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Serologic Analysis of Human Rotavirus Serotypes P1A and P2 by Using Monoclonal Antibodies

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Three human rotavirus (HRV) VP4 serotypes and one subtype have been described on the basis of a fourfold or an eightfold-or-greater difference in neutralization titer when tested with hyperimmune antisera to recombinant VP4 or VP8* (serotypes P1A, P1B, P2, and P3). To start to analyze the antigenic basis underlying serotype specificity, we produced a library of 13 VP4-specific neutralizing monoclonal antibodies (NMAbs) to two HRVs, the serotype P1A strain Wa and the serotype P2 strain ST3, and characterized the reactivity of these NMAbs with a panel of serotypically diverse HRV strains by neutralization assay and enzyme-linked immunosorbent assay (ELISA). We characterized the serotypic specificity of the NMAbs by using a fourfold or an eightfold-or-greater difference in titer against the homologous (i.e., immunogen) and heterologous strains as a criterion for serotype. Some ST3-derived NMAbs reacted specifically with serotype P2 HRVs by ELISA and/or neutralization assay, while some Wa-derived NMAbs reacted specifically by ELISA and/or neutralization assay with some or all serotype P1A HRVs. Other Wa- and ST3-derived NMAbs reacted with some or all serotype P1A and P2 HRV strains by neutralization assay and ELISA. Most NMAbs did not react with serotype P1B or P3 strains. In previous studies, three distinct operationally defined epitopes have been identified on VP4 by examining the reactivity patterns of selected antigenic variants of HRV strain KU. At least one of the NMAbs described here recognizes an epitope unrelated to these previously identified epitopes, since it neutralized both KU and its variants.

Fourteen different group A rotavirus serotypes, including nine that infect humans, have been identified to date on the basis of cross-neutralization assays with hyperimmune sera prepared in antibody-negative animals (1–4, 6, 32). Serotype specificity has been defined on the basis of a 20-fold difference in reciprocal antibody neutralization titers (11). Neutralization assays measure the combined antibody reactivity to both outer capsid proteins, VP4 (the product of gene 4) and VP7 (the product of gene 7, 8, or 9, depending on the strain) (21); however, the predominant serotype-specific response found in hyperimmune sera raised against whole virus is generally directed at VP7 rather than VP4 (15). The dual nature of rotavirus neutralization proteins prompted the suggestion of a binary classification system for rotavirus serotypes using the prefix G (for glycoprotein) to name the VP7 serotypes and the prefix P (for protease-sensitive protein) to name the VP4 serotypes (9).

VP4 from human rotavirus (HRV) has been classified into genetic groups by hybridization analysis (5, 7) and putative serotypes by neutralization with hyperimmune antisera to recombinant VP4 or VP8* (9, 14) (Table 1). This classification scheme includes strains from both symptomatic and asymptomatic infections but has not been broadly applied to field strains.

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Although neutralizing monoclonal antibodies (NMAbs) to VP7 have been widely used for serotypic classification of G serotypes, similar studies using NMAbs to VP4 have not been reported. NMAbs to VP4 of a number of human and animal strains have been produced, however. NMAbs to HRV strains have been described as (i) serotype G2 specific; (ii) widely cross-reactive among different G serotypes, including both symptomatic and asymptomatic strains; (iii) widely cross-reactive among symptomatic but not asymptomatic strains; and (iv) specific for HRV strain K8 (serotype G1, P3) (12, 13, 30, 32). NMAbs to animal rotavirus VP4 types have in turn been described as widely cross-reactive, strain specific, or narrowly cross-reactive when tested by neutralization assay versus a number of animal rotavirus strains (16, 18). However, cross-reaction of the animal VP4 NMAbs with human strains has been infrequent.

Some of the amino acids important for recognition by anti-VP4 neutralizing antibodies have been identified by sequencing of antigenic variants selected with NMAbs (12, 18, 29). Additionally, the minimum VP4 peptides required for antibody recognition have been identified by testing the reactivity of NMAbs with truncated polypeptides (19). Most NMAbs to VP8* of rhesus monkey rotavirus (RRV) are strain specific and map to the most prominent variable region of VP4, between amino acids 87 and 188 (18). Conversely, NMAbs to VP5* are primarily cross-reactive and have been

TABLE 1. Tentative VP4 classification of group A HRVs based on genomic and antigenic characteristics

VP4 genetic group[a]	VP4 serotype[b]	Associated VP7 serotype(s)	Symptomatic infection
1	P1A	G1, G3, G4, G9	+
2	P1B	G2, G12	+
3	P2	G1–G4	–
4	P3	G1	+
5	ND[c]	G8	+

[a] From references 7 and 25.
[b] From reference 9.
[c] ND, not determined.

mapped at amino acid 388, 393, or 441 of RRV (18) and at amino acid 305, 385, 392, 428, 433, or 439 of HRVs (12, 29).

