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Typing of Human Rotavirus VP4 by an Enzyme Immunoassay Using Monoclonal Antibodies

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Two different neutralization specificities exist on the outer capsid of group A rotaviruses. At least seven VP7 (G) antigenic types are distinguishable among human rotaviruses. Four distinct antigenic (P) types of human rotavirus VP4 corresponding to separate rotavirus gene 4 groups have been described. The aim of this study was to identify P types in clinical specimens by developing an enzyme immunoassay, using P-type-specific neutralizing monoclonal antibodies (N-MAbs). Three N-MAbs primarily or solely recognizing each of P types 4, 6, and 8 and binding to VP4 or its subunit VP5* were derived. These N-MAbs served as detector antibodies in an enzyme immunoassay P-typing system similar to that in use for G typing. P-type specificity was highest when the G-type specificity of the capture antiserum was matched to the G type of the rotavirus in the test sample. The method correctly identified the P types of 13 well-characterized, cell culture-adapted human rotaviruses and was used to classify a further six strains. P typing of 118 rotavirus-positive stools gave results consistent with the P type inferred from the G type for 98 (83%) samples. Twelve (10%) of the stools showed no reaction with any N-MAb and eight (7%) samples were untypeable because of cross-reactivity between N-MAbs or high background readings. This P-typing enzyme immunoassay system is economical and amenable to large-scale use in epidemiological studies. Its use will facilitate assessment of the distribution of P types worldwide and of the role of VP4 in eliciting protective immune responses.

Rotaviruses cause severe diarrhea in the young of humans and animals. The use of candidate vaccines has shown the importance of the outer capsid glycoprotein, VP7, in immunity to rotavirus. Both VP7 and the minor outer capsid protein, VP4, are capable of inducing rotavirus-neutralizing antibodies which passively protect against infection. At least seven serotypes (G types) exist among group A human rotaviruses (HRVs); the serotypes are based on the antigenic specificity of VP7 determined by cross-neutralization tests with hyperimmune antisera (1).

Four alleles of the group A HRV gene 4 (encoding VP4) have been defined by nucleotide sequence analysis. The proteins encoded by these alleles were tentatively assigned to protease-sensitive protein (P) types 4, 6, 8, and 9 (8). Three distinct HRV P types and one subtype, which correspond to these alleles, were more recently defined by using guinea pig antisera raised to baculovirus-expressed VP4 protein (13). The criterion for P-type specificity was a greater than eightfold difference in neutralizing antibody titer. By this classification, HRVs of previously defined P types 4, 6, 8, and 9 are equivalent to antigenic P types 1B, 2, 1A, and 3, respectively. Rotaviruses of P type 6 exclusively infected neonates, with few or no symptoms of disease, whereas rotaviruses of P types 4, 8, and 9 were capable of causing symptomatic infection in older children (13).

The existence of two neutralization specificities apparently circulating independently necessitates the development of a dual typing system. Determination of the G type has been achieved by using cross-neutralization assays, solid-phase immune electron microscopy (10), and the polymerase chain reaction (PCR) technique (15). However, monoclonal antibody (MAb)-based enzyme immunoassays (EIAs) are most widely used because of their simplicity, specificity, and applicability to rotavirus in stool specimens (7, 29, 34, 35). Rapid and efficient P typing has proven more difficult because of the predominant reactivity of hyperimmune antisera to VP7, the cross-reactivity of many VP4-neutralizing MAbs, and the low levels of discrimination of antisera to expressed VP4 (2, 11). Hybridization of PCR-derived probes (18) and PCR typing using nested primers (9) have been used to detect and classify alleles of gene 4. However, correlation of gene sequence with antigenic type is not fully established, and these techniques are not applicable to large-scale epidemiological studies.

We aimed to derive MAbs suitable for P typing in an EIA system. As conventional protocols for immunization of mice rarely yielded neutralizing MAbs (N-MAbs) to VP4 previously, novel protocols of oral immunization with live virus and intraperitoneal inoculation of virus grown in the absence of trypsin were examined. The characterization of four N-MAbs to VP4, the classification of HRV VP4 antigenic types, and the development of an EIA for P typing rotaviruses are the subjects of this report.

MATERIALS AND METHODS

Viruses and cells. The origin and source of the cultivable human and animal rotaviruses used were described previously (5, 6), as noted: human viruses M37, Wa, Ku, RV-4, and K8 (G1); DS-1, RV-5, RV-6, S2, and 1076 (G2); P, Yo, and RV-3 (G3); VA70, Hosokawa, and ST-3 (G4); B37 and 69M (G8); F45 and Wi61 (G9); porcine rotaviruses AT/76 and CRW-8 (G3); MDR-13 (G3/5); BEN-144 (G4); TRF-41 (G5); G3 simian rotaviruses SA11 and RRV; and canine rotavirus K9 (G3). Australian equine rotavirus E2 and bovine rotaviruses A3 and A5 were characterized previously (1). HRVs AU-1 (G3) and AU-32 (G9) were isolated from ill children in Japan (23, 24), and L26 was isolated from a child in the Philippines during a small rotavirus outbreak (36). The reassortant rotaviruses used were F45 × SA11 (gene encoding VP7 of F45 and gene 4 of SA11) and ST-3 × SA11 (gene encoding VP7 of ST-3 and gene 4 of SA11). All viruses were propagated in MA104 cells with trypsin as described previously (6), except for the ST-3 rotavirus used to immunize

mice, which was grown from high-titered virus stock (7.8×10^6 fluorescent cell-forming units/ml) activated with 2.5 μg of trypsin per ml and grown without the addition of further trypsin to give a trypsin concentration in the maintenance medium of <0.5 μg/ml.

