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Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity

(intertypic rotavirus/rotaviral gene 4/antigenic characterization/new classification system)

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ABSTRACT **Antiserum prepared against the M37 strain of rotavirus, recovered from an asymptomatic newborn infant in Venezuela, neutralized two prototype human rotaviruses that define two separate serotypes: serotype 1 (Wa) and serotype 4 (ST3). Thus, the M37 strain is a naturally occurring intertypic rotavirus. Analysis of reassortant viruses produced during coinfection *in vitro* indicated that the observed dual serotype specificity of M37 resulted from sharing a related outer capsid protein, VP3, with the ST3 virus and another related outer capsid protein, VP7, with the Wa virus. Analysis of single (VP3)-gene-substitution reassortants indicated that VP3 was as potent an immunogen as VP7. In addition, direct evidence was obtained that the serotype specificity of neutralizing antibody elicited by VP3 can differ from the serotype specificity of neutralizing antibody elicited by VP7, indicating the need for a dual system of rotavirus classification in which the neutralization specificity of both VP3 and VP7 outer capsid proteins are identified.**

Rotaviruses, members of the reoviridae family, are major etiologic agents of acute nonbacterial gastroenteritis of the young in a wide variety of mammalian and avian species including humans. The need for immunoprophylaxis against rotaviral gastroenteritis has stimulated interest in the biochemical, molecular, genetic, and clinical aspects of these agents with the aim of developing safe and effective vaccines (1–3).

The genome of rotaviruses consists of 11 discrete segments (genes) of double-stranded (ds) RNA. These genes reassort with high efficiency during coinfection, and this property has facilitated the selection of reassortant viruses with a mixed constellation of genes derived from two biologically and antigenically distinct rotaviruses. Analysis of reassortant viruses has provided much of our current understanding of gene–product relationships. For example, the fourth gene segment has been shown to code for the outer capsid hemagglutinin protein VP3 (4), which is also the site for protease activation of infectivity (4) and for host-range restriction of rotavirus infectivity (5, 6). The major subgroup antigen(s) was shown to be coded for by the sixth RNA genome segment (7, 8). The eighth or ninth genome segment, depending on the virus strain, was shown to code for a major neutralization antigen, VP7 (6, 7, 9).

Hybridoma technology has also aided the functional and structural analysis of the relatively complex rotaviruses. For example, some monoclonal antibodies directed against the fourth rotaviral gene product, VP3, exhibit both hemagglutination-inhibiting and neutralizing activity (8–10). Also, subgroup-specific monoclonal antibodies react with the sixth gene product, VP6 (11), whereas certain monoclonal antibodies directed against the eighth or ninth rotaviral gene product, VP7, exhibit a high level of neutralizing activity (9, 10, 12, 13). Until now the outer capsid VP7 protein has been considered the major neutralization antigen. The recent observation that some monoclonal antibodies directed against VP3 exhibit a moderate to high level of neutralizing activity was not surprising, however, because this antigen is also located on the outer capsid (9, 10, 13).

During the course of analyzing rotavirus isolates by the plaque-reduction neutralization (PRN) technique, we observed that hyperimmune guinea pig antiserum raised against the Venezuelan neonatal rotavirus isolate M37 neutralized both serotype 1 (strain Wa) and serotype 4 (strain St. Thomas no. 3) rotaviruses to the same degree (14). A combined genetic and serologic analysis of this "intertypic bridging" phenomenon indicated that the VP3 and VP7 outer capsid proteins of the M37 rotavirus each played a role in the observed dual serotype of the neonatal rotavirus isolate. This indicated that these neutralization specificities present on VP3 and VP7 segregate independently in nature.

MATERIALS AND METHODS

Viruses. The following cultivatable rotaviruses were used in this study: human rotavirus Wa (serotype 1); human rotavirus M37 (serotypes 1 and 4); human rotavirus St. Thomas no. 3 (ST3) (serotype 4); bovine rotaviruses UK and NCDV (serotype 6). All rotaviruses were triply plaque-purified in this laboratory before use in the current experiments.

