

Evaluation of three panels of monoclonal antibodies for the identification of human rotavirus VP7 serotype by ELISA

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Three panels of monoclonal antibodies used for rotavirus serotype identification by enzyme-linked immunosorbent assay (ELISA) were evaluated at the National Institutes of Health, USA, to identify antibodies suitable for distribution to laboratories involved in WHO-sponsored trials of rotavirus vaccines. Two of the panels were comparably effective in identifying the serotype of each of the human rotavirus reference strains of serotype 1, 2, or 3. In addition, one of the panels included a monoclonal antibody that was effective in identifying strains of serotype 4. However, two different lots of a third, commercially available panel were not effective in identifying the eight strains representing the four serotypes. A third shipment of this panel was therefore tested using revised instructions and, under these conditions, it was effective in serotyping seven of the eight reference strains. It appears that a battery of monoclonal antibodies for each serotype may be required to identify antigenic variants within a serotype. Additional studies are needed to assess the extent of antigenic variation in rotavirus field strains.

Introduction

Rotavirus is the single most important etiological agent of severe diarrhoea among infants and young children. The mortality rate from rotavirus diarrhoea is high in areas where rehydration therapy is not generally available. Because of the importance of this disease as a major global health problem, efforts are underway to develop a rotavirus vaccine (1). An effective, safe vaccine would probably reduce the number of deaths from severe rotavirus diarrhoea (estimated to be over 800 000 per year) and significantly decrease the incidence of hospitalization for this condition (2).

To be effective, a human rotavirus vaccine must induce protection against the four epidemiologically important serotypes of the virus (designated 1, 2, 3, and 4) (27). Two additional human rotavirus serotypes (designated 8 and 9) have been described recently, but their epidemiological importance is not known (3, 4). Rotavirus serotypes are defined primarily by the antigenic specificity of the outer capsid protein (VP7) in neutralization tests per-

med *in vitro* using hyperimmune sera raised against rotavirus reference strains (5–7). Additional epidemiological data are needed about the distribution, seasonality, and relative importance of each serotype.

Several laboratories have developed VP7 serotype-specific monoclonal antibodies (MAbs) for determining the serotype of rotavirus strains directly in stool specimens by conventional enzyme-linked immunosorbent assay (ELISA)—an approach that circumvents the labour-intensive and often difficult task of tissue-culture adaptation of each field strain before further characterization (8–11). The availability of the reagents required has been limited. WHO considers that a well-characterized panel should be made available to laboratories involved in WHO-sponsored vaccine studies worldwide. WHO therefore selected three panels of monoclonal antibodies for the evaluation of their efficiency and practicality and organized their shipment to the National Institutes of Health (NIH) from selected laboratories in Australia, Japan, and the USA. In the present study, we report the ability of these panels of antibodies to identify the serotype of human rotavirus reference strains whose serotype and genotype were known. The advantages and disadvantages of each panel and the performance of individual monoclonal antibodies are described.

Methods

Monoclonal antibody panels

The following panels of VP7 serotype-specific monoclonal antibodies were submitted for evaluation:

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panel 1 (developed by Greenberg et al. at Stanford University, Palo Alto, USA); panel 2 (developed by Taniguchi and Urasawa et al. at Sapporo Medical College, Sapporo, Japan); and panel 3 (developed by Bishop and Coulson et al. at the Royal Children's Hospital, Melbourne, Australia).^a In the evaluation, "capture" refers to the antibodies adsorbed on to the solid phase of the microtitration plate; "detector" refers to the antibodies used to determine the binding of rotavirus antigen; and "conjugate" refers to the enzyme-linked immunoglobulin used to assay the binding of the detector antibody. The data reported for monoclonal antibodies from different panels and within panels were not always obtained from tests performed concurrently.

Rotavirus reference strains

The following human rotavirus strains were used as test antigens: Wa (serotype 1), M37 (serotype 1), DS-1 (serotype 2), 1076 (serotype 2), P (serotype 3), YO (serotype 3), ST3 (serotype 4), VA70 (serotype 4), 69M (serotype 8), and WI61 (serotype 9). Serotypic characterization of each of these strains by neutralization with hyperimmune sera has been described previously (3, 4, 6, 12-16). In addition, the amino acid sequences of the VP7 of strains Wa, M37, DS-1, P, YO, ST3, VA70, 69M, and WI61 have been deduced (17-19). For production of rotavirus ELISA antigen, trypsin-activated virus was propagated in MA-104 cells in the presence of trypsin (0.1 µg/ml). The cells and supernatant fluid were frozen and thawed once before being stored at -70 °C. An uninfected tissue culture control consisted of MA-104 cells and supernatant fluid.