Virus preparation for animal immunization, EIA antigen, and immunoblotting. Rotavirus-infected or mock-infected cells were harvested by freeze-thawing. Harvested infected cells were used to immunize mice orally. For EIA antigen, MA104 cell control antigen, and intraperitoneal inoculation of mice, cell harvests were extracted with fluorocarbon and then concentrated 100-fold by ultracentrifugation (27). For rabbit immunization, the viruses were purified further by banding in cesium chloride gradients. Tris-buffered saline (pH 7.2) with 10 mM calcium chloride was used to stabilize the outer capsid layer.

Immunization of mice. (i) Oral. Specific-pathogen-free BALB/c mice 8 weeks old, which were seronegative by EIA and fluorescent focus neutralization (FFN) assay to the immunizing virus, were kept in isolator cages and fed by gavage with 0.3 ml of F45 rotavirus (5×10^4 fluorescent cell-forming units) three times each day for 2 consecutive days. This procedure was repeated every 3 weeks for 4 months (six times). Mice were housed in isolator cages for the duration of the experiments and were bled for antibody estimation at 1 and 3.5 months after the first inoculation. Neutralizing antibody responses to F45 virus were detectable in the sera of 50% of the mice at 1 month and in 100% of the mice at 3.5 months. Reciprocal titers ranged from 100 to 2,500. Heterologous neutralizing antibody responses to at least three of the RV-3, RV-4, RV-5, ST-3, and Wi61 rotaviruses were observed at 3.5 months, and responses to all of these above viruses were seen at the time of splenic fusion. Four days before fusion, mice were given 0.4 ml (6×10^5 fluorescent cell-forming units) of F45 virus (as EIA antigen) intraperitoneally.

(ii) Parenteral. Seronegative BALB/c mice 8 weeks old kept in isolator cages were immunized intraperitoneally with 0.5 ml of partially purified ST-3 rotavirus (2×10^3 fluorescent cell-forming units) grown with a trypsin level of <0.5 μg/ml. Mice were given an identical booster injection 50 days later. Homologous reciprocal neutralizing antibody titers in sera 2 months after the first inoculation ranged from 7,000 to 12,000.

Hybridoma production and characterization. Fusion of P3-X63-Age 8.653 (clone 653) myeloma cells with the immunized mouse splenocytes was carried out 3 days after the final immunization, and resulting hybridomas were screened for antibody production by FFN assay (4), using the homologous rotavirus. Positive hybridomas were subcloned at least twice by limiting dilution and grown as ascites tumors in Pristane-primed syngeneic mice (6). MAbs of immunoglobulin G subclasses 2 and 3 were partially purified from the resulting ascites fluid by affinity chromatography on *Staphylococcus aureus* protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) for some experiments or by ammonium sulfate precipitation. Immunoglobulin G1 class antibodies were purified by precipitation with ammonium sulfate. The working dilutions of antibody quoted represent the dilution relative to ascites fluid. Determination of titers in antisera and mouse ascites fluid was done by FFN assay (4) and by direct binding EIA. This EIA was performed as described previously (7) except that sheep anti-mouse immunoglobulins, affinity isolated and conjugated to horseradish peroxidase (Silenus Laboratories, Melbourne, Australia), were used to detect the MAb binding to cell culture-grown rotavirus EIA antigens or MA104 cell controls adsorbed to polystyrene trays, as for the VP4 and VP7 EIA systems. The specificity of the N-MAbs for VP4 was determined by reaction with appropriate rotavirus reassortants (6).

Immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of partially purified rotavirus proteins was carried out as previously described (4) under fully reduced or nonreducing conditions (6). Electrophoresed proteins were transferred electrophoretically to nitrocellulose (25). Blotted proteins were probed with dilutions of N-MAbs as ascites fluid and then by goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Promega Biotech, Madison, Wis.) at a 1:7,500 dilution and then were developed with 5-bromo-4-chloro-3-indolyl-phosphate-Nitro Blue Tetrazolium substrate (Promega) as described previously (25). The positions of viral proteins were determined by probing blotted proteins with hyperimmune antiserum to homologous virus, as described previously (25).

Radioimmunoprecipitation. ^{35}S-methionine-labeled, rotavirus-infected cell lysates were prepared as described earlier (6). Mixtures of 25 μl of the viral lysate and 25 μl of N-MAb ascites fluids diluted 1:50 to 1:500 were incubated overnight at 4°C. The immune complexes were precipitated with 50 μl of Pansorbin cells (Calbiochem) for 1 h at 20°C with frequent mixing. The precipitates were collected by centrifugation at 4,000 × *g* for 5 min and then washed three times with 0.05 Tris HCl buffer (pH 7.4) containing 0.15 NaCl, 1 mM EDTA, 0.25% bovine serum albumin (Sigma), 0.05% Triton X-100, and 0.02% NaN_3. The adsorbed labeled viral proteins were recovered by boiling in Laemmli sample buffer (6). Following centrifugation at 10,000 × *g* for 10 min, the supernatants were electrophoresed in a polyacrylamide gel as described previously (6, 25).

