JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1987, p. 509–515
0095-1137/87/030509-07$02.00/0

Vol. 25, No. 3

Simple and Specific Enzyme Immunoassay Using Monoclonal Antibodies for Serotyping Human Rotaviruses

BARBARA S. COULSON,[1]* LEANNE E. UNICOMB,[1] GRAHAM A. PITSON,[2] AND RUTH F. BISHOP[1]

Department of Gastroenterology, Royal Children's Hospital,[1] and Department of Paediatrics, University of Melbourne,[2] Parkville, Victoria 3052, Australia

Received 17 July 1986/Accepted 14 November 1986

An enzyme immunoassay for serotyping human rotaviruses in stools and in cell culture was developed. Hyperimmune rabbit antisera to rotaviruses were used as capture antibodies, and rotavirus-neutralizing mouse monoclonal antibodies specific for serotypes 1, 2, 3, and 4 were used as detection reagents. Partial purification of monoclonal antibodies and inclusion of skim milk powder in antibody diluents contributed to assay specificity. The sensitivity of this assay was greater than that of a direct enzyme immunoassay in which rotaviruses of the appropriate serotype were adsorbed directly to the solid phase. When fecal extracts were concentrated threefold, this serotyping enzyme immunoassay was of equal specificity and approached the sensitivity of electron microscopy for rotavirus detection. This assay is simple and rapid and is suitable for serotyping the large numbers of isolates obtained from epidemiological studies and vaccine trials.

The necessity for development of a simple, specific, and sensitive method for serotyping human rotaviruses is recognized (14). Rotaviruses are the single most important cause of infantile gastroenteritis worldwide, and vaccine development is recognized as imperative (7). However, successful immunoprophylaxis depends on a complete understanding of the extent and stability of the pool of virus-neutralizing antigens to which humans may be exposed and on the nature and level of cross-reaction among serotypes.

Two rotavirus outer capsid proteins are known to provoke a neutralizing antibody response. These are the glycosylated product of gene 7, 8, or 9 (2) of molecular weight approximately 34,000 (gp34) and the product of gene 4 (10) of molecular weight 80,000 to 90,000 (p84). Currently, the rotavirus serotype is determined for cultivable rotaviruses and some stool isolates of rotavirus by cross-neutralization assays with hyperimmune antisera (4, 12). However, since cross-reactions between serotypes have been observed (12) which may in some cases relate to the sharing of p84 among different serotypes (11), it may become necessary to define the neutralization of each of these proteins separately. This would make the use of monoclonal antibodies or gene probes essential for serotyping rotaviruses.

Although for research purposes human rotaviruses are now cultivable in cells (20), rotavirus serotyping by the conventional methods of plaque or fluorescent focus neutralization is still limited because these assays are slow, laborious, and suitable only for cultivable virus strains. Serotyping of both cultivable rotavirus and stool rotavirus has been achieved by enzyme immunoassay (EIA) (23) and solid-phase immune electron microscopy (EM) (9) with extensively cross-adsorbed hyperimmune antisera. These reagents are not readily generated, so these assays have not been widely adopted. In addition, serotyping by solid-phase immune EM is even more laborious than conventional methods and requires a skilled electron microscopist.

Previously, 10 neutralizing monoclonal antibodies to human rotaviruses RV-3 (serotype 3) (6), RV-4 (serotype 1), RV-5 (serotype 2), and ST-3 (serotype 4) (6a) were derived and characterized in our laboratory. All except two of these antibodies were directed to the major outer capsid glycoprotein, gp34. One antibody was directed to p84. The protein specificity of another antibody could not definitely be determined. In this paper, we report the development of a simple, specific, and sensitive EIA, based on use of these antibodies to serotype human rotavirus in culture and in stools.

MATERIALS AND METHODS

Viruses and cells. The cultivable human rotaviruses RV-3, RV-4, and RV-5 were isolated in our laboratory (1). The ST-3 human rotavirus was provided by R. G. Wyatt, Bethesda, Md., and the simian rotavirus SA11 (serotype 3) was provided by H. Malherbe, San Antonio, Tex.

All viruses were propagated in MA104 cells in the presence of porcine trypsin type IX (10 μg/ml to activate virus, 1 μg/ml in maintenance medium; Sigma Chemical Co., St. Louis, Mo.) as described previously (20). All virus stocks were identified as true to type by polyacrylamide gel electrophoresis of viral RNA (6) and coelectrophoresis of the RNA with that extracted from early-passage rotavirus stocks.

Virus preparation for rabbit immunization and for EIA antigen. Rotavirus-infected or mock-infected cells were harvested by freeze-thawing; virus was prepared by fluorocarbon extraction and then concentrated 100-fold by ultracentrifugation (19) for EIA antigen and MA104 control antigen. These were used at a dilution of 1:200 (human rotaviruses) or 1:400 (animal rotavirus). For rabbit immunization the viruses were purified further by banding in cesium chloride gradients (19). Tris-buffered saline (pH 7.2) with 10 mM calcium chloride was used to stabilize the outer capsid layer (5).

Antisera. Outbred New Zealand rabbits showing preinoculation rotavirus antibody titers by EIA of <1:100 were inoculated at three to six sites subcutaneously. The inoculum consisted of 1 ml of virus preparation containing 10^8 intact, double-shelled rotavirus particles emulsified in an equal volume (1 ml) of Freund complete adjuvant.

At 1 month postimmunization, rabbits were boosted with the viral preparation in Freund incomplete adjuvant and then again in 7 days with virus in saline. Serum was collected 14

* Corresponding author.