To start to analyze the antigenic basis underlying serotype specificity, we have produced a panel of NMAbs to VP4 of the serotype P1A symptomatic HRV strain Wa and the serotype P2 asymptomatic HRV strain ST3. The NMAbs were characterized by neutralization and enzyme-linked immunosorbent assay (ELISA) with a panel of serotypically diverse HRV and animal rotavirus strains. HRV serotypes P1A and P2 were found to contain both cross-reactive and serotype-specific neutralization epitopes.

MATERIALS AND METHODS

Viruses. The P serotype specificity of some of the strains used in this study has not been determined. The following cultivatable HRV strains were used: Wa and KU (serotype G1, P1A), S12 (serotype G1, P unknown), M37 (serotype G1, P2), K8 (serotype G1, P3), DS1 and S2 (serotype G2, P1B), Price (serotype G3, P1A), ITO (serotype G3, P unknown), McN13 (serotype G3, P2), ST3 (serotype G4, P2), VA70 (serotype G4, P1A), 69M (serotype G8, P unknown), and WI61 (serotype G9, P1A). Additionally, several animal rotavirus strains were used: RRV and SA11 (serotype G3), Gottfried (serotype G4, P2), OSU (serotype G5), UK (serotype G6), and YM (serotype G11). Trypsin-activated viruses were propagated in MA104 cells and harvested 3 to 4 days after infection.

Virus purification. After the viruses were harvested, the cells were freeze-thawed twice. Virus obtained at this stage was used without further purification for ELISA. For immunization, the virus suspension was fluorocarbon extracted and then concentrated by ultracentrifugation at 100,000 × *g* for 45 min. The virus pellet was resuspended in 1 ml of TNC buffer (50 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$ [pH 7.4]), layered on CsCl (ρ = 1.37 g/ml) in TC buffer (50 mM Tris, 2 mM $CaCl_2$ [pH 7.4]), and centrifuged at 100,000 × *g* for 18 h. The double-shelled virus band (ρ = 1.37 g/ml) was collected and dialyzed against TNC buffer.

Immunization. Two-month-old BALB/c mice (Institute for Medical Research, San Jose, Calif.) were immunized intraperitoneally with 20 μg of purified Wa or ST3 virus in 250 μl of TNC buffer mixed with an equal volume of complete Freund's adjuvant. The same dose mixed with incomplete Freund's adjuvant was used 1 month later. Mice were bled 1 week after the boost, and their sera were shown to have focus reduction neutralization titers in the range of 1:800 to 1:12,800. The mice with the highest titers were given intravenous booster injections 1 to 2 months after the second immunization with virus in TNC and sacrificed 4 days later.

Hybridoma production. Spleen fusion, cloning, and ascites production were done as previously described (26) with the following modifications: FOX cells were used instead of NS1 cells, and 7.5 × 10^{-2} mM adenine–8 × 10^{-4} mM aminopterin–1.6 × 10^{-2} mM thymidine was used as the selection medium. After fusion, cells were plated at a density of 1.75 × 10^6 spleen cells per ml. Hybridoma supernatants from mice immunized with HRV ST3 were screened by focus reduction neutralization against ST3 and by RRV-recombinant VP4 ELISA as recently described (23). Hybridoma supernatants from mice immunized with HRV Wa were screened by focus reduction neutralization against Wa and by ELISA against whole RRV. Positive hybridomas were cloned twice by limiting dilution with thymocytes or splenocytes as the feeder layer.

RIPA. The protein specificity of individual NMAbs was determined by immunoprecipitation of metabolically labeled rotavirus proteins as previously described (19, 28). The following nonneutralizing anti-Wa MAbs were used as controls in radioimmunoprecipitation assays (RIPAs): anti-VP6 631/9 (10) and 1E11 (selected from the Wa fusion by ELISA against whole RRV), anti-VP7 129 (27), and anti-VP2 3A8/6E8 (28).

Isotyping of MAbs. The isotypes of individual MAbs were determined with an EK-5050 mouse MAb subisotyping kit (Hyclone Laboratories, Logan, Utah).

Focus reduction neutralization test. The method described by Shaw et al. (26) was used to identify NMAb-producing hybridomas and to determine titers of NMAbs from ascitic fluid.

