JOURNAL OF CLINICAL MICROBIOLOGY, July 1977, p. 10-15

Vol. 6, No. 1
Printed in U.S.A.

Solid-Phase Radioimmunoassay for Detecting Bovine (Neonatal Calf Diarrhea) Rotavirus Antibody

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Received for publication 7 March 1977

An indirect solid-phase microradioimmunoassay is described for detecting antibodies against rotaviruses. The test involved ethanol fixation of microcultures of bovine rotavirus-infected BSC-1 cells and reaction with bovine anti-rotavirus serum, followed by ^{125}I-labeled rabbit anti-bovine immunoglobulin G. The technique was shown to be virus specific and highly sensitive. The fixed microcultures could be stored at 4°C for at least 2 months without affecting the sensitivity of the test. The application of this system for the detection of rotavirus antibodies in humans is briefly discussed.

Since the first description of a bovine rotavirus in 1969, substantial evidence has accumulated to implicate this group of viruses as a cause of neonatal diarrhea in calves, other animals, and also in humans (1, 3, 4, 7, 11, 12, 14, 15).

Current methods most frequently used to detect rotavirus antigen or antibody are agar gel diffusion or counterimmunoelectrophoresis (16), complement fixation (11), immunoelectron microscopy (17), immunofluorescence (17), and, most recently, hemagglutination and hemagglutination inhibition (M. Fauvel, L. Spence, L. A. Babiuk, R. Petro, and S. Bloch, Intervirology, in press). Even though these techniques have been used to demonstrate that rotaviruses are extremely prevalent in cattle populations in some areas (19, 22), detailed epidemiological studies have not been possible, because these techniques are laborious and lack sensitivity. Radioimmunoassays (RIAs) have several advantages over other techniques for detecting antigen or antibody since they are usually highly sensitive and specific, require small quantities of reagents, give objective end points, and are usually more convenient to perform (8, 10, 13, 21). The recent successful in vitro propagation of bovine rotavirus (6) has made it feasible to utilize rotavirus-infected cell cultures for the detection of antibody by an RIA. The present report describes one such RIA.

MATERIALS AND METHODS

Sera. Reference anti-rotavirus serum, prepared by inoculating a gnotobiotic calf with a strain of rotavirus isolated in England, was kindly provided by G. N. Woode (Institute for Animal Research, Compton, England). Test sera were obtained from conventionally raised steers (6 to 8 months old) given concentrated rotavirus antigen obtained from the feces of an infected calf. The concentrated antigen was prepared by two cycles of centrifugation at 3,000 × *g* for 20 min. The supernatant fluid, containing virus, was freed of bacteria and debris by passage through a 0.45-μm membrane filter (Millipore Corp.) The clear filtrate was then centrifuged at 55,000 × *g* for 3 h, and the pelleted virus was suspended in phosphate-buffered saline (pH 7.2) to 1/20 the original volume of feces. Four milliliters of the concentrated crude antigen was emulsified with an equal volume of Freund complete adjuvant and injected intramuscularly on two separate occasions 10 days apart. Immune serum was collected 3 weeks after the second injection.

Rabbit anti-rotavirus serum was prepared by intradermal and intramuscular inoculation of rabbits, on three separate occasions 2 weeks apart, with concentrated viral antigen in Freund complete adjuvant.

Parainfluenza-3 (PI-3) and infectious bovine rhinotracheitis (IBR) antisera were obtained from Miles Laboratories, Inc. (Elkhart, Ind.). Vaccinia antiserum was obtained from a heifer (8 months old) immunized intradermally on one occasion with 10^9 plaque-forming units of vaccinia virus. This animal had previously been immunized against IBR.

Purification and preparation of radiolabeled rabbit anti-bovine immunoglobulin. Rabbit anti-bovine immunoglobulin G (IgG) was prepared by four intradermal injections of purified bovine IgG at 3-week intervals. The first injection consisted of 400 μg/rabbit in Freund complete adjuvant; subsequent injections were at a concentration of 100 μg/rabbit in Freund incomplete adjuvant. When the rabbits had high levels of antibody, as determined by immunodiffusion, they were exsanguinated and the gamma globulin was precipitated two times with 50% ammonium sulfate before passage through a diethylaminoethyl-cellulose column as described previ-

