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Rescue and Serotypic Characterization of Noncultivable Human Rotavirus by Gene Reassortment

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Thirty-three of 50 noncultivable human rotavirus strains from a variety of locations were successfully rescued by gene reassortment. The serotype of each of the 33 strains was investigated by a qualitative cytopathic effect neutralization assay. Nineteen strains resembled the previously characterized human rotavirus serotype Wa, whereas three strains were serologically related to the DS-1 strain. Eleven strains appeared to be serotypically distinct from the Wa and DS-1 strains and thus apparently represent one or more new human rotavirus serotypes.

Rotaviruses are an important cause of infantile diarrhea in both humans and animals (11, 17). Unfortunately, the study of the epidemiology, biology, and immunity of human rotaviruses has been hindered by the difficulties encountered in propagating these fastidious viruses in tissue culture (4, 17). Recent studies indicate that at least some human rotavirus isolates may be cultivated directly (13, 14). As a strategy to circumvent the difficulties in cultivation encountered with human rotavirus, we have taken advantage of the segmented nature of the rotavirus genome and its high frequency of gene reassortment during coinfection to rescue noncultivable human rotaviruses by reassorting them with a cultivable *ts* mutant of a bovine rotavirus (5). In this manner, the genes of the noncultivable human rotavirus that restricted growth in vitro were replaced by the corresponding genes from a tissue culture-adapted *ts* bovine rotavirus (5). In our first study we successfully rescued two serotypically distinct human rotavirus strains, Wa and DS-1 (5). In this paper we have extended our preliminary rescue experiments to 50 additional noncultivable human rotavirus strains collected over the past 16 years from many parts of the world. Thirty-three of the strains have been successfully rescued by genetic reassortment.

MATERIALS AND METHODS

Human rotavirus specimens. The country of origin and the frequency of successful rescue of the human rotavirus strains studied are summarized in Table 1. Also included in this table are the laboratory designations and years of origin of the successfully rescued human rotavirus strains. The 50 human stool specimens were selected for study because they were known to contain rotavirus detectable by enzyme-linked immunosorbent assay or electron microscopy (or both); they came from widely diverse locations and were collected primarily from sick children. Specimens AS (no. 3) and 008a (no. 1) were specifically chosen because they were known to be subgroup 1 viruses (10). The four English strains were studied because they were derived from a well-studied outbreak of largely asymptomatic rotavirus infection in a nursery for newborns (1). Specimen F64-40K (BLD) was also obtained from a well child. Specimens 77-25, 77-12, 77-11, 75-82, and 77-28 were obtained from gnotobiotic calves experimentally infected with human rotavirus strains (8, 9, 17). All strains were derived from children less than 4 years of age. Specimens were provided either as stool samples or, in many cases, as rectal swabs or stool suspensions. Most specimens had been stored at −70°C before study.

Rescue of human rotavirus. Rescue experiments were carried out essentially as described previously (5). In cases where only a small amount of stool specimen was available (such as the Bangladesh strains which were all collected as rectal swabs), 0.3 to 1 ml of the sample was extracted once with Genetron 113 and then treated as described previously (5). Only bovine rotavirus UK strain *ts* mutants M7 and M11 were used for rescue in this study (5). Both *ts* bovine rotaviruses were used at a multiplicity of infection of 1 in the rescue experiments. Selection of the desired reassortants was accomplished as described previously in MA104 cells by using restrictive temperature and hyperimmune bovine rotavirus antiserum (5). In all studies, control cell cultures were inoculated with the human stool specimen alone. The growth yield from cultures coinfected with noncultivable human rotavirus and *ts* bovine virus or noncultivable human rotavirus alone was plaqued at 39°C with and without bovine rotavirus antiserum in the overlay. If reassortant plaques developed in the wells treated with antiserum, between one and five were picked and plaque purified one additional time at 39°C with antiserum in the overlay and then amplified in tissue culture for preparation of virus stock and virion RNA.