VP4 typing ELISA. Immulon II (Dynatech) microtiter plates were coated with 1:1,000 to 1:8,000 dilutions of ascitic fluid in phosphate-buffered saline (PBS)–0.05% sodium azide (PBS-Az). After overnight incubation at room temperature, the plates were washed twice with PBS-Az and blocked with 10% fetal calf serum (FCS) in PBS-Az for 12 to 24 h at 4°C (all further washings were done with PBS-Az, and all incubations were done with 5% FCS in PBS-Az, unless otherwise indicated). The plates were then washed twice and incubated for 2 h at 37°C with 75 μl of nonpurified virus. The amount of each virus used in the assay was adjusted to obtain an A_{410} of 1.0 ± 0.25 in the VP7 serotyping ELISA described by Padilla-Noriega et al. (22). After the plates were washed four times, a 1:1,000 dilution of an equivolumetric mixture of four rabbit hyperimmune antirotavirus sera (produced with the viruses Wa, DS1 × RRV [20], RRV, and ST3) was added and incubated for 1 h at 37°C. The plates were then washed four times and incubated for 1 h at 37°C with alkaline phosphatase anti-rabbit immunoglobulin G conjugate (Kirkegaard & Perry, Gaithersburg, Md.). Finally, the plates were washed four times, the substrate (1 mg of *p*-nitrophenyl phosphate per ml in 1 mM $MgCl_2$–1% diethanolamine buffer [pH 9.8]) was added, and the plates were incubated at 37°C until the A_{410} of the controls was approximately 1.0.

RRV VP4, VP5*, and VP8* ELISA and ELISA with whole RRV. An ELISA designed to detect antibodies reactive to recombinant baculovirus-expressed RRV VP4, VP5*, and VP8* was carried out as previously described (23). In addition, an ELISA using whole, nonpurified RRV was carried out as described by Losonsky et al. (17).

VP5* and VP8* NMAb specificity. The specificity of NMAbs to the Wa VP8* and VP5* polypeptides of VP4 was determined by DNA amplification-restricted transcription-translation (DARTT) (19). Briefly, total plus-stranded RNA was synthesized by the endogenous viral polymerase in an in vitro reaction from purified, single-shelled Wa virions. Sub-

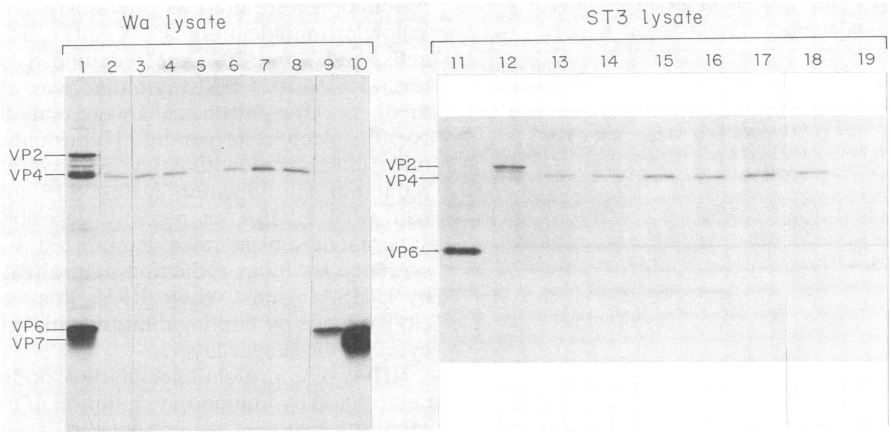

FIG. 1. Radioimmunoprecipitation of VP4 from Wa- or ST3-infected cell lysates by NMAbs. [^{35}S]methionine-labeled proteins of MA104 cells infected with rotavirus Wa or ST3 were immunoprecipitated with antibodies from murine serum or ascitic fluid and analyzed by 10% (Wa lysates) or 12% (ST3 lysates) PAGE. The antibodies tested against Wa lysates were anti-Wa serum (lane 1), anti-Wa NMAbs 1A10 (lane 2), 1E4 (lane 3), 2A3 (lane 4), 2A4 (lane 5), 2C11 (lane 6), 2G1 (lane 7), and 3D6 (lane 8), and control MAbs anti-VP6 631/9 (lane 9) and anti-VP7 129 (lane 10). The antibodies tested against ST3 lysates were control MAbs anti-VP6 1E11 (lane 11) and anti-VP2 3A8/6E8 (lane 12), anti-ST3 NMAbs HS3 (lane 13), HS6 (lane 14), HS7 (lane 15), HS8 (lane 16), HS11 (lane 17), and HS16 (lane 18), and normal ascitic fluid (lane 19). NMAb 1C6 is not shown.

