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Comparative Sensitivities of Solid-Phase Immune Electron Microscopy and Enzyme-Linked Immunosorbent Assay for Serotyping of Human Rotavirus Strains with Neutralizing Monoclonal Antibodies

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Suspensions of 24 rotavirus strains, 6 for each known human rotavirus serotype, were serially diluted and titrated by (i) enzyme-linked immunosorbent assay (ELISA) for rotavirus detection, using monoclonal antibodies (MAbs) specific for group-specific sites of the VP6 inner capsid protein; (ii) ELISA for subgrouping, using MAbs reactive with subgroup-specific determinants of rotavirus VP6; (iii) ELISA for serotyping, using MAbs directed to serotype-specific sites of the VP7 outer capsid glycoprotein; and (iv) solid-phase immune electron microscopy (SPIEM) for serotyping, using VP7-specific MAbs. In addition, in each preparation the proportion of double-shelled rotavirus particles was determined by direct electron microscopy. Results showed that SPIEM was 2- to 16-fold more sensitive than ELISA for serotyping of rotavirus. The titers in VP7-specific tests correlated well with the proportion of double-shelled virus particles in each of the samples. Titers obtained by ELISA for serotyping of suspensions containing 20% or fewer complete particles were up to 4,096-fold lower than those obtained by ELISA for detection. ELISA serotyping titers of samples containing 20 to 80% double-shelled rotavirus particles were up to 128-fold lower than ELISA detection titers, whereas preparations with nearly 100% complete particles had ELISA titers that were less different from each other. ELISA subgrouping titers were four- to eightfold lower than corresponding rotavirus detection titers. It was concluded that, although SPIEM appears to be more sensitive than ELISA, the amount of complete virus particles in the specimens is of critical importance for successful serotyping of human rotavirus strains. Samples rich in single-shelled particles but containing low amounts of VP7 outer capsid glycoprotein might even be strongly reactive in assays for rotavirus detection and subgrouping but virtually unreactive in tests for serotyping.

Group A human rotavirus (HRV) strains, the major etiologic agents of acute gastroenteritis in infants and young children, have been classified into two distinct subgroups, subgroup I including serotype 2, and subgroup II including serotypes 1, 3, and 4 (13, 14, 22). Subgroup is specified by VP6, the gene 6 product (14), and, to a lesser extent, by VP2, the gene 2 product (19), whereas serotype is specified by both outer capsid proteins VP7, the gene 9 or 8 product, and VP3, the gene 4 product (12).

During the past several years, the need for simple and rapid methods for the serotyping of HRV strains has been recognized. Initially, enzyme-linked immunosorbent assay (ELISA) (20) and solid-phase immune electron microscopy (SPIEM) (10, 11) were developed, using polyclonal antisera extensively absorbed with heterotypic rotavirus and reactive with both VP3 and VP7. However, some cross-reactivity could not be entirely avoided by ELISA, whereas SPIEM, although giving highly specific results, was somewhat cumbersome and required a skilled electron microscopist. Recently, neutralizing monoclonal antibodies (MAbs) specific for the four recognized HRV serotypes were obtained, and ELISA methods employing them were developed for the typing of HRVs in cell culture fluids (17) and even directly in stool samples (7, 18).

In the present study, we evaluated the sensitivity of both ELISA and SPIEM methods for the serotyping of HRVs based on the use of neutralizing MAbs directed to serotype-specific determinants of the rotavirus outer capsid glycoprotein VP7. The sensitivities of ELISA and SPIEM for serotyping were also compared with those of ELISA for rotavirus detection and subgrouping with VP6-specific MAbs. Results showed that SPIEM was more sensitive than ELISA for serotyping, but the reliability of both typing procedures appeared to depend on the proportion of double-shelled rotavirus particles in the samples.

