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# Serological Characterization of Human Reassortant Rotaviruses

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**We analyzed the serological properties of two human wild-type cell culture-adapted rotaviruses (strains 308 and 46) and of 308 × 46 reassortants which were previously obtained and genetically characterized. Strain 308, exhibiting a so-called long RNA pattern, was found to belong to human rotavirus subgroup II, serotype 1, whereas strain 46, exhibiting a so-called short RNA pattern, represented subgroup I, serotype 2. Among the 308 × 46 reassortants we analyzed, two belonged to subgroup II, serotype 1, and exhibited short RNA patterns. This showed that the correlation observed between human subgroups I and II rotaviruses and the short and long electrophoretic patterns is not supported by any molecular basis (i.e., gene segment 10 or 11 was not involved in the subgroup specificity).**

Recent research on human rotaviruses has provided a better understanding of their antigenic characterization, although the characterization remains far from complete. Progress has been made mainly by the following major advances: (i) the increasing number of well-characterized strains adapted to cell culture conditions (2, 5, 14, 30); (ii) the development of reliable assays allowing serological characterization of a large number of field isolates (3, 13, 23, 27, 30); (iii) the production of monoclonal antibodies directed at different antigenic specificities (6, 24–26); (iv) the extensive study of rotavirus RNA which has allowed the definition of new serological groups (pararotavirus) (19, 21); and (v) the use of gene reassortment techniques with strains of rotaviruses (8, 9, 12, 17).

Most rotaviruses isolated from different species share a group antigen; it has been proposed that these viruses be classified as group A rotaviruses (20).

Group A rotaviruses can be serologically characterized into serotypes and subgroups. Human rotaviruses have been divided into four and possibly five distinct serotypes (1–4) by plaque neutralization tests (28–30), immunofluorescence focus reduction assays (2, 3), or enzyme-linked immunosorbent assays (ELISA) (27). Subgroups I and II have also been defined by immune adherence hemagglutination assay (13) and ELISA by use of either polyclonal sera (27) or monoclonal antibodies (6, 26). In addition, it has been observed of human rotaviruses that serotype 2 is associated only with subgroup I (22).

The molecular bases of these antigenic specificities have been well established. Group and subgroup specificities are located on the 42,000-molecular-weight protein (42K protein) VP6 which is encoded by gene segment 6 (12, 16). Type specificity is located on VP7, the major neutralization antigen, which is encoded by gene segment 8 or 9 according to the genomic pattern of the strain (4, 7, 12). In addition, VP3, the product of gene segment 4, has been proposed as a minor neutralization antigen (6, 10, 17).

Based on studies of electrophoretic patterns and serological properties, it has been reported that subgroup I is related to the short pattern (i.e., slow migration in polyacrylamide gel electrophoresis of segments 10 and 11) and subgroup II is related to the long pattern (i.e., fast migration in polyacrylamide gel electrophoresis of gene segments 10 and 11) (11, 26). However, this relationship does not apply to rotaviruses of animal origin.

In the present report we analyzed the serological properties of two human wild-type cell culture-adapted rotaviruses, strain 308 (long RNA pattern) and strain 46 (short RNA pattern), and eight reassorted viruses obtained after in vitro mixed infection with strains 308 and 46. These reassortants were genetically characterized in a previous study (5). This allowed us to show that a reassorted virus can belong to subgroup II, serotype 1, even though it exhibits a short RNA pattern.