Fecal rotaviruses. Stools had been collected from neonates infected with rotavirus at the Royal Women's Hospital, (RWH) Melbourne, Australia, from 1977 through 1978 as part of previous studies (2, 20). Rotaviruses of a single type (P2 G3) and one or two electropherotypes (7, 26) were endemic in that hospital during that time (2, 20). From 10 to 50% (wt/vol) homogenates in phosphate-buffered saline, pH 7.2 (PBS), all stools had been concentrated by ultracentrifugation and examined for rotavirus (28) by electron microscopy. Homogenates were then stored at −20°C. From some stools, the supernatant above the virus pellet resulting from ultracentrifugation was also available for testing. Fecal homogenates were also available from children admitted with severe rotavirus gastroenteritis to the Royal Children's Hospital (RCH), Melbourne, Victoria, during 1983 to 1984 and 1989 to 1991; Fairfield Infectious Diseases Hospital (FIDH), Melbourne, Victoria, during 1990; Liverpool Hospital, New South Wales, during 1990; Alice Springs Hospital (ASH), Alice Springs, Northern Territory, during 1979, 1982 to 1984, 1986, and 1988 to 1990; and Gadjah Mada Hospital, Yogyakarta, Indonesia, from 1978 (29). These rotavirus infections were diagnosed by electron microscopy or EIA. Fecal homogenates which had been stored at −70°C were centrifuged at 2,000 × *g* for 10 min at 4°C. The resulting supernatant (clarified fecal homogenate) was used in the P-typing assay unless otherwise stated.

VP4 and VP7 serotyping EIA systems. The VP4 EIA typing method was adapted from the VP7 serotyping EIA described earlier (7). Hyperimmune rabbit antiserum to HRVs RV-4, RV-5, RV-3, ST3, and F45 and preimmune rabbit serum were produced in outbred New Zealand rabbits by subcutaneous inoculation (7). Homologous direct EIA titers ranged

from 1 in 80,000 to 1 in 250,000. These sera were diluted 1:6,000 to 1:8,000 in PBS. Sheep anti-rotavirus (RV-4, RV5, P, and ST-3 combined) hyperimmune antiserum (Silenus Laboratories, Victoria, Australia) was diluted 1:500 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, Roskilde, 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 (PBS-T-SMP; Carnation) was added to all wells, and then 25 μl of clarified fecal homogenate per well, rotavirus EIA antigen, or control antigen was added. The dilution of EIA antigen used was that which was optimal in the direct EIA method, and the control antigen was diluted to the same degree as the rotavirus EIA antigen. Each test sample was added to wells coated with antiserum matched for G type to the rotavirus in the sample, to wells coated with preimmune serum, and, as required, to wells coated with the sheep antiserum. Trays were left for 18 h at 4°C and then washed as described above. MAbs reactive with each P type, with G types 1 to 4, 8, and 9 as required, and with MAb 60 (detects VP7 of all G types tested) were diluted 1:500 to 1:5,000 in PBS-T-SMP and added to the trays (100 μl per well). The optimal dilution of each MAb was determined by titration with representative rotavirus strains within the P or G type detected by each MAb. The lowest dilution of antibody at which the least well-detected rotavirus strain was still saturated was used in the typing EIA. After trays were incubated at 37°C for 2.5 h and washed, 100 μl of a 1:8,000 dilution in PBS of sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (Silenus Laboratories) 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, plates were washed, and the tetramethyl benzidine-hydrogen peroxidase indicator system was added (7). The reaction was stopped after 10 min with 50 μl of 2 M H_2SO_4 per well, and then the absorbance of each well was recorded at 450 nm (OD_{450}). A fecal sample was considered positive for a particular P type if its OD_{450} with the P-typing MAb (P) was at least two times the OD_{450} of a pool of fecal extracts negative for rotavirus by MAb EIA (7) reacted with the same coating and detecting antibodies (N), i.e., a P/N value of ≥2.0. For cultivable rotaviruses, N was calculated with MA104 control OD_{450} values. When more than one P-typing MAb reacted with a sample containing rotavirus, doubling dilutions of the sample to 1:80 were tested in the P-typing EIA. The P-typing MAb which reacted specifically with the highest titer of virus was defined to represent the P type of the virus in the sample. Samples with the same titers to more than one P-typing MAb were deemed nontypeable.