ously (B. T. Rouse, R. C. Wardley, L. A. Babiuk, and T. K. S. Mukkur, J. Immunol., in press). The purified IgG was labeled with ^{125}I by the chloramine-T method of Greenwood et al. (9). The residual unbound ^{125}I was removed by passing the preparation through a Sephadex G-50 column previously treated with bovine serum albumin fraction V. The labeled protein was dialyzed for 3 days against several changes of tris(hydroxymethyl)aminomethane-saline [0.01 M tris(hydroxymethyl)aminomethane, 0.32 M NaCl]. Precipitation with 10% trichloroacetic acid indicated that >95% of the radioactivity was bound to the purified IgG (^{125}IRaBIgG).

Cells and viruses. Monkey kidney (BSC-1) and Georgia bovine kidney cells were cultured in Eagle minimal essential medium (MEM) as described previously (2; Fauvel et al., in press). Each liter was supplemented with 2 mmol of glutamine, 10 ml of nonessential amino acids (GIBCO, no. 114), 3.5 mg of gentamicin, and 2.5 g of sodium bicarbonate. For growth, the medium contained 10% fetal calf serum. Micro-cell cultures were prepared by seeding 5×10^4 cells into each of the 96 wells of a microtiter plate (Falcon Plastics, no. 3040). Monolayers were confluent within 24 h, at which time they were washed two times with Hanks balanced salt solution and infected at a multiplicity of infection of 0.5 with a strain of bovine rotavirus isolated in this laboratory from a calf suffering from diarrhea. A description of some of the properties of the virus has been reported previously (Fauvel et al., in press). After adsorption for 1 h at 37°C, unadsorbed virus was removed, and MEM containing 0.5% bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, Mo.) was added to each well. Twenty-four hours postinfection all cultures showed extensive cytopathology. The monolayers were fixed for 20 min with 95% ethanol, 100% methanol, or 10% Formalin and used in the micro-RIA. Bovine viral diarrhea (BVD), IBR, vaccinia, and PI-3 viruses were prepared as described previously (2; B. T. Rouse and L. A. Babiuk, J. Immunol., in press). Infected monolayers for use in the RIA were prepared as described above for rotavirus, except the Georgia bovine kidney cells rather than BSC-1 cells were used for infection with IBR and BVD viruses.

Indirect micro-RIA. The fixed cultures were washed three times with Hanks balanced salt solution. Serial dilutions of antisera were prepared in MEM and added in 50-μl volumes to each of six replicate wells. Controls included uninfected cell monolayers or infected monolayers incubated with MEM or MEM + 5% fetal calf serum. The fixed cells and antisera were incubated in a humidified atmosphere at 37°C for 1 h and washed four times with Hanks balanced salt solution, and 50 μl of ^{125}IRaBIgG in MEM was added. The plates were reincubated for a further 1 h at 37°C, washed four times with Hanks balanced salt solution, and allowed to air-dry. The bottoms of the wells were punched out, placed in 12- by 75-mm glass holders, and counted in a Nuclear-Chicago gamma counter. The amount of specific binding (binding ratio) was determined by the following formula: binding ratio = counts per minute bound by test sera/counts per minute bound by control. Binding ratios greater than 2 were considered positive, since they generally represented values greater than the control mean + 10 times the standard error. Therefore, the probability of recording false positives was very low (<99.99%).

Counterimmunoelectrophoresis. Counterimmunoelectrophoresis was performed on glass slides (3.8 by 7.5 cm) covered with 6.5 ml of 1% ion agar (Med-Ox Chemicals, Ottawa, Ontario) in 0.3 M Veronal buffer (pH 8.6). Three parallel rows of six wells (3 mm in diameter and 5 mm apart) were cut in the agar. Antigen was placed in the wells next to the cathode and antisera in the wells adjacent to the anode, as described by Middleton et al. (16). The two chambers of a Gelman electrophoresis apparatus were filled with 0.3 M Veronal buffer (pH 8.6), connected to the coated slides with filter paper, and subjected to electrophoresis for 2 h at 250 mV. The slides were read against a dark background.