Monoclonal antibodies directed against subgroup I (monoclone 255/60) and subgroup II (monoclone 631/9) (6) were used for subgroup determination by ELISA. Polyclonal rabbit hyperimmune antiserum (720) to human rotavirus (1) was used as capture antibody. All antisera were kindly supplied by T. Flewett. The ELISA used for subgroup determination was performed by the technique of Beards and Flewett (2). Strain 308 clearly reacted with subgroup II-specific monoclone 631/9 without cross-reaction with subgroup I-specific monoclone 255/60 (Table 1) and therefore could be classified as antigenic subgroup II. Furthermore, strain 46 could be classified as subgroup I. These results are in agreement with subgroup determination by the short or long RNA pattern. Subgroup determination was also performed for eight 308 × 46 reassortants. The parental origins of each RNA genomic segment for these viruses are given in Table 1, except those for segments 2, 3, and 6 whose parent strains were electrophoretically indistinguishable. Six reassorted viruses exhibited long RNA patterns (strains $A_2$, $A_{742}$, $A_9$, $B_{155}$, $C_1$, and $F_{148}$). Among these viruses, $A_{742}$ derived both genes 10 and 11 from parent strain 308. The other five reassortants received segment 11 from strain 308 and segment 10 from segment 11 of strain 46. This could occur because segments 10 and 11 of strain 308 have been shown to be genetically equivalent to segments 11 and 10, respectively, of strain 46 (5). Two reassorted viruses exhibited short RNA patterns ($D_1$ and $E_{757}$); these viruses retained both genes 10 and 11 from parent strain 46. Results of subgrouping by ELISA are summarized in Table 1. All reassortants were found to belong to subgroup II. Of interest was the fact that reassortants $D_1$ and $E_{757}$ exhibited short RNA patterns but belonged to subgroup II. This result clearly demonstrates that neither gene 10 nor gene 11 are involved in subgroup specificity.

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TABLE 1. Subgroup determination by ELISA of strains 308 and 46 and 308 × 46 reassortants

| Virus | Optical density with monocolonal antibody[a]: | | Subgroup by ELISA | RNA pattern | Parental origin of RNA segments in 308 × 46 reassortants[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 255/60 (anti-subgroup I) | 631/9 (anti-subgroup II) | | | 1 | 4 | 5 | 7 | 8 | 9 | 10 | 11 |
| Parent strains | | | | | | | | | | | | |
| 308 | 0.017 | 1.998 | II | Long | | | | | | | | |
| 46 | 1.879 | 0.226 | I | Short | | | | | | | | |
| Reassortants | | | | | | | | | | | | |
| $A_{742}$ | 0.049 | >2 | II | Long | L | L | S | $L_7$ | $L_8$ | $L_9$ | $L_{10}$ | $L_{11}$ |
| $A_2$ | 0.062 | 1.841 | II | Long | S | S | S | $S_7$ | $S_9$ | $L_9$ | $S_{11}$ | $L_{11}$ |
| $A_9$ | 0.028 | >2 | II | Long | S | L | L | $L_7$ | $L_8$ | $L_9$ | $S_{11}$ | $L_{11}$ |
| $B_{155}$ | 0.040 | 1.552 | II | Long | L | L | L | $L_7$ | $L_8$ | $L_9$ | $S_{11}$ | $L_{11}$ |
| $C_1$ | 0.034 | 1.655 | II | Long | S | L | S | $L_8$ | $S_9$ | $L_9$ | $S_{11}$ | $L_{11}$ |
| $F_{148}$ | 0.038 | 1.684 | II | Long | S | L | L | $S_7$ | $S_9$ | $L_9$ | $S_{11}$ | $L_{11}$ |
| $D_1$ | 0.035 | 1.700 | II | Short | S | L | S | $S_7$ | $S_9$ | $L_9$ | $S_{10}$ | $S_{11}$ |
| $E_{757}$ | 0.092 | 1.980 | II | Short | S | L | L | $S_7$ | $L_7$ | $L_9$ | $S_{10}$ | $S_{11}$ |
| Controls[c] | | | | | | | | | | | | |
| DS-1 | 1.672 | 0.150 | I | Short | | | | | | | | |
| By 1 | >2 | 0.186 | I | Short | | | | | | | | |
| Mc 2 | 0.050 | 1.482 | II | Long | | | | | | | | |

[a] Average optical density from two wells.