Qualitative CPE neutralization assay. Doubly cloned

TABLE 1. Human rotavirus strains successfully rescued by gene reassortment

Country of origin of human rotavirus strains studied by genetic reassortment	No. of human reassortants recovered/no. of human strains studied	Laboratory designation of successfully rescued human rotavirus strains	Year of collection	Serotype of human rotavirus reassortant as determined by qualitative neutralization assay		
				Wa	DS-1	Unclassified[a]
United States and Canada	7/13	77-25 (W)	1977	* (2)[b]		
		77-12 (M) (no. 5)	1976			* (2)
		77-11 (G)	1975	* (2)		
		AS (no. 3)	1977		* (1)	
		75-82 (D)	1974	* (2)		
		77-28 (Fh) (K)	1974	*		
		Mc (w)	1981	*		
Guatemala	2/2	6546 (E)	1966	*		
		7300 (F)	1966	*		
Venezuela	1/2	008A (no. 1)	1980		* (1)	
England	4/6	St. Thomas no. 1	1975			*
		St. Thomas no. 2	1975			*
		St. Thomas no. 3	1975			*
		St. Thomas no. 4	1975			*
Egypt	4/4	078-41 (C)	1980	*		
		04/62 (D)	1980	* (2)		
		017 (I)	1979	*		
		053 (J)	1979	*		
Japan	1/2	77-1955 (B)	1977			*
Hong Kong	4/4	NAHL no. 39 (no. 13)	1977	*		
		WALK no. 57 (no. 14)	1977			* (2)
		YIP no. 43 (no. 15)	1977			* (2)
		CMH no. 53 (no. 16)	1977	* (2)		
Malaysia	2/2	YSH (no. 11)	1978	* (2)		
		YCS (no. 12)	1978	*		
Bangladesh	8/15	013592 (BLA)	1978	*		
		006522 (BLB)	1978			* (2)
		F64-40K (BLD)	1978	* (2)		
		F109-41D (BL-E)	1978			* (2)
		F78-40 D (BL-F)	1978	* (2)		
		V14-33-40 (BL-G)	1979	* (2)		
		V14-5-41 (BL-J)	1978		*	
		F227-40D (BL-K)	1978			*

[a] Neither DS-1 nor Wa.

[b] Subgroup specificity determined by immune adherence hemagglutination or the enzyme-linked immunosorbent assay (or by both) given within parentheses. Asterisk indicates that the strain is of the indicated serotype.

reassortant stock or, in many cases, individual plaques diluted in 2 ml of Eagle minimal essential medium with 0.5% gelatin were inoculated into CV1 cell monolayers (24-well dish, 2 cm^2 per well). Each well contained 1 ml of maintenance medium and was inoculated with 25 to 100 μl of virus. CV1 cell maintenance medium consisted of Eagle minimal essential medium with antibiotics, glutamine, and 0.5 μg of trypsin per ml as previously described (5). In addition, selected wells contained a 1:2,000 dilution of specific hyperimmune neutralizing rotavirus antiserum. Control wells did not contain antiserum. Cultures were incubated at 39°C, fixed in Formalin, and stained with crystal violet when the control wells exhibited complete cytopathic effect (CPE), usually 3 to 5 days after inoculation. No attempt was made to standardize the virus inoculum; however, inoculum derived directly from plaques was not assayed on the same plates as the amplified stock inoculum.

Antiserum employed in serotype assay. The guinea pig hyperimmune bovine rotavirus (NCDV) and human rotavirus (Wa) antisera have been described previously (5). By plaque reduction these antisera both had homologous titers greater than 1:60,000 and heterologous titers of less than 1:100. The hyperimmune human rotavirus (DS-1) antiserum used in our first study (5) was replaced with a similarly prepared but more potent guinea pig antiserum. This antiserum has an homologous titer of over 1:31,000 and a heterologous titer of less than 1:100 versus bovine rotavirus (UK strain) and Wa human rotavirus.

Genotypic analysis of reassortant clones. Genomic double-stranded RNA from one or two clones of each human reassortant was prepared and analyzed on polyacrylamide gels as described previously (5, 7). In most cases, there was not a sufficient amount of human rotavirus parent to prepare virion RNA for coelectrophoresis with the reassortant. To further investigate the parental origin of reassortant genes, double-stranded reassortant virion RNA was hybridized to a single-stranded, ^{32}P-labeled probe derived from the cultivable bovine parent (5; J. Flores, submit-

ted for publication). These studies gave some information as to which genes in the reassortant were derived from the noncultivable human rotavirus parent and which were derived from the bovine parent.

Subgroup antigen assay. Selected human reassortant clones were subgrouped by using either the immune adherence hemagglutination assay or the ELISA (or both) as previously described (10).