RESULTS

Effect of fixation. To establish the conditions best suited for preserving the antigen on microtiter plates, various methods of fixation were tried. Plates were used directly, air-dried, air-dried and then fixed, or fixed without air-drying. Unfixed cultures always exhibited lower specific binding as well as poor reproducibility, presumably as a result of detachment of cells during the procedure. Air-drying, however, prevented cell detachment and consequently gave better reproducibility than unfixed cultures, even though the level of binding was lower than in fixed cultures (Table 1). A possible explanation for the lower binding in air-dried samples is that specific antibody reacts only with the antigen on the surface of virus-infected cells, whereas fixation allows reaction with intracellular virus as well.

Methanol and ethanol appeared to be comparable fixatives but better than Formalin, as evidenced by the higher degree of binding and greater reproducibility (smaller standard errors). As with unfixed cultures, Formalin fixation allowed the detachment of virus-infected cells, thus resulting in reduced binding and poor reproducibility. Therefore, in all subsequent experiments ethanol was used as a fixative.

Specificity. To establish the immunological specificity of the assay, several approaches were adopted. First, as presented in Table 2, specific anti-rotavirus serum prepared in a gnotobiotic calf reacted against rotavirus-infected cells but not against uninfected cells or cells infected with BVD, IBR, PI-3, or vaccinia virus. However, antisera to IBR, PI-3, BVD, and vaccinia viruses did react with cells infected with

TABLE 1. *Effect of various fixation methods on the variability and degree of ^{125}IRaBIgG binding to rotavirus-infected cultures*

Fixation[a]	cpm bound ± standard error					
	Positive sera[b]			Negative sera[c]		
	Infected	Uninfected	BR[d]	Infected	Uninfected	BR
Unfixed	1,261 ± 273	207 ± 71	6.1	296 ± 71	214 ± 67	1.4
Air-dried	1,537 ± 144	309 ± 33	4.9	321 ± 37	292 ± 32	1.1
Air-dried, methanol	4,385 ± 141	615 ± 51	7.1	712 ± 53	697 ± 43	1.0
Air-dried, ethanol	4,226 ± 173	624 ± 43	6.7	770 ± 49	721 ± 39	1.1
Air-dried, Formalin	1,252 ± 307	380 ± 124	3.3	200 ± 104	306 ± 107	0.6
Methanol	5,584 ± 123	609 ± 52	9.1	709 ± 46	597 ± 53	1.2
Ethanol	5,713 ± 142	614 ± 49	9.3	615 ± 41	662 ± 40	0.9
Formalin	705 ± 401	226 ± 127	3.1	324 ± 126	294 ± 96	1.1

[a] Air-dried for 1 h. Fixation with fixatives was for 20 min.
[b] Reference sera obtained from G. N. Woode (Compton, England) used at a 1/180 dilution.
[c] Fetal calf serum used at a 1/50 dilution.
[d] Binding ratio (BR) of counts per minute bound to infected cultures divided by that bound by uninfected cultures.

TABLE 2. *Specificity of the solid-phase RIA*

Micro-cultures infected[b] with:	Test antisera[a]									
	Rotavirus		PI-3		Vaccinia		IBR		BVD	
	cpm	BR[c]	cpm	BR	cpm	BR	cpm	BR	cpm	BR
Rotavirus	5,503	8.59	649	1.00	84	1.15	673	1.11	ND[d]	ND
IBR	604	0.94	653	1.00	ND	ND	5,121	8.41	594	0.94
PI-3	670	1.04	2,569	3.92	741	1.01	ND	ND	603	0.95
BVD	623	0.97	632	0.97	694	0.95	659	1.08	4,193	6.63
Vaccinia	637	1.00	643	0.99	3,419	4.67	662	1.09	ND	ND
Control	641		652		732		609		632	

[a] All antisera were tested at a 1/100 dilution.
[b] Rotavirus, PI-3, and vaccinia viruses were cultured in BSC-1 cells, whereas IBR and BVD viruses were grown in Georgia bovine kidney cells.
[c] Binding ratio (BR) calculated as in Table 1. The homologous antisera-culture system is underlined.
[d] ND, Not determined.

the respective viruses (Table 2). As a second specificity control, rotavirus-infected fixed cultures were pretreated with rabbit anti-rotavirus serum, prior to reaction with the bovine anti-rotavirus serum. This procedure gave a marked reduction of binding, whereas normal rabbit serum failed to block binding of bovine anti-rotavirus serum (Fig. 1). Finally, after inoculation of an 8-month-old calf with purified rotavirus, it was possible to demonstrate a 60-fold increase in anti-rotavirus antibody titer, from a level of 40 before inoculation to over 2,500 after immunization (Fig. 2). Similar seroconversion was also demonstrated by counterimmunoelectrophoresis but titers were markedly lower, changing from negative to only one in two. The corresponding titers detected by RIA were 1/2,500, indicating that RIA was at least 1,000-fold more sensitive that the commonly used counterimmunoelectrophoresis assay.