sequently, 5 μg of total Wa RNA was reverse transcribed in the presence of a 3′-end, gene 4-complementary oligonucleotide (CAATCTACATTGTAGTATTAACTGTTC). A polymerase chain reaction (PCR) of full-length gene coding sequences was performed by adding a 5′-terminal oligonucleotide containing a T3 RNA polymerase sequence (GAAT TAACCCTCACTAAAGGGATGGCTTCACTCATTTAT) to the reaction mixture. The PCR was run under the following conditions: denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and polymerization at 72°C for 3 min for 35 cycles. Full-length gene 4 sequences were gel purified and used as a template for subsequent PCRs of VP8*- and VP5*-containing DNA fragments under the same conditions. VP8* amplifications were performed in the presence of the 5′-end oligonucleotide above and the 4Wa747N oligonucleotide (TCTCTTATACTGTATCGA) at the 3′ end of VP8*. VP5*-encoding sequences were amplified by using a new 5′-end oligonucleotide containing a T3 polymerase recognition sequence, 4WaVP5T3 (GAATTAACCCTCACTAAAG GGATGGCACAAGTTAATGAA) and the 3′-end terminal sequence above. The amplified fragments encoding Wa VP4, VP8*, and VP5* proteins were transcribed into RNA by T3 RNA polymerase and subsequently translated in a rabbit reticulocyte lysate system as previously described (19) in the presence of 1 mCi of L-[^{35}S]methionine (Amersham) per ml. Translated products were immunoprecipitated by MAbs in the presence of RIPA buffer as previously described (19), separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

RESULTS

Specificity of NMAbs for VP4, VP5*, or VP8*. We isolated a total of 14 hybridomas secreting NMAbs to two HRVs that differed in their P serotype specificity, the serotype P1A strain Wa (8 hybridomas) and the serotype P2 strain ST3 (6 hybridomas). Of the 14 NMAbs, 13 were able to immunoprecipitate VP4 from radiolabeled cell lysates infected with the homologous strain (Fig. 1). The single nonimmunoprecipitating anti-Wa MAb 2A4 (Fig. 1, lane 5) was shown to be directed at VP7 by measuring hemagglutination inhibition with single-gene rotavirus reassortants that differed in the gene coding for VP7 (24). In addition, we isolated two

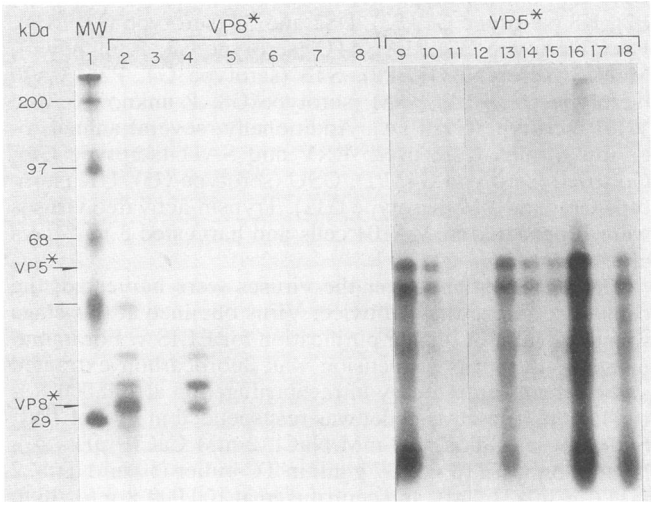

FIG. 2. Radioimmunoprecipitation of in vitro-translated VP8* or VP5* by selected NMAbs. Selected regions (VP8* or VP5*) of Wa gene 4 cDNA were amplified by PCR with simultaneous addition of T3 polymerase transcription signals and transcribed into RNA by T3 RNA polymerase. The mRNA produced was translated in a rabbit reticulocyte lysate system with [^{35}S]methionine. The translated VP8* (lane 2) or VP5* (lane 18) was immunoprecipitated with MAbs and analyzed by 12% PAGE. Lane 1 contains molecular weight (MW) markers. The NMAbs tested against VP8* were HS3 (lane 3), HS6 (lane 4), HS7 (lane 5), HS8 (lane 6), HS11 (lane 7), and HS16 (lane 8). The NMAbs tested against VP5* were HS3 (lane 9), HS8 (lane 10), HS11 (lane 11), HS16 (lane 12), 1A10 (lane 13), 2A3 (lane 14), 2C11 (lane 15), 1C6 (lane 16), and 1E4 (lane 17). The positions of VP5* and VP8* are indicated by arrows. The VP5*-reactive NMAbs HS7, 2G1, and 3D6 are not shown.