Panel 1 method

Samples of ascites fluids and rotavirus hyperimmune guinea-pig 962 serum were obtained from Greenberg. The assay method used was similar to that described by Shaw et al. (8), using the monoclonal antibody as capture antibody and the rotavirus hyperimmune serum as detector antibody. The wells of a 96-well polyvinyl chloride microtitration plate^b were coated with 50 µl of one of the following monoclonal antibodies at the dilution shown in phosphate-buffered saline (PBS), pH 7.2: 2C9 (serotype-1-specific), 1:5000 dilution; 5E8 (serotype-1-specific), 1:8000; 2F1 (serotype-2-specific), 1:3000; 1C10 (serotype-2-specific),

1:3000; 4F8 (serotype-3-specific), 1:5000; 159 (serotype-3-specific), 1:5000; and 129 (anti-VP7 common-reactive), 1:2000. Some tests were also performed using Immulon 1 microtitration plates.^b The plates were incubated at 4 °C overnight, and the wells were subsequently washed twice with PBS, and 200 µl of blocking buffer (PBS containing 5% fetal calf serum (FCS)) was added. The plates were incubated at 4 °C overnight and washed three times with PBS. A 1:2 dilution of rotavirus antigen or uninfected tissue culture control was made in PBS-10% FCS, and 50 µl was added to the wells. After the plates had been incubated at 37 °C for 1 hour, each well was washed four times with PBS and 50 µl of a 1:4000 dilution of guinea-pig 962 rotavirus hyperimmune serum in PBS-5% FCS was added. After a further 1 hour at 37 °C, the wells were washed four times with PBS, and 50 µl of goat anti-guinea-pig IgG conjugated with alkaline phosphatase^c diluted 1:2000 in PBS-5% FCS was added to each well. The plates were then incubated for 1 hour at 37 °C, the wells washed four times, and the substrate was added (50 µl per well; Sigma 104 substrate tablets dissolved in diethanolamine buffer (1% diethanolamine, 1 mmol/l magnesium chloride solution, 0.029% (w/v) sodium azide, pH 9.8)). The absorbance at $\lambda=405$ nm was determined spectrophotometrically^d and the result expressed as the average (A_{405}) in duplicate wells. The background absorbance of a control well was subtracted automatically by the spectrophotometer from that of each of the other wells.

Panel 2 method

Ascites fluids, pooled rabbit hyperimmune sera against rotavirus serotypes 1-4, and goat anti-rabbit IgG that was labelled with peroxidase were obtained from Taniguchi & Urasawa. The method used was similar to that described by Taniguchi et al. (11), with the monoclonal antibody functioning as capture antibody and the rotavirus hyperimmune serum as detector antibody. The wells of a polyvinyl chloride microtitration plate^b were coated with 100 µl of one of the following monoclonal antibodies in ascites fluid (diluted to 1:10 000 in PBS) and kept overnight at 4 °C: KU6BG, S2-2G10, YO-1E2, and ST-2G7, which were specific for rotavirus strains of serotype 1, 2, 3, and 4, respectively. After the plates were washed

^a Available from Silenus Laboratories, Pty. Ltd, Hawthorn, Victoria, Australia.

^b Dynatech Laboratories, Inc., Chantilly, VA, USA.

^c Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA.

^d Molecular Devices spectrophotometer, Menlo Park, CA, USA.

twice with PBS that contained 0.05% Tween (PBS-T), 200 μ l of blocking buffer (PBS-T-2.5% skimmed milk) was added, and the plates were then incubated at 37 °C for 2 hours. Subsequently, the plates were washed again, rotavirus antigen or uninfected tissue culture control (50 μ l per well) was added, and the plates were incubated for 12-18 hours at 4 °C. Following three washes with PBS-T, pooled rotavirus hyperimmune rabbit serum diluted 1:16 000 in PBS-T-2.5% skimmed milk was added (50 μ l per well) and allowed to incubate at 37 °C for 1 hour. After the wells had been washed three times with PBS-T, goat antibody to rabbit IgG that was labelled with peroxidase was added (50 μ l per well of a 1:10 000 dilution in PBS-T-2.5% skimmed milk). Following incubation at 37 °C for 1.5 hours, the substrate (0.1 mg of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) per ml of 0.1 mol/l citrate buffer, pH 5.0, in 0.012% v/v hydrogen peroxide) was added (100 μ l per well), and the absorbance at $\lambda=405$ nm was measured. The results were expressed as described above for panel 1.

