Phenotypic Mixing during Coinfection of Cells with Two Strains of Human Rotavirus

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Coinfection of MA-104 cells with serotype 1 (Wa strain) and serotype 3 (P strain) human rotaviruses resulted in progeny viruses that were phenotypically mixed in their major outer-shell structural-protein VP7 and in the genome segment that encodes this protein (segment 9). Segments from the Wa virus predominated in these progeny whether they were of parental or reassortant genotype. Neutralization with monoclonal antibodies specific for VP7 proteins of the coinfecting viruses caused an alteration in genomic distribution favoring the strain heterologous to the neutralizing monoclonal antibody. Because the percentage of change in distribution of segment 9 was similar to that of the other combined segments, there appeared to be no greater association between VP7 and segment 9 than with other segments of the homologous virus during encapsulation. From these results, it was calculated that the progeny of coinfection with P segment 9 were 77.4% mosaic structures and 14.8% pseudotypes; progeny of coinfection with Wa segment 9 were 40.2% mosaic structures and 1.3% pseudotypes. Similar determinations were made for the reassortant progeny alone.

Rotavirus particles are composed of 11 segments of double-stranded RNA packaged within a double-shelled capsid containing at least six different viral proteins (2). Two outer capsid proteins, VP3 and VP7, contain neutralization epitopes used to make serotype determinations (6, 15). A unique property of viruses with segmented genomes is the ability to form reassortants upon coinfection of cells with viruses of different strains. Reassortants, viruses with genome segments from both coinfecting strains, have been used in a variety of studies. After coinfection, selection of the desired reassortants by picking plaques and electrophoretotyping the isolates can be extremely time-consuming. Certain manipulations have been used to minimize these efforts. A method used to force selection of reassortants with genome segments which code for specific VP3 or VP7 proteins has been the treatment of the progeny of coinfection with neutralizing monoclonal or polyclonal antibodies directed at the neutralization proteins of the coinfecting strains (3, 5, 12-14, 19, 21).

Progeny viruses formed after coinfection of cells with two virus strains do not necessarily contain capsid proteins encoded by the genome they encapsulate. Coinfection with viruses of various genera has resulted in the formation of mosaics (virions with capsid proteins encoded by both coinfecting strains) and pseudotypes (virions whose genomes and capsid proteins are derived from separate coinfecting parents) (1, 7, 9, 10, 16, 18, 22). If phenotypically mixed particles are dominant after coinfection with two strains of rotavirus, selection of reassortants through the use of neutralizing antibodies may be of little value unless these antibodies are added only to the overlay agar during plaque formation. Addition of antibody to the progeny of the coinfection prior to inoculation of the cell monolayer could result in the loss of the most desired reassortants. Both methods have been used (3, 5, 6, 12-14, 19, 21), but addition of antibody prior to inoculation is more common because it requires considerably less neutralizing antibody. The pur-

pose of this study was to determine the prevalence of phenotypically mixed particles after coinfection of cells with two strains of human rotavirus as identified by their abilities to be neutralized by monoclonal antibodies specific for the coinfecting strains.

The strains chosen for this study were Wa and P, representatives of serotypes 1 and 3, respectively. Both viruses were triply plaque purified, grown, and analyzed for infectious particles by plaque formation in MA-104 cells, as previously described (20). Coinfection was performed in tube cultures at a high multiplicity of infection (i.e., 10) for each virus. These cultures were frozen at 19 h postinfection, when cytopathic effect was nearly complete. The genotypes of progeny viruses were determined by polyacrylamide gel electrophoresis after phenol extraction of virion RNA. As previously noted (20), the parental origins of all segments except segment 6 could be identified in progeny viruses obtained after coinfection with these two human rotavirus strains. The neutralizing monoclonal antibodies used for this study, 2C9 and 159, were from mouse ascites fluids and were specific for epitopes on VP7 of serotype 1 and serotype 3 rotaviruses, respectively (4, 17).