MATERIALS AND METHODS

Rotavirus strains. Twenty-four rotavirus strains were used in this study. Among them, the following were cultivable strains: HRV strains DS-1, P, and ST3 (kindly provided by R. G. Wyatt and Y. Hoshino, National Institute of Allergy and Infectious Diseases, Bethesda, Md.); strains S2 and YO (provided by S. Urasawa, University of Sapporo, Sapporo, Japan); strains OS1486 and OS1488 (provided by I. Orstavik, Ulleval Hospital, Oslo, Norway); strains RV4 and RV5 (1); strain Hochi (provided by Y. Inaba, National Institute of Animal Health, Ibaraki, Japan); HRV strains GF561 and BD27 (provided by G. Zissis, St. Pierre Hospital, Brussels, Belgium); and simian rotavirus SA11 (provided by S. Kalter, Southwest Foundation for Animal Research, San Antonio, Tex.). HRV strain VA70, serotype 4B (11), was previously adapted to growth in cell culture as described before (9). All

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strains were propagated in MA104 cell cultures in the presence of trypsin (9). The other 10 rotavirus strains used were extracts of stools collected in Italy from infants and young children with acute gastroenteritis. The infectious titer of cell culture-adapted strains was determined by immunoperoxidase staining of infected cells 18 h after inoculation (9).

Rotavirus suspensions were serially diluted and then titrated by SPIEM for serotyping, as well as by ELISA for detection, subgrouping, and serotyping. In addition, the proportion of complete rotavirus particles was determined in each of the samples by direct electron microscopy (EM).

Murine MAbs. The preparation and characterization of MAbs RV-4:2 (anti-serotype 1), RV-5:3 (anti-serotype 2), RV-3:1 (anti-serotype 3), and ST-3:1 (anti-serotype 4) have been previously reported (6, 7). The neutralizing MAb directed to serotype 4B was selected from MAbs obtained in our laboratory from BALB/c mice immunized with HRV strain VA70. VA70 MAb as well as rotavirus group- and subgroup-specific MAbs were produced and characterized essentially as described elsewhere for SA11 MAbs (11a).

ELISA for rotavirus detection and subgrouping. For rotavirus detection, an ELISA recently developed in our laboratory (G. Gerna, A. Sarasini, A. Di Matteo, M. Torsellini, and M. Battaglia, manuscript in preparation) was used. The assay was based on the use of MAbs directed to group-specific determinants of the VP6 inner capsid protein. Samples were treated with EDTA, which converts double-shelled particles into single-shelled particles (5). Briefly, serial twofold dilutions of virus suspensions in phosphate-buffered saline containing 0.01 M EDTA, as well as negative controls, were mixed with peroxidase-labeled group-specific MAbs and incubated for 1 h at 37°C in microtiter plate wells precoated with rabbit antirotavirus or preimmune immunoglobulin. After a 1-h incubation at 37°C, the wells were washed and *o*-phenylenediamine was added as a chromogenic substrate. After a 30-min incubation at room temperature, the optical density (OD) at 492 nm was determined with a microtiter plate reader (Titertek Multiskan; Flow Laboratories, Inc., Irvine, Calif.). Negative control samples were consistently unreactive (OD < 0.05). A cutoff value of 0.10 was adopted for determination of endpoint titers.

Subgrouping was done by a double-antibody sandwich ELISA with MAbs reactive with subgroup-specific epitopes of VP6 (G. Gerna et al., in preparation). Briefly, virus dilutions were incubated for 1 h at 37°C in wells precoated with rabbit preimmune immunoglobulin or immunoglobulin specific for either rotavirus subgroup, followed by a 1-h incubation with peroxidase-labeled subgroup-specific MAbs. Reading of results was as reported above for rotavirus detection, using a cutoff value of 0.10.