Panel 3 method

The monoclonal antibodies used have been described previously by Coulson et al. (10) and are available in a commercial kit.* The serotyping ELISA was carried out using the kit, following the manufacturer's instructions and the procedure described by Coulson et al. (10). Four guinea-pig hyperimmune sera (prepared by Bishop & Coulson et al. (not included in the kit), anti-RV4, anti-RV5, anti-RV3, and anti-ST3, which were raised against rotavirus strains of serotype 1, 2, 3, or 4, respectively, and a Silenus kit containing one vial each of the commercially available monoclonal antibodies RV-4:1, RV-5:2, RV-3:3, and ST-4:4 that were specific for rotavirus strains of serotype 1, 2, 3, or 4, respectively, were obtained from Bishop & Coulson. The following lots ("batches") of protein-A-purified monoclonal antibodies[†] were tested separately at different times: the first lot (LF03, LF04, LF05, and LF06), "panel 3A"; and the second lot (MD27, MD28, MD29, and MD30), "panel 3B". The lyophilized monoclonal antibodies were reconstituted in 0.5 ml sterile distilled water immediately before use, as recommended by the manufacturer. Hyperimmune serum, which was used in conjunction with the monoclonal antibody of the same serotype specificity, was diluted in PBS as recommended by Bishop & Coulson (anti-RV4, 1:8000; anti-RV5, 1:6000; anti-RV3, 1:6000; and anti-ST3, 1:8000) and 100 μ l was added to the wells of a 96-well polyvinyl chloride

microtitre plate[‡] as capture antibody. Some tests with this panel were also performed using Immulon 1 microtitre plates.[‡] After incubation at 37 °C for 2 hours, the wells were washed three times with PBS-T. Blocking buffer (PBS-T-2.5% skimmed milk) was added (75 μ l per well) and allowed to incubate at 37 °C for 2 hours. Rotavirus antigen or uninfected tissue culture control (25 μ l per well) was then added and the plates were left at 4 °C overnight. After the wells had been washed three times with PBS-T, the reconstituted protein-A-purified monoclonal IgG of the same serotype-specificity as the hyperimmune capture antibody was added (detector antibody) at the recommended dilution (1:200 each) in 0.1 mol/l Tris, pH 7.4 (100 μ l per well), and the plates were kept at 37 °C for 2.5 hours. The plates were then washed three times with PBS-T, and bound monoclonal antibody was detected using sheep anti-mouse IgG conjugated with peroxidase[§] at a 1:1000 dilution in PBS-T-2.5% skimmed milk (100 μ l per well). The procedure used to develop the substrate, determine the absorbance values, and express the results was the same as described for panels 1 and 2.

Criteria for determining positive reactivity with monoclonal antibodies

The following criteria, which are similar to those used by Ahmed et al. (20), were employed to assign the serotype of a strain: an average absorbance value of ≥ 0.2 in the duplicate wells for the reaction of the virus with the monoclonal antibody of corresponding serotype specificity; and the absorbance value for the reactivity of the virus with the monoclonal antibody of that serotype had to be twice that of the value of the reactivity with other monoclonal antibodies in the panel.

Use of different hyperimmune sera and conjugates with panels of monoclonal antibodies

Because usually only the monoclonal antibodies would be distributed to laboratories, we determined whether variability among the hyperimmune sera and antibody conjugates available in different laboratories could affect the ability of panels of monoclonal antibodies to identify serotype, by examining the combinations outlined below.

- Panel 1 as the capture monoclonal antibodies at the recommended dilutions using rabbit immunoglobulins against human rotavirus^b (1:500 dilution)

[†] See footnote b, p. 602.

[‡] Sigma Chemical Company, St. Louis, MO, USA.

[§] Dako Corporation, Glostrup, Denmark.

* See footnote a, p. 602.

Table 1: Comparison of the reactivities of the three panels of monoclonal antibodies used for rotavirus