[b] S, RNA segment derived from strain 46 (short RNA pattern); L, RNA segment derived from strain 308 (long RNA pattern). In some cases, a segment from a reassortant was equivalent to a parental segment of a different number. The equivalent segment number in the parent strain is indicated by a subscript. Segments 2, 3, and 6 of the parent strains were electrophoretically indistinguishable.

[c] Reference strain DS-1 (subgroup I, serotype 2) and two well-characterized human isolates, By 1 (subgroup I, serotype 2) and Mc 2 (subgroup II, serotype 3) (26), were used as controls.

Immunoprecipitation by subgroup II-specific monoclone 631/9 was done on [$^{35}$S]methionine-labeled cell lysate infected by strains 308 and 46 and by reassortants $A_2$, $A_9$, $C_1$, $D_1$, and $E_{757}$. This monoclonal antibody has been shown to immunoprecipitate the 42K major inner-shell protein VP6 (6). Monoclone 631/9 recognized the 42K proteins from strain 308 and from the five 308 × 46 reassortants, including those exhibiting short RNA patterns ($D_1$ and $E_{757}$) (Fig. 1). The 42K protein from strain 46 was not immunoprecipitated, however, by this subgroup II-specific monoclonal antibody. These results are in agreement with subgroup determinations obtained by ELISA. Furthermore, because the 42K protein VP6 is encoded by gene 6, immunoprecipitation provided indirect evidence that gene 6 was derived from parent strain 308 in all the reassortants tested. This assignment could not have been made by electrophoresis, because segments 6 of the parent strains were electrophoretically indistinguishable.

Serotypic characterization of strains 308 and 46 and the 308 × 46 reassortants was performed by a plaque reduction neutralization test with polyclonal antibodies against both human rotavirus serotypes 1 (antiserum 281) and 2 (antiserum 146) (27). The genomic patterns of the viruses and the results of the plaque reduction neutralization test are given in Table 2. By using a 90% plaque reduction method, strains 308 and 46 could be classified, respectively, as serotypes 1 and 2 based on a 20-fold difference in antibody titers. The four 308 × 46 reassortants ($A_2$, $C_1$, $D_1$ and $E_{757}$) assayed in the plaque reduction neutralization test were chosen because genetic reassortment had occurred between segments 7, 8, or 9 (segments 8 or 9 encode for the major neutralization antigen). In a previous study we showed that the genes contained in RNA segments 7, 8, and 9 differ between the long and short electrophoretype (5). Thus, RNA segments 7, 8, and 9 of strain 308 are genetically equivalent, respectively, to segments 9, 7, and 8 of strain 46. This explains why segment 7 in reassortant $C_1$ and segment 8 in reassortants $A_2$, $D_1$, and $E_{757}$ could not be detected from any of the parental viruses; the genetically equivalent segments from the other parent strain had been substituted for these segments.

The four reassortants tested, including those with short RNA patterns ($D_1$ and $E_{757}$), were found to belong to serotype 1 on the basis of at least a 20-fold difference in reciprocal neutralizing antibody titers. However, a low-level neutralization was noted for reassortant $A_2$ in the presence of antiserum to human serotype 2. It should be noticed that all these reassortants derived segment 9 from parent strain 308. Some of them derived segment 7 ($E_{757}$) or 9 ($C_1$) or both segments 7 and 9 ($A_2$ and $D_1$) from parent strain 46, but none had received segment 8 from strain 46. These results demonstrate that segment 9 of human rotavirus 308 codes for serotype 1 specificity, whereas segment 8 of human rotavirus 46 codes for serotype 2 specificity. The results also confirm the genetic equivalence of segment 8 from strain 308 and segment 9 from strain 46, which had been predicted by analyzing the genotypes of the 308 × 46 reassortants. These findings are in agreement with the gene coding assignments of serotype specificity obtained in previous studies with reassortants between animal and human rotaviruses (12, 17).