ELISA for serotyping. Rotavirus suspensions, as well as negative controls, diluted in phosphate-buffered saline (pH 7.2) containing 0.1% (vol/vol) Tween 20 and 10% fetal calf serum were incubated for either 2 h at 37°C or overnight at 4°C in microtiter plate wells precoated with homotypic rabbit polyclonal antibody (diluted 1:10,000) or with preimmune immunoglobulin. After being washed, optimal dilutions of mouse MAbs specific for HRV serotypes 1 (1:5,000), 2 (1:10,000), 3 (1:40,000), 4A (1:40,000), and 4B (1:30,000), as determined by checkerboard titration against homologous strains, were added and incubated for 2 h at 37°C. Biotinylated antibody to mouse immunoglobulin was then added and incubated for 90 min at 37°C. The bound biotinylated antibody was detected by adding a preformed complex of avidin with biotinylated peroxidase (Vectastain ABC Kit; Vector Laboratories, Burlingame, Calif.). Test results were read as reported above. Negative control specimens were consistently unreactive (OD < 0.05). The reactivity of specimens in wells coated with preimmune immunoglobulin was also negligible (OD < 0.05). Rotavirus strains were considered typed when the differential OD between immune and preimmune immunoglobulin-coated wells was >0.10.

Direct EM and SPIEM. The SPIEM technique was done essentially as previously described (11). Optimal working dilutions of each serotype-specific MAb, as determined by SPIEM titration against homologous strains, were used (1:1,000 for serotype 1 and 1:5,000 for serotype 2, 3, 4A, and 4B MAbs). Briefly, carbon-Formvar-coated 400-mesh grids were treated first with protein A and then with the type-specific MAbs. Previous incubation of protein A-coated grids with rabbit anti-mouse immunoglobulin for 30 min at room temperature was required for binding of VA70 MAb (immunoglobulin G1 isotype). After negative staining, 20 squares for each grid were examined with a Philips EM201 EM at ×45,000, and the average number of virus particles per grid square was determined (11). A cutoff value of five particles per grid square was used for determination of the endpoint titers of rotavirus samples by SPIEM.

For direct EM, grids were floated on a drop of each rotavirus preparation for 10 min and then stained for 10 s with a 2% aqueous solution of uranyl acetate. The percentage of double-shelled virus particles was determined for each sample after examination of at least 200 particles.

RESULTS

Selection and characterization of rotavirus for the comparative study. Among hundreds of strains belonging to the four recognized HRV serotypes, previously typed by neutralization or by SPIEM with animal hyperimmune sera or by both methods, 24, 6 for each serotype, were selected for the comparative study. Three strains for each of serotypes 1, 2, and 4 were stool extracts, whereas the other three were infected culture fluids. Among the six serotype 4 strains, five were subtype 4A and one was subtype 4B. Only one stool specimen (PCP5) was available for serotype 3, owing to the poor circulation of serotype 3 HRVs in Italy in the period 1981 to 1987. Criteria for selection were the presence in each sample of a high number of virus particles (at least five per grid square by direct EM) and the purity of each rotavirus strain, as determined by RNA electropherotype analysis (8) and by specific reactivity of each specimen in immunological tests for rotavirus subgrouping and serotyping.

Titration by ELISA for detection and for subgrouping confirmed that the specimens selected for the study were rich in rotavirus. The ELISA titers of samples reactive with MAbs directed to group-specific determinants of rotavirus VP6 ranged from 1:640 (BD27 strain) to 1:1,310,720 (strains PCP5, PV12091, and PV11586) (Table 1). The titers obtained by ELISA for subgrouping were four- to eightfold lower than those obtained by ELISA for rotavirus detection and varied between 1:80 (strain BD27) and 1:163,840 (strains PCP5, PV12091, and PV11586).

Comparative sensitivity of ELISA and SPIEM for serotyping. The relative sensitivities of ELISA and SPIEM are reported in Table 1. The ELISA titers of samples with serotype-specific MAbs directed to rotavirus outer capsid glycoprotein VP7 ranged from 1:10 (strain PA15) to 1:40,960 (strain PCP5) and appeared to be 2- to 4,096-fold lower than the corresponding ELISA detection titers. The endpoint titers determined by SPIEM with the same MAbs used for

TABLE 1. Relative sensitivity of SPIEM and ELISA for serotyping of rotavirus strains with MAbs

