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Specific Enzyme-Linked Immunoassay for Rotavirus Serotypes 1 and 3

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We prepared monoclonal antibodies against two serotypically distinct rotavirus strains: Wa, a serotype 1 virus of human origin, and rhesus rotavirus, a simian serotype 3 virus. Monoclonal antibodies which react specifically with VP7 of each serotype were identified by hemagglutination inhibition tests, plaque reduction neutralization studies, and solid-phase immunoassays which used wild-type and reassortant strains of rotavirus. An enzyme-linked immunoassay was designed which utilizes two of these antibodies to correctly identify serotype 1 and serotype 3 viruses.

Rotaviruses have been established as an important cause of severe gastroenteritis in humans as well as in other mammalian and avian species (5, 12; A. Z. Kapikian et al., *in* R. Lerner et al., ed., *Modern Approaches to Vaccines: Molecular and Chemical Basis of Resistance to Viral, Bacterial, or Parasitic Diseases*, in press). Rotaviral disease is most significant in children under 2 years of age, but it also affects the elderly and the immunocompromised (18, 29). Recently, an antigenically distinct rotavirus has been described in association with epidemic diarrheal illness in normal adults (14). The public health and economic impact of rotaviral disease has made the need for a vaccine apparent, especially in less developed regions where insufficient medical care often results in infant mortality.

Immunoprophylaxis requires an understanding of the serotypic diversity of rotaviruses and the nature of the host immunologic response to infection. Until recently, research on these aspects of human rotaviral disease had been limited by the inability to grow human strains in cell culture. Despite breakthroughs permitting human rotavirus cultivation in cell culture (1, 22, 26), investigation of rotavirus serotype diversity is still limited by slow and expensive serotyping assays requiring in vitro viral replication. The possibility of a simple serotyping test was demonstrated by Thouless et al. (25), who used polyclonal serotyping reagents in an enzyme-linked immunosorbent assay (ELISA) to distinguish human rotavirus serotypes 1, 2, and 3. However, the production of these reagents required a cumbersome and inexact absorption process and failed to provide the sensitivity needed to serotype cell culture specimens (2). Other workers have not easily generated similar reagents, and this assay has not come into widespread use. In an attempt to develop a simple, sensitive, and specific serotyping test for human rotaviruses we undertook the production of monoclonal antibodies with serotype-specific characteristics.

In this paper, we report the isolation and characterization of two monoclonal antibodies with specificity for serotype 1 and serotype 3 rotaviruses, respectively. These serotypes were chosen for initial study because they reportedly constitute a large proportion of human isolates and because previously described monoclonal subgrouping reagents have been reasonably effective for indirectly assigning human isolates to serotype 2 (8, 11, 13). We also report the use of a unique hybridoma screening process using human reassortant viruses to identify antibodies reacting specifically with VP7, the surface glycoprotein which contains the major serotype determinants. In addition, the utility of these monoclonal reagents in a simple serotyping ELISA is demonstrated.

MATERIALS AND METHODS

Cells. An established fetal rhesus monkey kidney cell line (Ma 104) was used throughout this study. The cells were propagated as previously described (1) except that the medium used was M199 supplemented with 5% fetal bovine serum, L-glutamine (2 mM), penicillin (250 U/ml), and streptomycin (250 U/ml). Viral stocks were propagated in these cells using 0.5 μg of trypsin per ml in M199 without fetal bovine serum.

Viruses. The single-gene reassortant viruses used were described previously as noted: D × RRV 6-1-1 and DS1 × RRV 4-1-1 (19); B3 × RRV 39-2-1 (K. Midthun and H. Greenberg, unpublished data); P × UK 22-1-1 (9, 19); and Ban × UK (11). Rhesus rotavirus (RRV), NCDV, OSU, Wa, SA-11, CU-1, and UK strains of rotavirus were all from pools which have been previously described (13). Animal and human strains of known titer and serotype (2, 21, 28) were cultivated at Baylor College of Medicine and sent to Stanford for testing as coded specimens. The strains Tx-1111, Tx-1258, and Col 546 were adapted to cultivation from human stool specimens in Texas.

