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Identification of Serotype 9 Human Rotavirus by Enzyme-Linked Immunosorbent Assay with Monoclonal Antibodies

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Hybridomas producing monoclonal antibodies to VP7, a major neutralizing protein of serotype 9 rotavirus (strain WI61), were prepared. One monoclonal antibody, WI61-6A1, was shown to neutralize only serotype 9 rotavirus strains and reacted specifically with serotype 9 rotaviruses in an enzyme-linked immunosorbent assay. The development of an immunoassay for detection of serotype 9 rotaviruses should facilitate epidemiologic studies.

Rotavirus is the major etiologic agent of severe diarrhea in infants and young children worldwide, and the development of an effective vaccine against rotavirus has become an important public health goal (10). Evaluation of several vaccine candidates is currently under way and will be greatly facilitated by defining the relative importance of different serotypes in the epidemiology of rotavirus. Four serotypes of rotavirus (serotypes 1, 2, 3, and 4) have been well recognized in the human population and characterized by neutralization assays (1, 17–19). Human rotavirus isolates belonging to two new serotypes, serotype 8 (69M strain) and serotype 9 (WI61 and F45 strains), have now been described (3, 7, 11). The development of an enzyme-linked immunosorbent assay (ELISA) using serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies has made it possible to type large numbers of human rotavirus isolates belonging to these four serotypes (2, 4, 5, 14, 16). This report describes the development of serotype 9-specific monoclonal antibodies and their application to ELISA.

The WI61 strain of human rotavirus (serotype 9) was grown in MA104 cell culture without trypsin, and the cell culture supernatant was centrifuged through a sucrose cushion. The pellet was extracted with fluorocarbon and examined by electron microscopy to verify the presence of double-capsid particles. Six-week-old female BALB/c mice were immunized intraperitoneally with this preparation three times over the course of several months. Three days after the last immunization, the mouse spleen cells and NS-1 myeloma cells were fused with polyethylene glycol as described previously (8). Hybridomas secreting neutralizing antibodies were identified by screening the culture fluid with a fluorescent focus neutralization assay and were cloned twice by limiting dilution (15). Ascitic fluid was made by inoculating 10^7 hybridoma cells intraperitoneally into pristane-primed BALB/c mice. The monoclonal antibodies were further characterized by plaque reduction neutralization assay (PRNA), fluorescent focus neutralization assay, and ELISA as described previously (9, 15, 16). The isotypes of the monoclonal antibodies were determined by Ouchterlony double-immunodiffusion tests with rabbit antisera to mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, IgA, and IgM.

To define the specificity of the monoclonal antibodies, the following human, animal, and reassortant rotavirus strains, propagated in MA104 cell culture, were used in PRNA, fluorescent focus neutralization assay, or ELISA: (i) Wa, M37, KU, and D × RRV 6-1-1 (serotype 1); (ii) DS-1, 1076, and DS-1 × RRV 240-2-1 (serotype 2); (iii) P, McN, RRV, and P × UK 22-1-1 (serotype 3); (iv) ST3 and ST3 × RRV 39-2-1 (serotype 4); (v) OSU (serotype 5); (vi) NCDV and UK (serotype 6); (vii) Ch-2 (serotype 7); (viii) 69M (serotype 8); and (ix) WI61, F45, WI61 × UK 22-1, and F45 × UK 8-1 (serotype 9). Reassortant rotaviruses D × RRV 6-1-1, DS-1 × RRV 240-2-1, ST3 × RRV 39-2-1, and P × UK 22-1-1 have been characterized previously (12, 13). They derive the gene coding for VP7 from their human rotavirus parents (D, DS-1, ST3, and P, respectively) and their remaining genes from their animal rotavirus parent (RRV or UK). Reassortant rotaviruses WI61 × UK 22-1 and F45 × UK 8-1 were prepared by coinfection of cell cultures with human rotavirus strain WI61 or F45 and bovine strain UK as described previously (12). These reassortants were selected in the presence of hyperimmune guinea pig antiserum to the bovine strain NCDV; by PRNA, they were similar, if not identical, to their serotype 9 human rotavirus parent. Their genotypes were determined by RNA-RNA hybridization, and they were shown to derive the gene coding for VP7 from their human rotavirus parents but the remaining 10 genes from their animal rotavirus parents.