TABLE 2. Focus reduction neutralization titers of Wa or ST3 VP4-specific NMAbs against human symptomatic, human asymptomatic, and animal rotavirus strains

Rotavirus strain	VP4 serotype	Titer of MAb (immunogen)[a]: 1A10 (Wa)	1C6 (Wa)	1E4 (Wa)	2A3 (Wa)	2C11 (Wa)	HS3 (ST3)	HS7 (ST3)	HS6 (ST3)	HS8 (ST3)	HS11 (ST3)	HS16 (ST3)	2G1 (Wa)	3D6 (Wa)
Symptomatic[b]														
Wa	P1A	**25,600**	**25,600**	**102,400**	**25,600**	**6,400**	**1,600**	6,400	—	—	—	—	**400**	**100**
Price	P1A	**409,600**	**102,400**	—	—	—	**6,400**	**25,600**	**400**	100	—	—	—	—
WI61	P1A	**102,400**	**25,600**	—	—	—	100	100	—	—	—	—	—	—
VA70	P1A	—	—	6,400	**6,400**	**1,600**	—	—	—	—	—	—	—	—
KU	P1A	800	100	100	100	—	400	400	—	—	—	—	—	—
S2	P1B	1,600	100	100	100	—	100	—	100	—	100	100	—	**100**
DS1	P1B	400	100	100	100	400	—	—	—	—	—	—	**100**	**100**
S12	ND[c]	**25,600**	**25,600**	100	100	—	400	1,600	—	—	—	—	—	—
ITO	ND	400	100	400	400	**1,600**	400	400	**400**	100	—	—	**100**	**100**
Asymptomatic														
M37	P2	—	—	**25,600**	**6,400**	**1,600**	**6,400**	**≥102,400**	**400**	**102,400**	**1,600**	**102,400**	—	—
McN13	P2	—	400	**25,600**	**6,400**	**1,600**	**1,600**	**≥102,400**	**1,600**	**≥102,400**	**6,400**	—	—	—
ST3	P2	—	100	1,600	**6,400**	**1,600**	**6,400**	**102,400**	**1,600**	**102,400**	**6,400**	**102,400**	—	—
Animal[b] (Gottfried)	P2	—	—	400	—	—	400	**25,600**	**1,600**	6,400	—	—	—	—
NMAb Group		A	A	B	B	B	B	B	B	C	C	C	D	D
Protein specificity[d]		VP5*	VP5*	NP	VP5*	VP5*	VP5*	VP5*	VP8*	VP5*	NP	NP	VP5*	VP5*

[a] Titers are expressed as the reciprocal of the highest dilution that neutralized 60% of the infectious virus. Boldface numbers indicate that the difference between the titers of the MAb to the homologous (i.e., immunogen) and heterologous viruses was eightfold or less. —, neutralizing titer of <100.

[b] Symptomatic HRV strains K8 (serotype P3) and 69M (P serotype unknown) and animal rotavirus strains RRV, SA11, OSU, and YM were not neutralized (titer, <200) by any NMAb.

[c] ND, the P serotype specificity has not been determined.

[d] The VP5* or VP8* specificity was determined by in vitro transcription of the VP5* or VP8* region of Wa gene 4 cDNA followed by translation and radioimmunoprecipitation by MAbs from ascitic fluid. NP, nonprecipitating.

nonneutralizing anti-VP4 MAbs from the ST3 fusion. These MAbs were selected because they cross-reacted with baculovirus-expressed RRV VP8* (HS1) or VP5* (HS2) in an ELISA (23). All of the hybridomas secreted immunoglobulin G except for hybridoma 1C6, which secreted an immunoglobulin M antibody.

We attempted to determine whether our VP4 NMAbs specifically bound to either VP8* or VP5* by the DARTT technique (19). This specificity was determined for 10 of the 13 NMAbs by RIPA of in vitro-translated VP5* and VP8* of HRV strain Wa (Fig. 2 and Table 2). Of the 13 NMAbs, one precipitated VP8* and two larger-than-full-length polypeptides (HS6 [Fig. 2, lane 4]), nine precipitated VP5* and three smaller-than-full-length polypeptides (1A10, 1C6, 2A3, 2C11, HS3, HS7, HS8, 2G1, and 3D6 [Fig. 2 and data not shown]), and three did not precipitate either of the subunits of VP4 (1E4, HS11, and HS16 [Fig. 2 and data not shown]). The two larger-than-full-length VP8* products are likely to originate during PCR amplification by downstream hybridization of the 3′-end oligonucleotide, while the three smaller-than-full-length VP5* products are likely to originate during PCR amplification by downstream hybridization of the 5′-end oligonucleotide, upstream hybridization of the 3′-end oligonucleotide, or premature termination of translation of full-length VP5* mRNA. The larger- and smaller-than-full-length products were shown to be related to VP8* and VP5*, respectively, since they were precipitated by control nonneutralizing, cross-reactive MAbs HS1 (VP8* specific) and HS2 (VP5* specific), which have been shown to be directed to VP8* and VP5* by ELISA with recombinant RRV proteins (data not shown).

