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Stool Desorbing Activity: a Possible Cause of False-Positive Reactions in Competitive Enzyme Immunoassays

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We have developed a competitive enzyme immunoassay for the measurement of purified toxin A of *Clostridium difficile*. However, when we applied this assay to the detection of *C. difficile* toxin in stool specimens, we noted a high rate of nonspecific activity in fecal specimens which did not contain toxin. We found that the low specificity (26%) of the assay was due to the presence in stool specimens of interfering factors which desorbed the antigen coated on the solid-phase surface. These factors could be detected by measurement of the desorption of biotin-labeled proteins attached to the solid-phase surface. In addition, these interfering factors were partially inactivated by heating at 56°C for 10 min and partially inhibited by phenylmethylsulfonyl fluoride (2 mM) or soybean trypsin inhibitor (10 mg/ml). These data suggested that the desorbing activity was due to proteolytic activity in the fecal specimens. Fetal calf serum (50%) was found to be the most effective measure in preventing the interfering effect. By using 50% fetal calf serum as a diluent, we increased the specificity of the antibody inhibition enzyme immunoassay to 93%. Interfering factors in stool specimens could be a cause of false-positive results in other competitive immunoassay systems. The use of diluents which neutralize protease activity can result in a marked improvement in the specificity of competitive immunoassay systems.

Solid-phase enzyme immunoassays (EIAs) are becoming widely used for the detection of microbial antigens in body fluids. EIAs can be performed in a variety of reaction formats. Many EIA systems involve the sequential binding of antigen and antibodies to a solid-phase surface coated with specific antibody. However, these non-competitive, "binding" EIAs require that the antigen have multiple binding sites to allow for the binding of antigen to both solid-phase and labeled antibodies (14). For this reason, competitive formats have been devised in which the presence of antigen is indicated by an inhibition of the interaction between labeled antibody and solid-phase antigen. Although such competitive assays are often less sensitive than corresponding binding EIA systems, they offer the advantage that they require only a single antigenic binding site on the antigen and thus might be advantageous for the detection of low-molecular-weight antigens. In addition, competitive formats involve the use of fewer reaction steps and do not require multiple sources of highly purified antibodies (14, 15). Despite these advantages, there are very few reports in the literature of competitive immunoassays for the detection of microbial antigens in body fluids (3, 4, 9, 11). One explanation for this may be provided by our recent observation that fecal specimens contain a proteolytic activity capable of desorbing solid-phase immunoreactants (12). The desorption of solid-phase antigen would decrease the amount of antigen on the solid phase and the labeled antibody bound in subsequent steps. This decrease would result in a false-positive reaction indistinguishable from competitive inhibition of the labeled antibody by antigen in the specimen. We investigated this possibility by attempting to develop a competitive EIA for the detection of toxin A of *Clostridium difficile* in fecal specimens.

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MATERIALS AND METHODS

Stool specimens. A total of 111 frozen human fecal specimens previously submitted to this laboratory for *C. difficile* cytotoxin assay and 36 frozen fecal specimens from healthy persons were diluted 1:4 (wt/vol) in phosphate-buffered saline (PBS; pH 7.4), blended vigorously in a Vortex mixer, and clarified by centrifugation at 12,000 × *g*. The supernatant fractions were filter sterilized through a 0.45-μm nitrocellulose membrane filter, assayed for cytotoxicity as previously described (1), and stored frozen at −20°C until assayed in the EIA.

Preparation of biotinillated immunoglobulin. Rabbit antiserum to toxin A was prepared as previously described (8). The immunoglobulin G (IgG) fraction was purified by DEAE–Affi-Gel Blue chromatography according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). IgG-containing fractions were pooled and concentrated on an Amicon YM 30 ultrafiltration membrane (Amicon Corp., Lexington, Mass.) to a concentration of 10 mg/ml in 0.1 M bicarbonate buffer (pH 8.3). Biotinyl-*N*-hydroxysuccinimide (E-Y Laboratories, Inc., San Mateo, Calif.) was used to covalently link biotin to the rabbit IgG by a method modified from that of Bayer and Wilchek (2). A 40-μl portion of biotinyl-*N*-hydroxysuccinimide (50 mg/ml in dimethyl sulfoxide) was added dropwise to 1 ml of the IgG solution. The mixture was incubated for 4 h at room temperature and dialyzed extensively against PBS at 4°C. The biotin-labeled antibody was stored at 4°C until used.