Hybridoma production. Mice used for hybridoma production and ascites amplification of monoclonal antibodies were adult female BALB/c mice (Institute for Medical Research, San Jose, Calif.). Five-week-old BALB/c mice were immunized with RRV or human rotavirus strain Wa. Virus for immunization was partially purified by high-speed centrifugation, fluorocarbon extraction, and either rate-zonal sedimentation through a 20 to 40% sucrose gradient or isopycnic banding in cesium chloride. The immunization schedule and fusion technique were similar to those described previously (8).

After fusion to NS-1 myeloma cells with 50% polyethylene glycol, the cells were suspended in HAT medium and directly plated in 96-well Costar plates. Hybridomas were refed with HAT medium as evaporation necessitated. After

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TABLE 1. HI by serotype-specific monoclonal antibodies

Virus (species of origin)	Serotype	HI titer[a] (reciprocal) of each serotyping monoclonal antibody versus given virus	
		4F8/E1/B3	2C9/D8/D7
D × RRV 6-1-1[b]	1	<10	50,000
DS1 × RRV 4-1-1[b]	2	<10	<10
RRV (simian)	3	100,000	<10
B3 × RRV 39-2-1[b]	4	<10	<10
NCDV (bovine)	5	<10	<10
OSU (porcine)	6	<10	<10

[a] Titer of mouse ascites fluid. Preinoculation serum titers were <10.
[b] Human × simian reassortants.

selection of hybridomas by hemagglutination inhibition (HI) assay (see below), cells of interest were cloned twice by limiting dilution using thymocyte feeder layers. Selected monoclonal antibodies were amplified in mouse ascites. A dose of 10^7 cells was injected intraperitoneally 10 days after the priming of each mouse with an intraperitoneal injection of 0.5 ml of Pristane (Aldrich Chemical Co.).

HI. The HI test was performed as previously described (17). Viruses harvested from Ma 104 cell cultures were diluted in Dulbecco phosphate-buffered saline (PBS) (Irvine Scientific, Santa Ana, Calif.) and 1% bovine serum albumin to a concentration of 8 hemagglutination units per microtiter well in 25 μl of buffer. Virus and antibody were incubated for 1 h at room temperature before the addition of 0.5% human type O erythrocytes in PBS–1% bovine serum albumin.

Plaque reduction neutralization test. The plaque reduction neutralization test has also been described previously (7, 27). Titers were determined by 60% plaque reduction. Approximately 30 PFU were inoculated per well of a six-well cell culture plate.

Immunochemical focus neutralization test. For the immunochemical focus neutralization test we used a procedure modified from one recently described by Pauli et al. (20). Briefly, viral samples were activated by the addition of 5 μg of trypsin (Sigma) per ml and incubated at 37°C for 30 min. Appropriate dilutions of monoclonal antibodies in M199 medium were prepared in a 96-well microtiter plate and incubated with diluted activated virus at 37°C for 1 h. The dilution of virus used produced approximately 100 stainable Ma 104 cells per well of a 96-well tissue culture plate. The virus-antibody mixture was inoculated on Ma 104 cells in a 96-well plate and incubated overnight. The cells were then washed once with PBS and fixed for 10 min with 100% methanol chilled to −20°C (200 μl per well). The plate was then washed twice with PBS and incubated with goat anti-rotavirus hyperimmune serum (100 μl of a 1:1,000 dilution) (3) for 30 min at 37°C. After two washings with PBS, rabbit anti-goat immunoglobulin G conjugated to horseradish peroxidase was applied (100 μl of a 1:500 dilution; Accurate Chemicals, Westbury, N.Y.) and incubated for 30 min at 37°C. The substrate, 3-amino-9-ethyl-carbazole (Sigma), was prepared as a stock solution of 4 mg/ml of *N,N*-dimethylformamide. A 1.5-ml volume of the stock solution was combined with 3.5 ml of a 0.05 M acetate-acetic acid buffer (pH 5), containing 10 μl of 30% H_2O_2, and filtered through a 0.45-μm filter before being used. After 15 min of incubation at 37°C in the dark, the plate was washed with PBS–0.1% sodium azide to stop the reaction, and the monolayer was examined. A complete absence of stainable cells was considered indicative of the presence of neutralizing antibody in the test sample. Wells containing the viral inoculum incubated with medium alone were always included to provide a comparison.