RESULTS

Derivation and antigenic specificity of N-MAbs to VP4. From two cell fusion experiments each using one mouse immunized with rotavirus grown with low levels of trypsin, one N-MAb directed to VP4 of ST-3 was derived (ST-3:3). Two N-MAbs to VP4 of F45 rotavirus (F45:3 and F45:4) were obtained from two fusions using splenocytes pooled from two mice immunized orally. As shown in Table 1, use of these immunization protocols correlated with an increased success rate in deriving stable N-MAbs to VP4. These new N-MAbs, and one to VP4 of RV-5 rotavirus (RV-5:2) previously produced from fusion of splenocytes from intraperitoneally inoculated mice (6), were reacted with a panel of 30 cultivable rotaviruses by FFN and direct binding EIA. The two N-MAbs to F45 virus showed similar patterns of EIA binding and neutralization, but titers of N-MAb F45:4 (Tables 1 and 2) were 1 to 3 logs higher than those of N-MAb F45:3 (data not shown). N-MAb F45:3 was therefore unsuitable for typing and was not tested further. N-MAb F45:4 neutralized all of the P8 rotaviruses tested to low or moderate titer and four of the five P6 rotaviruses tested to low titer (Table 1). However, as shown in Table 2, this N-MAb showed high EIA titers to all P8 viruses (at least 1:260,000) and moderate titers with three P6 viruses (1:16,000 to 1:64,000). Viruses of P types 2, 3, 4, 7, and 9 were not detected by this N-MAb. MAb RV-5:2 neutralized and bound to the P4 rotaviruses to high titer, to L26 rotavirus to moderate titer, and to 1076 rotavirus to a very low titer. This MAb did not recognize any other rotavirus tested. MAb ST-3:3 neutralized the homologous virus only to high titer, but showed moderate to high EIA titers with three P6 viruses (ST-3, RV-3, and M37) and low titer with the fourth virus (1076).

TABLE 1. Effect of immunization protocol on N-MAb specificity

Immunization protocol	No. of fusions	N-MAb specificity	
		VP4	VP7
Intraperitoneal, with trypsin[a]	15	2	16
Intraperitoneal, with low-level trypsin	2	1	2
Oral	2	2	5

[a] Data from previous studies (4–6) and unpublished work.

Polypeptide specificities of the N-MAbs. N-MAb F45:4 radioimmunoprecipitated the VP4 and the VP5* fragment of the VP4 of rotavirus RV-4 (data not shown) and was not tested by immunoblotting. N-MAb RV-5:2 radioimmunoprecipitated VP4 of RV-5 virus (6) and bound to unreduced VP4 of RV-5 virus only in immunoblots (data not shown). Earlier testing by an alternative Western blotting (immunoblotting) technique had shown no binding to reduced or unreduced RV-5 VP4 (6). N-MAb ST-3:3 immunoprecipitated the VP5* fragment of the VP4 of ST-3 rotavirus and bound to the VP4 of ST-3 virus in immunoblots of proteins run under nonreducing, but not reducing, conditions.

Development of the VP4 typing EIA. Optimal dilutions of the N-MAbs for VP4 typing were determined from the highest dilution of N-MAb at which the OD_{450} was still maximal (OD_{max}) for each cultivable rotavirus within the VP4 type predominantly detected by the N-MAb. For example, N-MAb F45:4 showed OD_{max} values of 0.5 to 1.8, at dilutions ranging from 1/5,000 to 1/512,000. Therefore, the purified N-MAb was used for VP4 typing at a dilution of 1/5,000. Purified N-MAbs RV-5:2 and ST-3:3 were reacted at dilutions of 1/20,000 and 1/500, respectively.

The G-type specificity of the coating antiserum used in the VP4 typing EIA had an effect on the EIA reactivity. As shown in Table 4, for rotaviruses of P types 4 and 8, coating antisera raised to a rotavirus of the same G type, rather than P type, generally gave the highest specific OD_{450} with cultivable rotaviruses. Rotaviruses of the G1P8 type (Wa, Ku, and RV-4) were best typed with G1P8- or G3P8-specific antiserum or antiserum to G types 1 to 4 combined, although all antisera tested gave a positive VP4 typing result with these viruses. G3P8 rotaviruses (Yo and P) were best typed

TABLE 2. Neutralization specificities of N-MAbs to VP4

Virus	P type by[a]:		Reciprocal FFN titer of given N-MAb		
	Neutralization[b]	Gene 4 sequence[c]	F45:4	RV-5:2[d]	ST-3:3
SA11	ND[e]	2	—[f]	—	—
RRV	ND	3	—	—	—
DS-1	1B	4	—	200,000	—
S2	1B	4	—	360,000	—
RV-5	1B[g]	4	—	1,000,000	—
RV-6	1B[g]	SND[h]	—	500,000	ND
L26	1B[g]	4	—	11,000	—
M37	2	6	3,300	—	400
1076	2	6	700	240	150
RV-3	2[g]	6	800	—	150
ST-3	2	6	—	—	600,000
BEN144	?2[i]	?6[i]	6,000	—	—
AT/76	OSU-like[i]	?7	—	ND	—
CRW8	OSU-like[i]	?7	—	ND	—
MDR13	OSU-like[i]	?7	—	ND	—
TFR41	OSU-like[i]	?7	—	ND	—
Wa	1A	8	4,500	—	—
Ku	1A	8	26,000	—	—
RV-4	1A[g]	SND	320	—	—
Yo	1A	8	7,300	—	—
P	1A	8	6,200	—	—
VA70	1A	8	78,000	—	—
Hoso	1A	8	36,000	—	—
F45	1A	8	10,000	—	—
Wi61	1A	8	4,400	—	—
AU32	1A[g]	SND	140,000	—	—
K8	3	9	—	—	—
B38	?	?	—	—	—
69M	?	?	—	ND	—
K9	?	?	—	—	—
AU-1	?	?	—	300	—