RESULTS

Of the 50 human strains studied, 33 (66%) were successfully rescued (Table 1). A phenotypically human rotavirus reassortant as defined by neutralization specificity could not be isolated from the other 17 strains. However, in 8 of these 17 strains there was some evidence for limited reassortment of the human rotavirus parent since the coinfection growth yield grown without bovine rotavirus antiserum produced plaques at the restrictive temperature, whereas the bovine rotavirus *ts* mutant alone did not. These plaques were always serotypically bovine when tested by the qualitative CPE neutralization assay. In positive reassortment experiments the fraction of the growth yield that was ts^+ ranged from 0.0001 to 0.01. The fraction of the growth yield that was both ts^+ and phenotypically human rotavirus was generally 10-fold less. There was no clear indication as to why virus in some of the stool specimens did not appear to reassort with high efficiency. Cultivable human rotavirus reassortants were derived with approximately equal efficiency with either *ts* bovine rotavirus parent. Human stool specimens with only small quantities of detectable virus, such as many of the rectal swab specimens, could be rescued successfully. Cultivable human rotavirus reassortants were obtained from each of the geographic areas studied (Table 1). Although most of the rotavirus strains studied were obtained during the last 6 years, two successfully rescued strains from Guatemala (Table 1) had been collected over 16 years ago. The ability of the parental human virus to replicate abortively in the African green monkey kidney (AGMK) cells, as measured by immunofluorescent staining, did not correlate well with the success of rescue (16). Under the conditions of these experiments, the human rotavirus parent was never cultivable by itself.

The principal purpose of this study was to investigate the extent of serotypic diversity of human rotavirus strains. In previous studies from our laboratory with a plaque reduction neutralization assay we described two distinct human rotavirus serotypes (Wa and DS-1) (5). The large number of individual viral plaques generated in this study precluded the rapid analysis of all isolates by plaque reduction. To screen potential human reassortants and to classify them serotypically before quantitative analysis, we developed a qualitative CPE neutralization assay (Fig. 1). The serotype of a rescued virus was determined by the ability of a single dilution of specific hyperimmune rotavirus antiserum to neutralize viral CPE which, in most cases, consisted of the total destruction of the cell sheet (Fig. 1). In the representative figure (Fig. 1), previously characterized Wa reassortant 9-2, DS-1 reassortant, 41-2 and the UK bovine parent virus are shown neutralized by the appropriate homologous antiserum (5). In addition, reassortant WALK no. 57 (no. 14) (Table 1), an unclassified serotype, is included. This strain was not neutralized by the three hyperimmune antisera used in our analysis. As presented in the accompanying paper, plaque reduction neutralization assays have been carried out on representative suspensions of the first 20 human strains rescued (15). These studies confirmed, and in some cases extended, the serotypic designations shown here. The qualitative neutralization assay was used to characterize the 33 rescued human rotavirus strains (Table 1). Fifty-eight percent of the isolates were Wa-like, 9% were DS-1-like, and the remaining 33% were unclassified since neither human rotavirus antiserum neutralized them. These 11 strains represent potential new human serotypes.

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The double-stranded virion RNA of one or two doubly plaque-purified isolates from each of the 33 reassorted human strains was examined on ethidium bromide-stained polyacrylamide gels. There was no evidence to suggest that any of the isolated clones contained a mixed population of reassortants. Because in most cases the human parental RNA was not available for study, detailed genotypic analysis of the reassortants was difficult. However, direct comparison of the reassortant virion RNAs with the virion RNAs of the UK parent and hybridization studies of labeled UK rotavirus single-stranded mRNA to reassortant virion RNA were instructive (Fig. 2). Among all of the cultivatable reassortants, including the four representative examples in Fig. 2, gene 4 was the only gene which was always derived from the bovine rotavirus parent. Reassortants that were serotypically Wa-like (Fig. 2, lanes C and D) all appeared to have a ninth gene segment that was not derived from the bovine parent. Unfortunately, the origin of the seventh, eighth, and ninth genes of the DS-1-like and unclassified serotypes (Fig. 2, lanes B and E) could not be accurately assigned without concurrent analysis of the human rotavirus parent. All of the 14 reassortants tested that were subgroup 2, including CMH no. 53 (no. 16) and 77-12 (M) (no. 5) in Fig. 2, had a