ELISA. Ascites fluid containing each monoclonal antibody was diluted 1:5,000 in carbonate buffer (pH 9.6). A 100-μl volume was placed in each well of Immulon II 96-well plates (Dynatech) and incubated at 4°C overnight. The plates were then postcoated with PBS–5% fetal bovine serum at 4°C overnight. Viral samples (100 μl per well) were placed in each antibody-coated well for 2 h at 37°C and washed three times with PBS. Detection of bound virus was accomplished by an indirect ELISA. Guinea pig anti-Wa rotavirus hyperimmune serum (National Institutes of Health reference reagent V710-511-558) was incubated for 1 h at 37°C (100 μl per well, 1:5,000 dilution), washed three times, and replaced with 100 μl of goat anti-guinea pig antibody conjugated to alkaline phosphatase (Kirkegaard & Perry, Gaithersburg, Md.) per well. After 1 h of incubation at 37°C and three washes in PBS, the substrate *p*-nitrophenyl phosphatase (Sigma 104 alkaline phosphatase substrate) in diethanolamine buffer (pH 9.8) was added. Optical density (OD) at 405 nm was measured approximately 30 min later. Zero calibration was performed using the lowest value of several wells incubated with M199 medium alone.

RESULTS

The major serotype antigen of rotavirus has been shown to reside on the 36,000-molecular-weight surface glycoprotein (VP7) by both genetic and monoclonal antibody studies (6, 10, 16). Previous experience indicated that isolation of monoclonal antibodies directed at this protein was difficult because solid-phase immunoassays preferentially selected antibodies directed at other viral proteins (8). Therefore, we utilized an HI assay with a combination of wild-type and single-gene reassortant rotavirus strains to specifically select monoclonal antibodies directed to VP7. For example, RRV is a serotype 3 simian rotavirus (13). Human reassortant D × RRV 6-1-1 is a single-gene reassortant (gene 9) which differs from the RRV wild type by only the gene 9 product: the glycoprotein VP7. In the reassortant, this protein is derived

TABLE 2. Plaque reduction neutralization titers of the two serotype-specific monoclonal antibodies

Virus (species of origin)	Serotype	Neutralization titer[a] (reciprocal) of each serotyping monoclonal antibody versus given virus	
		4F8/E1/B3	2C9/D8/D7
Wa (human)	1	<100[b]	25,600[b]
D × RRV 6-1-1[c]	1	<100	6,400
DS1 × RRV 66-1-1[c]	2	<100	<100
RRV (simian)	3	25,600	<100
SA-11 (simian)	3	25,600	<100
P × UK 22-1-1[d]	3	1,600	<100
CU-1 (canine)	3	25,600[b]	<100[b]
B3 × RRV 39-2-1[c]	4	<100	<100
UK (bovine)	5	<100	<100
OSU (porcine)	6	<100	<100

[a] Titer of mouse ascites fluid. Preinoculation titers of mouse sera were 1:100 or less.
[b] Immunochemical focus neutralization test. Titer shown is highest dilution of mouse ascites fluid resulting in total absence of detectable virus. A sample of 10^3 PFU was inoculated per well of a 96-well tissue culture plate.
[c] Human × simian reassortant.
[d] Human × bovine reassortant.

TABLE 3. ELISA serotype assay of selected strains of human and animal rotavirus strains using serotype-specific monoclonal antibodies

Virus (species of origin)	Known subgroup	Known serotype	OD[a]		Ratio[b]	ELISA serotype assignment
			4F8/E1/B3	2C9/D8/D7		
Wa (human)	2	1	0.079	1.340	0.06	1
D × UK 47-1-1[c]	1	1	0.093	1.464	0.06	1
D × RRV 6-1-1[d]	1	1	0.087	0.745	0.12	1
DS1 (human)	2	2	0.074	0.099	0.75	NA[e]
DS1 × UK 66-1-1[c]	1	2	0.120	0.138	0.87	NA
DS1 × RRV 4-1-1[d]	1	2	0.105	0.170	0.62	NA
RRV (simian)	1	3	0.704	0.069	10.20	3
SA-11 (simian)	1	3	1.471	0.066	22.29	3
CU-1 (canine)	1	3	0.776	0.128	6.06	3
Feline (feline)	1	3	0.502	0.116	4.33	3
P × UK 22-1-1[c]	1	3	0.505	0.103	4.90	3
P (human)	2	3	0.846	0.005	179.20	3
Gottfried (porcine)	2	4	0.171	0.131	1.40	NA
Ban × UK 2-1[c]	1	4	0.114	0.153	0.75	NA
St. Thomas no. 4 (human)	2	4	0.032	0.069	0.46	NA
B3 × RRV 39-2-1[d]	1	4	0.026	0.069	0.38	NA
NCDV (bovine)	1	5	0.062	0.077	0.81	NA
UK (bovine)	1	5	0.020	0.053	0.38	NA
OSU (porcine)	1	6	0.093	0.175	0.53	NA