TABLE 1. Neutralizing monoclonal antibodies to rotavirus evaluated as serotyping reagents

Monoclonal antibody	Immunoglobulin class[a]	Specific for serotype:	Homologous reciprocal direct EIA titer (10^5)	Virus protein specificity
RV-4:1	G1	1	20	gp34
RV-4:2[b]	G3	1	1.3	gp34
RV-4:3	G2b	1 (and 3)[c]	20	gp34
RV-5:1	A	2	2.0	gp34
RV-5:2	G2b	2	16	p84
RV-5:3	G3	2	2.0	gp34
N3/B7	G2a	2	4.0	gp34
RV-3:1	G2b	3	2.0 (6)	gp34 region A/C (8)
RV-3:2	G2b	3	2.0 (6)	gp34 region B (8)
ST-3:1	G3	4	20	ND[d]

[a] Determined by EIA kit with subclass- and isotype-specific antisera (Commonwealth Serum Laboratories, Parkville, Victoria, Australia).

[b] Antibodies eventually selected for use in the serotyping EIA are in boldface type.

[c] Reacts to a 10 to 100 times lesser extent with human serotype 3 rotaviruses than with serotype 1 rotoviruses (6a).

[d] ND, Not determined.

days later. Homologous EIA titers ranged from 1:50,000 to 1:100,000. Antisera were prepared to SA11, RV-3, RV-4, RV-5, and ST-3 rotaviruses.

The anti-human rotavirus hyperimmune serum 720 xii, prepared by repeated inoculation of a rabbit with purified virions from three fecal isolates representing three different serotypes by virus neutralization, was a gift of T. H. Flewett, Birmingham, England.

Monoclonal antibodies. The derivation and characterization of the serotype-specific, rotavirus-neutralizing monoclonal antibodies have been the subjects of previous reports (6, 6a, 8, 15). Antibody designation, immunoglobulin class, specificity, and homologous reciprocal EIA titers are summarized in Table 1. A high-affinity monoclonal antibody, RVA, directed to the major inner capsid protein p42 of rotavirus and reactive by EIA with all group A rotaviruses tested (unpublished results), was included in the serotyping EIA protocol as a positive control for the presence of rotaviral antigen. This immunoglobulin G2b (IgG2b) class antibody was derived from fusion of myeloma cells with mouse splenocytes immunized with RV-3 rotavirus (6). All hybridoma cell lines were inoculated into syngeneic BALB/c mice with antirotaviral antibody titers in serum of <1:100 by EIA. Mice were primed with Pristane (ICN Pharmaceuticals, Plainview, N.J.). Monoclonal antibodies of IgG subclasses 2 and 3 were partially purified from the resulting ascities fluid by affinity chromatography on *Staphylococcus aureus* protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) (6) or by ammonium sulfate precipitation for some experiments. IgA and IgG1 class antibodies were purified by precipitation with ammonium sulfate. The working dilutions of antibody quoted represent the dilution relative to ascites fluid.

Fecal extracts. Stools had been collected from neonates at the Royal Women's Hospital, Melbourne, from 1975 through 1977 as part of a previous study. Rotaviruses of a single type (serotype 3) and one or two electropherotypes (6, 18) were endemic in that hospital during that time. From 10 to 50% (wt/vol) homogenates in phosphate-buffered saline, pH 7.2 (PBS), all stools had been concentrated by ultracentrifugation, examined by EM for rotavirus (22), and then stored at −20°C. Fecal homogenates were also available from children attending or admitted to the Royal Children's Hospital, Melbourne, during 1983 and 1984, with acute gastroenteritis; some of these infections were diagnosed as rotaviral by EM, EIA with polyclonal antiserum (17), and EIA with monoclonal antibody RVA as the detector antibody. For both EM and serotyping by EIA, homogenates were clarified by centrifugation at 2,000 × *g* for 10 min. Clarified homogenates were concentrated approximately 100-fold with polyacrylamide hydrogel (Lyphogel; Gelman Sciences, Inc.) for EM and concentrated 2.5-fold by the same method, when necessary, for testing in the serotyping EIA (24). Concentration was used for approximately 30% of samples.

Serotyping EIA. The EIA serotyping method was developed from the assay for detection of rotavirus group antigen described by Beards et al. (3).

