toxin A (●), toxin B (○), and proteins derived from a nontoxigenic strain of *C. difficile* (▲).

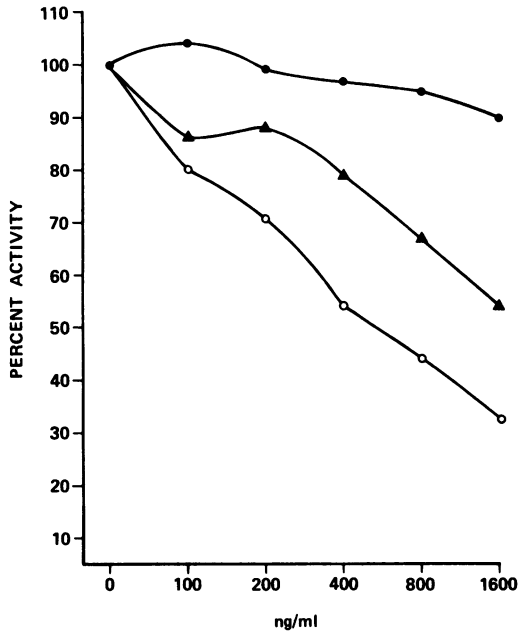

FIG. 4. Percent activity for toxin B-coated plates of the positive control serum preincubated with increasing concentrations of toxin A (●), toxin B (○), and proteins derived from a nontoxigenic strain of *C. difficile* (▲).

the toxins and indicates that the toxin A preparation is not significantly contaminated with toxin B or nontoxin proteins. Finally, levels of toxin A-binding immunoglobulin G antibody measured by ELISA correlated closely with titers of toxin-neutralizing antibody ($P < 0.001$).

The toxin B preparation resolved into three protein bands by polyacrylamide gel electrophoresis. The ELISA blocking test showed that anti-B activity could not be inhibited by toxin A, but it was partially inhibited by proteins from a nontoxigenic strain. Thus, the anti-B activity measured by ELISA in part reflects a response to nontoxin proteins. On the other hand, the ELISA activity correlated highly ($P < 0.001$) with titers in the cytotoxicity neutralization assay. The exact nature of the ELISA activity should be examined in future studies.

Hamsters immunized with a crude culture filtrate toxoid developed cytotoxicity neutralizing antibodies to both toxins after five parenteral doses. The ability of hamsters to develop neutralizing antibodies has been previously demonstrated with a slightly different immunogen (14). In the ELISA assay serum samples from immunized animals demonstrated a significantly increased reactivity to both toxins compared with the low levels (net absorbance, 0.035) seen in serum samples before immunization and samples from control, sham-immunized hamsters. Antibody levels by ELISA progressively increased with additional booster doses of the immunogen.

Cytotoxicity neutralization has been described previously as a method to demonstrate antibody to toxin B (4). However, this is technically difficult and time consuming and requires tissue culture facilities. The enzyme immunoassay method has proven to be a sensitive and specific technique for the detection of a variety of viral and bacterial antibodies (5, 11). A method similar to ours has been developed to detect antibody to cholera toxin, using purified cholera enterotoxin on the solid phase (6, 18). An ELISA assay to detect antibody to *C. difficile* antigen has also been reported (D. M. Lylerly, J. M. Libby, and T. D. Wilkins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B120, p. 38). The technical advantages of ELISA over other assay methods have been amply documented (16). The ELISA assay presented here could be readily applied to human sera by using a different enzyme conjugate. This should be a sensitive and specific assay to define the prevalence of

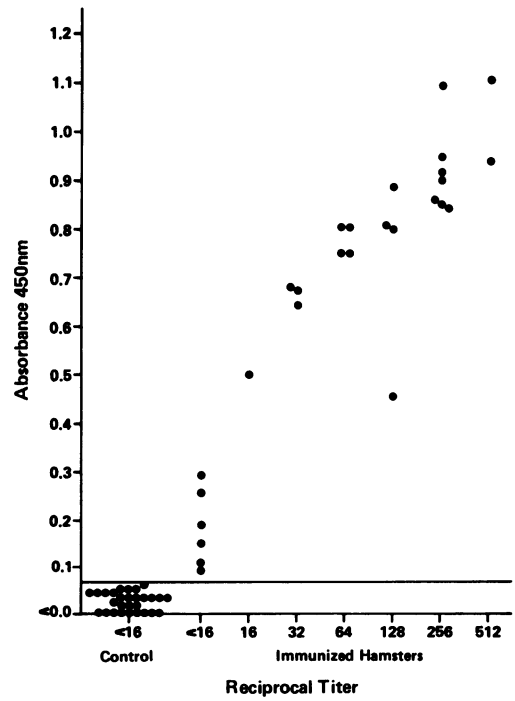

FIG. 5. Relationship between ELISA value (corrected absorbance) and titer measured by the cytotoxicity neutralization assay for anti-B antibody in 27 sera from immunized hamsters and 28 sera from control hamsters. The solid horizontal line represents the absorbance corresponding to 2 standard deviations above the mean of a negative control serum. The Spearman rank correlation coefficient is 0.98 ($P < 0.001$).

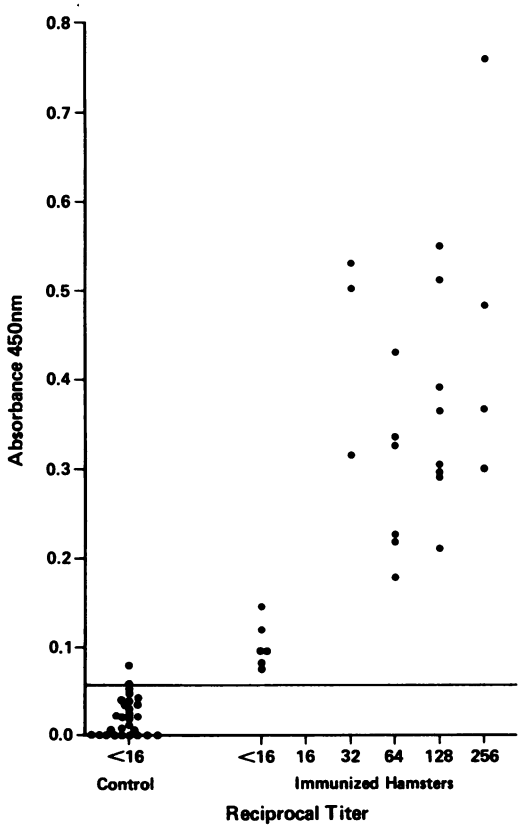

FIG. 6. Relationship between ELISA value (correlated absorbance) and titer measured by the cytotoxicity neutralization assay for anti-A antibody in 27 sera from immunized hamsters and 28 sera from control hamsters. The solid horizontal line represents the absorbance corresponding to 2 standard deviations above the mean of a negative control serum. The Spearman rank correlation coefficient is 0.97 ($P < 0.001$).

antibody to *C. difficile* toxins and to study the immune response of patients with *C. difficile*-induced disease.

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