[a] Serotype-specific monoclonal antibodies were used as capture antibodies. Mouse ascites fluid was used at a 1:5,000 dilution.
[b] (OD of 4F8 − blank)/(OD of 2C9 − blank). A ratio value of >2.5 determines serotype 3; <0.4 determines serotype 1. If the highest OD value was <0.200, the serotype was considered not assignable.
[c] Human × bovine reassortant.
[d] Human × simian reassortant.
[e] NA, Not assignable.

from the D strain, a serotype 1 human rotavirus (9, 19). Because RRV and D × RRV 6-1-1 differ by only VP7, antibodies which react to only one of this pair of viruses would be expected to be directed at VP7. The monoclonal antibody 4F8/E1/B3 (a fusion product derived from an RRV-immunized mouse) inhibited hemagglutination of RRV but not D × RRV 6-1-1, identifying the target of this antibody as VP7 in a serotype 3 virus (Table 1). The monoclonal antibody 2C9/D8/D7 (derived from a mouse immunized with Wa, a human serotype 1 virus) showed the opposite HI pattern and can therefore be shown to react to VP7 of a serotype 1 virus. Because VP3 has been shown to be the viral hemagglutinin (15), antibodies to VP7 are presumed to produce HI through steric interference (10).

The obvious speed, simplicity, and specificity of the HI assay allowed the screening of over 1,000 clones in a few hours. Using this screening procedure, we were able to isolate seven putative serotype 3 monoclonal antibodies from three fusions and three putative serotype 1 monoclonal antibodies from two fusions. We then used representative rotaviruses from six serotypes in an HI assay to provide preliminary data indicating the serotype specificity of these reagents. Two monoclonal antibodies were chosen for further study (Table 1; 4F8/E1/B3 and 2C9/D8/D7) because of high-titer serotype-specific reactivity in HI and reactivity in pilot ELISA assays (data not shown). Gel immunodiffusion studies revealed both monoclonal antibodies to be of the immunoglobulin G1 subclass (data not shown).

The serotypic specificity of these reagents was demonstrated in neutralization assays against rotaviruses representing six serotypes (Table 2). Both monoclonal antibodies demonstrated activity against all viruses of their respective serotypes, although titers tended to be higher against the virus to which the antibody was originally formed. Neutralization activity against any heterologous serotype was not detected with either antibody. These results strongly suggested the interaction of each monoclonal antibody with an epitope which was serotype specific and shared (at least partially) by viruses of the same serotype.

We then evaluated the serotype-specific reactivity of these monoclonal antibodies in a serotyping ELISA. Preliminary experiments indicated that ascites fluid derived from inoculation of BALB/c mice with 4F8/E1/B3 and 2C9/D8/D7 cells would have greater sensitivity when used as a capture antibody in a solid-phase ELISA. Therefore, we tested cultivatable rotavirus strains representative of six serotypes (including the four known human serotypes) in such an ELISA (Table 3). Crude cell culture harvests with titers of 10^5 to 10^8 PFU/ml were used as antigen samples. We used the data from this test of rotavirus samples of known serotype to set the parameters used in subsequent serotype assays of coded unknown rotavirus specimens. ELISA activity greater than 0.200 OD unit was considered necessary, but not sufficient, to determine an unequivocally specific reaction between a test sample and one of the serotyping monoclonal antibodies. As a further measure of serotype-specific reactivity, a ratio was calculated that compared reactivity of a test sample with the serotype 3-specific monoclonal antibody with reactivity with the serotype 1-specific antibody. The known rotavirus samples that reacted with either monoclonal antibody to produce activity greater than 0.200 OD unit segregated clearly into a serotype 1 group (no sample in this group had a ratio exceeding 0.120) or a serotype 3 group (no sample in this group had a ratio less than 4.33). Samples from other serotypes failed to produce more than 0.171 OD unit. We arbitrarily chose a ratio of 2.5 as the lowest limit indicating serotype 3 assignment and 0.4 as the upper limit indicating serotype 1 assignment. Using these parameters, all samples tested were correctly assigned to serotype 1 or serotype 3 or were considered not assignable. Further experience with these assays will establish the best determination of these limits to balance sensitivity and specificity.