Rotavirus strain	Endpoint titer of rotavirus strain[a]				Infectivity[b]	% Complete virus particles[c]
	ELISA			SPIEM serotype		
	Detection	Subgroup	Serotype			
Serotype 1						
RV4	5,120	1,280	160	2,560	1.6×10^4	95
OS1486	5,120	1,280	320	1,280	6.0×10^3	90
OS1488	2,560	640	80	320	1.0×10^3	80
PV11197	81,920	10,240	20,480	81,920	ND[d]	98
PV12210	5,120	1,280	160	1,280	ND	85
PV12292	10,240	2,560	320	1,280	ND	80
Serotype 2						
DS-1	10,240	2,560	320	2,560	2.0×10^6	85
S2	10,240	1,280	320	2,560	2.0×10^3	90
RV5	5,120	1,280	20	320	4.0×10^3	70
PA15	40,960	5,120	10	160	ND	5
BR-1	40,960	5,120	1,280	10,240	ND	90
MI106/83	40,960	5,120	160	640	ND	20
Serotype 3						
P	20,480	5,120	1,280	5,120	2.0×10^3	85
YO	40,960	5,120	1,280	2,560	2.5×10^5	60
SA11	40,960	5,120	5,120	20,480	3.0×10^7	100
GF561	5,120	1,280	1,280	5,120	6.4×10^5	100
BD27	640	80	160	2,560	1.2×10^2	100
PCP5	1,310,720	163,840	40,960	327,680	ND	90
Serotype 4						
ST3	5,120	1,280	80	640	8.2×10^3	75
Hochi	40,960	5,120	640	1,280	4.0×10^3	50
VA70	2,560	640	1,280	5,120	2.0×10^6	100
PV12306	40,960	10,240	320	2,560	ND	60
PV12091	1,310,720	163,840	10,240	40,960	ND	40
PV11586	1,310,720	163,840	320	1,280	ND	2

[a] Titers are expressed as reciprocals. Detection and subgrouping were done with MAbs directed to group- and subgroup-specific sites of VP6, respectively. For serotyping by ELISA and SPIEM, the same MAbs reactive with rotavirus VP7 were used.
[b] Infectious titers, expressed as focus-forming units per 25 μl, were determined for cell culture-adapted strains only.
[c] Approximate percentage of double-shelled rotavirus particles determined by direct EM.
[d] ND, Not determined.

serotyping by ELISA ranged from 1:160 (strain PA15) to 1:327,680 (strain PCP5). Titers by SPIEM for serotyping were between 4-fold higher and 1,024-fold lower than the corresponding ELISA detection titers. Thus, overall, SPIEM appeared to be 2- to 16-fold more sensitive than ELISA for serotyping of rotavirus.

However, when the serotyping titers of each sample were considered with respect to ELISA detection and subgrouping titers, as well as to the proportion of double-shelled rotavirus particles as determined by direct EM (Table 1), it was found that they did not differ greatly for each of the specimens with nearly 100% complete virus particles (PV11197, SA11, GF561, BD27, and VA70). It was observed that, regardless of serotype, when there were more than 80% double-shelled virus particles, the ELISA serotyping titers were 2- to 32-fold lower than their respective rotavirus detection titers, whereas the SPIEM serotyping titers were between 4-fold higher and 4-fold lower than ELISA detection titers. When the proportion of complete particles was 20 to 80%, the SPIEM and ELISA serotyping titers became 8- to 64-fold and 32- to 256-fold lower than the ELISA detection titers, respectively. Rotavirus preparations with these characteristics in Table 1 are OS1488 and PV12292 for serotype 1; RV5 for serotype 2; YO for serotype 3; and ST3, Hochi, PV12306, and PV12091 for serotype 4. Samples containing fewer than 20% double-shelled virus particles had SPIEM and ELISA typing titers 64- to 1,024-fold and 256- to 4,096-fold lower, respectively, than those obtained by ELISA for rotavirus detection. Specimens of this kind in Table 1 are PA15 and MI106/83 for serotype 2 and PV11586 for serotype 4. The ratios obtained by dividing the ELISA detection titer by the ELISA or the SPIEM typing titer inversely correlated with the number of double-shelled virus particles in the samples (Fig. 1). In addition, infectious titers appeared to be directly correlated with SPIEM typing titers (Fig. 2). That was observed for all but three cell culture-adapted strains (S2, P, and BD27), which showed very low levels of infectivity.