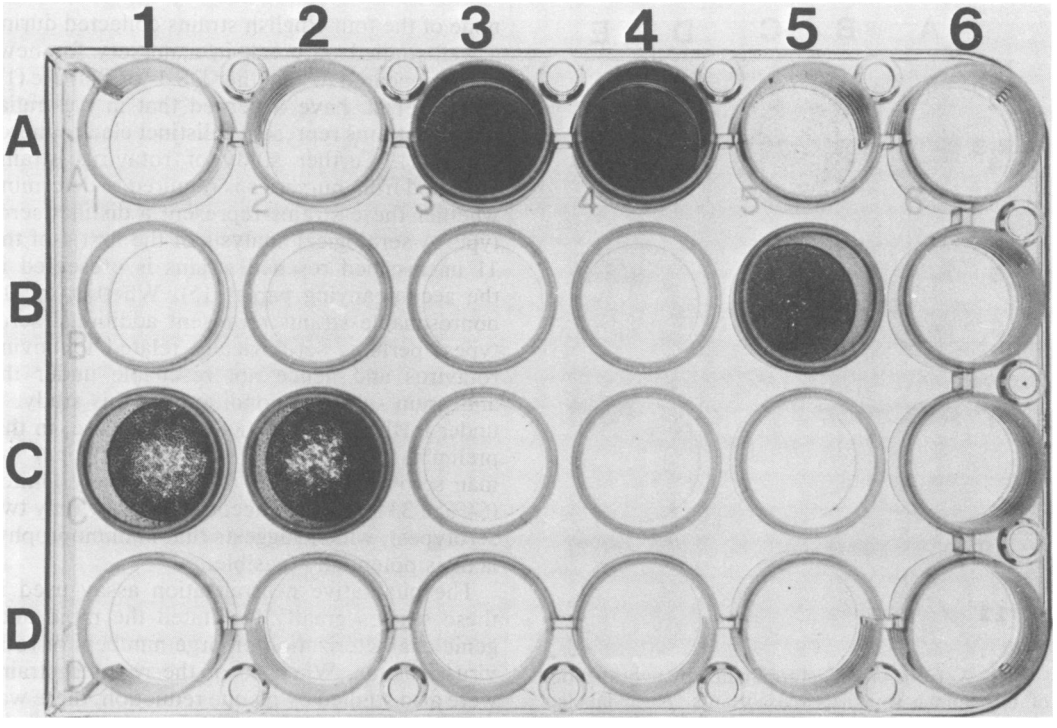

FIG. 1. Qualitative CPE neutralization assay. CV1 cell monolayers were maintained as described in the text. In rows 1 and 2 the medium contained a 1:2,000 dilution of human rotavirus (Wa) antiserum. In rows 3 and 4 the medium contained a 1:2,000 dilution of human rotavirus DS-1 antiserum. In row 5 the medium contained a 1:2,000 dilution of bovine rotavirus (NCDV) antiserum. Row 6 wells did not receive antiserum. Row A wells were inoculated with 50 μl of DS-1 human rotavirus reassortant 41-2, row B wells were inoculated with 50 μl of UK bovine rotavirus, row C wells were inoculated with 50 μl of Wa human rotavirus reassortant 9-2, and row D wells were inoculated with unclassified human rotavirus reassortant WALK no. 57 (no. 14) (Table 1) (6).

sixth gene segment apparently derived from the human rotavirus parent. In addition, subgroup 1 reassortants As (no. 3) and 008A (no. 1) had sixth genes derived from their human rotavirus parents.

DISCUSSION

The difficulty of cultivating human rotavirus has greatly hampered our ability to investigate this important pathogen. Although very recent studies indicate that some strains of human rotavirus may be amenable to direct cultivation, the applicability of this technique to all strains and to small quantities of virus has yet to be explored (13, 14). In this study we have shown that rescue of noncultivable human rotavirus isolates by genetic reassortment is a relatively efficient method for rendering human isolates cultivable. Thirty-three of the 50 specimens which we studied were cultivated. In many cases only small amounts of virus were available. Two of the specimens were over 16 years old. An advantage of the reassortment rescue technique is that all the cultivable isolates grew well in tissue culture (approximately 10^6/ml or greater), plaqued easily, and caused extensive CPE. All of these qualities made serotype analysis relatively easy.

Initial qualitative serological analysis of the 33 human reassortants is revealing. Over 50% of the rescued isolates were grouped as a single serotype, i.e., Wa-like. The Wa serotype was detected in virtually all geographic areas (Table 1) and, interestingly, appears to have circulated for at least 16 years. The other well-characterized human serotype, DS-1, was detected less frequently, but was also isolated from a variety of locations. This serotype appears to be closely related to the so-called short 10 and 11 genotype (6). If this genotype-serotype linkage continues to hold true, the DS-1 serotype may circulate with greater frequency than is indicated by this study (2, 3). Genotypic analysis of the nonrescuable strains in this study would shed light on this question. It is conceivable that DS-1 serotypes reassort less efficiently than do other human isolates. Flores et al. have recently shown that DS-1-like human strains represent a