Previous reports have underlined an apparent correlation between human subgroup I and II rotaviruses and the so-called short and long electrophoretic patterns (11, 26). In the present work we demonstrated that it was possible to obtain viruses belonging to subgroup II with short electrophoretypes by in vitro reassortment between two human rotaviruses of subgroups I (strain 46) and II (strain 306). Moreover, this result provides evidence that there is no molecular basis for any association between gene segment 10 or 11 and the subgroup antigenic specificity which is encoded by gene segment 6. Thus, in the 308 × 46 reassortants that were analyzed, some had derived gene segment 11 from short-patterned subgroup I parent strain 46, and others had derived both genes 10 and 11 from strain 46, but none of these reassortants belonged to subgroup I. In addition,

immunoprecipitation clearly showed that VP6 and therefore gene segment 6 was derived from subgroup II strain 308. The reason why such a correlation between subgroup and electrophoretype seems to exist in vivo for human rotaviruses remains obscure. However, human isolates with short electrophoretypes are much less common than those with long electrophoretypes (15, 19). If the association between short electrophoretype and subgroup II is very uncommon in nature, then a great number of field isolates will have to be analyzed before such a virus is found. Therefore, as previously pointed out (22), it is probably unwise to infer subgroup from electrophoretype. However, it should be possible to find a human rotavirus of subgroup I associated with a long electrophoretype, as has recently been demonstrated by Nakagomi et al. in a single case (18), but we did not obtain this type of reassortant in vitro. It has been hypothesized that such a virus would very likely be an animal rotavirus (6). In vivo reassortment between two human rotaviruses of different subgroups might also yield this type of virus.

When analyzing the serotypes of 308 × 46 reassortants, we found that they all belonged to serotype 1, the serotype of parent strain 308. Serotype 1 specificity was related to gene segment 9 of strain 308, whereas serotype 2 specificity was related to gene segment 8 of strain 46. Since, as for subgroup I, only serotype 2 viruses have been found associated with the short electrophoretype, one could in this case infer serotype from electrophoretype. We showed that among the serotype 1 reassortants, two exhibited short RNA patterns. If the occurrence of such wild-type viruses is possible, perhaps as the result of an in vivo reassortment, it is then predictable that the correlations between electrophoretype, subgroup, and serotype will not hold true. Although 308 × 46 reassortants clearly belonged to serotype 1, a low-level neutralization was noted for reassortant $A_2$ in the presence of antiserum to human serotype 2. In this reassortant, gene 4 was derived from parent strain 46 (serotype 2), whereas other reassortants ($C_1$, $D_1$, and $E_{757}$) received gene 4 from parent strain 308 (serotype 1). VP3, the product of gene segment 4, has been shown to play a role as a minor neutralization antigen (6, 10, 17). Thus, the presence of antibodies directed against VP3 in the antiserum to human rotavirus serotype 2 could explain the cross-neutralization observed with reassortant $A_2$.