Patterns of reactivity of anti-VP4 NMAbs by neutralization against HRV and animal rotavirus strains. The interserotypic cross-reactivity of the 13 VP4-specific NMAbs was determined by a neutralization assay with a panel of 20 separate rotavirus strains, including 11 symptomatic human strains, 3 asymptomatic human strains, and 6 animal strains (Table 2). These strains included representatives from P serotypes 1A, 1B, 2, and 3 as well as untyped strains. The NMAbs showed a wide range of neutralization titers to the strains tested (Table 2). We considered an eightfold or greater difference in the titers of an NMAb to the homologous (i.e., immunizing) and heterologous strains as indicative of type differences, since this criterion was previously chosen by Gorziglia et al. to characterize VP4 serotypes (9). The NMAbs were classified into four reactivity groups (A, B, C, and D) according to their neutralization patterns versus serotype P1A and serotype P2 HRVs (Table 2). NMAbs in groups A and C showed the highest specificity to serotype P1A and P2 HRV strains, respectively. Group A NMAbs neutralized specifically a subset of serotype P1A strains, while group C NMAbs neutralized specifically either all three or two of the three serotype P2 strains of human origin but not the serotype P2 animal rotavirus strain Gottfried. NMAbs in group B showed substantial interserotypic (P1A-P2) cross-reactivity, since they neutralized one or two of the five serotype P1A HRVs and all three or two of the three serotype P2 HRVs. Two of the group B NMAbs (HS6 and HS7) were also able to neutralize, at homotypic levels, the serotype P2 Gottfried rotavirus strain. Group B NMAbs did not neutralize any serotype P1B or P3 HRVs or animal strains other than Gottfried. The two group D NMAbs neutralized the parental

TABLE 3. ELISA reactivity patterns of Wa or ST3 VP4-specific NMAbs against human symptomatic, human asymptomatic, or animal rotavirus strains

Rotavirus strain	VP4 serotype	Mean A_{410} for MAb (immunogen)[a]:												
		1A10 (Wa)	1C6 (Wa)	1E4 (Wa)	2A3 (Wa)	2C11 (Wa)	HS3 (ST3)	HS7 (ST3)	HS6 (ST3)	HS8 (ST3)	HS11 (ST3)	HS16 (ST3)	2G1 (Wa)	3D6 (Wa)
Symptomatic[b]														
Wa	P1A	**1.11**	**1.22**	**1.14**	**1.17**	**0.92**	**0.32**	**1.00**	0.19	—	—	—	**0.88**	**0.90**
Price	P1A	**1.20**	**1.61**	**1.60**	**1.37**	**0.64**	**0.56**	**1.31**	—	0.18	—	—	0.13	0.20
WI61	P1A	**1.04**	**1.21**	**0.98**	**0.97**	**0.60**	**0.22**	**1.15**	—	—	—	—	**0.93**	**0.91**
VA70	P1A	**0.60**	**0.85**	**1.05**	**1.09**	**0.58**	**0.33**	**0.92**	0.13	**0.46**	—	0.13	**1.43**	**1.41**
KU	P1A	**0.76**	**0.85**	**0.76**	**0.76**	**0.37**	**0.20**	0.28	—	0.10	0.10	—	—	—
K8	P3	0.27	—	—	—	—	—	—	—	—	—	—	**0.45**	**0.48**
S12	ND[c]	**0.71**	**0.84**	**0.71**	**0.78**	**0.25**	0.14	0.26	0.10	—	—	—	—	—
ITO	ND	**0.33**	0.10	**1.09**	**1.05**	**0.78**	0.12	—	0.12	—	—	0.11	**0.74**	**0.54**
69M	ND	—	—	—	—	—	—	—	0.14	—	—	0.13	**0.32**	**0.29**
Asymptomatic														
M37	P2	—	—	—	0.11	0.14	**0.26**	0.33	**1.11**	**0.39**	**1.38**	**0.44**	—	—
McN13	P2	—	0.30	0.27	0.24	**0.30**	**0.37**	**0.65**	**1.30**	**0.67**	**1.73**	—	**0.22**	**0.28**
ST3	P2	—	0.17	**0.70**	**0.46**	0.17	**0.73**	**1.51**	**1.79**	**1.16**	**0.87**	**1.37**	—	—
Animal[b]														
Gottfried	P2	—	—	**1.55**	**1.28**	0.11	**0.59**	**1.92**	**0.60**	**2.07**	0.11	0.14	—	—
SA11	ND	**0.47**	—	—	—	—	—	—	—	**0.30**	—	—	0.15	0.21
NMAb group		A	A	B	B	B	B	B	B	C	C	C	D	D

[a] Each value is the average A_{410} of two duplicate wells. Boldface numbers indicate that the difference between the A_{410}s for the homologous (i.e., immunogen) and heterologous viruses was fourfold or less. —, A_{410} of <0.1.