Antibody inhibition EIA for toxin A. An antibody inhibition EIA was formulated by the method of Rissing et al. (10), with toxin A of *C. difficile* prepared as described previously (13). The wells of polyvinyl microtiter plates (no. 220-24; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of purified toxin A (0.1 μg/ml) diluted in PBS.

TABLE 1. Comparison of two diluents in the toxin A antibody inhibition EIA with 97 negative stool specimens[a]

Result in EIA	No. (%) of specimens giving indicated result with the following diluent:	
	PS-T-GP	50% FCS-T-GP
Positive	72 (74)	7 (7)
Negative	25 (26)	90 (93)

[a] Stool specimens were diluted 1:40 in PBS–0.05% Tween 20–1% normal guinea pig serum (PB-T-GP) or 1:8 in 50% FCS–0.05% Tween 20–1% normal guinea pig serum (50% FCS-T-GP). The 97 stool specimens were negative in tissue culture assays and included 61 specimens submitted to our laboratory for *C. difficile* cytotoxin assay and 36 specimens from healthy persons.

After incubation for 16 h at 4°C, the plates were either used immediately or stored at 4°C. Samples (50 μl) of stool or positive controls were mixed with an equal volume of rabbit antiserum to toxin A diluted 1:1,000 in PBS or fetal calf serum (FCS) containing 0.1% Tween 20 and 2% normal guinea pig serum and incubated for 1 h at 37°C. The antigen-antibody mixture was then transferred to plates coated with toxin A antigen, as described above. The plates were sealed with Parafilm and incubated for 1 h at 37°C. After the plates were washed five times with phosphate buffer–0.05% Tween 20 (PB-T), 100 μl of alkaline phosphatase-labeled goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.) diluted 1:1,000 in PBS–0.05% Tween 20 and 0.5% gelatin was added to each well. After incubation for 1 h at 37°C, the plates were washed five times with PB-T, substrate solution (100 μl per well) was added, and the plates were incubated for 1 h at room temperature. The substrate was a 1-mg/ml solution of *p*-nitrophenyl phosphate prepared in diethanolamine buffer (pH 9.8) by using Sigma 104 phosphate substrate tablets (Sigma Chemical Co., St. Louis, Mo.). The absorbance at 405 nm was measured with a microplate colorimeter (MR 480; Dynatech Laboratories, Inc.). The result for stool specimens was also expressed as a percent inhibition: $(1 - \text{mean absorbance}_{\text{stool sample}}/\text{mean absorbance}_{\text{PBS control}}) \times 100$. A positive assay was defined as a mean absorbance more than 2 standard deviations below the mean absorbance obtained in control wells containing PBS in place of a stool sample.

Desorption assay of biotinillated protein. Each well of polyvinyl microtiter plates (Dynatech Laboratories, Inc.) was coated with 100 μl of biotin-labeled IgG antibody to toxin A (8.2 mg/ml) diluted 1:50,000 in 0.05 M carbonate buffer (pH 9.6). After incubation for 16 h at 4°C, the plates were either used immediately or stored at 4°C. Before use, the plates were washed five times with PB-T, and 100-μl portions of a stool supernatant diluted 1:40 in PBS or 1:8 in 50% FCS were added to duplicate wells. The plates were sealed with Parafilm and incubated for 1 h at 37°C. After the plates were washed five times with PB-T, each well received 100 μl of the avidin-biotin complex (Vector Laboratories, Burlingame, Calif.) prepared as a 1:800 dilution in PBS–0.05% Tween 20 and 0.5% FCS of avidin DH and biotin-labeled horseradish peroxidase. After the plates were incubated for 45 min at 37°C, they were washed five times with PB-T. The substrate solution (100 μl per well) was added, and the plates were incubated for 45 min at room temperature. The 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) immediately before use. The absorbance at 450 nm was measured with a microplate colorimeter. The percent desorption of biotin-labeled IgG was determined by the following formula: $(1 - \text{mean absorbance}_{\text{stool sample}}/\text{mean absorbance}_{\text{PBS control}}) \times 100$.