To confirm the serotyping specificity and sensitivity dem-

TABLE 4. ELISA serotype assay of coded specimens of human and animal rotavirus strains using serotype-specific monoclonal antibodies

Virus (species of origin)	Known subgroup	Known serotype	Optical Density[a]		Ratio[b]	ELISA serotype assignment
			4F8/E1/B3	2C9/D8/D7		
Wa (human)	2	1	0.010	1.022	0.01	1
KU (human)	2	1	0.144	1.343	0.08	1
K8 (human)	2	1	0.139	0.853	0.16	1
DS1 (human)	1	2	0.031	0.042	0.74	NA[c]
S2 (human)	1	2	0.104	0.105	1.0	NA
Tx-1258 (human)	1	Probable 2	0.050	0.076	0.66	NA
YO (human)	2	3	0.073	0.090	0.81	NA
Nemoto (human)	2	3	0.684	0.266	2.57	3
Ito (human)	2	3	0.536	0.099	5.41	3
P (human)	2	3	0.835	0.159	5.25	3
Hosokawa (human)	2	4	0.079	0.123	0.6	NA
St. Thomas no. 4 (human)	2	4	0.096	0.080	1.20	NA
Col 546 (human)	2	Unknown	0.109	0.008	13.63	NA
Tx-1111 (human)	2	Unknown	0.260	0.154	1.70	NA
SA-11 (simian)	1	3	1.008	0.049	20.57	3
RRV-2 (simian)	1	3	0.702	0.170	4.13	3
CU-1 (canine)	1	3	0.357	0.088	4.06	3
OSU (porcine)	1	5	0.073	0.085	0.86	NA
BO 2352 (bovine)	1	Not 1 or 3	0.057	0.067	0.85	NA
BO 486 (bovine)	1	Not 1 or 3	0.025	0.071	0.35	NA

[a] Serotype-specific monoclonal antibodies were used as capture antibodies. The OD was calculated by subtracting the lowest value of three control wells with the medium only well as a blank.

[b] The ratio is calculated as described in Table 3, footnote *b*. Serotype assignments are made to 3 when the ratio is >2.5 and to 1 when <0.4, assuming that the greatest OD value is <0.200.

[c] NA, Not assignable.

onstrated in Table 3, we next tested a coded group of rotavirus strains. These unknown strains represented a wide variety of cultivatable rotaviruses of human and animal origin with titers of 10^3 to 10^8 PFU/ml (Table 4). The subgroup of the specimen was determined using the previously described monoclonal antibodies 255/60 and 631/9 (serogroups 1 and 2, respectively) in an ELISA (8). All samples clearly belonged to either subgroup 1 or 2 (data not shown) except the specimen later identified as the human strain YO, which was considered only a "probable" subgroup 2 because of low reactivity in the subgroup test. These samples were then tested in the serotyping ELISA. Correlation of the ELISA serotype assignment with the known serotypes revealed that all coded samples were correctly assigned to serotype 1, 3, or neither, except YO. Presumably, YO was not assignable because of very low antigen titer (3×10^4 PFU/ml), as there was barely enough antigen to detect by using the subgrouping monoclonal antibodies which are directed at the most abundant viral protein, VP6. Note that human strain P (from the coded specimens in Table 4) as well as P × UK (Table 3) reacted clearly in the serotyping ELISA with 4F8/E1/B3 despite the relatively low (1:1,600) neutralization titer against P × UK in the plaque reduction neutralization assay (Table 2).