DISCUSSION

Serotyping of rotavirus strains is important for both epidemiological surveys and studies for the evaluation of rotavirus vaccines in field trials. Subgrouping is also useful for differentiation of simian rotavirus vaccine (3), a subgroup I strain of serotype 3, from serotype 3 HRVs which, as a rule, belong to subgroup II (13). Reliable serotyping of HRV strains was initially done only by serum neutralization after adaptation of field strains to growth in cell cultures, which is cumbersome, time consuming, and often unsuccessful (9, 21). Thus, for some years few serotyping attempts were made. Subsequently, using cross-absorbed animal immune sera, HRV strains could be serotyped directly in stools by ELISA (20) and by SPIEM (10). The absorption procedure required large amounts of purified virus of different serotypes and did not allow adequate standardization of type-

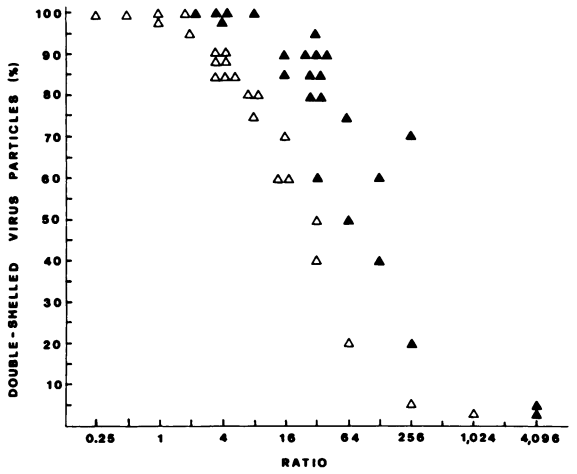

FIG. 1. Correlation between the percentage of double-shelled rotavirus particles and the ratios obtained by dividing the ELISA detection titer by the ELISA (▲) or the SPIEM (△) typing titer.

specific polyclonal antisera among different preparations. In addition, some strains could not be unequivocally typed, especially by ELISA. This was attributed to circulation of new serotypes and to poor sensitivity of the assay. When neutralizing MAbs directed to VP7 of each HRV serotype were produced, ELISAs were developed for direct serotyping of rotavirus directly in stools (7, 18). However, even by ELISA with MAbs some strains were untypable, possibly owing to a narrow range of reactivity of MAbs and, again, to the circulation of new, unknown serotypes.

To investigate whether other reasons could be responsible for the problems encountered in the serotyping of HRVs, we performed experiments aimed at determining the relative amounts of VP6 and VP7 in rotavirus samples. In parallel, the proportion of double-shelled virus particles was determined in each sample by direct EM, using a negative staining procedure which is likely to cause only minimal degradation

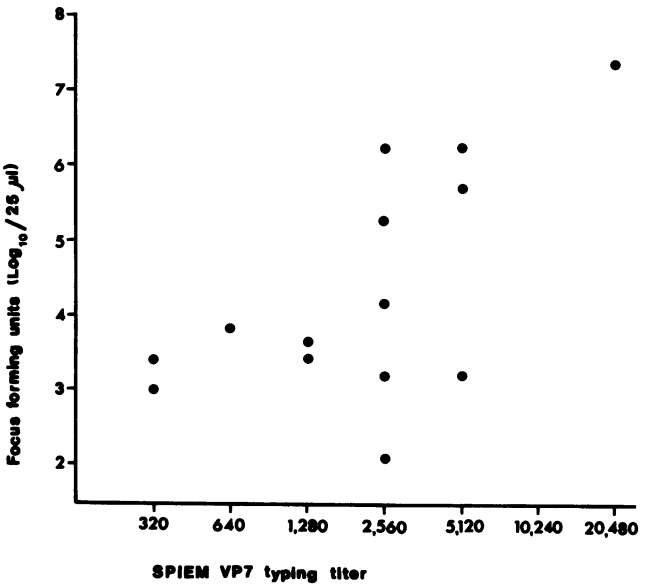

FIG. 2. Correlation between infectivity and SPIEM VP7 typing titer of 14 cell culture-adapted rotavirus strains.