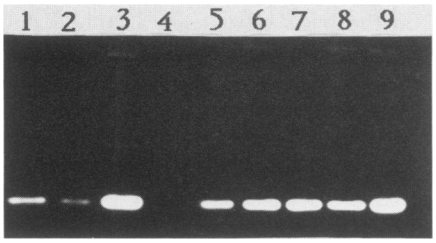


FIG. 1. Immunoprecipitation by 631/9, a subgroup II-specific monoclonal antibody (lanes 3 to 9), of [$^{35}$S]methionine-labeled cell lysate infected with human rotavirus 308 or 46 or with 308 × 46 reassortants. MA 104 cell culture was infected with 5 PFU of rotavirus per cell. The cell culture was labeled with [$^{35}$S]methionine (40 μCi/ml; Amersham Corp.) 6 h at postinfection. Cells were harvested at 18 h postinfection. The soluble proteins were extracted with 50 mM Tris hydrochloride (pH 8)–150 mM NaCl–5 mM EDTA–1% Nonidet P-40–1% deoxycholate and spun at 100,000 × *g* for 1 h. The soluble protein extract was incubated with 50 μl of *Staphylococcus aureus* Cowan. The supernatant was then incubated with the monoclonal antibodies followed by the addition of *S. aureus*. Immune precipitates were washed three times with 50 mM Tris hydrochloride (pH 7.4)–0.5 M NaCl–5 mM EDTA–1% Nonidet P-40–5% sucrose, suspended in sodium dodecyl sulfate sample buffer, boiled for 3 min, and clarified by centrifugation. The supernatant was analyzed by polyacrylamide gel electrophoresis. Lanes: 1 and 2, control immunoprecipitation with a monoclonal antibody directed at the group specificity of major inner-capsid protein VP6; 1, immunoprecipitation of strain 308-infected cell lysate; 2, immunoprecipitation of strain 46-infected cell lysate; 3 to 9, immunoprecipitation with 631/9 subgroup II-specific monoclonal antibody; 3, strain 308; 4, strain 46; 5, $A_2$ reassortant; 6, $A_9$ reassortant; 7, $C_1$ reassortant; 8, $D_1$ reassortant; 9, $E_{757}$ reassortant. Among these reassortants, $D_1$ and $E_{757}$ exhibited short RNA patterns. Complete segment assignments for these reassortants are given in Table 1.

TABLE 2. Serotype determination of strains 308 and 46 and 308 × 46 reassortants[a]

| Virus | Reciprocal titer obtained with antiserum | | Ascribed serotype | Origin of RNA segments[b] | | | Subgroup by ELISA | RNA pattern |
|---|---|---|---|---|---|---|---|---|
| | 281 (against serotype 1) | 146 (against serotype 2) | | 7 | 8 | 9 | | |
| 308 | 10,240 | 40 | 1 | | | | II | Long |
| 46 | <40 | 1,280 | 2 | | | | I | Short |
| $A_2$ | 10,240 | 320 | 1 | $S_7$ | $S_9$ | $L_9$ | II | Long |
| $C_1$ | 10,240 | 80 | 1 | $L_8$ | $S_9$ | $L_9$ | II | Long |
| $D_1$ | 10,240 | 80 | 1 | $S_7$ | $S_9$ | $L_9$ | II | Short |
| $E_{757}$ | 10,240 | 80 | 1 | $S_7$ | $L_7$ | $L_9$ | II | Short |

[a] The plaque reduction neutralization test was performed in six-well plates (Becton Dickinson Labware) of MA 104 cell monolayer. Equal volumes of viruses were diluted in minimal essential medium with 2 μg of trypsin per ml (type III; Sigma Chemical Co.) to yield 50 to 90 PFU per well, and serial twofold serum dilutions (1:40 to 1:40,960) were incubated for 1 h at 37°C. Before inoculation, cells were washed three times with serum-free medium. After 1 h of adsorption, the cells were washed twice and overlaid with minimal essential medium and 0.7% agarose (SeaPlaque; FMC Corp.) containing 1 μg of trypsin per ml. Neutral red staining was performed, usually within 3 days, when an optimal number of plaques (50 to 90) developed in the control wells. Antibody titers were determined by a 90% plaque reduction method. A 20-fold-greater reciprocal difference in antibody titers was considered significant.

[b] Complete segment assignments for these reassortants are given in Table 1.

Recently Midthun et al. (17) have proposed that rotaviruses from the reassortment of wild-type animal viruses and noncultivable human viruses are potential live-vaccine strains. The subgroup II, serotype 1, short-electrophoretype reassortants described in this report may also be promising as vaccines if they are shown to lack pathogenicity for humans after an attenuation step. Three major advantages will then be provided by these reassortants. (i) They contain human rotavirus genes only. (ii) Unlike temperature-sensitive mutants, they have not been exposed to any mutagenic agent. (iii) They possess markers usually not found among human rotavirus isolates, allowing them to be easily distinguishable from wild-type strains. However, additional reassortants of different serotype specificities but with these unusual markers will be needed for use as vaccines.

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