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Analysis of Nonspecific Reactions in Enzyme-Linked Immunosorbent Assay Testing for Human Rotavirus

ROBERT H. YOLKEN* AND PETER J. STOPA

Eudowood Division of Infectious Diseases, Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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Solid-phase enzyme immunoassays can be utilized to detect antigens directly in clinical specimens. However, a small number of stools which we tested for human rotavirus by enzyme-linked immunosorbent assay (ELISA) were found to have nonspecific activity in the absence of rotaviral antigen. Similar nonspecific activity was found in eight of eight sera which contained rheumatoid factor. This nonspecific activity was markedly reduced by pretreatment of the specimens with reducing agents, normal goat serum, and anti-human immunoglobulin M (IgM). Thus, it is likely that these specimens contain an IgM antibody capable of reacting nonspecifically with the other components of the assay. Although pretreatment with the mild reducing agent *N*-acetylcysteine markedly reduced this nonspecific activity, such treatment did not reduce the specific ELISA activity due to rotavirus. Other treatments did produce a reduction in specific activity. Thus pretreatment with *N*-acetylcysteine offers a practical means to increase the specificity of ELISA systems without reducing their sensitivity.

Human rotavirus is an important cause of gastroenteritis in infants and young children (12, 14, 21). Since human rotavirus does not grow efficiently in tissue culture systems (24), other methods, such as immune electron microscopy (13), complement fixation (2), counterimmunoelectrophoresis (2, 15), immunofluorescence (27), radioimmunoassay (11, 16), and enzyme-linked immunosorbent assay (ELISA) (7, 25, 29) have been utilized to detect antigen in clinical specimens. Since ELISA does not require radioactive reagents or specialized equipment, it is particularly suited to the study of large numbers of specimens in a wide range of laboratory conditions (22, 23).

In the process of testing large numbers of stool suspensions for human rotavirus by ELISA, we and others have found that a small number of such suspensions react nonspecifically with the goat antiserum used to coat the solid phase, giving rise to false-positive reactions (6, 28). These false-positive reactions can be distinguished from true positive reactions in that the activity will not be blocked by preincubation of the specimen with specific anti-rotavirus antiserum and in that the specimens will yield positive reactions when the solid phase is coated with nonimmune as well as hyperimmune anti-rotavirus goat antiserum. We have shown previously that this nonspecific activity can be reduced by the preincubation of specimens with nonimmune goat serum, suggesting that the nonspecific activity was due to anti-immunoglobulin antibodies present in the stool specimens (10, 28). However, the difficulty in obtaining goat serum free from naturally occurring rotaviral antibody has limited this approach (24).

Although the exact nature of the antibodies in stools is not known, it is possible that these antiglobulins are analogous to immunoglobulin M (IgM) rheumatoid antiglobulins present in some serum specimens (3, 20). If this were the case, the nonspecific activity might be diminished by regimens such as treatment with a reducing agent (1, 17). We therefore investigated the nature of the nonspecific activity present in some stool suspensions and the ability of chemical and immunological treatments to diminish such activity.

MATERIALS AND METHODS

Specimens. Stool suspensions were prepared by adding approximately 1 g of stool to 10 ml of phosphate-buffered saline and shaking to achieve a homogeneous suspension. Clarified suspensions were prepared by centrifuging the above suspension at 9,000 × *g* for 20 min and saving the supernatant fluid. Stool filtrates were prepared by passing the clarified suspensions through a 0.22-μm filter (Millipore Swinnex-25) pretreated with 1% bovine serum albumin. Sera containing rheumatoid factor were obtained from patients being screened for rheumatic diseases. All sera had titers of rheumatoid factor of greater than 1:64 by latex fixation (20).

The 30 stools tested were selected from a group of

855 specimens tested for rotavirus by ELISA. The 30 specimens were selected because they had nonspecific activity with nonimmune goat serum as described below. Nine came from patients living in the Baltimore area, and 21 came from patients living in rural Bangladesh. None of these specimens had specific rotavirus activity by the ELISA blocking test (27, 28), and none had viral particles detectable by electron microscopy. In addition, 18 stools positive for rotavirus by electron microscopy were tested for ELISA specific activity. Eight of these were from patients living in Baltimore, and 10 were from patients living in Bangladesh.