RESULTS

Ninety-seven stool specimens which were negative for *C. difficile* toxins in the tissue culture assay were tested for toxin A in the antibody inhibition EIA by using PBS supplemented with 1% normal guinea pig serum as a diluent; stool specimens were from patients suspected of having antibiotic-associated diarrhea, and 36 specimens were from healthy persons. The stool supernatants were tested at a 1:40 dilution. Of the 97 specimens which were negative for toxin by the tissue culture assay, 72 (74%) gave a positive result in the inhibition EIA as defined by an activity which was 2 standard deviations less than the activity of buffer alone (Table 1). This included 30 (83%) of the 36 stool specimens from healthy persons.

One explanation for the false-positive results with PBS as a diluent was suggested by our previous studies, showing that some stool specimens contained substances capable of desorbing solid-phase immunoreactants (12). The relationship between the false-positive results in the antibody inhibition assay with PBS–1% normal guinea pig serum as a diluent and the ability of stool supernatants to desorb solid-phase immunoreactants was therefore investigated by examining the interaction of the 97 tissue-culture-negative stool supernatants with microtiter plates coated with rabbit IgG labeled with biotin. The 97 stool supernatants diluted 1:40 in PBS desorbed 0 to 81% (mean, 48%) of the solid-phase biotinillated IgG. The correlation for individual stool samples between the nonspecific inhibition obtained in the antibody inhibition assay and the ability of the stools to desorb solid-phase immunoreactants is shown in Fig. 1. The results are expressed as percent desorption for the desorption assay and as percent inhibition for the antibody inhibition assay. The correlation coefficient between the two

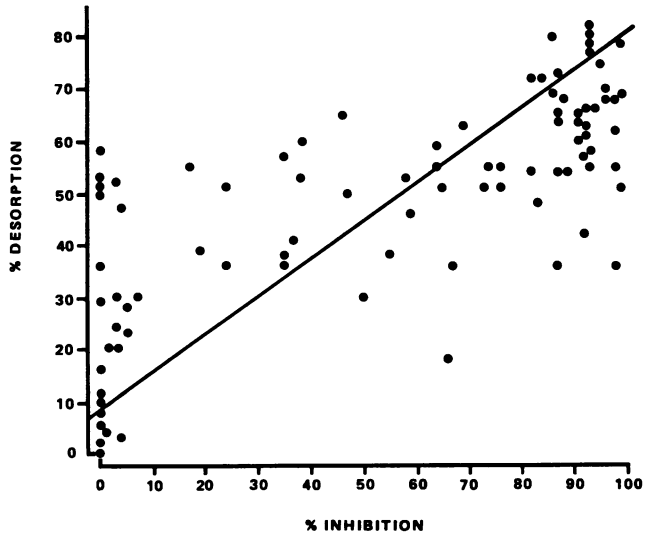

FIG. 1. Correlation between percent desorbing activity and percent inhibition in toxin A antibody inhibition EIA of 97 tissue-culture-negative stool specimens. Linear regression performed on the data indicated a significant linear association (——) between the two variables. The correlation coefficient (r) was 0.74 ($P < 0.001$), and the Spearman rank correlation coefficient (r^s) was 0.69 ($P < 0.001$).

TABLE 2. Percent inhibition of 16 tissue-culture-negative fecal specimens in antibody inhibition EIA with different treatments[a]

Fecal specimen no.	% Inhibition with following treatment						
	PBS	Soybean trypsin inhibitor (10 mg/ml)	2 mM PMSF[b]	Bovine serum albumin (50 mg/ml)	Heat inactivation		50% FCS
					56°C for 30 min	80°C for 10 min	
1	99	50	9	92	72	0	27
2	92	54	36	87	69	24	14
3	87	95	82	77	88	12	0
4	87	45	37	87	67	25	0
5	87	95	39	64	69	4	15
6	93	24	18	94	88	1	2
7	93	51	62	93	88	0	0
8	93	50	100	92	88	0	0
9	95	50	17	93	88	0	5
10	93	53	63	92	94	32	15
11	99	46	31	93	70	0	3
12	76	70	39	63	49	0	0
13	98	56	48	85	69	4	1
14	76	95	0	76	69	0	0
15	98	51	83	92	72	0	0
16	88	95	63	87	70	25	0
	91 ± 7	61 ± 22	45 ± 28	85 ± 10	76 ± 12	8 ± 12	5 ± 8

[a] Results are expressed as percent inhibition: (1 − mean absorbance $_{\text{stool sample}}$/mean absorbance $_{\text{PBS control}}$) × 100. Values at the foot of each column are mean ± standard deviation.