of complete rotavirus particles (16). For some samples, VP6 and VP7 titers were comparable, whereas for others, VP7 titers were moderately to markedly lower than those determined in VP6-specific assays. Examination of the specimens by direct EM showed that titers in VP7-specific tests were directly related to the proportion of double-shelled rotavirus particles. In other words, rotavirus preparations containing nearly 100% complete particles had similar VP6 and VP7 titers, whereas in samples with fewer than 20% double-shelled virus particles, VP7 titers were markedly lower than VP6 titers. This suggests that the quality of the specimens is of crucial importance for the serotyping of HRVs, and it should be considered along with other factors, such as circulation of unknown serotypes and low sensitivity of the assays, when serotyping is unsuccessful. When the difference between VP6 and VP7 titers is small, nearly all rotavirus strains of serotypes 1 to 4 should be readily serotyped and untypable strains may represent genuine new serotypes. On the other hand, when the difference in titer is higher, serotyping of rotavirus becomes increasingly difficult. Samples containing fewer than 20% complete virus particles by direct EM might even be highly reactive in rotavirus detection and subgrouping assays but virtually unreactive in tests for serotyping. Thus, most untypable samples are likely to possess insufficient quantities of double-shelled rotavirus particles. This conclusion is also indirectly supported by the good correlation between infectivity and SPIEM typing titers observed for most of the culture-adapted strains. Only three HRV strains which showed very low infectious titers did not correlate, probably because of poor adaptation to growth in cell cultures and the presence of high particle-to-infectivity ratios. We would recommend, whenever possible, examination of all untypable strains by EM for the presence of complete virus particles, using suitable staining procedures (16), before considering other possible factors. Preliminary data on two presumptive new HRV serotypes (2, 4, 15) showed that the prototype strains examined, 69M and WI61, were strongly reactive by ELISA for rotavirus detection and subgrouping, but although showing a proportion of double-shelled particles higher than 80%, they did not react by ELISA or by SPIEM with any of the MAbs specific for serotypes 1 to 4 (G. Gerna, A. Sarasini, and M. Parea, unpublished observations).

SPIEM appears to be 2- to 16-fold more sensitive than ELISA for serotyping of rotavirus. This means that, according to our SPIEM cutoff value (five particles per grid square), samples containing at least 10 to 80 double-shelled particles per grid square by SPIEM should readily by typed by ELISA. Although SPIEM and ELISA were evaluated in this study with samples preselected for specific reactivity, data on randomly selected specimens suggest that a low, and comparable, proportion of equivocal results (less than 5%) is obtained by both techniques (Gerna et al., unpublished observations). Our preliminary data suggest that a few rapid freeze-thawings cannot greatly reduce the sensitivity of rotavirus-typing assays. However, in our experience, freeze-thawings should be avoided whenever possible, and samples should be stored at −80°C after preparation to obtain the best typing results. Once serotyped, a rotavirus strain might not even be retyped after thawing. On the other hand, some strains may be serotyped only by ELISA because of the presence of immune aggregates, a condition previously shown to hamper serotyping by SPIEM (11). Thus, we recommend that, when possible, both SPIEM and ELISA techniques should be performed to obtain the highest serotyping rates. However, since most HRV samples can readily

be typed by ELISA (with working dilutions of MAbs two- to eightfold higher than those used in SPIEM), it would be convenient to attempt serotyping initially by ELISA and then examine, if required, untypable strains by SPIEM.

In conclusion, both ELISA and SPIEM with neutralizing MAbs directed to rotavirus VP7 are highly sensitive for the serotyping of HRV strains, even though the latter technique appears to be slightly superior. Within serotypes 1 to 4, the quality of samples with respect to the relative amount of double-shelled particles represents a critically important factor for successful typing. HRV strains readily detected by immunological methods based on rotavirus group-specific reactivity might be serotyped only if they contain a sufficiently high number of complete virus particles.

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