Hyperimmune rabbit antiserum to human rotaviruses RV-4, RV-5, RV-3, and ST-3 and simian rotavirus SA11 and preimmune rabbit sera were diluted 1:6,000 to 1:8,000 in PBS. The optimal antibody dilution was determined by checkerboard titration. Samples of 100 μl were added to wells of polystyrene Immuno I microtiter trays (Nunc, Denmark) and incubated at 37°C for 2 h. After trays were washed three times with PBS containing 0.05% (vol/vol) Tween 20 (PBS-T), 75 μl of PBS-T containing 2.5% (wt/vol) skim milk powder (SMP; Carnation) (PBS-T-SMP) was added to all wells, followed by 25 μl of 10% (wt/vol) fecal extract per well, rotavirus EIA antigen, or control antigen. Rotavirus EIA antigen was diluted 1:400 (RV-3, RV-4, ST-3) or 1:800 (SA11). Each test sample was added to wells coated with antiserum to each of the human rotavirus serotypes, to wells coated with preimmune serum, and to wells coated with anti-SA11 serum. Trays were left for 18 h at 4°C and then washed as above. Monoclonal antibody reactive with the same virus serotype as the coating serum or to the rotavirus group antigen was diluted 1:2,000 to 1:60,000 in PBS-T-SMP and added to the trays (100 μl per well). The group antigen-specific antibody RVA was added to wells coated with preimmune serum and anti-SA11 serum. After incubation at 37°C for 2.5 h and washing, 100 μl of a 1:800 dilution in PBS-T-SMP of goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (DAKO Immunoglobulins) was added to each well. The optimal conjugate dilution was determined by checkerboard titration; the conjugate was incubated at 37°C for 1.5 h and then washed. A sample (100 μl) of 0.01 M sodium citrate-acetate buffer (pH 6.0) containing 0.1 mg of 3,3′, 5,5′-tetramethyl benzidine (Sigma) per ml and 1.3 mmol of 30% (vol/vol) H_2O_2 per liter was added to each well. The blue reaction changed to yellow on stopping after 10 min with 50 μl 2 M H_2SO_4 per well, and then the absorbance of each well was recorded at 450 nm. A fecal sample was considered positive for a particular serotype if its optical density at 450 nm (OD_{450}) against sera produced to that serotype (*P*) was at least 2.0 times the mean of the OD_{450} of that sample reacted against sera to the other nonreacting serotypes (*N*), i.e., a *P*/*N* value of ≥2.0. For cultivable rotaviruses, N was calculated with the MA104 control values. Positive-minus-negative (*P* − *N*) values were used to construct antibody-binding curves.

FFN assay. Since 10% (wt/vol) fecal extracts contained insufficient numbers of infectious virus particles, sample concentration was required. A 2.0-ml sample of fecal extract was spun for 1 min at 10,000 × *g* in an Eppendorf centrifuge to pellet bacteria. The supernatant was diluted with Dulbecco modified Eagle medium to fill SW40 rotor tubes (Beckman Instruments, Inc., Fullerton, Calif.) and

ultracentrifuged at 90,000 × *g* for 75 min at 4°C. The pellet was suspended in 100 μl of Dulbecco modified Eagle medium, and the number of rotaviral fluorescent-cell-forming units was estimated by indirect immunofluorescent staining of infected MA104 cells as described previously (6). Only suspensions containing at least 100 fluorescent-cell-forming units in 5 μl could be serotyped successfully. Appropriate dilutions of these suspensions were incubated with 10-fold dilutions of rabbit hyperimmune antisera to Wa (serotype 1), RV-5 (serotype 2), RV-3 (serotype 3), and ST-3:1 (serotype 4) rotaviruses in the fluorescent focus neutralization (FFN) assay, as described previously (6). The neutralization titer of each serum was expressed as the highest reciprocal of the dilution giving a 50% reduction in the number of fluorescent cells. The serotype of the virus was determined as the specificity of the antiserum which gave the greatest titer by at least 10-fold.

RESULTS

Development of the serotyping EIA. Preliminary experiments with monoclonal antibodies RV-3:1, RV-3:2, and N3/B7 and rabbit antiserum 720 to coat the trays established that the overnight incubation of stool samples at 4°C produced higher specific absorbance readings than did incubation at 37°C for 1 to 2.5 h. Saturation of the monoclonal antibody binding at 37°C and of enzyme conjugate was reached by 2.5 and 1.5 h, respectively, thus fixing their incubation times. Incubation of monoclonal antibody or conjugate (or both) at 4°C for 18 h did not significantly alter absorbance readings. Similar results were obtained by using the group antigen reactive monoclonal antibody RVA, and anti-SA11 serum to coat the wells.

Initial assays were performed with 1% (wt/vol) bovine serum albumin in the PBS-T diluent to block nonspecific binding. However, some monoclonal antibodies showed low levels of binding to some cultivable and fecal rotaviruses of other serotypes under these conditions. A comparison of the blocking efficiencies of bovine serum albumin and SMP,

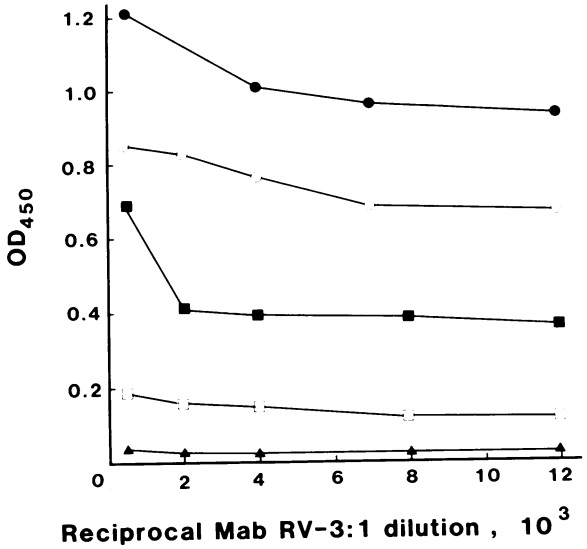

FIG. 1. Comparison of the effectiveness of BSA and SMP in reducing nonspecific binding in the serotyping EIA with Protein A affinity-purified monoclonal antibody RV-3:1. Symbols: ●, SA11 plus BSA; ○, SA11 plus SMP; ■, RV-5 or MA104 control plus BSA; □, RV-5 or MA104 control plus SMP; ▲, pooled rotavirus-negative fecal extract plus BSA or SMP.