Reproducibility and stability of prepared plates. To determine if the assay was reproducible from day to day, one standard serum was titrated on separate days using plates of fixed rotavirus-infected cultures prepared at different times. Three such representative experiments are presented in Table 3. In all instances, the titer remained constant (within a fivefold range), suggesting that the variability in the assay, which might arise as a result of different degrees of virus replication and consequently different quantities of antigen, would not be a major problem.

Experiments were performed to determine if rotavirus-infected cultures could be stored and used subsequently in the RIA. For this purpose, infected monolayers were fixed with ethanol and stored at 4°C, and the RIA was performed at intervals during a 2-month period. The results (Table 4) show that the sensitivity of the test did not change during the storage period.

DISCUSSION

Rotaviruses are associated with many outbreaks of diarrhea in human infants and other neonatal mammals, including calves (1, 3, 4, 12). The virus has been difficult to detect because primary isolates grow poorly in tissue culture; consequently, the bovine virus was not described until 1969 and the human virus was not described until 1973 (4, 15). Both were initially detected by electron microscopic observation (4, 15). Since these viruses do not adapt rapidly to growth in cell culture, most serological assays of the virus have relied on concentrated virus particles obtained from fecal samples (16). Because of the difficulties encountered in obtaining adequate concentrations of antigen, these assays are usually tedious and insensitive and have not proven convenient for use in diagnostic laboratories or in epidemiological surveys to measure the prevalence of the virus. In the present report we have exploited the recent finding that the bovine rotavirus can be adapted to grow in cell culture (6) and have used microcultures of such virus-infected cells to detect antibody by an indirect solid-phase RIA. The assay proved to be highly sensitive, detecting antibody at dilutions of test sera of 1/2,000. The same sera gave a titer of one in two by counterimmunoelectrophoresis, a commonly used serological technique for detecting rotavirus antibody (16). Specificity was shown by the failure of preinfection sera and sera prepared against other viruses to bind to rotavirus-infected microcultures and by the ability of specific rabbit anti-rotavirus sera to block the binding of the bovine antiserum. Furthermore, bovine rotavirus serum failed to bind to cells infected with other bovine viruses—PI-3, IBR,

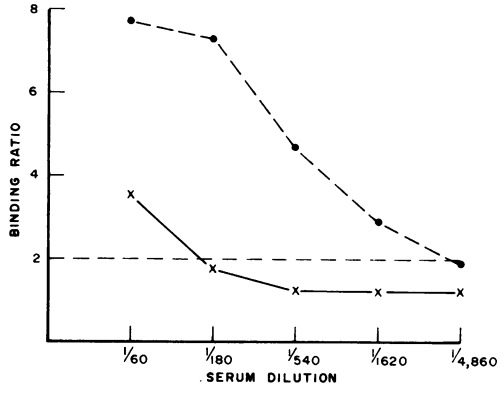

FIG. 1. *Effect of pretreatment of rotavirus-infected microcultures with rabbit anti-rotavirus antisera. Rotavirus-infected cultures were pretreated with a 1/20 dilution of rabbit anti-rotavirus antibody or with normal rabbit serum for 60 min. Cultures were then washed and reacted with various dilutions of bovine anti-rotavirus antibody and ^{125}IRaBIg in the usual manner. Binding ratios of the rabbit anti-rotavirus sera (×) and normal serum (○) pretreatment were calculated from the mean counts per minute of six replicate control cultures and six replicate test cultures as described in the text.*