Incubation (37°C, 30 min) of the Wa strain with a 1:200 dilution of 2C9 reduced its titer from 4.1×10^7 to 1.6×10^4 PFU/ml, a 2,500-fold decrease (Table 1); similarly, incubation with a 1:1,000 dilution of monoclonal antibody 159 reduced the titer of the P strain from 1.1×10^7 to 2.5×10^3 PFU/ml, a 4,400-fold reduction. Neither monoclonal antibody had any detectable effect on the infectivity of the opposite, or heterologous, strain. These reductions were consistently observed during three repeated experiments. When the progeny of the Wa $\times$ P coinfection were neutralized under the same conditions, an initial titer of 2.5×10^7 PFU/ml was reduced to 7.5×10^5 or 1.2×10^7 PFU/ml by monoclonal antibody 2C9 or 159, respectively (Table 1). These were decreases of 97 and 52%, respectively, or a combined total of 149%. This result suggested that approximately 50% of progeny viruses formed during the Wa $\times$ P

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TABLE 1. Effect of neutralizing monoclonal antibodies 2C9 and 159 on rotavirus strains Wa and P and the progeny of their coinfection

Virus	Monoclonal antibody	Viral titer (PFU/ml)		% Survival
		Before treatment	After treatment	
Wa	2C9	4.1×10^7	1.6×10^4	0.04
	159	4.1×10^7	4.1×10^7	100.0
P	2C9	1.1×10^7	1.1×10^7	100.0
	159	1.1×10^7	2.5×10^3	0.02
Wa $\times$ P progeny	2C9	2.5×10^7	7.5×10^5	3.0
	159	2.5×10^7	1.2×10^7	48.0

coinfection were mosaics; i.e., they contained both Wa and P VP7 proteins at sites available for neutralization.

Phenotypic mixing within these progeny viruses was further examined in relation to their genotypic distributions. A total of 50 plaque-purified isolates were obtained without further treatment and examined by polyacrylamide gel electrophoresis (Table 2). After neutralization with either 2C9 or 159 monoclonal antibody, 43 and 54 isolates, respectively, were obtained and examined. Progeny obtained without further treatment contained an excess of Wa segments in both parental and reassortant genotypes. This is in agreement with the observation that the Wa parent also grew to higher titers than the P parent (Table 1). Treatment with monoclonal antibody 2C9 resulted in significant increases in both the percentage of isolates with the parental P genotype ($P = 0.0001$), as determined by a two-tailed Fisher exact test, and percentage of P segments in reassortants ($P = 0.008$) (Table 2). However, it should be noted that the percentage of reassortants with P segment 9, the segment that encodes VP7 (8, 11, 12), did not increase relative to that of the mean of the other segments. Similarly, treatment with monoclonal antibody 159 caused a significant overall increase in the percentage of progeny with the parental Wa genotype ($P = 0.01$) and with Wa segments within reassortants ($P = 0.005$). However, the increase of Wa segment 9 in reassortants was no greater than that of the mean for the other combined segments. This result indicates that there was a relationship between the viral origin of the VP7 proteins and genome segments in these particles, possibly due to partial compartmentalization of viral molecules within the infected cell. However, the relationship was no greater for the segment coding for the VP7 protein than for the other nine segments that could be analyzed. Greater numbers of progeny would have to be examined to confirm these suggestions.

From these results, it was possible to calculate the percentage of progeny viruses produced after Wa $\times$ P coinfection

TABLE 3. Relationship between VP7 proteins and genome segment 9 in the progeny of Wa $\times$ P coinfection

Origin of segment 9	% Particles with specified VP7 from ^a :		
	P only	P and Wa ^b	Wa only
Reassortants			
P	4.2	79.6	16.2 ^c
Wa	2.6 ^c	63.6	33.8
Total progeny			
P	8.4	76.8	14.8 ^c
Wa	1.3 ^c	40.2	58.5

^a Method of calculation is described in the text.

^b Mosaics.

^c Pseudotypes.

tion that contained either Wa or P segment 9 and that were phenotypic mosaics or pseudotypes. For example, to determine these percentages for reassortants with P segment 9, the following calculation was made. Of the original 2.5×10^7 PFU/ml, 11 of 50 were reassortants with P segment 9, a concentration of 5.5×10^6 PFU/ml. After treatment with monoclonal antibody 2C9, a titer of 7.5×10^5 PFU/ml was observed, and 13 of 43 isolates were reassortants with P segment 9, a titer of 2.3×10^5 PFU/ml. Thus, it appeared that 2.3×10^5 (4.2%) of 5.5×10^6 reassortants with P segment 9 contained only P VP7. The remaining 95.8% contained at least some Wa VP7 at a neutralization site. Similarly, when the progeny of coinfection were treated with monoclonal antibody 159, the titer was reduced to 1.2×10^7 PFU/ml and 4 of 54 isolates were reassortants that contained P segment 9, a concentration of 8.9×10^5 PFU/ml. Thus, of the original 5.5×10^6 reassortants with P segment 9, it appeared that 16.2% were pseudotypes containing only Wa VP7 and 79.6% (95.8% - 16.2%) were mosaics containing both Wa and P VP7 proteins at neutralization sites. These results are summarized in Table 3, along with similar calculated values for reassortants with Wa segment 9 as well as for total progeny (parental and reassortant genotypes) with either Wa or P segment 9.