DISCUSSION

Reassortant rotaviruses provide a novel and efficient mechanism for screening monoclonal antibodies for serotype-specific activity. Selected reassortants, differing from wild type only in the eighth or ninth gene product, VP7, enabled us to use a simple and rapid HI test to identify antibodies directed at the protein that confers serotype assignment. Using wild-type viruses alone, antibodies inhibiting hemagglutination could be directed at either VP3 (the most frequent target) or VP7, and further evaluation would be required. Using wild-type human viruses alone is not feasible because of the difficulty in demonstrating hemagglutination with these strains (23).

While the monoclonal serotype-specific antibodies are generally effective when used as detection antibodies (R. Shaw and H. Greenberg, unpublished data), we chose to construct a serotyping test that utilized them as capture antibodies because our pilot studies indicated minor improvements in sensitivity with this format. In addition, it was convenient to prepare batches of the microtiter plates with bound capture antibody in advance to screen viral samples rapidly as they became available.

When a serotyping ELISA is performed on unknown specimens it would be useful to have a control present that would detect rotavirus serotype determinants from any serotype. We used the subgrouping monoclonal antibodies in an ELISA to document the presence of rotavirus antigen in test specimens. However, these antibodies, directed at the major inner capsid protein VP6, may react with specimens when antibodies directed at VP7 would not. This may be due to the greater sensitivity obtained when using monoclonal antibodies directed at the more abundant protein VP6. Alternatively, the outer capsid (containing VP7) may have disintegrated or degraded, possibly affecting the antigenicity of the sample. Disintegration is known to occur during prolonged storage at room temperature or in low-calcium environments (4, 24). To avoid the possibility of a false-negative result, we have been seeking a monoclonal antibody directed at a domain of VP7 common to all (or many) serotypes. In preliminary experiments we have isolated two monoclonal antibodies that react with VP7 of several serotypes but do not neutralize rotavirus, and we are currently studying the suitability of these agents as controls in ELISA serotyping tests.

Previous studies have grouped rotaviruses into two sub-

groups and up to seven serotype groups (13). Human rotavirus disease is associated with both of the subgroups and serotypes 1 through 4 (28). In very limited studies it seems that serotypes 1, 2, and 3 make up the great majority of human isolates (11). This serotypic diversity has been elucidated primarily in slow, labor-intensive, and expensive neutralization assays requiring a cell culture facility. Previous attempts to serotype specimens in ELISA using absorbed polyclonal sera showed some utility for concentrated fecal extracts (2), but the lack of sensitivity and the difficulty of generating consistent reagents by using an inexact absorption process are notable drawbacks. The reagents described in this report are a significant advance in the design of a serotyping ELISA. The extent to which low-titer fecal samples can be accurately assayed has yet to be determined. However, there is no doubt that high-titer fecal specimens, which often contain virus in concentrations far exceeding those in the cell culture specimens in this report, will be easily within the sensitivity limits of this test. Specimens with viral concentrations less than 10^4 PFU/ml may require virus concentration or alterations in the detection technique to increase the sensitivity of the ELISA.

We are currently pursuing the isolation of monoclonal antibodies to identify serotype 2 and 4 rotaviruses. Pending the isolation of such reagents, the probes described in this test, used in conjunction with subgrouping monoclonal antibodies, are capable of assigning known human rotavirus isolates to one of the human serotypes, 1, 2, or 3. Indirect assignment of human strains to serotype 2 is possible because of the high degree of association of subgroup 1 and serotype 2 in human isolates. The availability of serotype 2-specific reagents will permit the exact extent of this association to be tested rigorously.

Recent findings have shown that human and animal rotaviruses may share serotypic antigens (13). Hoshino et al. have shown human serotype 4 (St. Thomas no. 4) to be similar if not identical to three porcine rotaviruses by plaque reduction neutralization assays using hyperimmune antisera. Serotype 3, originally defined for human rotaviruses, now includes simian, canine, equine, and feline strains. Reactivity of the anti-VP7 monoclonal antibody 4F8/E1/B3 with human strains P, Nemoto, and Ito as well as simian, canine, and feline strains in the ELISA is further evidence of the sharing of a region of VP7 between human and animal rotavirus. This overlap of human and animal virus neutralizing regions is important for the possible development of human rotavirus vaccines derived from animal rotaviruses and vice versa.

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