Reagents. Goat anti-rotavirus, guinea pig anti-rotavirus, and alkaline phosphatase-labeled goat anti-guinea pig sera were prepared as previously described (11, 28). The globulin fraction of the goat serum was prepared by precipitation with saturated ammonium sulfate, and the IgG fraction was prepared by diethylaminoethyl-Sephadex chromatography (9, 17). Buffered *N*-acetylcysteine (Mucomist) was purchased from Mead Johnson, and 2-mercaptoethanol was obtained from Kodak. Both were used as 20% solutions. Low-antibody normal goat serum was obtained from a yearling goat. This goat serum was selected from a total of 11 available goat sera because it had the least amount of antibody as measured by complement fixation and the ELISA blocking test (27). Normal rabbit serum was obtained from Flow Laboratories, and normal human serum was obtained from a volunteer. Rabbit anti-human IgM (heavy chain specific) and rabbit anti-secretory component were purchased from Dako (Copenhagen). All sera were heat inactivated at 57°C for 40 min.

ELISA assays. The ELISA assay for human rotavirus was performed as previously described with goat anti-rotavirus antiserum used to coat the wells of a polyvinyl microtiter plate (Dynatech 220-24) and, after incubation with the specimen, successive applications of guinea pig anti-rotavirus serum, enzyme-labeled goat anti-guinea pig serum, and alkaline phosphatase substrate (Sigma 104-5) (28). These antisera were diluted to their optimal concentrations in phosphate-buffered saline (pH 7.4) containing 1% fetal calf serum and 0.05% Tween 20. The amount of yellow color caused by the reaction of the substrate and enzyme bound in the above steps was measured in a spectrophotometer which is capable of determining absorbance directly in microtiter plates (5). Visual determinations were performed by comparing the color with that produced by five known negative controls.

Nonspecificity tests were performed as above except that preimmunization serum from the same goat was used to coat the wells (Fig. 1 and 2). This preimmunization serum did not have any detectable specific anti-rotavirus activity as evaluated by its failure to react with high concentrations of purified human rotavirus. Each specimen was reacted in two wells coated with preimmunization serum and in two coated with postimmunization serum. Nonspecific activity was defined as the mean absorbance at 405 nm of the specimen in the wells coated with nonimmune, preimmunization goat serum. Rotavirus specific activity was defined as the mean absorbance of the specimen in the wells coated with hyperimmune, postimmunization anti-rotavirus serum minus the absorbance of the same specimen in wells coated with the preimmunization goat serum.

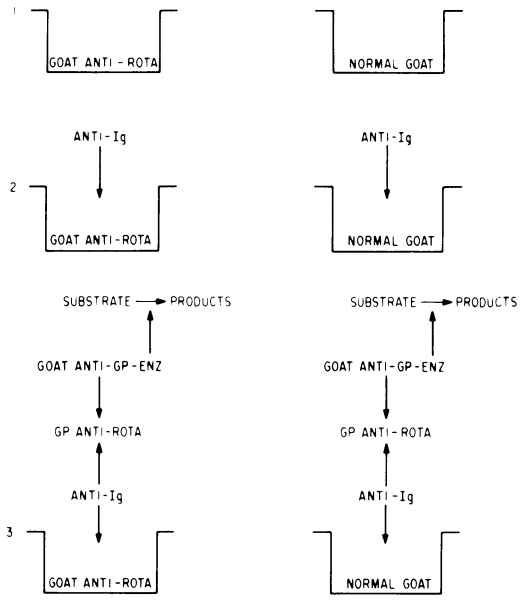

FIG. 1. *Cross-reactivity due to anti-immunoglobulin activity in clinical specimens. (1) Alternate rows of microtiter plates are coated with goat anti-rotavirus immunoglobulin and an equal concentration of immunoglobulin from the serum of a goat without demonstratable anti-rotavirus antibody. (2) The specimen is added to both wells. If a nonspecific anti-immunoglobulin is present it will bind to both the goat anti-rotavirus immunoglobulin and the normal goat immunoglobulin. (3) This anti-immunoglobulin will react with the subsequent antisera leading to a reaction in both wells. If rotavirus is also present, there will be an increased reaction in the well coated with the goat anti-rotavirus immunoglobulin.*

Preincubation experiments were performed by adding an equal volume of stool suspension and either serum or reducing agent in the wells of a hard, uncoated microtiter plate (Dynatech Microelisa). After incubation for 2 h at 37°C, the contents of the wells were transferred into coated plates and processed and interpreted as described above.