The calculations used to determine the percentage of mosaics found after coinfection with Wa and P strains are presumed to denote minimum values. Quite possibly, viral particles with both Wa and P VP7 proteins may have been neutralized by only one of the two monoclonal antibodies. For example, the epitopes on VP7 molecules recognizable by one of the monoclonal antibodies may have been inaccessible because of configurational restrictions within the capsid. Alternatively, these VP7 proteins may have been present either at sites not involved in neutralization or in amounts too low to represent a sufficient portion of a neutralization site. Other explanations are also possible.

TABLE 2. Genotypic analysis of the progeny of Wa $\times$ P coinfection plaque picked either after no treatment or after neutralization with monoclonal antibody 2C9 or 159

Treatment	Total no. of isolates	Parental origin of segment	No. with parental genotype	No. of reassortants with segment:											Total segments in reassortants (%)
				1	2	3	4	5	6	7	8	9	10	11	
None	50	Wa	25	15	14	17	20	19	ND ^a	18	16	13	22	14	168 (70)
		P	1	9	10	7	4	5	ND	6	8	11	2	10	72 (30)
2C9	43	Wa	4	12	11	13	20	7	ND	15	15	10	16	15	134 (58)
		P	16	11	12	10	3	16	ND	8	8	13	7	8	96 (42)
159	54	Wa	40	11	6	13	12	14	ND	11	14	10	14	11	116 (83)
		P	0	3	8	1	2	0	ND	3	0	4	0	3	24 (17)

^a ND, Not determinable.

TABLE 4. Genotypic analysis of the progeny of Wa $\times$ P coinfection plaque picked from plates with agarose overlay containing either monoclonal antibody 2C9 or 159

Monoclonal antibody	Total no. of isolates	Parental origin of segment	No. with parental genotype	No. of reassortants with segment:											Total segments in reassortants
				1	2	3	4	5	6	7	8	9	10	11	
2C9	59	Wa	0	29	26	27	33	26	ND ^a	23	29	0	27	21	241
		P	20	10	13	12	6	13	ND	16	10	39	12	18	149
159	29	Wa	22	2	6	7	5	6	ND	7	6	7	2	5	53
		P	0	5	1	0	2	1	ND	0	1	0	5	2	17

^a ND, Not determinable.

It is highly unlikely that these results could have been confounded by a low percentage of progeny viruses that contained sufficient VP7 molecules at a neutralization site and that these molecules were available to bind neutralizing antibody but the viruses still escaped neutralization by the homologous monoclonal antibody. As already noted, the reduction in titer for Wa and P by their homologous monoclonal antibodies was $\geq 2,500$ -fold (Table 1). This level of escape would have been undetectable in these experiments.

To further establish that these observations were due to phenotypic mixing and not some unusual property of the progeny of coinfection, the analysis was repeated after the addition of either 2C9 (1:1,000 dilution) or 159 (1:5,000 dilution) only to the agarose-containing medium used to overlay the culture for plaque picking of viruses. The combined total reduction in PFU by these monoclonal antibodies was approximately 100%, as anticipated. Furthermore, only viruses with segment 9 heterologous to the specificity of the monoclonal antibody in the agarose overlay were found in genotypic analysis of isolates picked in the presence of either 2C9 or 159 (Table 4). Thus, the phenotypes of isolates picked under this condition matched their genotypes.

The results of this study indicate that both pseudotypes and mosaics of the capsid protein VP7 readily formed after coinfection of cells with rotavirus strains Wa and P. Under the conditions of this experiment, the probability was somewhat greater that genome segments of these two viruses would be packaged in capsids containing the homologous rather than the heterologous VP7 (Tables 2 and 3). Therefore, neutralization of the progeny of coinfection before inoculation of cells for plaque picking should cause some increase in the probability of selecting viruses with the desired genotypes. However, this probability should be greatly magnified if plaques are selected with neutralizing antibody only in the overlay medium. Thus, the latter procedure is clearly the method of choice for selection of viruses with VP3 and VP7 proteins from the desired parent.

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