The serotyping ELISA was adapted from Taniguchi et al. (16). Briefly, the wells of polyvinyl chloride plates were coated with the serotype 9 specific monoclonal antibody (ascitic fluid diluted 1:1,000 in phosphate-buffered saline) for 1 day at 4°C. The plates were washed twice with phosphate-buffered saline–0.05% Tween (PBST) and blocked with 1% bovine serum albumin in PBST for 1 day at 4°C. They were washed again, and rotavirus-infected culture fluids or rotavirus-positive diarrheal-stool specimens (50 μl per well) were added and incubated for 12 to 18 h at 4°C. After three washes, rabbit antiserum to human rotavirus (50 μl per well of a 1:10,000 dilution in PBST) was added for 1 h at 37°C. The antiserum was a mixture of hyperimmune rabbit antisera to serotype 1, 2, 3, and 4 human rotavirus strains. This antiserum is the same as that used in conjunction with the serotype-specific monoclonal antibodies in the detection of serotypes 1 to 4 by ELISA, as described previously (16). After the plates were washed, horseradish peroxidase-conjugated goat IgG antibody to rabbit IgG (50 μl per well of a

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TABLE 1. Reactivity pattern of neutralizing monoclonal antibodies to WI61 by fluorescent focus neutralization

Hybridoma[a] (ascites)	Neutralizing titer[b] to:		
	Serotypes 1–4[c]	Serotype 9	
		WI61	WI61 × UK 22-1
WI61-6AI	<5	81,920	81,920
WI61-4B4	<5	81,920	327,680
WI61-3D4	<5	320	320
WI61-2B1	<5	20	20
WI61-4A8	<5	81,920	81,920
WI61-5F9	<5	81,920	81,920
WI61-3C3	<5	81,920	81,920
WI61-4F3	<5	81,920	327,680
WI61-4G9	<5	327,680	327,680

[a] All hybridomas were isotype IgG1.
[b] Expressed as the reciprocal of the highest dilution of ascitic fluid that resulted in 60% fluorescent focus reduction.
[c] Strains D × RRV 6-1-1 (serotype 1), DS1 × RRV 240-2-1 (serotype 2), P × UK 22-1-1 (serotype 3), and ST3 × RRV 39-2-1 (serotype 4).

1:1,000 dilution in PBST) was added for 1 h at 37°C. After the plates were washed, 100 μl of freshly made substrate [0.1 mg of 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) per ml of 0.1 M citrate buffer (pH 5.0)–0.012% hydrogen peroxide] was added to each well. The A_{410} was measured, and results were expressed as 1,000 × the sum of the optical densities at 410 nm in duplicate wells. Values of >300 in each ELISA were considered positive reactions. Serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies provided by K. Taniguchi were also used in the ELISA and reacted specifically with serotypes 1 to 4, respectively.

Nine hybridomas producing neutralizing monoclonal antibodies to human rotavirus WI61 strain were identified. All of these monoclonal antibodies belonged to the IgG1 isotype and were directed at the VP7 protein of WI61, as demonstrated by the fact that they neutralized both WI61 and the WI61 × UK reassortant that derives only the gene encoding VP7 from WI61. None of these monoclonal antibodies neutralized reassortant rotaviruses that derived their VP7 genes from serotype 1 (D strain), serotype 2 (DS-1 strain), serotype 3 (P strain), or serotype 4 (ST3 strain) (Table 1). The monoclonal antibody designated WI61-6A1 was characterized more extensively by PRNA and ELISA (Table 2). Ascitic fluid containing WI61-6A1 neutralized serotype 9 human rotaviruses (WI61 and F45 strains) and human-bovine reassortants, WI61 × UK 22-1 and F45 × UK 8-1, to high titer. There was no cross-reactivity with rotavirus strains belonging to serotypes 1 to 8, including bovine strain UK (serotype 6). Likewise, by ELISA, WI61-6A1 reacted only with serotype 9 human rotaviruses (WI61 and F45 strains) and the reassortants WI61 × UK 22-1 and F45 × UK 8-1 (Table 2). These results indicate that this neutralizing monoclonal antibody is specifically directed at the VP7 protein of serotype 9 strains. This specificity for VP7 is supported by the high neutralization titer and ELISA reactivity to the reassortants which derive only the VP7 gene from their serotype 9 human rotavirus parent (WI61 or F45) and their remaining genes from their serotype 6 bovine rotavirus parent (UK). Finally, the applicability of WI61-6A1 for serotyping rotavirus-positive stool specimens by ELISA was examined. Stool specimens from 70 patients with rotavirus diarrhea diagnosed by confirmatory ELISA were tested by ELISA with WI61-6A1 and the serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies. The majority of these specimens were identified as serotype 1, 2, 3, or 4, and