RESULTS

The effect of preincubation of 30 stool suspensions and 8 rheumatoid factor-containing sera is presented in Table 1. The nonspecific activity was markedly reduced by preincubation with the reducing agents 2-mercaptoethanol and *N*-acetylcysteine. With these reagents, none of the 30 stool specimens and 1 of the 8 sera specimens yielded false-positive reactions by visual determination. The nonspecific reactivity was also reduced by preincubation with normal goat serum, although two stools gave false-positive vis-

ual readings with this method. The nonspecific activity was reduced to a lesser extent by preincubation with human and rabbit serum but not by fetal calf serum. In the case of preincubation with the specific anti-immunoglobulin sera, the nonspecific activity was reduced by preincubation with anti-human IgM but not by preincubation with anti-human secretory IgA. In addition, the nonspecific activity was not reduced by the substitution of the goat and guinea pig sera with the respective IgG fractions.

The effect of preincubation with *N*-acetylcysteine, 2-mercaptoethanol, and 1% goat serum on specific rotavirus activity was investigated by examining the effect of these preincubations on 18 stool specimens known to contain rotavirus. The *N*-acetylcysteine had no demonstratable effect on the specific activity of the specimen. However, both the 2-mercaptoethanol and the 1% goat serum led to a diminution of specific activity in all of the specimens, resulting in three and two false-negative reactions, respectively.

DISCUSSION

Solid-phase immunoassays such as radioimmunoassays and ELISA have the capability of detecting small quantities of antigen directly in clinical specimens. However, if the labeled reagent is bound nonspecifically to the solid phase and the other reactants, a false-positive reaction may occur (25). One potential cause of such nonspecific binding is an antiglobulin in the specimen which is capable of binding both to the immunoglobulin on the solid phase and the label (6, 28).

We found such activity in 30 stools which we had tested for rotavirus. The nonspecificity of these reactions was discovered because the specimens yielded ELISA activity in wells coated with preimmunization, nonimmune goat serum and the immunoglobulin and IgG fraction prepared from this serum. The color in these wells was equal to that in wells coated with anti-rotavirus serum. This method of testing specimens in wells coated with immune and nonimmune sera, which was devised by Greenberg et al. for the system of radioimmunometric detection of Norwalk virus in stools, provides a simple, rapid method for distinguishing specific from nonspecific activity in solid-phase assays (8).

The fact that the nonspecific activity was greatly reduced by treatment with reducing agents and preincubation with anti-human IgM suggests that this activity was largely caused by an IgM antibody (1, 17). The fact that the activity was reduced by preincubation with goat and other immunoglobulin-containing animal sera suggests that this antibody is directed against a

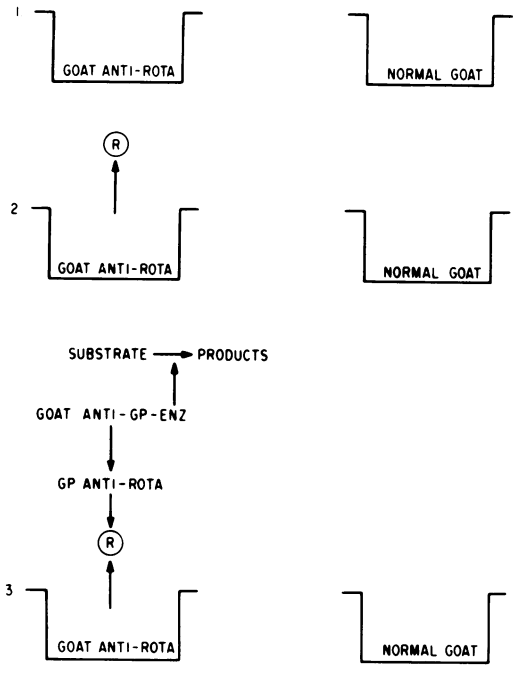

FIG. 2. *Confirmatory ELISA to distinguish specific rotavirus activity from nonspecific activity due to anti-immunoglobulin activity in the clinical specimens. (1) Alternate rows of microtiter plates are coated with goat anti-rotavirus immunoglobulin and an equal concentration of immunoglobulin from the serum of a goat without demonstrable anti-rotavirus antibody. (2) The specimen is added. If it contains rotavirus it will react with the goat anti-rotavirus immunoglobulin. Nonspecific anti-immunoglobulin will react with both the goat anti-rotavirus immunoglobulin and the normal goat immunoglobulin. (3) Guinea pig anti-rotavirus, enzyme-linked goat anti-guinea pig immunoglobulin, and substrate are sequentially added with washing steps in between incubations. Specific rotavirus activity is manifested by a difference in color between the wells coated with goat anti-rotaviurs serum and the wells coated with normal goat serum.*