[b] Symptomatic HRV strains S2 and DS1 (serotype P1B) and animal rotavirus strains RRV, OSU, and YM did not react with any NMAb; i.e., the A_{410} for the homologous virus was at least fourfold higher than that for the heterologous virus.

[c] ND, the P serotype specificity has not been determined.

strain Wa to a very low titer; hence, it was not possible to analyze eightfold or greater differences in titers of these NMAbs to other strains.

Patterns of reactivity of anti-VP4 NMAbs by ELISA against HRV and animal rotavirus strains. The VP4-specific NMAbs were used as capture antibodies in an ELISA against the same rotavirus strains that were studied by neutralization (Table 3). The amount of double-shelled virus used in the assay was standardized by selecting viral preparations that gave A_{410} readings in the range of 0.75 to 1.25 in the VP7 serotyping ELISA described by Padilla-Noriega et al. (22) when run simultaneously.

We analyzed the serotype specificity of the ELISA results by the same criteria used by Larralde et al. for the neutralization assay with hyperimmune anti-VP8* sera (14). The reactivity by ELISA was considered to be significant if the difference in A_{410} between the homologous and heterologous viruses was fourfold or greater. NMAbs showed broader type-specific reactivity by ELISA than by neutralization (Table 3). However, the two group A NMAbs (1A10 and 1C6) reacted specifically with P1A strains, while the three group C NMAbs reacted predominantly with some or all of the P2 strains but not with strains of other putative P serotypes. One of the group A NMAbs (1A10) and one of the group C NMAbs (HS8) cross-reacted with the animal strain SA11, a reactivity not seen in the neutralization assay.

The group B NMAbs showed a higher degree of cross-reactivity by ELISA than either the group A or group C NMAbs. This result is similar to the neutralization data. Most of the group B NMAbs reacted with virtually all of the P1A strains as well as some or all of the P2 strains. They did not react with animal strains other than Gottfried or with the P1B or P3 isolates. Of interest is NMAb HS6, which appears to be highly type specific for P2 strains in ELISA although it cross-reacted with two P1A strains by the neutralization assay. Finally, the group D NMAbs also showed interserotypic cross-reactivity by ELISA, since they reacted with at least three serotype P1A HRV strains, one P2 HRV strain, and the only P3 strain tested.

Neutralization of KU and KU variants by NMAbs. An operational antigenic map of the KU strain has been constructed by examining the reactivity patterns of its antibody-selected antigenic variants with several cross-reactive NMAbs (30). On the basis of this method, at least three distinct cross-neutralizing epitopes representing two separate domains were identified on VP4. Sequence analysis of the KU VP4 variants demonstrated that all the NMAbs appeared to be directed at epitopes on VP5* (12, 29).

The 13 NMAbs characterized in this study either did not neutralize KU or neutralized it to a low titer; i.e., there was a greater-than-eightfold difference in comparison with the titer of the antibody to the parental strain Wa or ST3. This suggests that the epitopes recognized by these NMAbs are not related to the three cross-neutralization epitopes on KU that have been described (12, 29, 30). For the single NMAb (1A10) with the highest neutralization titer to KU (1/800), it was possible to further study the relationship between the epitope recognized by 1A10 and the three cross-neutralization epitopes on KU. There was no significant difference between the titer of NMAb 1A10 against KU (1/800) and the titer of 1A10 (1/400 in all cases) to any of the previously described KU variants V-KU-4D7, V-KU-6B11, V-YO-1E6, V-YO-1S3, V-YO-2C2, and V-ST-1F2, which represent functional regions C3, C2, and C1. These data indicate that the epitope recognized by NMAb 1A10 is not operationally

related to the three cross-neutralization epitopes on strain KU.

DISCUSSION

VP4 serotypes of HRVs have been recently characterized by a neutralization assay with hyperimmune antisera to recombinant VP4 or VP8*; however, the fine antigenic structure of the neutralization epitopes that contribute to HRV VP4 serotype specificity is not clear. The reactivity of previously described anti-human VP4 NMAbs has been most frequently reported in relationship to the G serotype specificities of the rotaviruses being tested, since a potential P serotyping scheme has become available only recently (4). Accordingly, the various human VP4 NMAbs have been described as serotype G2 specific or widely cross-reactive among several G serotypes (12, 30, 31). It is likely that the previously described serotype G2-specific VP4 NMAbs correspond to NMAbs specific for serotype P1B VP4, since this serotype has been found to segregate with symptomatic serotype G2 strains. Among the cross-reactive VP4 NMAbs previously described, those produced against strain KU (serotype P1A) react with serotype P1A strains and with either serotype P1B or serotype P2 strains (12, 30). The single NMAb that had previously been produced against a serotype P2 HRV strain was widely cross-reactive between serotype P1A and P2 strains (30), while a single NMAb made against the VP4 of the P3 strain K8 reacted only with K8 in a neutralization assay but did cross-react with SA11, NCDV, and 69M in an ELISA (13).