		Absorbance ($\lambda=450$ nm)										
		Panel 1 MAb: ^a						Panel 2 MAb: ^a				
		Serotype 1		Serotype 2		Serotype 3		Common serotype 1		Serotype 2	Serotype 3	Serotype 4
Rotavirus strain	Serotype	2C9	5E8	2F1	1C10	4F8	159	129	KU6BG	S2-2G10	YO-1E2	ST-2G7
Wa	1	1.53^b	1.438	0.148	0.338	0 ^c	0.061	0.559	1.163	0.040	0.051	0.038
M37	1	0	0.566	0	0.178	0	0	0.352	0.911	0.076	0.031	0.027
DS-1	2	0	0	0.316	1.242	0	0.061	0.252	0.040	1.213	0.071	0.044
1076	2	0	0	0	0.453	0	0	0.013	0.021	0.984	0.029	0.043
P	3	0	0	0.043	0.268	2.214	>3.0	2.287	0.051	0.105	1.395	0.055
YO	3	0.056	0.081	0.063	ND	0.098	1.264	0.426	0.168	0.079	1.216	0.148
ST3	4	0	0	0	0.205	0	0	0.689	0.038	0.114	0.067	1.349
VA70	4	0	0.345	0	0.144	0	0.040	0.320	0.165	0.062	0.006	0.473
69M	8	0	0	0.031	0.258	0	0.198	0.379	0.046	0.294	0.060	0.039
W161	9	0.216	0.451	0.094	0.430	0	0.114	1.143	0.051	0.093	0.067	0.041
Uninfected control	-	0	0	0	0.205	0	0	0.037	0	0.070	0.027	0.030

^a MAb=monoclonal antibody.

^b Figures shown in bold are the average absorbances in duplicate wells; these represent positive reactivity with

^c 0=average absorbance in duplicate wells <0 after the background absorbance in the negative control well had been

^d NT=no test because the variability between duplicate wells was >50%.

^e ND=not determined.

as the detector antibody. The conjugate was goat anti-rabbit IgG labelled with peroxidase at a 1:10 000 dilution (Taniguchi & Urasawa).

● Panel 1 as the capture monoclonal antibodies at the recommended dilutions and NIH guinea-pig human rotavirus (strain "D") hyperimmune serum (1:10 000 dilution) as the detector antibody. The conjugate was goat anti-guinea-pig IgG labelled with peroxidase^f at a 1:2000 dilution.

● Panel 1 as the capture monoclonal antibodies at the recommended dilutions and the recommended guinea-pig 962 rotavirus hyperimmune serum (1:4000 dilution) that accompanied panel 1 as described above. The conjugate was goat anti-guinea-pig IgG labelled with peroxidase^f at a 1:2000 dilution.

● Panel 2 as the capture monoclonal antibodies at the recommended dilutions using guinea-pig 962 rotavirus hyperimmune serum (provided by Greenberg) at 1:4000 dilution as the detector antibody. The

conjugate was goat anti-guinea-pig IgG labelled with peroxidase^f at a 1:2000 dilution.

● Panel 2 as the capture monoclonal antibodies at the recommended dilutions using rabbit immunoglobulins against human rotavirus^f (1:500 dilution) as the detector antibody. The conjugate was goat anti-rabbit IgG labelled with peroxidase^f at a 1:1000 dilution.

Results

The ability of each panel of monoclonal antibodies to identify the serotype of nine rotavirus reference strains that represented serotypes 1-4, 8, and 9 was examined (serotypes 8 and 9 were included as "negative controls" since ELISA reagents for identifying these serotypes were not available). The protocols from each investigator were followed closely

^f See footnote c, p. 602.

^f See footnote h, p. 603.

^g Cappel Laboratories, Cochranville, PA, USA.

serotype identification by ELISA

Absorbance ($\lambda = 450$ nm)											
Panel 3A, 3B, and 3C MAb: ^a											
Serotype 1			Serotype 2			Serotype 3			Serotype 4		
RV-4:1			RV-5:2			RV-3:3			ST-3:4		
Panel 3A	Panel 3B	Panel 3C	Panel 3A	Panel 3B	Panel 3C	Panel 3A	Panel 3B	Panel 3C	Panel 3A	Panel 3B	Panel 3C
0.020	0.020	0.297	0	0	NT ^c	0.025	0.006	NT	0.017	0.013	0.032
0.014	ND ^b	0.295	0	ND	0.013	0	ND	0.008	0	ND	0.002
0.011	0.011	0.01	0	0	0.428	0	0.004	0.033	0	0.012	0.022
0.020	ND	0	0	ND	0.002	0	ND	0	0	ND	0
0.065	0.01	0.037	0.004	0	0.030	0	0.004	0.652	0	0.002	0.018
ND	ND	0.017	ND	ND	0	ND	ND	0.495	ND	ND	0.012
0	0.016	0.024	0	0	0.084	0	0.005	0.084	0.106	0.014	0.680
ND	ND	0.053	ND	ND	0.011	ND	ND	0.051	ND	ND	0.307
ND	ND	0.017	ND	ND	0.042	ND	ND	0.076	ND	ND	0.031
ND	ND	0.027	ND	ND	0.040	ND	ND	0.045	ND	ND	0.107
0.012	0.018	ND	0	0	ND	0	0.013	ND	0.014	0.023	ND

monoclonal antibodies, as defined in Methods, subtracted by the spectrophotometer.

with respect to the recommended reagents, dilutions, and incubation times. Table 1 shows the average absorbances in duplicate wells (using polyvinyl chloride microtitration plates) for each of the monoclonal antibodies versus each of the rotavirus test antigens.