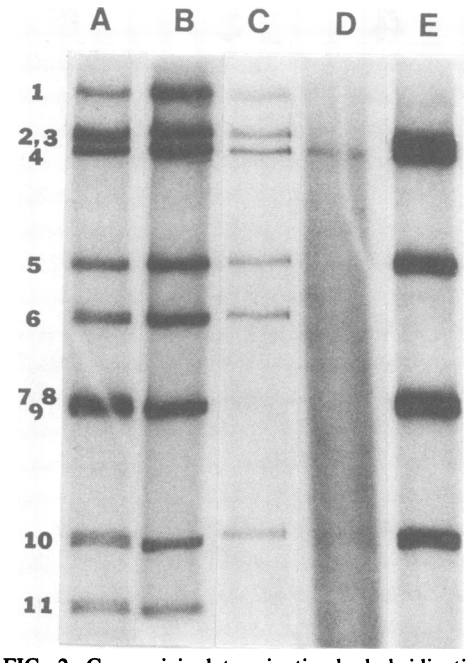

FIG. 2. Gene origin determination by hybridization of UK human rotavirus reassortants. A ^{32}P-labeled, single-stranded mRNA probe of the UK rotavirus was produced in vitro by utilizing the virion RNA polymerase. Double-stranded RNA from some reassortants was denatured with dimethyl sulfoxide and hybridized to the UK probe. Hybrid RNAs were then treated with S1 nuclease, electrophoresed overnight in 7.5% polyacrylamide gels at a constant current of 12 mA as described previously (7; J. Flores, H. B. Greenberg, A. R. Kalica, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock, Fifth International Virology Congress, abstr. no. P47, **10**:429, 1981), and autoradiographed. Double-stranded RNA was derived from the following: lane A, from the UK virus (homologous hybridization); lane B, from reassortant AS (no. 3); lane C, from reassortant 078-41 (C); lane D, from reassortant CMH no. 53 (no. 16); and lane E, from reassortant 77-12 (M) (no. 5). UK genes in the reassortants are visualized as radioactive bands. Genes derived from the noncultivable human parents were not detected since they do not hybridize to the UK probe. The numbers on the left side of the figure represent the double-stranded segment order of the UK rotavirus genome.

genetically distinct family when studied by cross-hybridization (submitted for publication).

From the qualitative serotypic studies done here, it became clear that a substantial proportion (33%) of the rescued isolates apparently represented one or more additional human serotypes since they were not neutralized by either Wa or DS-1 anti-human rotavirus hyperimmune antiserum. Evidence for additional human rotavirus serotypes was found in most locations studied (Table 1). Of note was the finding that none of the four English strains collected during a well-studied outbreak in a nursery for newborns appeared to be either DS-1- or Wa-like (1). Rogers et al. have observed that in Australia, nursery strains represent a distinct electropherotype (12). Further study of rotavirus strains collected from nurseries is required to determine whether these strains represent a distinct serotype. A serological analysis of the first 4 of the 11 unclassified rescued strains is presented in the accompanying paper (15). Whether the 17 nonrescuable strains represent additional serotypes, perhaps serologically related to bovine rotavirus and hence not rescuable under the antiserum selection conditions of this study, is under further investigation. In any case, in this preliminary survey the number of distinct human serotypes appears to be relatively limited (64% of 33 strains are accounted for by only two serotypes), which suggests that immunoprophylaxis is potentially feasible.

The qualitative neutralization assay used in these studies greatly facilitated the rapid antigenic characterization of large numbers of rotavirus isolates. When 20 of the rescued strains were also studied by plaque reduction, there was complete concordance with the results of the CPE neutralization assay (15). The CPE neutralization assay allowed us to detect rapidly new human serotype candidates that merited characterization in greater detail. As monospecific hyperimmune antisera become available for various unclassified human strains, these sera can then be added to the qualitative assay.

The genotypic analysis of the rescued strains was less than ideal since the majority of parental human rotavirus specimens were in such limited supply they could not be studied by gel electrophoresis. However, several interesting findings did emerge from our studies: As reported (Flores et al., Virology, in press; Greenberg et al., submitted for publication), gene 4 appears to be the gene that restricts cultivation of human rotavirus since it is the only bovine gene found in all the cultivatable reassortants. Gene 9 codes for the neutralization antigen of the Wa virus (7). Although this coding assignment could not be definitively established for the 19 Wa serotypes isolated in this study, in all cases these reassortants appeared to have a ninth gene derived from their human rotavirus parent. The gene coding for neutralization in the DS-1-like strains as well as the unclassified serotypes is less clear-cut and is under study. In all of the reassortants tested that had a subgroup 2 antigen, including both Wa and unclassified serotypes, gene 6 appeared to be derived from the human parent. This finding is consistent with our previous assignment, in the Wa virus, of subgroup antigenic specificity to gene 6 (7).

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