TABLE 2. Reactivity pattern of neutralizing monoclonal antibody WI61-6A1 by PRNA and ELISA

Serotype	Rotavirus strain[a]	Neutralizing titer[b]	ELISA[c]
1	Wa	<80	119
	M37	NT	119
	KU	NT	50
2	DS-1	<80	87
	1076	NT	81
3	P	<80	40
	McN	NT	65
	RRV	<80	
4	ST3	<80	64
5	OSU	<80	83
6	NCDV	<80	150
	UK	<80	55
7	Ch-2	<80	87
8	69M	<80	135
9	WI61	81,920	404
	F45	81,920	624
	WI61 × UK 22-1	81,920	913
	F45 × UK 8-1	81,920	924

[a] All of these strains were grown in MA104 cell culture.
[b] Expressed as the reciprocal of the highest dilution of ascitic fluid that resulted in 60% plaque reduction. NT, Not tested.
[c] The ELISA was performed by using the monoclonal antibody as a capture antibody. The value is the sum of the optical densities in duplicate wells × 1,000. Values of >300 in each test were considered positive reactions. Strains belonging to serotypes 1 to 4 were tested with serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies and shown to have a positive reaction with their respective monoclonal antibodies by the criteria described above (data not shown).

none reacted with WI61-6A1. These results indicated that nonspecific reactivity of serotype 1 to 4 rotavirus-positive stool specimens did not occur with neutralizing-monoclonal-antibody WI61-6A1 in ELISA. The avidity of WI61-6A1 for serotype 9-positive stool specimens could not be assessed because of the current paucity of such specimens. However, past experience with neutralizing monoclonal antibodies to serotype 1, 2, 3, or 4 rotaviruses has shown a good correlation between the avidity of these antibodies for cell culture and stool specimens containing rotavirus strains of the same serotype (4, 16).

Serotype identification of human rotaviruses is needed for any comprehensive study of the epidemiology of infection and diarrheal disease caused by these viruses. It is also needed for evaluating the efficacy of different rotavirus vaccine candidates, especially with regard to the question of heterotypic immunity. Identification of serotype has been accomplished by various neutralization assays and solid-phase immune electron microscopy (1, 6, 18). Neutralization assay requires the adaptation of human rotaviruses to growth in cell culture and is time-consuming and sometimes unsuccessful because of the fastidious nature of human rotaviruses. Immune electron microscopy is also time-consuming and requires an experienced operator and expensive equipment. Therefore, these techniques are not practical in typing large numbers of human rotavirus isolates, in contrast to ELISA, which uses serotype-specific monoclonal antibodies. However, different studies using ELISA for serotype identification have noted that a certain proportion of isolates are untypeable (2, 5, 14, 16). In an Australian study, 62% of 552 fecal specimens positive for human rotavirus, collected over an 11-year period, were identified as belonging to serotype 1, 2, 3, or 4, whereas the remaining specimens were untypeable (2). In the present study, a neutralizing monoclonal antibody that specifically reacts with

serotype 9 human rotavirus by PRNA and ELISA has been identified. The two serotype 9 strains, WI61 and F45, were isolated in the United States and Japan, respectively. This suggests that serotype 9 has a broad geographic distribution, similar to that of other human rotavirus serotypes. The serotype 9-specific monoclonal antibody described in this study should allow the rapid identification by ELISA of other serotype 9 isolates. When added to a panel of monoclonal antibodies directed at serotypes 1 to 4, it may be useful in typing isolates previously classified as untypeable and should be a useful adjunct in studying the relative importance of different serotypes in the epidemiology of rotavirus infection.

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