Cell Cultures. Primary African green monkey kidney (AGMK) cell cultures (M. A. Bioproducts, Walkersville, MD) were used for genetic reassortment. An established cell line of fetal rhesus monkey kidney cells, MA104, was used for selection of reassortant viruses, for virus propagation, and for PRN assay.

Hyperimmune Antisera. All hyperimmune antisera were prepared in guinea pigs as described (15, 16). Hyperimmune antisera were heat-inactivated at 56°C for 30 min before use.

Genetic Reassortment and Selection of Reassortants. AGMK cell monolayers were infected with human rotavirus [pretreated with trypsin (2 μg/ml)] at a multiplicity of infection (moi) of $\approx$1, after which the cultures were incubated for 60 min at 37°C. The monolayers were then washed once and infected with the UK strain of bovine rotavirus (also pretreated with trypsin) at a moi of $\approx$1, and the cultures then were incubated for 60 min at 37°C. The cell cultures were washed twice and fed with Eagle's minimal essential medium (MEM) supplemented with glutamine, trypsin (0.5 μg/ml), and antibiotics. The cultures were frozen and thawed once

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Abbreviations: PRN, plaque-reduction neutralization; ds RNA; double-stranded RNA.

and then harvested when 50–80% of the cells showed cytopathic effects.

Selection of the desired reassortants was accomplished by the method described by Midthun *et al.* (17). Briefly, the coinfected cell culture lysates were inoculated onto MA104 cell monolayers in the presence of hyperimmune guinea pig antiserum raised against the NCDV strain of bovine rotavirus. This antiserum has a high titer of neutralizing antibodies directed against VP7 of the UK bovine rotavirus but lacks detectable neutralizing antibodies directed against VP3 of the UK strain. Each reassortant clone was plaque-purified three times in MA104 cell monolayers.

PRN Assay. Antigenic characterization of selected reassortant rotaviruses was carried out by PRN assay using 4-fold dilutions of serum and MA104 cell monolayers in Costar plastic 6-well plates as the assay system, as described (15, 16). Sixty percent plaque-reduction end points were calculated. A 20-fold reciprocal difference between homologous and heterologous neutralizing antibody titers was considered significant.

Polyacrylamide Gel Electrophoresis (PAGE) of Genomic ds RNA. Genomic ds RNAs were extracted from lysates of MA104 cells infected with reassortant or parental rotavirus, using phenol-chloroform as detailed previously (17). RNAs were analyzed by PAGE on 10% slab gels with 4% stacking gels, using a Tris/glycine buffer system as described by Laemmli (18). After electrophoresis, the ds RNA bands were visualized under UV light after staining with ethidium bromide.

Genotype Analysis of Reassortants by RNA·RNA Hybridization Assay. RNA·RNA hybridization assays were performed to determine the parental origin of each of the genes present in each reassortant rotavirus, as described previously (19). Briefly, ^{32}P-labeled transcription probes, prepared *in vitro* from purified single-shelled rotavirus particles, were hybridized to heat-denatured genomic ds RNAs. The RNA hybrids that formed were analyzed by PAGE followed by ethidium bromide staining and visualization under UV light. The slab gels were then dried and autoradiographed on Kodak X-Omat AR film.

RESULTS

Dual Serotype Specificity of M37 Rotavirus. The M37 strain of rotavirus was recovered from a newborn infant who underwent an asymptomatic infection. Antisera prepared in guinea pigs against the M37 rotavirus neutralized the serotype 1 prototype strain (Wa) and the serotype 4 prototype strain (ST3) to the same extent and to the same titer as the homologous virus (M37) (Table 1). Antiserum to Wa virus neutralized M37 virus to high titer, whereas antiserum to ST3 virus had a neutralizing antibody titer for M37 that was significantly lower than its homologous titer (Table 1). This indicated that the crossneutralization relationship between M37 and ST3 viruses was not completely reciprocal.