TABLE 2. Comparison of coating sera in the serotyping EIA with the serotype-3 specific monoclonal antibodies

Virus	Monoclonal antibody	*P* − *N* value with the following coating serum[a]:		
		Anti-RV-3	Anti-SA11	Serum 720
RV-3	RV-3:1	1.094	0.226	0.744
	RV-3:2	0.685	0.262	0.721
SA11	RV-3:1	0.979	0.460	0.230
	RV-3:2	0.520	0.160	0.232

[a] The *P* − *N* value is the OD_{450} of the virus sample minus the OD_{450} of the MA104 control.

illustrated with monoclonal antibody RV-3:1 on plates coated with hyperimmune serum 720, is shown in Fig. 1. The high level of binding of the antibody to both the RV-5 rotavirus and the MA104 cell control was reduced to background levels by using SMP in the diluents; although the specific binding to SA11 virus was also reduced, the *P/N* value was either maintained or increased over the range of dilutions tested. The absorbance of a pool of 10 rotavirus-negative fecal extracts was also reduced by using SMP.

Initially, mouse ascites fluids containing monoclonal antibodies RV-3:1, RV-5:1, and RV-5:3, each of which was shown previously to be specific for a single human rotavirus serotype by FFN and direct EIA (6, 6a), cross-reacted with cultivable rotaviruses of other serotypes in this serotyping EIA, even when SMP was included in the diluents. This background reaction was removed by partial purification of the antibody from ascites fluid by ammonium sulfate precipitation or protein A affinity chromotography. Monoclonal antibody RV-5:1 showed additional cross-reactivity with uninfected MA104 wells; this cross-reactivity also was removed by ammonium sulfate precipitation. The remaining antibodies to RV-4, RV-5, RV-3, and ST-3 were specific for the virus against which they were raised, without purification of ascites fluid. Purification did not alter the EIA binding of these antibodies and was routinely performed.

The rotavirus strain against which the coating antiserum was produced had a marked effect on EIA absorbance. For example, serum to RV-3 rotavirus was superior to anti-SA11 serum in detection of both SA11 and RV-3 with monoclonal antibody RV-3:1 (Table 2). Serum 720, raised in the laboratory of T. Flewett to a mixture of human rotaviruses of three different serotypes, was of intermediate usefulness. In detecting RV-5, ST-3, and RV-4 rotaviruses, the homologous antiserum gave 50 to 90% greater specific absorbances than did the 720 serum.

Selection of monoclonal antibodies for the serotyping EIA. To assess the performance of the monoclonal antibodies in the serotyping EIA, the titer of each purified antibody was determined against the homologous rotavirus strain, using antiserum to this strain as the capture antibody and SMP to block nonspecific reactions. The results of titration of the serotype 1-, 2-, and 4-specific antibodies are presented in Fig. 2. The serotype 1-specific antibodies showed similar slopes and maximum *P* − *N* values, although monoclonal antibody RV-4:2 showed the highest maximum *P* − *N* value. Among the serotype 2-specific antibodies, *P* − *N* values were highest over the full range of dilutions for monoclonal antibody RV-5:3, suggesting that this antibody would be the best for detection of serotype 2 viruses. Monoclonal antibody N3/B7 showed *P* − *N* values approaching those of RV-5:3. However, monoclonal antibodies RV-5:1 and

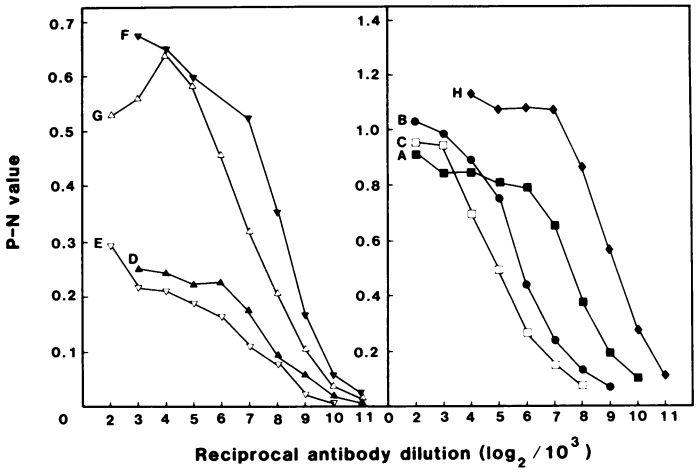

FIG. 2. Titration curves of the serotype 1-, 2-, and 4-specific monoclonal antibodies to the homologous cultivable rotavirus. Curves: A through C, titrated to RV-4 virus; A, RV-4:1; B, RV-4:2; C, RV-4:3; D through G, titrated to RV-5 virus; D, RV-5:1; E, RV-5:2; F, RV-5:3; G, N3/B7; H, ST-3:1 titrated to ST-3 virus.

RV-5:2 gave shallow titration curves and low $R - N$ values, so that their use would produce an EIA of reduced sensitivity. The single serotype 4-specific antibody available showed the highest maximum $P - N$ value and the greatest endpoint of all antibodies tested, in addition to a steep gradient on the linear portion of the curve. This antibody therefore appeared suitable for serotyping. The lowest dilution of antibody at which the antigen was still saturated was used in the serotyping EIA, except for monoclonal antibody ST-3:1, which reacted to the cultivable serotype 4 rotavirus VA70 to a 100-fold lesser extent than to ST-3 virus and so was used at a 100-times-greater concentration than that calculated with ST-3 virus as the antigen.