[a] Question marks indicate uncertainty and incomplete data.
[b] Classification by Gorziglia et al. (13), determined by neutralization titration with antisera to baculovirus-expressed VP4.
[c] Classification adapted from Estes and Cohen (8), determined by comparison of nucleic acid sequences of VP4.
[d] Titers to all viruses except F45, Wi61, AU32, L26, M37, 1076, BEN144, 69M, AT/76, CRW-8, MDR13, TFR41, and AU-1 are from reference 6.
[e] ND, not determined.
[f] —, ≤100.
[g] Determined in this study.
[h] SND, nucleotide sequence of VP4 not determined.
[i] From reference 33.

TABLE 3. Binding of N-MAbs to rotavirus measured by EIA

Virus	Reciprocal EIA titer of given N-MAb		
	F45:4	RV-5:2	ST-3:3
SA11	—[a]	—	—
RRV	—	—	—
DS-1	—	2,000,000	—
S2	—	2,000,000	—
RV-5	—	1,000,000	—
RV-6	—	2,000,000	—
L26	—	128,000	—
M37	64,000	—	64,000
1076	32,000	2,000	2,000
RV-3	—	—	500,000
ST-3	—	—	260,000
BEN144	16,000	—	—
AT/76	—	—	—
CRW8	—	—	—
TFR41	—	—	—
Wa	1,000,000	—	—
Ku	2,000,000	—	—
RV-4	260,000	—	—
Yo	500,000	—	—
P	>2,000,000	—	—
VA70	2,000,000	—	—
Hoso	2,000,000	—	8,000
F45	>2,000,000	—	—
Wi61	4,000	—	—
AU32	512,000	—	—
K8	—	—	—
B38	—	—	—
69M	—	—	—
K9	ND[b]	—	ND

[a] —, ≤1,600.
[b] ND, not done.

with G3P6-specific antiserum and were not detected with G1P8- or G4P6-specific antisera. G9P8 rotaviruses F45 and Wi61 were typed by antisera to G type 2, 3, or 9 and by the antiserum to G types 1 to 4 combined. P6 rotaviruses RV-3 and ST-3 were best typed with homologous antisera (Table 5). When P type only was matched, these viruses were detected poorly or not at all. M37 virus was typed by all antisera, but best with the serum to G1 to -4 viruses combined. Titration of each of the RV-3 and M37 viruses in doubling dilution to their endpoint with each N-MAb showed that they reacted to N-MAb ST-3:3 to higher titer than N-MAb F45-4 (data not shown). Rotavirus 1076 was not readily typeable with these N-MAbs, as it reacted either to a similar level with both N-MAbs F45:4 and ST-3:3 (coating antiserum to RV-5 or to G types 1 to 4) or to N-MAb ST-3:3 only (coating antiserum to RV-4 or ST-3).

Evaluation of the VP4 typing EIA with coded fecal and tissue culture specimens containing rotavirus. As coating antisera to RV-5 virus or to RV-4, RV-5, P, and ST-3 viruses combined were suitable if not ideal for VP4 typing all cultivable rotaviruses tested except 1076 (Tables 4 and 5), 30 samples containing rotavirus were analyzed by using those two coating antisera without prior knowledge of the rotavirus VP7 serotype (Table 6). Insufficient samples were available for titrating samples showing dual VP4 N-MAb reactivity. On breaking the code, it was clear that N-MAbs F45:4 and ST-3:3 were each cross-reacting with 30 to 40% of samples likely to be of the other's P type, to give false-positive results. In all cases, the mistyped samples showed dual reactivity with N-MAbs F45:4 and ST-3:3. N-MAb RV-5:2 was specific for viruses likely to be P4. However, a single G2 rotavirus from the stools of an Indonesian infant (11 months old) with rotavirus gastroenteritis typed strongly as P6 and showed no reaction with N-MAb RV-5:2. None of the N-MAbs reacted with the four nonhuman rotaviruses tested.

Evaluation of the VP4 typing EIA with rotavirus-containing stool extracts using coating antisera matched to G type. Stool extracts (n = 118) from Melbourne, Victoria (n = 71), and Alice Springs, Northern Territory (n = 47), containing rotavirus of G types 1 to 4 previously determined by N-MAb EIA (RCH, ASH, and FIDH) or inferred (RWH) were analyzed by VP4 typing EIA. Coating antiserum was matched to G type, and the 15 samples showing dual reactions with N-MAbs F45:4 and ST-3:3 (13%) were titrated. As shown in Table 7, the inferred and measured P types for all typeable rotavirus-containing stools concurred. P/N ratios for the P4 viruses averaged 5.8 (range, 4.1 to 7.2). The P6 viruses showed a mean P/N value of 3.9 (range, 2.0

TABLE 4. Effect of coating antiserum on VP4 typing result[a] with cultivable rotaviruses of P types 4 and 8