Generation of Human (M37 or ST3 Strain) × Bovine (UK Strain) Rotavirus Reassortants. Reassortant viruses were prepared from the M37 or ST3 human rotavirus and the bovine UK rotavirus (serotype 6) in order to define the rotavirus proteins responsible for the dual serotype of the neonatal rotavirus isolate. Two classes of human rotavirus (M37 or ST3 strain) × bovine rotavirus (UK strain) reassortants were selected under the differential pressure of hyperimmune antiserum raised against the NCDV (serotype 6) strain of bovine rotavirus. One group of reassortants derived 10 gene segments from a human rotavirus (M37 or ST3 strain) and a single gene segment (the fourth gene) from the UK bovine rotavirus (Fig. 1 and Table 1). The other group derived 10 gene segments from the UK bovine rotavirus and a single gene segment (the ninth gene, which codes for the major neutralization protein) from a human rotavirus (M37 or ST3 strain) (Table 1).

Antigenic Analysis of Human × Bovine Rotavirus Reassortants by PRN. A single-gene-substitution M37 × UK reassortant, 11-1, which received only VP7 from its M37 parent, induced a high titer of neutralizing antibodies against M37, confirming that VP7 bears major antigenic determinants involved in virus neutralization. However, the ST3 human rotavirus strain (serotype 4), which was neutralized to a high titer by hyperimmune antiserum (1:20,480) to the M37 human rotavirus strain (serotypes 1 and 4), was not neutralized significantly by antiserum raised against reassortant 11-1, indicating that the major neutralization VP7 protein of M37 does not play a role in the bridging phenomenon between ST3 and M37 (Table 1). The high titer of neutralizing antibodies against M37 in the 11-1 reassortant antiserum indicates that the M37 VP7 protein present in this reassortant is highly antigenic.

Substitution of the UK bovine rotavirus VP3 for the corresponding protein of the ST3 human rotavirus in reassortant 55-1 abrogated the high level of reactivity of ST3 with neutralizing antibodies directed against the M37 strain. This suggests that the VP3 of M37 plays a major role in the bridging phenomenon between ST3 and M37. Also, antiserum against a M37 × UK reassortant, 10-1, which received only its VP3 gene from the UK parent, exhibited considerably less neutralizing activity against ST3 virus than M37 (Table 1). Thus, substitution of bovine VP3 for M37 VP3 in a reassortant used

Table 1. Antigenic characterization of single-gene-substitution reassortant rotaviruses by PRN assay

	Origin of gene			Reciprocal of 60% PRN antibody titer of hyperimmune antiserum*						
Rotavirus	VP3	VP7	All others	Wa	M37	ST3	UK	NCDV	M37 × UK (10-1)	M37 × UK (11-1)
Human										
Wa (serotype 1)	Wa	Wa	Wa	*40,960*	20,480	80	<80	<80	20,480	20,480
M37 (serotype 1 [and 4])	M37	M37	M37	5,120	*40,960*	640	<80	<80	40,960	40,960
ST3 (serotype 4)	ST3	ST3	ST3	160	20,480	*20,480*	<80	<80	160	80
Bovine										
UK (serotype 6)	UK	UK	UK	<80	<80	<80	*40,960*	40,960	10,240	10,240
NCDV (serotype 6)	NCDV	NCDV	NCDV	<80	<80	80	20,480	*40,960*	<80	<80
Reassortant										
M37 × UK (10-1)	UK	M37	M37	10,240	40,960	80	10,240	<80	*40,960*	40,960
M37 × UK (11-1)	UK	M37	UK	5,120	20,480	<80	5,120	<80	20,480	*81,920*
ST3 × UK (55-1)	UK	ST3	ST3	<80	80	40,960	10,240	<80	10,240	10,240
ST3 × UK (52-1-1)	UK	ST3	UK	<80	80	10,240	5,120	<80	5,120	20,480

*Values derived from seven different tests, each of which involved nine different viruses and a single hyperimmune antiserum (indicated above each column). Homologous titers are given in italics.