Panel 1, which used two individual serotype-1-specific monoclonal antibodies, two serotype-2-specific monoclonal antibodies, two serotype-3-specific monoclonal antibodies, and one common VP7-reactive control monoclonal antibody, identified the serotype of human rotavirus reference strains of serotype 1, 2, or 3. However, three monoclonal antibodies (2C9, serotype-1-specific; 2F1, serotype-2-specific; and 4F8, serotype-3-specific) failed to recognize one of two homotypic strains. For example, MAb 2C9 did not recognize the serotype 1 strain M37, although serotype-1-specific MAb 5E8 did, while both 2C9 and 5E8 recognized the serotype 1 strain Wa. Serotype-2-specific MAb 1C10 recognized strains DS-1 and 1076, while serotype-2-specific MAb 2F1 recognized only strain DS-1. Also, serotype-3-specific MAb 159 recognized strains P and YO, while

MAb 4F8 recognized only strain P. In addition, low-level cross-reactivity was observed with the following monoclonal antibodies: MAb 2C9 (serotype-1-specific) and strain WI61; MAb 5E8 (serotype-1-specific) and strains VA70 and WI61; MAb 1C10 (serotype-2-specific) and strains Wa, P, ST3, and 69M and the uninfected tissue culture control. The common reactive VP7-specific MAb 129, which was included as a control for the presence of VP7 antigen, recognized all strains tested with the exception of serotype 2 strain 1076.

Panel 2, which used four monoclonal antibodies that were specific for serotype 1, 2, 3, or 4, identified the serotype of human rotavirus reference strains of serotype 1, 2, 3, or 4 (Table 1). MAb KU6BG (serotype-1-specific) recognized homotypic strains M37 and Wa; MAb S2-2G10 (serotype-2-specific), homotypic strains DS-1 and 1076; MAb YO-1E2 (serotype-3-specific), homotypic strains P and YO; and MAb ST-2G7 (serotype-4-specific), homotypic strains ST3 and VA70. Low-level cross-reactivity was observed with MAb S2-2G10 (serotype-2-specific) and serotype 8 strain 69M.

Table 2: Ability of different combinations of reagents to determine the serotype of rotavirus reference strains Wa, DS-1, P, and ST3

Capture monoclonal antibodies	Detector	Conjugate	Rating
Panel 1	Rabbit immunoglobulins to human rotavirus (Dako Corporation)	Goat anti-rabbit IgG labelled with peroxidase (Taniguchi & Urasawa)	Satisfactory
Panel 1	Guinea-pig human rotavirus hyperimmune serum (NIH)	Sheep anti-guinea-pig IgG labelled with peroxidase (Kirkegaard & Perry Laboratories)	Satisfactory
Panel 1	Guinea-pig 962 rotavirus hyperimmune serum (Greenberg)	Goat anti-guinea-pig IgG labelled with peroxidase (Kirkegaard & Perry Laboratories)	Satisfactory
Panel 2	Guinea-pig 962 rotavirus hyperimmune serum (Greenberg)	Goat anti-guinea-pig IgG labelled with peroxidase (Kirkegaard & Perry Laboratories)	Satisfactory
Panel 2	Rabbit immunoglobulins to human rotavirus (Dako Corporation)	Goat anti-rabbit IgG labelled with peroxidase (Cappel Laboratories)	Satisfactory

Unexpectedly, panel 3A, which included the initial lot of commercially available monoclonal antibodies from Silenus Laboratories, did not recognize any of the strains tested—although the serotype-4-specific monoclonal antibody (ST-3:4) exhibited weak reactivity with the serotype 4 strain ST3 (Table 1). Since the monoclonal antibodies were not effective, a second shipment of Silenus monoclonal antibodies from a different lot (panel 3B) was tested, although the results obtained were similar (Table 1). Repetition of some tests with panel 3B using Immulon 1 plates produced similar negative results (data not shown). The MAbs obtained from Silenus were developed originally by Coulson et al., who used them successfully in several epidemiological surveys. After completion of the evaluation study, we therefore carried out additional tests using a new shipment of the following monoclonal antibodies: MD27, MD28, MD29, and MD30 (designated panel 3C, but from the same lot as panel 3B). In addition, Bishop & Coulson made available a different set of rotavirus serotype-specific hyperimmune sera. The following modifications were made in the experimental procedure with panel 3C: Nunc polystyrene "Maxisorp" Immuno-plates[†] were used (kindly provided by Dr R. Bishop); the second set of serotype-specific hyperimmune sera received from Bishop & Coulson was used as capture antibodies; the lyophilized monoclonal antibodies were reconstituted in 0.5 ml of distilled water, as before, but 0.5 ml of water that contained 10 mg/ml of bovine serum albumin was also added—the recommended dilution of 1:200 for each MAb as detector antibody