wide range of immunoglobulins, thus cross-reacting with the goat serum coating the plate and the other reagents participating in the reaction (6, 10). Although the exact nature of this stool antiglobulin is not known, the fact that all sera from patients with rheumatoid arthritis exhibited similar nonspecific responses suggests that this stool antibody might be a rheumatoid-like factor. An additional explanation is that it is an IgM heterophil antibody reacting with antigen from a number of animal species (10, 18). The characterization and significance of this stool antiglobulin should be the subject of further investigation.

TABLE 1. *Effect of pretreatment on nonspecific ELISA activity*

Treatment	Stool suspensions ($N = 30$)		Sera ($N = 8$)	
	Nonspecific activity[a]	No. visually positive	Nonspecific activity[b]	No. visually positive
No treatment	0.532	30/30	0.611	8/8
Preincubation				
1% Fetal calf serum	0.521	30	0.608	8
1% Human serum	0.454	27	0.563	8
1% Rabbit serum	0.249	14	0.302	4
1% Goat serum	0.141	2[c]	0.145	1[c]
20% *N*-Acetylcysteine	0.072	0[c]	0.113	1[c]
20% 2-Mercaptoethanol	0.067	0[c]	0.107	1[c]
Anti-human IgM	0.163	3[c]	0.115	1[c]
Anti-human SC[d]	0.511	30	0.609	8
Pretreatment				
Clarification	0.461	28	0.610	8
Filtration[e]	0.326	17	0.599	8

[a] Mean of duplicate determinations of 30 specimens. Absorbancy at 405 nm.
[b] Mean of duplicate determinations of 8 specimens. Absorbancy at 405 nm.
[c] $P < 0.01$ compared with that of no treatment.
[d] Secretory component.
[e] Filter of 0.22-μm pore size (Millipore).

From a practical standpoint it would be desirable to eliminate or reduce the nonspecific responses without reducing the specific reaction for the test antigen. In the case of the ELISA for rotavirus in stool specimens, this goal was achieved by preincubation with 20% *N*-acetylcysteine. Such pretreatment provided a simple means of avoiding false-positive reactions in 30 stools without resulting in any reduction of specific activity when rotavirus-containing stools were tested. On the other hand, although preincubation of stools with goat serum led to a diminution of nonspecific activity, specific rotavirus ELISA activity was also diminished, probably due to the presence of small amounts of blocking antibody in the serum (26). Pretreatment regimens also reduced the amount of nonspecific activity in sera containing rheumatoid factor. However, the reduction was incomplete, with a single serum yielding a visually positive, nonspecific reaction in spite of pretreatment with reducing agent or animal serum.

Preincubation with *N*-acetylcysteine is thus the method of choice for reducing nonspecific activity in solid-phase assays in which the antigen is resistant to the mild reducing agent. *N*-Acetylcysteine has also been used to increase the sensitivity of an ELISA for the detection of respiratory syncytial virus in nasal secretions, presumably by increasing the concentration of available antigen and reducing background activity (4). Thus, reducing agents should prove to be useful reagents in solid-phase assays. However, to insure maximum specificity, all specimens which yield a positive reaction should be verified in a confirmatory test. Although this can be done in a neutralization test as described previously (11, 25), it is simpler to run each specimen in parallel wells coated with immune and nonimmune sera as described above and to define positivity in terms of a difference in activity between the parallel reactions.

Solid-phase immunoassays offer a number of advantages such as sensitivity, rapidity, and low cost. However, they are also subject to occasional false-positive reactions due to nonspecific interactions. The use of simple pretreatments and control reactions as described in this report should markedly increase the specificity of solid-phase immunoassays and allow for the development of detection systems for a number of antigens in crude clinical specimens.

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