Virus	OD_{450} (10^2)[b] with given antiserum to rotavirus					
	RV-4	F45	RV-5	RV-3	ST-3	RV-4, RV-5, P, ST-3
Wa	40	33	37	46	21	79[c]
Ku	129	ND[d]	ND	161	ND	ND
RV-4	33	21	22	27	19	49
Yo	12(−)[e]	28	63	99	14(−)	ND
P	11(−)	19(−)	85	139	11(−)	102
F45	32	83	105	90	36	123
Wi61	30	81	101	81	39	ND
RV-5	10(−)	9(−)	29	9(−)	8(−)	6(−)
DS-1	8(−)	7(−)	65	10(−)	10(−)	ND

[a] Data are for the N-MAb matched to the P type of the indicated virus, i.e., N-MAb F45:4 for Wa, Ku, RV-4, Yo, P, F45, and Wi61 and N-MAb RV-5:2 for RV-5 and DS-1 viruses. No reactivity, using any coating antiserum, of the N-MAbs of heterologous P type with the indicated virus was observed.
[b] Unless otherwise indicated, the OD_{450} is a positive reading.
[c] The highest positive OD_{450} reading with each virus is underlined.
[d] ND, not determined.
[e] (−), OD_{450} was below the positive/negative cutoff.

to 11.7), and the mean P/N value for the P8 viruses was 10.2 (range, 2.1 to 34.7). The VP4 typing rates varied from 50 to 100%. Lower typing rates resulted from testing stools not reactive with any N-MAb tested and stools showing background color in all wells of the test plate. The P-typing rates were similar to the G-typing rates obtained at the same time, with two exceptions. G2P4 viruses from ASH showed a 17% G-typing rate but a 50% P-typing rate. Neonatal G3P6 viruses (RWH B) tested as subviral particles (not sedimented at 60,000 × *g*) showed a 57% G-typing rate and a 93% P-typing rate, with little dual reactivity compared with the fecal extracts from the same origin (RWH A).

Effect of freeze-thawing fecal extracts on VP4 typing EIA results. Fifteen fecal extracts containing typed rotaviruses of G types 1 to 4 and P type 4 ($n = 3$) or 8 ($n = 12$) were frozen at −18°C, thawed, and retyped by EIA. Nine (60%) of these showed a cross-reactivity with N-MAb ST-3:3 which was absent in the first typing experiment. One of the 15 G2P4 rotaviruses was not reactive with the G2 typing N-MAb RV-5:3 on retyping. The remaining five samples showed no change in their VP4 or VP7 reaction.

DISCUSSION

Success in VP4 typing by EIA using N-MAbs depends on the availability of P-type-specific N-MAbs. In this study, derivation of such N-MAbs appeared to be facilitated by oral immunization of mice or parenteral inoculation with virus grown with low levels of trypsin, suggesting that these inoculation strategies enhanced VP4-specific responses. VP4 appeared to have an important role in inducing a neutralizing response after oral infection, as opposed to systemic inoculation, in mice inoculated with SA11 rotavirus (28). Success in deriving N-MAbs to VP4 with virus grown in low levels of trypsin has been reported previously (32). However, why trypsin-cleaved VP4 in infectious virus should be less likely to elicit VP4-neutralizing antibodies than uncleaved VP4 in noninfectious virus is unclear. Possibly, VP4-neutralizing antibodies elicited by cleaved VP4 of HRVs are more likely to be strain specific (4), whereas P-type-specific and cross-reactive (32) antibodies are preferentially produced to uncleaved VP4.

Two of the three P-typing N-MAbs (F45:4 and ST-3:3)

TABLE 5. Effect of coating antiserum on VP4 typing result with cultivable rotaviruses of P type 6

Virus	N-MAb[a]	OD_{450} (10^2)[b] with given antiserum to rotavirus					
		RV-4	F45	RV-5	RV-3	ST-3	RV-4, RV-5, P, ST-3
RV-3	F45:4	8(−)[c]	6(−)	7(−)	27[d]	7(−)	21
	ST-3:3	19(−)	22	27	96	19(−)	63
ST-3	ST-3:3	31	66	44	66	127	119
M37	F45:4	42	34	40	42	26	67
	ST-3:3	32	22	27	26	25	57
1076	F45:4	11(−)	6(−)	62	9(−)	14(−)	20
	RV-5:2	19(−)	20	15(−)	25	15(−)	6(−)
	ST-3:3	22	14(−)	23	18	27	19

[a] No reactivity with any coating antiserum was observed with N-MAb RV-5:2 and viruses RV-3, ST-3, and M37. N-MAb F45:4 did not react with ST-3 virus with any coating antiserum.
[b] Unless otherwise indicated, the OD_{450} is a positive reading.
[c] (−), OD_{450} was below the positive/negative cutoff.
[d] The highest positive OD_{450} reading with each virus is underlined.