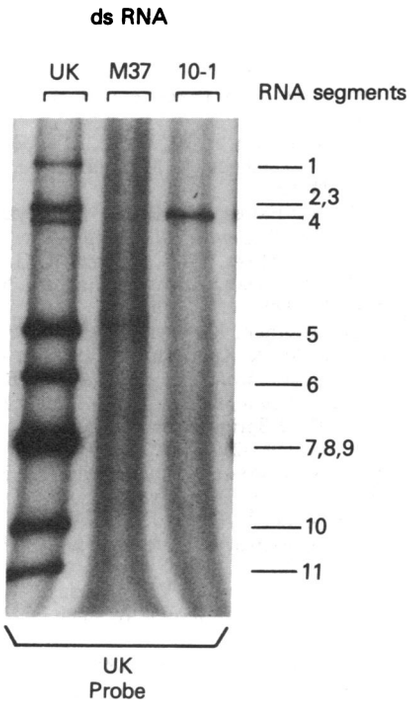

FIG. 1. Genotype analysis of the M37 × UK rotavirus reassortant 10-1 by RNA·RNA hybridization and PAGE. The fourth gene of reassortant 10-1 was derived from the bovine UK rotavirus parent while the remaining 10 genes were derived from the human M37 rotavirus parent.

for immunization abrogated most of the neutralizing antibody response against the human rotavirus ST3. This observation adds further support to the view that the basis for the predominantly unidirectional neutralization relationship between human rotaviruses M37 and ST3 is shared antigenic determinants on VP3.

A single human rotavirus gene substitution ST3 × UK reassortant, 52-1-1, containing the VP7 gene of ST3 and the remaining 10 genes from the UK bovine rotavirus, was not neutralized to a significant degree by antiserum to M37, confirming that the VP7 neutralization protein of ST3 does not play a role in the bridging phenomenon between ST3 and M37 (Table 1).

Specificity of Antibodies Directed at VP3 of the UK Bovine Rotavirus. Substitution of bovine UK rotavirus VP3 for the corresponding VP3 of the M37 or ST3 human rotavirus conferred on the resulting single-gene-substitution reassortants (10-1 and 55-1) the capacity to be neutralized efficiently by antibodies induced by the bovine UK rotavirus, but not by antibodies induced against another bovine rotavirus, NCDV, which has been classified as serotype 6 (Table 1). This further supports the view that VP3, in addition to VP7, elicits and reacts with antibodies that neutralize virus infectivity. Furthermore, this suggests that the high level of crossneutralization observed between UK and NCDV cannot be ascribed to shared antigenic determinants on VP3. It is very likely that the shared antigenic determinants responsible for the crossneutralization of these viruses are present on VP7, but this remains to be established. In any case, it should be noted that these two bovine rotaviruses possess antigenically distinct VP3 antigens.

DISCUSSION

Following the discovery that growth of wild-type rotaviruses *in vitro* was facilitated by trypsin treatment of virus, incorporation of trypsin in the maintenance medium, and incubation of inoculated cell cultures on a roller apparatus, it became possible to isolate and grow virtually any rotavirus in cell culture and to identify its serotype specificity (1–3). Systematic characterization of rotaviruses isolated from humans and animals indicated that a proportion of strains appeared to possess dual serotype specificity. Such strains were neutralized by two distinct antisera that defined two separate serotype specificities (14, 20, 21). In the present report, we have characterized one such intertypic rotavirus isolate, the M37 strain derived from an asymptomatic human neonate in Venezuela. Antiserum prepared against M37 virus neutralized both the prototype serotype 1 Wa virus and the prototype serotype 4 ST3 virus (21). Analysis of reassortant viruses prepared from the M37 strain and the prototype serotype 4 ST3 virus indicated that the former virus shared VP3 antigenic specificity with the latter virus, whereas VP7 antigenic specificity of M37 strain was shared with the prototype serotype 1 Wa virus. These findings provide an explanation for the dual serotype specificity of the M37 strain and indicated that VP3 also possesses determinants that induce and react with neutralizing antibodies.