[b] PMSF, Phenylmethylsulfonyl fluoride.

assays was 0.74 ($P < 0.001$), and the Spearman rank correlation coefficient was 0.69 ($P < 0.001$).

Sixteen tissue-culture-negative stool specimens were tested with different diluents in the antibody inhibition EIA (Table 2). When protease inhibitors, such as soybean trypsin inhibitor (10 mg/ml) or phenylmethylsulfonyl fluoride (2 mM, prepared from 0.1 M stock solution in 70% alcohol), were used as diluents, the mean percent inhibitions were 61% (range, 24 to 95%) and 45% (range, 9 to 100%), respectively. These protease inhibitors were partially effective when compared with PBS (mean percent inhibition of 91%, with a range of 76 to 99%). The interfering factors in these stool supernatants were inactivated by heating to 80°C for 10 min and were partially inactivated by heating to 56°C for 30 min, with mean percent inhibitions of 8% (range, 0 to 32%) and 76% (range, 49 to 94%), respectively. The percent inhibition of these stool supernatants was markedly decreased (mean, 5%, with a range of 0 to 27%) when 50% FCS was used as a diluent. In addition, when all 97 tissue-culture-negative stool specimens were diluted in 50% FCS, they desorbed 0 to 19% (mean, 7.5%) of the biotinillated IgG.

When the 97 negative specimens were tested in the antibody inhibition EIA diluted 1:8 in 50% FCS–0.5% Tween 20 containing 1% normal guinea pig serum, only seven specimens (7%) gave a false-positive result (Table 1). The specificity of the assay was therefore increased from 26 to 93% by the use of an FCS-supplemented diluent. Of the 50 tissue-culture-positive specimens tested, 21 (42%) were positive in the EIA. By titration of purified toxin A, we found that the antibody inhibition EIA was capable of detecting 20 ng (200 ng/ml) of toxin A. The addition of 50% FCS slightly decreased the sensitivity of the assay to 30 ng (300 ng/ml) (Fig. 2); 1 μg (10 g/ml) of toxin B and 1 μg (10 g/ml) of protein from the toxin-negative strain 1924 did not react in the assay.

DISCUSSION

We used standard techniques to develop an antibody inhibition EIA for the detection of toxin A of *C. difficile*. However, when the test was applied to the detection of toxin in stool specimens, its specificity was only 26%. One explanation for the large number of false-positive results was suggested by our previous studies, which showed that stool specimens contain substances capable of desorbing solid-phase immunoreactants (12). The desorption of solid-phase antigen would decrease the amount of labeled antibody capable of binding in subsequent steps. This decrease would

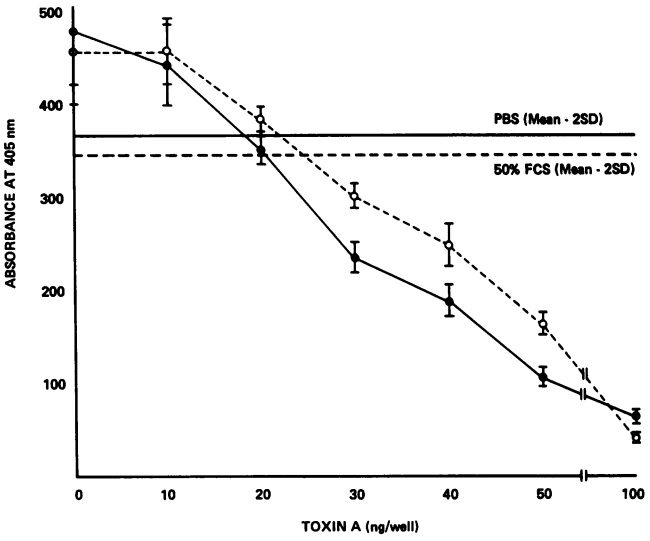

FIG. 2. Standard curves of toxin A in antibody inhibition EIA when diluted in PBS–0.05% Tween 20–1% normal guinea pig serum (●, mean absorbance ± standard deviation) and 50% FCS–0.05% Tween 20–1% normal guinea pig serum (○, mean absorbance ± standard deviation). A positive assay was defined as a mean absorbance more than 2 standard deviations below the mean absorbance obtained in control wells. The solid line represents the mean absorbance minus 2 standard deviations for PBS–0.05% Tween 20–1% normal guinea pig serum, and the dotted line represents the mean absorbance minus 2 standard deviations for 50% FCS–0.05% Tween 20–1% normal guinea pig serum.