TABLE 6. VP4 typing of stool and cultivable rotaviruses under code using capture antiserum to RV-5 virus and to combined rotaviruses (RV-4, RV-5, P, and ST-3)

Origin	G type	P type by inference	No. with given P type by EIA					Total no. tested
			4	6	8	NR[a]	NT[b]	
Indonesia, RCH	2	4	3	1	0	1	0	5
RWH[c]	3	6	0	6	2	0	0	8
RCH, LH[d]	1	8	0	2	4	1	0	7
RCH	3	8	0	0	1	0	1	2
RCH, LH	4	8	0	2	1	1	0	4
Equine A2[e]	3	Other	0	0	0	1	0	1
Bovine A3, A4, A5[e]	6	Other	0	0	0	3	0	3

[a] NR, no reaction.
[b] NT, not typeable because of high-background OD_{450} readings.
[c] One sample was cell culture-adapted RV-3 rotavirus.
[d] LH, Liverpool Hospital, New South Wales.
[e] Cell culture-adapted rotaviruses.

recognized epitopes on the VP5 subunit of VP4. N-MAbs ST-3:3 and RV-5:2 detected epitopes present only on unreduced VP4, suggesting that the VP5 subunit epitope of N-MAb ST-3:3 is conformation dependent, as is the VP4 epitope (on either VP5* or VP8*) detected by N-MAb RV-5:2. N-MAb S2-2F2, which has antigenic specificity similar to that of N-MAb RV-5:2 (32), selected a mutation in epitope II, antigenic region C2, on the VP5* of the VP4 of DS-1 (17, 31). This is the only antigenic region on VP4 recognized by N-MAbs which has been shown to be dependent on protein folding (17), so it is a likely binding site for N-MAb RV-5:2. Nucleic acid sequencing of antigenic variants selected with these N-MAbs is in progress to determine the antigenic region recognized by the N-MAbs. The dependence of N-MAbs to VP4 and VP5* with serotyping potential on protein conformation may help explain why *Escherichia coli*-expressed VP5*, which may not be in the correct conformation (19), did not elicit antibodies useful for VP4 typing by plaque reduction neutralization (19).

N-MAb RV-5:2 bound to and neutralized all known and inferred G2P4 rotaviruses to a similar degree, with EIA titers 10-fold greater than FFN titers. This N-MAb also bound to and neutralized the G12 virus L26 to titers approximately 50-fold less than the G2P4 viruses. On the basis of nucleotide sequence, this virus was related to G2P4 viruses via VP4 (33). This study shows that this relationship is also an antigenic one, although the classification of L26 as G12P4 should remain tentative until criteria for discrimination between P types are decided and additional typing reagents become available. It may be that, although this N-MAb recognizes VP5*, its virus binding and neutralization are affected by the particular VP7 with which the VP5* is associated, so that G12P4 rotaviruses are detected suboptimally. Interactions between VP4 and VP7 affecting expression of a cross-reactive N-MAb 2G4 which selected antigenic mutants with a single amino acid mutation in antigenic region C2 have been reported (2). It is possible that VP7 is directly involved in the interaction of N-MAb RV-5:2 with virus, given that the only virus tested of G type 2 but P type other than 4 (virus 1076) was also neutralized by this N-MAb, albeit to a very low titer.

N-MAb ST-3:3 was strain specific by FFN but detected all cultivable P6 viruses except 1076 efficiently by EIA. Virus 1076 was untypeable by VP4 typing EIA, consistent with its

TABLE 7. VP4 typing of stool rotaviruses with coating antiserum matched to VP7 type

Origin	G type	P type by inference	No. (%) G typed this time	No. (%) with given P type by EIA					Total no. tested
				4	6	8	NR[a]	NT[b]	
ASH	2	4	2 (17)[c]	6 (50)	0	0	1 (8)	5 (42)[d]	12
RWH[e]									
A	3	6	12 (86)	0	13 (93)	0	1 (7)	0	14
B	3	6	8 (57)	0	13 (93)	0	1 (7)	0	14
RCH	1	8	24 (96)[c]	0	0	20 (71)	7 (25)	1 (4)[f]	28
	3	8	7 (78)[c]	0	0	9 (100)	0	0	9
FIDH	3	8	6 (100)[c]	0	0	6 (100)	0	0	6
ASH	1	8	23 (100)[c]	0	0	21 (90)	1 (5)	1 (5)[f]	23
	4	8	10 (83)[c]	0	0	10 (82)	1 (9)	1 (9)[f]	12

[a] NR, no reaction.
[b] NT, not typeable.
[c] After the original G-typing EIA, stools were frozen and thawed once before P typing and repeat G typing.
[d] Not typeable because of high-background OD_{450} readings.
[e] A, stools tested as fecal extracts. P type was inferred from origin and RNA electropherotype. B, stools tested as the supernatant above the virus pellet obtained by ultracentrifugation at 60,000 × *g* of fluorocarbon-extracted fecal extracts (28). P type was inferred from origin and RNA electropherotype.
[f] Not typeable because of equal reactivity of the virus with the P6- and P8-reactive N-MAbs.

unusual reactivities with the N-MAbs. This reactivity may be due to interaction of VP7 with VP4 in 1076 affecting binding of the N-MAbs (see above) or to amino acid sequence variation between the VP4s of P6 rotaviruses. ST-3 and RV-3 rotaviruses show 97.4% amino acid homology in VP4, whereas homology of ST-3 with M37 and 1076 viruses is less (96.3 and 95.7%, respectively) (12). This ranking in amino acid homology correlates with the degree of neutralization and EIA binding of N-MAb ST-3:3 to each virus and inversely correlates with these properties of N-MAb F45:4.