was again used; the conjugate used was rabbit anti-mouse IgG labelled with peroxidase[‡] at a 1:800 dilution; and tetramethylbenzidine (TMB) was used as the substrate. With these modifications, all but one (serotype-2-strain 1076) of the eight reference strains was recognized by panel 3C (Table 1).

The availability of the reagents used in conjunction with serotype-specific monoclonal antibodies can vary among laboratories. It was therefore important to examine the effect of using hyperimmune sera or antibody conjugates that were different from those evaluated here for use with each of the panels of monoclonal antibodies. Using reagents available in our laboratory or purchased commercially, we performed serotype immunoassays with various combinations of capture antibodies, detector antibodies, and conjugates. The antigens included were: Wa (serotype 1), DS-1 (serotype 2), P (serotype 3), and ST3 (serotype 4). The performance of each set of conditions with individual panels of monoclonal antibodies is rated in Table 2. Here, "satisfactory" indicates that the serotype of all four reference strains was identified correctly (in the interpretation of the rating of panel 1, which did not include a serotype-4-specific monoclonal antibody, a "not serotype 1, 2, or 3" reactivity was considered to be a correct result for a tentative identification of serotype 4). In general, it was possible to vary the reagents with different panels of monoclonal antibodies and obtain similar results.

Discussion

We evaluated three panels of rotavirus VP7-specific

[†] Nunc, Roskilde, Denmark.

[‡] See footnote *h*, p. 603.

monoclonal antibodies as reagents for serotyping rotaviruses by immunoassay. The goal of this study was not to examine the efficiency of these panels in serotyping large numbers of field strains, but to examine the specificity of the reagents against human rotavirus reference strains that have been well characterized. It was important to determine whether there are major differences in reactivities among the monoclonal antibodies used for serotype analysis in order to identify reagents suitable for distribution by WHO. Although these monoclonal antibodies were evaluated overall as "panels", the specificities of individual monoclonal antibodies were also assessed. It is important to note that we evaluated the reactivity of these monoclonal antibodies against only human rotavirus strains.

At present, only one panel of monoclonal antibodies for the identification of rotavirus serotype can be purchased commercially. The potential advantages of marketing these reagents include widespread availability, standardization, and consistency of performance; however, the first set of monoclonal antibodies that we tested from this commercial source (Silenus, panel 3A) did not function satisfactorily under any of the conditions used. Subsequently, a second set of antibodies (panel 3B) from a different lot was tested, but this set also failed to function satisfactorily in our tests. Ascites fluid from Silenus that contained the serotype-specific monoclonal antibodies (without protein A chromatography) detected test antigens when used as the capture antibody (data not shown) and thus, the failure of the lyophilized monoclonal antibodies was not caused by an inherent inability to recognize the test antigens used in the study. Furthermore, the hybridomas from which the Silenus antibodies were derived have been used by Coulson et al. to generate monoclonal antibodies that function satisfactorily (10). In an attempt to account for our lack of success with these antibodies, a third set of Silenus monoclonal antibodies (panel 3C) was received and tested successfully using several modifications to the procedure described above for panels 3A and 3B. Because several modifications were made in the evaluation of panel 3C, we are not certain which particular condition was required for its success. However, it appears that the addition of bovine serum albumin to the reconstituted monoclonal antibodies (which was suggested by the manufacturer, in the revised instructions included in this third set, to be important for stabilizing and extending the storage period of these antibodies) may be important and that the use of Nunc microtitration plates, which have a high binding capacity, may also aid in achieving successful typing with these antibodies. It is possible that the problems encoun-

tered with the Silenus antibodies in this study might have been avoided if the assay conditions had been clarified in the instructions provided with the first two lots. In addition, the inclusion of serotype-specific hyperimmune sera (which are not part of the Silenus kit) would be useful for laboratories that do not have the facilities to generate such reagents.