Sensitivity of the serotyping EIA compared with a direct EIA. Previously, these monoclonal antibodies were reacted with the homologous rotavirus strains in an EIA system in which rotavirus antigen at the same dilution as used in the serotyping EIA was adsorbed directly onto the solid phase (6, 6a). A comparison of the endpoint titers obtained with the two EIA systems and the maximum $P - N$ values is shown in Table 3. Endpoint titers were increased 2- to 10-fold, and maximum $P - N$ values rose by 37 to 165% in the serotyping assay, over direct assay results. Hence the serotyping assay was of considerably greater sensitivity than the direct EIA, as measured by monoclonal antibody binding for a fixed amount of cultivable rotavirus added.

Evaluation of monoclonal antibody RVA for detection of rotavirus group antigen in stools. The sensitivity and specificity of monoclonal antibody RVA for the detection of rotavirus antigen in 151 stools, relative to EM and a polyclonal EIA, is illustrated in Table 4. Fifty stools were rotavirus positive by EM. The monoclonal EIA showed greater sensitivity and specificity than the polyclonal EIA and equal specificity to the reference method (EM). The sensitivity of the monoclonal EIA was greater than EM when each stool was examined once only and slightly lower when the same stools were examined by EM twice. In general, the P/N values of monoclonal antibody RVA with cultivable rotaviruses (Table 5) and rotavirus-positive stools tended to be greater than the ratios obtained with the serotyping monoclonal antibodies.

Evaluation of the serotyping EIA with cultivable rotaviruses. The results of the serotyping EIA with cultivable rotavirus strains and optimal dilutions of the coating antibodies, enzyme conjugate, and monoclonal antibodies are shown in Table 5. Each of the antibodies was totally specific for the representative virus of the given serotype at the dilutions used and gave P/N values of 6.7 to 64. Background absorbances were all less than 0.050. However, monoclonal antibody RV-4:3, which has been shown to bind to both serotype 1 and 3 rotaviruses by FFN and direct EIA (6a), gave a positive P/N value when reacted with RV-3 rotavirus (data not shown).

Evaluation of the serotyping EIA with stool specimens. The specificity of the serotyping assay for rotaviruses in stools was examined by using the four selected monoclonal antibodies described in Table 1 on 23 stools in which no rotavirus had been detected by EM or EIA; all four gave a negative result with each of the 23 rotavirus-negative stools. With the selected antibodies, all of 30 stools tested which contained serotype 3 (neonatal) rotavirus were determined to be positive for group antigen with monoclonal antibody RVA and 97% were serotyped as 3, providing sufficient extract (1.0 ml) was available for concentration (Table 6). The one untypable neonatal rotavirus sample did not react with any of the serotyping antibodies but showed a P/N ratio of 24 with monoclonal antibody RVA. Of stools containing >20 virions per grid square by EM, 93% could be typed

TABLE 3. Comparison of the sensitivity of the direct and serotyping EIA systems by endpoint titration of monoclonal antibodies

Monoclonal antibody	Direct EIA		Serotyping EIA	
	Endpoint[a] (10^5)	Maximum $P - N$ value[b]	Endpoint (10^5)	Maximum $P - N$ value
RV-4:1	20	0.71	40	1.1
RV-4:2	1.3	0.41	10	1.0
RV-4:3	20	0.49	40	1.3
RV-5:3	2	0.49	10	0.67
N3/B7	4	0.39	10	0.64
RV-3:1	2	0.82	10	1.2
RV-3:2	2	0.61	10	1.1
ST-3:1	20	0.49	30	1.1

[a] Reciprocal titer.
[b] Maximum value observed over a range of dilutions of 1:1,000 to 1:4,000,000 of the monoclonal antibody.

without concentration of the stool extract. The discrimination between positive and negative results was good; the lowest positive *P/N* value was 2.3, and 57% were greater than 6.0. Similarly, 92% (12 of 13) of the stools containing short RNA pattern (serotype 2) rotaviruses were successfully serotyped with *P/N* values of 2.8 to 40 (monoclonal antibody RV-5:3), and 18 to 22 (RVA). Insufficient material was available to allow concentration of the one stool containing presumptively serotype 2 rotavirus which did not react with any of the serotyping monoclonal antibodies.

To test the specificity and sensitivity of serotype 1- and 4-specific monoclonal antibodies, 24 rotavirus-containing stools, collected from children visiting the Royal Children's Hospital with acute gastroenteritis as outpatients and inpatients in 1983 and 1984, were processed for FFN. Sufficient fluorescent-cell-forming units were present in five serotype 1 and two serotype 4 rotavirus-containing stools for FFN serotyping. The serotyping EIA result corresponded to the FFN result in all cases. In addition to these 24 stools, a further 56 rotavirus-positive stools collected from the same group of children were also serotyped by EIA (Table 7). All were positive for rotavirus group antigen with monoclonal antibody RVA. Of the 80 stools examined, 72 (95%) reacted in the serotype EIA as either serotype 1 (75%) or serotype 4 (25%). Concentration of 5 samples (6%) was required to obtain a serotyping result. A clear-cut result was obtained with monoclonal antibodies RV- 4:2 and ST-3:1 when reacted with stools, with mean *P/N* ratios of 23 and 30, respectively. By *P/N* ratio, levels of group antigen detected in at least three of the four untypable stools were similar to levels in the stools in which serotyping was possible.

Regardless of the rotavirus serotype present in stools, reactions were observed only with antibody directed to the major glycoprotein of one serotype. All results obtained with stool extracts could be successfully read by eye by an independent observer.