The Gottfried strain of porcine rotavirus may be a natural reassortant between pig rotaviruses and HRVs on the basis of reciprocal cross-reactivity of N-MAbs between Gottfried virus and HRVs (14, 16). Australian porcine rotavirus isolate BEN144 shares P- and G-type specificity with Gottfried virus (21, 22), and VP4 of BEN144 virus showed some antigenic relationships with ST-3 and RV-3 rotaviruses (22). The cross-reactivity of N-MAbs F45:3 and F45:4 with BEN144 virus is therefore consistent with the antigenic cross-reactivity of this virus with P6 HRVs.

The VP4 types predicted from G typing and determined experimentally using the N-MAbs generally concurred. The P types suggested by gene 4 sequence homology (8, 18) and PCR typing (9) were also predictable from G typing, so these methods show general agreement to date. However, the N-MAbs showed P-type 4 and 8 viruses to be distinct, whereas antisera to expressed VP4 and VP8 suggested that these viruses are subtypes A and B of the same P type (13). It was also proposed that N-MAbs to the VP8 subunit, rather than VP5, will be more strain specific and hence more suitable for P typing. Indeed, antiserum to *E. coli*-expressed VP8 rather than to VP5 showed the better discrimination between P types (19). However, in this study, at least two of the three N-MAbs recognized the VP5 subunit of VP4 but were sufficiently P type specific to be of value for VP4 typing (13). Further testing of both N-MAbs and antisera to expressed VP8 and VP4 may resolve these issues.

Three N-MAbs to rotavirus VP4 proved to be capable of serotyping both cultivable and stool rotavirus strains in an EIA format used extensively for VP7 typing of HRVs (7, 10, 35). The VP7 typing EIA was most sensitive and specific when each hyperimmune capture antiserum was raised to an HRV homologous in G type to the test virus (35). Interestingly, the VP4 typing EIA also often gave optimal results when the capture antiserum was matched in G type to the test virus. This was clearly evident for G2 and G3 rotaviruses, irrespective of P type. VP7 constitutes approximately 30% by mass of rotavirus protein, whereas VP4 is much less abundant (1.5% [8]). Antiserum matched for VP4 would not trap intact virions or subviral particles containing VP4 as efficiently as VP7-matched antiserum. Capture with antiserum matched for VP7 should result in cocapture of VP4 because of their close proximity on the virion.

It was noteworthy that stool extracts from which intact neonatal rotavirus particles had been removed proved suitable for both VP4 and VP7 typing. Subviral particles containing antigenically reactive VP4 and VP7 may have been present. It is unlikely that individual VP7 molecules retain the correct conformation for N-MAb binding, although VP4 molecules may be more stable, as shown by the successful expression of antigenically active VP4 in baculovirus (13) and *E. coli* (19) systems and the ability to isolate antigenically active VP4 during virus purification (1). The lower success of typing VP7 in these stool extracts may relate to the conformation dependence of VP7 N-MAb epitopes.

Typing of VP4 by EIA was facilitated by avoidance of multiple freeze-thawing of stool extracts which led to increased N-MAb cross-reactivity between P types, rather than to a total loss of specific reaction as found with VP7 typing (35). This may also relate to the different conformational stabilities of these two viral proteins. Dilution of stool extracts which showed reaction with more than one N-MAb was sometimes also required for VP4 typing. However, a few P4, -6, and -8 fecal extracts are likely to be untypeable with the three N-MAbs described here because of the cross-reactivity of the N-MAbs between P types. In this study, using VP7 matched antiserum, 1076 virus and three fecal rotaviruses were untypeable because of cross-reactivity, representing 2.9% of HRVs tested. Derivation and inclusion of alternative VP4 N-MAbs may improve this VP4 typing specificity. To widen the range of P types detected, additional N-MAbs to VP4, with specificities including HRV K8 (P9) and B38 and animal rotaviruses, could be included.

A single isolate with P-type 6 and G-type 2 was identified in this study from an Indonesian infant aged approximately 11 months and excreting rotavirus symptomatically. Two rotavirus isolates containing a gene 4 which hybridized with P-type 6 gene 4, also from older children infected symptomatically, have been detected in Venezuelan hospitals (14). However, strains of P-type 2 have been isolated almost exclusively from asymptomatically infected newborn infants in neonatal wards and share a highly conserved gene 4 postulated to account for their avirulent phenotype (8, 12, 13, 18). It is important to determine the frequency of occurrence of type P6 rotaviruses in infants or older children with gastroenteritis to further examine this hypothesis.

VP4 typing by EIA using specific N-MAbs provides a direct method of assessing the antigenic diversity of VP4. VP4 gene typing by PCR recently also has been achieved. As suggested by Gentsch et al. (9), gene typing is a valid proxy method for P typing by EIA and will be particularly useful in typing VP4 in the relatively small number of rotaviruses untypeable by EIA. Its use will help distinguish between rotaviruses nonreactive by EIA because of antigen degradation or N-MAb cross-reactivity and viruses with novel VP4 types. EIA typing is less expensive and laborious, uses more basic equipment and reagents, and requires less highly skilled staff than PCR typing. This extends its usefulness to large-scale epidemiological studies and to VP4 typing of community rotavirus strains in field trials of candidate vaccine strains of rotavirus.

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