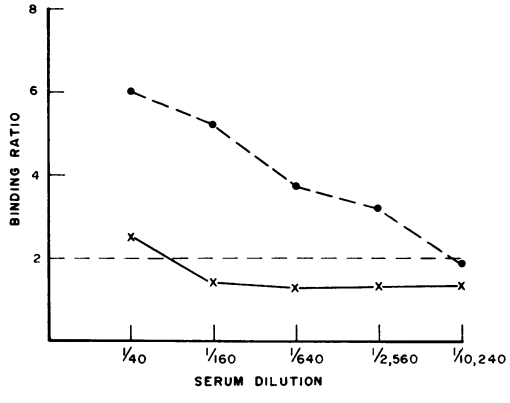

FIG. 2. *Titration of preimmunization and postimmunization sera. The binding ratios of preimmunization sera (×) and postimmunization sera (○) were calculated as described in Fig. 1.*

TABLE 3. *Reproducibility of titration of anti-rotavirus serum on three separate days using plates prepared on different occasions*

Anti-rotavirus serum dilution	Expt 1			Expt 2			Expt 3		
	Infected[a]	Uninfected	BR[b]	Infected	Uninfected	BR	Infected	Uninfected	BR
1/50	5,932	1,251	4.73	8,003	1,276	6.51	5,121	974	5.26
1/150	5,247	1,030	5.12	6,043	1,100	5.52	4,956	963	5.15
1/500	3,789	1,125	3.36	5,670	1,066	5.36	4,321	945	4.57
1/1,500	2,450	1,096	2.23[c]	2,608	1,048	2.43[c]	2,272	899	2.53[c]
1/4,500	1,720	1,121	1.47	1,981	1,046	1.89	1,473	873	1.64
1/13,500	1,271	1,031	1.23	1,276	999	1.27	1,143	878	1.30

[a] Mean counts per minute bound of six replicate cultures.
[b] Binding ratio (BR) = counts per minute infected/counts per minute uninfected.
[c] The last dilution considered positive since greater dilutions had a binding ratio of less than 2.

TABLE 4. *Effect of storage of rotavirus-prepared microcultures on the reproducibility of the RIA*[a]

Fixation	Anti-rotavirus serum dilution	Mean cpm bound after storage of:								
		No storage			3 Weeks			9 Weeks		
		Infected cultures	Uninfected cultures	BR[b]	Infected cultures	Uninfected cultures	BR	Infected cultures	Uninfected cultures	BR
Ethanol	1/150	3,929	994	3.9	4,206	979	4.3	3,572	687	5.2
	1/500	3,150	931	3.4	3,849	952	4.0	2,958	711	4.2
	1/1,500	2,242	904	2.5	2,614	893	2.9	2,167	692	3.1
	1/6,000	1,467	849	1.7	1,315	906	1.4	1,084	697	1.6
Air-dried[c]	1/150	3,204	849	3.8	3,197	851	3.8	2,403	539	4.5
	1/500	2,942	790	3.7	2,704	705	3.8	1,973	574	3.4
	1/1,500	2,172	698	2.7	1,922	796	2.4	1,242	543	2.3
	1/6,000	1,306	784	1.7	1,314	804	1.6	762	531	1.4

[a] BSC-1 cells were infected with rotavirus, fixed or air-dried, and stored at 4°C until used.
[b] Binding ratio (BR) calculated as in Fig. 1.
[c] Cultures were fixed with ethanol just before performing the RIA.

BVD, and vaccinia. The assay system was highly reproducible, and microcultures could be prepared, stored, and used for several weeks without loss in sensitivity. Since the assay is also objective, convenient to perform, and inexpensive in reagents, it is ideally suited for use by diagnostic laboratories and especially by those processing large numbers of samples. Furthermore, it may also be possible to automate the assay.

The observation that human rotavirus and other mammalian rotaviruses share group-specific antigens with the bovine virus means that it should be possible to adapt the assay to detect antibody to these viruses in other animals (7, 11, 12, 20). The human rotavirus does not grow in cell culture and can only be detected by counterimmunoelectrophoresis, electron microscopy, or immunoelectron microscopy and complement fixation (4, 11, 12, 16). However, human anti-rotavirus antibodies should bind to bovine rotavirus and this bond will be detectable by RIA. To further simplify the assay so as to avoid the need to prepare labeled anti-IgG reagents for each species, we are currently determining if labeled staphylococcus A protein can be used to detect bound antibody (5, 18).

ACKNOWLEDGMENTS

We express our appreciation to Elaine Van Morrlehem for enthusiastic and competent technical assistance, to Joyce Sander for typing the manuscript, and to K. Mohammed for providing the pre- and postimmunization sera.

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