DISCUSSION

Ten neutralizing monoclonal antibodies derived to the four main serotypes of human rotavirus all proved to be capable of serotyping both cultivable and stool rotavirus strains in an indirect EIA. Initial problems of elevated background color and cross-reactivity among rotaviruses were overcome by preliminary purification of the monoclonal antibodies and the introduction of SMP into the assay. SMP is known to effectively control nonspecific reactions in both EIA and Western blotting (13). However, the reason for the beneficial effect of antibody purificaiton is not entirely apparent. In some cases crude monoclonal antibody bound to a component in rotavirus samples only, so that the reaction was not due to nonspecific binding to a component of feces. Possibly, the mice used to produce ascites fluids experienced a rotavirus infection during the period of ascites accumulation and produced IgM antibodies to rotavirus. However, this does not fully explain the effectiveness of ammonium sulfate precipitation in removing the reaction, since IgM would have been retained at the 50% saturation level used for purification.

The assay was most sensitive when the hyperimmune coating serum used had been raised to the homologous serotype and even to the same rotavirus strain. This suggests that antigenic determinants other than the group and subgroup antigens already recognized on the product of gene 6 (p42) are involved in EIA binding of rotaviruses. This effect would not be evident in EIA systems for group antigen detection developed to date, in which both capture and detector antibodies are polyclonal, or in the serotyping EIA with a cross-adsorbed detector antiserum (23). Assays in which a combination of polyclonal antiserum and a monoclonal group-antigen or subgroup antigen-specific antibody is used (3) would also not be expected to show evidence of this previously undetected antigenic variation, which appears to occur both between and within a serotype.

Comparison of endpoint titers and maximum *P/N* values for each monoclonal antibody in the serotyping EIA, with a direct EIA in which rotavirus antigen was passively adsorbed onto the solid phase (6), suggested that the serotyping assay, in which antigen is bound with an antibody coating the solid phase, is the more sensitive one. It is impracticable, in any case, to serotype rotaviruses in stools with the direct assay. The serotyping monoclonal antibodies will attach to the solid phase (unpublished observation) and so could be

TABLE 4. Sensitivity and specificity of monoclonal and polyclonal EIA systems relative to EM for detection of rotavirus antigen in 151 stools

Detection method	Sensitivity[a]	Specificity[b]
Electron microscopy	100 (90)[c]	100
EIA, polyclonal[d]	92	95
EIA, monoclonal antibody RVA	96	100

[a] Sensitivity: (number of stools positive by both EM and EIA/number of stools positive by EM) × 100.
[b] Specificity: (number of stools negative by both EM and EIA/number of stools negative by EM) × 100.
[c] Sensitivity of 90% on a single examination, but 100% when stools positive by either EIA were reexamined by EM.
[d] EIA with guinea pig hyperimmune serum to SA11 rotavirus as the capture antibody and rabbit hyperimmune serum to SA11 rotavirus as the detector antibody (17).

TABLE 5. Serotyping cultivable human rotaviruses by EIA with monoclonal antibodies

Monoclonal antibody	Reciprocal dilution	*P/N* value with rotavirus (serotype):			
		RV-4 (1)	RV-5 (2)	SA11 (3)	ST-3 (4)
RV-4:1	60,000	**40**[a]	1.2	1.5	1.1
RV-4:2	4,000	**46**	1.4	1.5	1.0
RV-4:3	2,000	**64**	1.2	1.8	1.2
RV-5:1	500	1.2	**6.7**	1.5	1.8
RV-5:2	9,000	1.0	**11**	1.8	1.0
RV-5:3	20,000	1.3	**32**	1.9	1.4
N3/B7	20,000	1.3	**28**	1.7	1.5
RV-3:1	20,000	1.1	1.3	**34**	1.1
RV-3:2	2,000	1.3	1.5	**14**	1.5
ST-3:1	160,000	1.1	1.1	1.2	**12**
RVA	50,000	**58**	**56**	**52**	**30**

[a] Values in boldface type are from the homologous reaction.

TABLE 6. Effect on serotyping EIA sensitivity of amount of rotavirus present in stool sample, using Mab RV-3:1 and serotype 3 rotaviruses

Amt of virus by EM[a]	*n*	Sample treatment	No. (%) of positive samples	Mean (range) *P/N*
1–20	16	No concn	10 (63)	9.3 (2.3–24)
		Concn ×2.5	6[b] (38)	8.6 (3.9–16)
>20	14	No concn	13 (93)	13 (3.6–25)
		Concn ×2.5	0[b] (0)	

[a] Number of virus particles per grid square.
[b] Data for concentrated samples are numbers (percentages) of additional positive samples.

TABLE 7. *P/N* values obtained from EIA serotyping of rotaviruses in fecal extracts from 76 children with acute gastroenteritis

No. of stools	RV-4:2		RV-5:3		RV-3:1		ST-3:1		RVA	
	Mean *P/N* (range)	Result	Mean *P/N* (range)	Result	Mean *P/N* (range)	Result	Mean *P/N* (range)	Result	Mean *P/N* (range)	Result
54	23 (2.6–46)	+	0.8 (0.6–1.4)	–	1.2 (0.7–1.6)	–	1.4 (0.8–1.9)	–	25 (7.1–49)	+
18	1.1 (0.8–1.3)	–	1.1 (0.8–1.5)	–	1.2 (1.0–1.8)	–	20 (2.1–44)	+	26 (4.1–58)	+
4	1.0 (0.7–1.3)	–	1.2 (1.0–1.5)	–	1.4 (1.0–1.9)	–	1.4 (0.7–1.7)	–	23 (4.9–39)	+

used as capture antibodies, as described by Shaw et al. (21) for serotype 1 and 3 rotaviruses. However, in preliminary experiments, use of the monoclonal antibodies, singly or in combination, as both capture and detecting antibodies appeared to reduce assay sensitivity (unpublished observation).