Panel 1 performed well using the recommended protocol and reagents for the human reference strains of serotypes 1, 2, and 3. Its advantages include the use of two monoclonal antibodies per serotype (which takes into consideration the possibility of antigenic variation among strains of like serotype) and the inclusion of a common VP7 monoclonal antibody control to confirm the presence of VP7 antigen. The performance of panel 1 with respect to background absorbance values, low-level cross-reactivity, and speed of development of the substrate signal was improved by the use of a peroxidase-labelled anti-guinea-pig immunoglobulin conjugate (in general, peroxidase-linked conjugates functioned well in all the tests in the study and exhibited lower background levels than phosphatase-linked conjugates). One major disadvantage with panel 1 was the lack of a serotype-4-specific monoclonal antibody. For the identification of the serotype of field strains this could be problematic, as illustrated by the cross-reactivity of the serotype 4 strain VA70 with the serotype-1-specific MAB 5E8. VA70 was, however, identified as serotype 1 using the criteria employed in the study. The presence of a serotype-4-specific monoclonal antibody in the panel might have avoided this problem. It should be noted, however, that a dual reactivity for certain strains with serotype-1- and serotype-4-specific monoclonal antibodies has been reported (20).

Panel 2 functioned well in serotyping the reference strains of serotypes 1, 2, 3 and 4 used in this study and can be recommended as a sensitive, specific, practical assay for general use because it contains a monoclonal antibody that recognizes serotype 4 and yields consistent results. With the reference strains, the specific absorbances were high and background levels low, although in one case cross-reactivity was observed (serotype 8 strain 69M cross-reacted with the serotype 2-specific MAB). The presence of a serotype-8-specific monoclonal antibody may have circumvented this problem. The panels evaluated did not include serotype-8- or serotype-9-specific monoclonal antibodies. The epidemiological importance of these two "new" human rotavirus serotypes is not known because reagents for their study have only recently become available (21, 22).

Antigenic variation that is detectable using

certain monoclonal antibodies can occur within rotavirus strains of the same serotype (23–25). The biological significance of this antigenic variation and its role in the epidemiology of rotavirus is, however, not known. The term “monotypes” has been applied by Coulson to rotaviruses that show this variation within a serotype (23), and their existence suggests that the monoclonal antibodies used in a serotyping test should optimally react with a wide range of viruses that belong to the same serotype. Alternatively, monoclonal antibodies could be included in a panel that recognized every known monotype within each serotype. At present, such monoclonal antibodies are not available. However, MAb 2C9 (panel 1) appeared to recognize a monotype of serotype 1. Previously, we have reported that the amino acid in VP7 to which this MAb maps (as determined by sequence analysis of neutralization escape mutants) was residue 94 (25). Strain Wa, which is recognized by MAb 2C9, contains asparagine at this residue, and strain M37, which is not recognized by MAb 2C9, contains serine. Field strains that contain a VP7 sequence similar to the M37 strain may not be detected with MAb 2C9. Thus, for the identification of serotype 1 rotaviruses, monoclonal antibodies such as 5E8 in panel 1 and KU6BG in panel 2, which recognized both Wa and M37, would be desirable.

It should be noted that certain epitopes are apparently shared or are similar in some rotavirus strains that have a different serotype (26). The monoclonal antibodies used for analysis of the serotype of human rotavirus strains may not be suitable for identifying the serotype of some animal strains. For example, the porcine strain Gottfried (serotype 4) cross-reacts with serotype-3-specific MAb 159, and porcine strain OSU (serotype 5) cross-reacts with serotype-3-specific MAb YO-1E2 (K.Y. Green et al., unpublished observations). Hence, careful assessment of monoclonal antibodies is required for serotype analysis of rotavirus strains.

As more is understood about the epitopes that form the serotype-specific antigenic structure of VP7, it may be possible to expand the number of monoclonal antibodies included in ELISA serotype identification to define the type of reactivity that would specify the genotype of a particular VP7. For example, Nishikawa et al. found an absolute correlation between the presence of asparagine at residue 94 in the VP7 of 26 serotype-3 strains and the reactivity of these strains with MAb 159 (19). Only one serotype-3 strain contained lysine at position 94, and this strain was not recognized in an ELISA by MAb 159. In addition, the presence of threonine at position 221 correlated with the inability of MAb YO-1E2 to neutralize or bind certain serotype-3 rotaviruses.

Interestingly MAb YO-1E2 reacted in an ELISA with 21 of 27 (78%) serotype-3 strains, while MAb 159 reacted with 26 of 27 (96%). Such molecular studies (although exceedingly time consuming) are undoubtedly the most rational method of characterizing the monoclonal antibodies used in serotype analysis of rotaviruses. Clearly, a battery of monoclonal antibodies for each serotype may be required to allow for antigenic variation within a given serotype, and, in addition, more sequence data are needed to assess the extent of this genetic variation in field strains of rotavirus.