result in a false-positive reaction indistinguishable from competitive inhibition of the labeled antibody by antigens in the specimens. This possibility was supported by an analysis of 97 tissue-culture-negative stool specimens in an assay to measure desorption of a solid-phase biotin-labeled protein. A biotinillated protein was selected as a solid-phase label because biotin is easily conjugated to protein and is readily detectable in a single step with an avidin enzyme conjugate (5). When PBS was used as a diluent, the stool supernatant desorbed 0 to 81% (mean, 48%) of the biotinillated protein from the solid phase. Furthermore, the correlation between the desorbing activity of individual stool specimens and their inhibitory activity in the antibody inhibition assay was highly significant. The specimens which desorbed the greatest percentage of solid-phase biotinillated protein caused the greatest degree of nonspecific reactivity in the antibody inhibition assay.

Since our previous studies suggested that stool proteases were responsible for the desorbing activity, we evaluated several measures which inhibit endogenous proteolytic activity for their ability to improve the specificity of the antibody inhibition assay. Phenylmethylsulfonyl fluoride, a specific serine protease inhibitor which has been shown to prevent interfering factors in fecal specimens (6, 7), and soybean trypsin inhibitor were included in diluents for the antibody inhibition EIA. Both were partially effective in preventing the interfering factors. The high protein concentration was not responsible for the results, because 50 mg of bovine serum albumin per ml was without effect. Heat inactivation was also tested, and it showed that the interfering factors were heat labile at 80°C for 10 min and partially heat labile at 56°C for 10 min. The most effective measure in neutralizing this activity was a high concentration of FCS. When a diluent containing 50% FCS was included in the assay to measure desorption of a biotinillated protein, the 97 stool specimens desorbed only 0 to 19% (mean, 7.5%) of the solid-phase label. In addition, the specificity of the antibody inhibition assay was dramatically improved from 26 to 93% when a diluent containing 50% FCS was used.

The overall sensitivity of the assay was 42% for 50 tissue-culture-positive specimens tested. The poor sensitivity is not surprising, given the fact that the assay could only detect purified toxin A at a concentration of 300 ng/ml. By using an indirect sandwich EIA, we have been able to detect toxin A concentrations as low as 1 ng/ml and have achieved a sensitivity of 91% for tissue-culture-positive stool specimens (8). The increased sensitivity of sandwich assays over competitive assays is consistent with studies of other infectious agents (15; J. E. Sippel and A. Voller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C119, p. 330).

Competitive solid-phase assays for antigen detection offer a number of advantages in terms of convenience and reagent preparation. However, our studies document that stool specimens have a proteolytic activity which can decrease the amount of antigen on the solid phase and thus yield a false-positive reaction in such formats in the absence of specific antigen. The results of competitive assays should therefore be interpreted with caution when they are used to detect antigens in body fluids. Preliminary studies also indicate that this desorbing material can be encountered in other body fluids, such as nasal washes and pleural fluids. However, our studies indicate that the desorbing activity can be detected by a simple method consisting of the measurement of the desorption of biotin-labeled proteins attached to the solid-phase surface. In addition, the effect can be neutralized by materials such as FCS, presumably as a result of the presence of multiple protease inhibitors in animal sera. In the case of assays for stable antigens such as microbial polysaccharides (11), it is possible that the desorbing activity can also be removed by denaturing treatments, such as boiling, which destroy the proteolytic activity without altering the antigenic nature of the antigen. When competitive assays are used to detect microbial antigens in body fluids, we recommend that the presence of solid-phase reagent-desorbing materials be examined by the assay described above and that appropriate measures be undertaken to prevent the occurrence of false-positive reactions due to these materials. The use of these techniques should markedly improve the specificity of competitive assays for the detection of microbial antigens in body fluids.

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