In this article, we have described the production and characterization by neutralization and ELISA of a panel of NMAbs directed at a P1A HRV strain (Wa) and a P2 HRV strain (ST3). One group of the NMAbs (group C) neutralizes only serotype P2 rotavirus strains of human origin and reacts by ELISA with serotype P2 rotavirus strains of human and, in one case, animal origin. This is the first described group of NMAbs that is specific for serotype P2 rotaviruses. Another group of NMAbs (group A) is highly specific by neutralization assay for a subset of serotype P1A strains and reacts with all serotype P1A strains by ELISA. The failure of group A NMAbs to neutralize all serotype P1A strains reveals a hitherto unknown antigenic heterogeneity within this serotype.

The remaining two groups of NMAbs are more cross-reactive. One group (B) of NMAbs neutralized some but not all strains of serotype P1A and P2 and reacted by ELISA with most serotype P1A and P2 strains, hence confirming a previous report by Kobayashi et al., who also found a group of NMAbs reactive with serotype P1A and P2 but not P1B HRV strains (12). The fourth NMAb group (D) could not be analyzed by the neutralization assay because of the low titers against the parental Wa strain. However, by ELISA, the group D NMAbs were also found to be cross-reactive among strains of different P serotypes, including serotypes P1A, P2, and P3. None of the group A, B, or C NMAbs neutralized either serotype P1B or P3 strains, suggesting that HRV serotype P1A and P2 strains are more closely related to each other than to either serotype P1B or P3 strains. The serotypic distinctness between subtypes P1A and P1B has been noted by others (29) and should probably lead to consideration of these subtypes as entirely different serotypes.

We have isolated NMAbs that react with either serotype P1A or P2 strains as well as NMAbs that react with strains from both groups. Hence, HRV serotypes P1A and P2 clearly contain several cross-reactive neutralization epitopes as well as type-specific regions. It is not clear whether these different VP4 serotypes induce type-specific or cross-reactive antibodies following natural infection. In addition, it is not known which regions of VP4 are most immunogenic during natural infection. If regions held in common between types P1A and P2 are more immunogenic than the type-specific epitopes, then classifying P1A and P2 VP4s into different serogroups might be misleading.

Interestingly, the P2 strains have been linked specifically to asymptomatic rotavirus infections (8), and it has been postulated that genetic information encoded in the P2 VP4 is responsible for attenuating rotaviruses isolated from asymptomatically infected neonates. If this hypothesis is true, one should not find the P2 VP4 on isolates from ill children or the P1A VP4 on isolates from asymptomatic neonates. The apparent specificity of group A and C NMAbs for P1A and P2 strains should permit an efficient and systematic search for the presence or absence of these two serotypes in various epidemiologic settings.

With only one exception, all of the HRV-neutralizing MAbs that we have isolated appear to be directed at the VP5* region of VP4. A similar predominance of VP5*-specific NMAbs has been observed by others attempting to produce antibodies to HRV strains (29). On the other hand, at least two separate groups have reported the isolation of predominantly VP8*-specific NMAbs when animal rotaviruses were studied (16, 18). It is clear that neutralizing antibodies can be directed at the VP8* part of human rotavirus VP4 (14). However, at least in mice hyperimmunized with purified HRV, as opposed to recombinant VP4, neutralizing antibodies directed at VP5* seem far more common. At present, it is not clear whether the observed differences in VP8* and VP5* specificity between animal and human strains represent a fundamental difference in the immunogenicity of VP4s from these viruses or represent simply a sampling error or an artifact of the NMAb screening process, which in most cases has been a neutralization assay for human viruses and hemagglutination inhibition and neutralization for animal viruses. In any case, the serotypic classification of VP4 will almost certainly be dependent on which region of the VP4 molecule (VP5* or VP8*) is the target of typing antisera. Therefore, it will be important to determine whether the immune response to VP8* or VP5* predominates during natural infection and/or vaccination.

In this preliminary study, we have not localized the specific regions on VP5* or VP8* that are the target of NMAb binding. However, we have provisionally determined that at least one of our NMAbs (1A10) appears to be directed at a region that is distinct from the three neutralization epitopes previously described by Taniguchi and colleagues (30); hence, the antigenic structure of VP4 may be more complex than previously thought. Whether the NMAbs described here will map to entirely new domains on VP5* or VP8* or whether they will map to regions adjacent to those already characterized remains to be determined.

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