One practical finding that should be emphasized is that it is possible to substitute reagents used in conjunction with the monoclonal antibodies. Several reagents that were substituted in this study are available commercially. For example, rabbit anti-human rotavirus serum (Dako Corporation) functioned well as a detector antibody if monoclonal antibodies were used as capture antibodies. Conjugates used in rotavirus immunoassays must be tested for the presence of rotavirus antibody that can result in high “background” levels. Goat anti-guinea-pig IgG labelled with peroxidase (Kirkegaard & Perry Laboratories) and goat anti-rabbit IgG labelled with peroxidase (Cappel Laboratories) performed satisfactorily as conjugates in this regard. Rabbit anti-mouse IgG labelled with peroxidase (Dako Corporation) also performed satisfactorily as a conjugate if monoclonal antibodies were used as the detector. Serotype-specific reagents should be tested and titrated by individual laboratories to determine the optimum conditions and dilutions.

Expanded global surveillance of rotavirus serotypes should yield additional information of relevance to the design and development of successful rotavirus vaccine strategies. In addition, current trials of several rotavirus vaccine candidates should benefit from the availability of a method for monitoring the serotype of circulating strains. As a group of monoclonal antibodies, panels 1 and 2 performed satisfactorily for the identification of rotavirus reference strains. In addition, the following individual monoclonal antibodies performed satisfactorily: KU6BG and 5E8 (for serotype 1); S2-2G10 and IC10 (for serotype 2); YO-1E2 and 159 (for serotype 3); and ST-2G7 (for serotype 4). Based on the reagents evaluated in this study, an ELISA for identification of rotavirus serotype would ideally consist of the following components: the individual monoclonal antibodies noted above as capture antibodies, including the VP7 cross-reactive MAb 129 as a control for the presence of VP7 antigen; rabbit rotavirus hyperimmune serum (Dako Corporation) as detector; and goat anti-rabbit immunoglobulins labelled with peroxidase (Cappel

Laboratories) as conjugate. Alternatively, because the amount of stool specimen is often limited, it may be possible to screen initially with one panel, e.g., panel 2. Strains that are not identified with this panel could then be analysed further with a different set of monoclonal antibodies that recognize other serotype-specific epitopes.

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Résumé

Evaluation des collections d'anticorps monoclonaux utilisées pour identifier le sérotype VP7 du rotavirus humain par la technique ELISA

Les diarrhées à rotavirus constituent un grave problème de santé pédiatrique et des essais cliniques sont en cours pour trouver un vaccin efficace contre les principaux sérotypes circulants. Le sérotypage du rotavirus est important pour l'évaluation de l'efficacité des vaccins et pour les études épidémiologiques.

On dispose pour cela d'une technique ELISA qui consiste à faire réagir des anticorps monoclonaux spécifiques des différents sérotypes avec la protéine de la capsid externe (VP7). Dans la présente étude, nous avons évalué trois collections d'anticorps monoclonaux destinés à cet usage qui ont été mises au point par différents laboratoires. Dans un premier temps, deux de ces collections se sont révélées d'une efficacité comparable pour identifier les souches de rotavirus humain appartenant aux sérotypes 1, 2 ou 3. Une de ces collections comportait en outre un anticorps monoclonal permettant d'identifier les souches de sérotype 4. Par contre, avec deux lots différents d'une troisième collection disponible dans le commerce, il a été impossible d'identifier les huit souches représentées par les quatre sérotypes. Un troisième lot de cette collection a donc été évalué selon un mode opératoire révisé qui nous a permis d'identifier sept des huit souches de référence. La

réactivité différente de certains anticorps monoclonaux à l'égard des différentes souches d'un même sérotype confirme l'existence de variations antigéniques intrasérotypiques chez le rotavirus; il est donc important que les anticorps monoclonaux destinés au sérotypage soient dirigés contre les épitopes généralement présents dans les différentes souches d'un sérotype donné. Certains anticorps monoclonaux sont dirigés contre des épitopes qui se retrouvent sous une forme identique ou très voisine dans des souches appartenant à différents sérotypes et ne doivent par conséquent pas être utilisés pour le sérotypage. La collection établie par Taniguchi et collaborateurs s'est révélée pratique pour une utilisation générale, car elle a permis d'identifier de façon constante les quatre sérotypes. Toutefois, il peut être nécessaire de disposer d'une batterie d'anticorps pour chaque sérotype.

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