The specificity observed for each of the monoclonal antibodies with cultivable rotaviruses in this serotyping EIA corresponded to their previously determined specificities by FFN and direct EIA (6, 6a). When stool extracts containing rotavirus of a predetermined serotype were analyzed, 92 to 100% of viruses were correctly serotyped, and results could be read by eye. The incorrectly typed stools were all false-negatives. Sometimes concentration of stool extracts was necessary to obtain a result. None of the rotavirus-negative stools gave a false-positive reading with any of the monoclonal antibodies tested.

Although we were able to test 30 stools containing rotavirus already serotyped as 3, only small number of stool extracts carrying known serotype 1, 2, and 4 viruses were readily available to us. However, preliminary results from an exchange of 54 coded rotavirus-containing fecal extracts between ourselves and G. Gerna, Pavia, Italy, to compare the solid-phase immune EM (9) and serotyping EIA systems confirm our specificity rates of the serotyping EIA for the four rotavirus serotypes (G. Gerna, personal communication).

T. H. Flewett, Director, Regional Virus Laboratory, Birmingham, England, has been supplied with serotype 2- and 3-specific monoclonal antibodies. In a serotyping EIA with the monoclonal antibodies at a dilution of 1:10,000 as detector antibodies he has reported (personal communication) that monoclonal antibody RV-3:1 reacted with 15 of 16 (94%) of serotype 3 stool viruses, but none of 196 serotype 1, 5 serotype 2, 4 serotype 4, and 1 bovine isolate (from a calf in the United Kingdom). In Flewett's laboratory, monoclonal antibody RV-5:3 detected 13 to 16 (81%) of serotype 2 rotaviruses but none of 15 serotype 1, 4 serotype 3, 1 serotype 4, or the United Kingdom bovine rotaviruses. In his hands, monoclonal antibodies RV-5:1 and RV-5:2 appeared to cross-react between serotypes. Our own evaluation of the sensitivity and specificity of these monoclonal antibodies has therefore substantially been confirmed in either or both of two other independent laboratories, including the World Health Organization Collaborating Centre for Reference and Research on Rotaviruses in Birmingham.

In addition to serotype-specific antibodies, a group antigen-specific antibody RVA, directed to the major inner shell protein p42, was incorporated into the assay. For detection of rotavirus in stools, this antibody gave the EIA greater sensitivity and specificity, compared with an EIA with polyclonal antisera. Since antibody RVA may detect viral antigen when little or no serotyping antigen is present, a monoclonal antibody capable of detecting the gp34 of any rotavirus serotype would be a valuable inclusion. This would make it possible to distinguish between stools containing potential new serotypes and those in which there is insufficient antigen for serotyping. Although we have derived noneutralizing monoclonal antibodies to gp34 of human rotavirus, none of these reacted with all human rotaviruses tested, even in combination (6a).

Four specimens contained rotaviruses that could not be serotyped. As the RNA of these rotaviruses coelectrophoresed with RNA from rotaviruses which were successfully serotyped (unpublished results), these untypable viruses are likely to have contained insufficient outer capsid glycoprotein to be serotyped. As we observed no difference between the *P/N* ratios of monoclonal antibody RVA with serotypable and untypable rotaviruses, it is likely that the outer capsid in the untypable stools had disintegrated or been degraded. Although it has been postulated that serotyping is likely to be less sensitive than group antigen detection (21) by virtue of the greater amount of group antigen per virion, our limited experience suggests that outer capsid degradation is the major factor in the occurrence of false-negative serotyping results.

This serotyping EIA is simple and specific and shows a sensitivity approaching that of rotavirus detection by EM. Monoclonal antibodies to new serotypes, such as the Indonesian "super short" strain (16), could be readily incorporated into the assay. Its application in epidemiological studies and vaccine trials in humans is likely to provide valuable insights into the relationship between RNA electropherotype and serotype and into the cross-protection, if any, afforded by immunization or infection with rotaviruses of different serotypes.

ACKNOWLEDGMENTS

We thank Jennifer Tursi, Lorraine Adams, and Sandra Lawrance for their expert assistance with this research and S. Sonza for the N3/B7 antibody. The secretarial services of Val Williams and Jane Lee were much appreciated, as was the photography of Anneke Veenstra.

This project was supported by the Royal Children's Hospital Research Foundation and the National Health and Medical Research Council of Australia.

LITERATURE CITED

1. **Albett, M. J., and R. F. Bishop.** 1984. Cultivation of human rotaviruses in cell culture. J. Med. Virol. **13**:377–384.
2. **Bastardo, J. W., J. L. McKimm-Breschkin, S. Sonza, L. D. Mercer, and I. H. Holmes.** 1981. Preparation and characterization of antisera to electrophoretically purified SA 11 virus polypeptides. Infect. Immun. **34**:641–647.
3. **Beards, G. M., A. D. Campbell, N. R. Cottrell, J. S.M. Peiris, N. Rees, R. C. Sanders, J. A. Shirley, H. C. Wood, and T. H. Flewett.** 1984. Enzyme-linked immunosorbent assays based on polyclonal and monoclonal antibodies for rotavirus detection. J. Clin. Microbiol. **19**:248–254.
4. **Beards, G.M., J. N. Pilford, M. E. Thouless, and T. H. Flewett.** 1980. Rotavirus serotypes by serum neutralization. J. Med. Virol. **5**:231–237.
5. **Cohen, J., J. La Porte, A. Charpillienne, and R. Scherrer.** 1979.