In a recent report describing the development and serotype characterization of reassortant rotaviruses as potential vaccine strains, Midthun *et al.* (17) observed low-level neutralizing crossreactivity between single human rotavirus gene substitution reassortants and hyperimmune antisera to their animal rotavirus parent and speculated that the fourth-gene product, VP3, was a likely candidate for the observed minor neutralization specificity. Using a genetic approach, we have now provided direct evidence that the VP3 gene is responsible for the predominantly one-way crossneutralizing reactivity between M37 (serotypes 1 and 4) and ST3 (serotype 4) rotaviruses.

Preliminary observations suggest that VP3 also plays a role in the one-way crossneutralizing reactivity, reported previously (21), between canine rotavirus strain CU-1 (serotype 3) and porcine rotavirus strains Gottfried (serotype 4), SB-1A (serotypes 4 and 5), and OSU (serotype 5). Although direct evidence for genetic reassortment of rotaviruses under natural conditions has not been obtained, it is likely that this occurs because of the high efficiency with which rotavirus genes reassort during coinfection *in vitro*. The M37 strain may be an example of a naturally occurring reassortant between serotype 1 and serotype 4 rotaviruses. Similarly, the CU-1, Gottfried, SB-1A, and OSU animal rotavirus interrelationships probably also reflect gene reassortment in nature.

Several monoclonal antibodies directed against VP3 have been shown to have neutralizing activity (9, 10, 13). However, the number of neutralization epitopes on VP3 and the extent of the antibody response directed against it during natural infection are not known. In the present study, single bovine rotavirus gene reassortants that derived only VP3 from the UK bovine rotavirus (serotype 6) and the remaining 10 genes from the M37 or ST3 human rotavirus were prepared and employed to study the antigenicity of this outer capsid protein. Hyperimmune antiserum raised against the M37 × UK reassortant 10-1 provided information concerning the antigenicity of VP3. Previously it had been shown that VP7 contains major determinants of neutralization specificity (6, 7, 9), and hence the observation that a rotavirus can exhibit two distinct neutralization specificities was surprising. The hyperimmune antiserum to the M37 × UK reassortant 10-1 neutralized both the M37 human rotavirus and the UK bovine rotavirus (serotype 6) to the same degree (Table 1). This suggests that VP3 may be as potent an immunogen as VP7, which was previously identified as a major neutralization antigen. It is not known at present, however, at which stage(s) of infection antibodies against those two proteins interact with and neutralize virus. The role of the VP3 protein in inducing protective antibody and its importance in the

development of an effective human rotavirus vaccine merit further study.

Although antiserum to the single bovine rotavirus gene (VP3) substitution M37 × UK reassortant 10-1 neutralized the UK strain to high titer, it did not recognize another well-studied bovine rotavirus, the NCDV strain, which has also been assigned to serotype 6 (Table 1). This indicates that, although UK and NCDV appear to share highly related VP7 antigens, their VP3 antigens are quite distinct. This situation, the reciprocal of the M37–ST3 relationship, also suggests that a reevaluation of the serotype designation and classification of rotaviruses is indicated.

In a previous report describing antigenic relationships of human and other mammalian and avian rotaviruses as defined by neutralization, it was suggested that it may be necessary in a future system of rotavirus classification to identify the distinct antigenic specificities present on the VP3 and VP7 outer capsid proteins of each rotavirus isolate (21). We have now presented an example of a rotavirus that requires this form of classification in order to explain its neutralization specificities. The M37 human rotavirus was demonstrated to have two distinct neutralization specificities: serotype 4 based on VP3 (the fourth-gene function), and serotype 1 based on VP7 (the ninth-gene function). Thus, a unified system for the classification and nomenclature of the two distinct surface proteins of rotaviruses now becomes a necessity. Obviously, much more information on antigenic specificity of the VP3 of various rotaviruses is necessary in order to implement the desired classification scheme. Nonetheless, a binary system similar to that used for the influenza A viruses would seem needed. Rather than designate a single serotype assignment, it would seem desirable to identify both neutralization proteins VP3 and VP7. For example, the Wa virus, the prototype of serotype 1 in the current classification, would be designated VP3 serotype 1 (VP3:1), VP7 serotype 1 (VP7:1). ST3 would be designated VP3:4, VP7:4, whereas the intertypic M37 virus would be designated VP3:4, VP7:1.

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