Activation of RNA polymerase by calcium chelation. Arch. Virol. **60**:177–186.

6. **Coulson, B. S., K. J. Fowler, R. F. Bishop, and R. G. H. Cotton.** 1985. Neutralizing monoclonal antibodies to human rotavirus and indications of antigenic drift among strains from neonates. J. Virol. **54**:14–20.

6a. **Coulson, B. S., J. M. Tursi, W. J. McAdam, and R. F. Bishop.** 1986. Derivation of neutralizing monoclonal antibodies to human rotaviruses and evidence that an immunodominant neutralization site is shared between serotypes 1 and 3. Virology **154**: 302–312.

7. **de Zoysa, I., and R. G. Feacham.** 1985. Interventions for the control of diarrhoeal diseases among young children: rotavirus and cholera immunization. Bull. W.H.O. **63**:569–583.

8. **Dyall-Smith, M. L., I. Lazdins, S. Sonza, G. Tregear, and I. H. Holmes.** 1985. Antigenic determinants coded by rotavirus genes, p. 215–220. *In* S. Tzipori, G. Barnes, R. Bishop, I. Holmes, R. Robins-Browne (ed.), Infectious diarrhoea in the young. Elsevier Sciences Publishing, Inc., New York.

9. **Gerna, G., N. Passarani, M. Battaglia, and E. Percivalle.** 1984. Rapid serotyping of human rotavirus strains by solid-phase immune electron microscopy. J. Clin. Microbiol. **19**:273–278.

10. **Greenberg, H. B., J. Valdesuso, K. van Wyke, K. Midthun, M. Walsh, V. McAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47**:267–275.

11. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82**: 8701–8704.

12. **Hoshino, Y., R. G. Wyatt, H. B. Greenberg, J. Flores, and A. Z. Kapikian.** 1984. Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque-reduction neutralization. J. Infect. Dis. **149**:694–702.

13. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1**:3–8.

14. **Kapikian, A. Z., Y. Hoshino, J. Flores, K. Midthun, R. I. Glass, O. Nakagomi, T. Nakagomi, R. M. Chanock, L. Potash, M. M. Levine, R. Dolin, P. F. Wright, R. E. Belshe, E. L. Anderson, T. Vesikari, L. Gothefors, G. Wadell, and I. Perez-schael.** 1986. Alternative approaches to the development of a rotavirus vaccine, p. 192–214. *In* J. Holmgren, A. Lindberg, and R. Mollby (ed.), Development of vaccines and drugs against diarrhea. Studentlitteratur, Lund, Sweden.

15. **Lazdins, I., S. Sonza, M. L. Dyall-Smith, B. S. Coulson, and I. H. Holmes.** 1985. Demonstration of an immunodominant neutralization site by analysis of antigenic variants of SA11 rotavirus. J. Virol. **56**:317–319.

16. **Matsuno, S., A. Hasegawa, A. Mukoyama, and S. Inouye.** 1985. A candidate for a new serotype of human rotavirus. J. Virol. **54**:623–624.

17. **Roberton, D. M., M. Harrison, C. S. Hosking, L. C. Adams, and R. F. Bishop.** 1979. Rapid diagnosis of rotavirus infection: comparison of electron microscopy and enzyme linked immunosorbent assay (Elisa). Aust. Paediatr. J. **15**:229–232.

18. **Rodger, S. M., R. F. Bishop, C. Birch, B. McLean, and I. H. Holmes.** 1981. Molecular epidemiology of human rotaviruses in Melbourne, Australia, from 1973 to 1979, as determined by electrophoresis of genome ribonucleic acid. J. Clin. Microbiol. **13**:272–278.

19. **Rodger, S. M., R. D. Schnagl, and I. H. Holmes.** 1975. Biochemical and biophysical characteristics of diarrhea viruses of human and calf origin. J. Virol. **16**:1229–1235.

20. **Sato, K., Y. Inaba, T. Shinozaki, R. Fujii, and M. Matumoto.** 1981. Isolation of human rotavirus in cell cultures. Arch. Virol. **69**:155–160.

21. **Shaw, R. D., D. L. Stoner-ma, M. K. Estes, and H. B. Greenberg.** 1985. Specific enzyme-linked immunoassay for rotavirus serotypes 1 and 3. J. Clin. Microbiol. **22**:286–291.

22. **Soenarto, Y., T. Sebodo, R. Ridho, H. Alrasjid, J. E. Rohde, H. C. Bugg, G. L. Barnes, and R. F. Bishop.** 1981. Acute diarrhea and rotavirus infection in newborn babies and children in Yogyakarta, Indonesia, from June 1978 to June 1979. J. Clin. Microbiol. **14**:123–129.

23. **Thouless, M. E., G. M. Beards, and T. H. Flewett.** 1982. Serotyping and subgrouping of rotavirus strains by the ELISA test. Arch. Virol. **73**:219–230.

24. **Whitby, H. J., and F. G. Rodgers.** 1980. Detection of virus particles by electron microscopy with polyacrylamide hydrogel. J. Clin. Pathol. **33**:484–487.