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Selection of Cold-Adapted Mutants of Human Rotaviruses That Exhibit Various Degrees of Growth Restriction In Vitro

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Group A human rotavirus strains D, Wa, DS-1, and P were originally recovered from children with diarrhea. In an attempt to attenuate virulent, wild-type human rotaviruses of major epidemiological importance for use in a live oral vaccine, two reference rotavirus strains, D and DS-1, and two laboratory-generated reassortants, Wa × DS-1 and Wa × P, were subjected to cold adaptation. Collectively, these viruses provide antigenic coverage for both of the clinically important rotavirus VP4 antigens and three of the four important rotavirus VP7 antigens. Mutants of each of these rotaviruses were selected during successive serial passage in primary African green monkey kidney cells at progressively lower suboptimal temperatures (30, 28, and 26°C). The genotype of each mutant appeared to be indistinguishable from that of its wild-type, parental virus. The mutants recovered after 10 serial passages at 30°C exhibited both temperature sensitivity of plaque formation (i.e., a *ts* phenotype)and the ability to form plaques efficiently at suboptimal temperature (i.e., a cold adaptation [*ca*] phenotype), in contrast to parental wild-type rotavirus. The succeeding set of 10 serial passages at 28°C selected mutants that exhibited an increased degree of cold adaptation, and three of the mutants exhibited an associated increase in temperature sensitivity. Finally, in the case of three of the strains, the third successive serial passage series, which was performed at 26°C, selected for mutants with an even greater degree of cold adaptation than the previous series and was associated with greater temperature sensitivity in one instance. It appeared that each of the viruses sustained a minimum of four to five mutations during the total selection procedure. The ultimate identification of candidate vaccine viruses that exhibit the desired level of attenuation, immunogenicity, and protective efficacy needed for immunoprophylaxis will require clinical evaluation of these mutants in susceptible humans.

Acute nonbacterial gastroenteritis has long been recognized throughout the world as one of the most frequent human diseases. Within this broad disease complex, group A rotaviruses are the single most important etiologic agents of severe and often life-threatening diarrhea of infants and young children worldwide (11). The global impact of rotavirus diarrhea on public health has stimulated major research efforts to develop a vaccine for prevention and control of this disease.

The initial three candidate live oral rotavirus vaccines evaluated in various parts of the world were developed by an approach originally devised by Jenner for the prevention of smallpox. Jenner used an antigenically related, attenuated live virus derived from a nonhuman host as a vaccine for immunization against its human virus counterpart. The initial vaccines contained bovine or rhesus monkey rotavirus (RRV) (1, 2, 7, 12, 13, 25, 26). The protective efficacy of the monovalent bovine or simian rotavirus vaccines proved to be variable. As a consequence, a modified Jennerian approach was used to formulate a quadrivalent RRV vaccine which was recently shown to exhibit a high degree of protection against severe rotavirus diarrhea (3, 13). This modified vaccine contained RRV which has VP7 serotype 3 specificity and three human rotavirus-RRV reassortants, each possessing 10 RRV genes and a single human rotavirus gene that encodes VP7 serotype 1, 2, or 4 specificity, thereby providing coverage for the four clinically important human rotaviruses (13, 15, 16).

Group A rotaviruses possess two outer capsid proteins that function as independent neutralization antigens, namely, VP4 (encoded by genome segment 4) and VP7 (encoded by genome segment 7, 8, or 9 depending on the strain) (10, 19). Although initially VP7 was thought to be the dominant neutralization antigen, recent studies have shown that VP4 is as effective as VP7 in inducing neutralizing antibodies following infection of experimental animals (9) or susceptible infants or young children (5). Also, antibodies to VP4 or VP7 are independently associated with resistance of gnotobiotic piglets to experimental challenge with virulent rotavirus (9). However, VP7 is the only relevant rotavirus protective antigen present in candidate vaccines that have been evaluated for protective efficacy in humans. This is because these vaccines contain the VP4 of an animal rotavirus that is not related antigenically to the VP4 of any of the clinically important human rotaviruses.

In an attempt to maximize the immunogenicity of live rotavirus vaccines by incorporating both protective antigens (i.e., VP4 and VP7) of clinically important human rotaviruses, we pursued another approach to vaccine development in which cold-adapted (*ca*) and temperature-sensitive (*ts*) mutants of human rotaviruses were sought. This strategy, which entails the selection of such mutants during adaptation of a virus to grow efficiently at suboptimal temperatures, has been successfully employed to select various attenuated candidate human viral vaccine strains for other viruses (17, 18, 22). Matsuno et al. (14) have also described the selection of a *ca* human rotavirus strain, IGV-80-3 (VP7 serotype 1), which grew efficiently at 25°C, but further characterization has not been provided. In this report, we describe the recovery of *ca*, *ts* mutants of human rotaviruses that belong to three of the four clinically important VP7 serotypes (G1, G2, and G3) and both of the clinically important VP4 serotypes (P1A and P1B).

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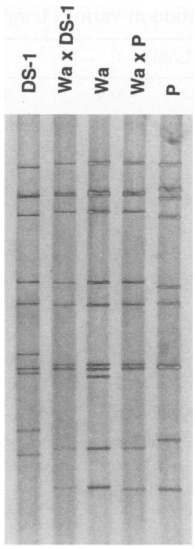

FIG. 1. Electrophoretic migration patterns of genomic RNAs of human rotaviruses DS-1, Wa × DS-1 reassortant, Wa, Wa × P reassortant, and P. Genomic RNAs were electrophoresed at 13 mA for 16.5 h, and the resulting migration patterns were stained by silver nitrate.

Viruses and cell cultures. D, Wa, DS-1, and P strains of rotavirus were recovered from children with diarrhea. The D and DS-1 strains that were cold adapted had been initially recovered by experimental infection of a gnotobiotic calf followed by one or two additional passages in gnotobiotic calves and five serial passages in African green monkey kidney (AGMK) cells (Whitaker Bioproducts, Walkersville, Md.). Two reassortant viruses were also cold adapted. These reassortants were prepared from the Wa strain, which had been passaged 18 times in primary AGMK cells (including plaque purification), and the DS-1 strain, which had been passaged 5 times in primary AGMK cells (without plaque purification), or the P strain, which had been passaged 12 times in primary AGMK cells (without plaque purification). The reassortants derived 10 genes from the Wa strain and only the VP7 gene from the DS-1 or P strain (Fig. 1). After genetic reassortment of Wa × DS-1 or Wa × P, the resulting reassortants were passaged 8 or 12 times, respectively, in primary AGMK cells prior to cold adaptation. The relevant protective antigens of these viruses and reassortants were VP4 1A (P1A) and VP7 1 (G1) for D or Wa; VP4 1B (P1B) and VP7 2 (G2) for DS-1; VP4 1A (P1A) and VP7 3 (G3) for P; VP4 1A (P1A) and VP7 2 (G2) for Wa × DS-1; and VP4 1A (P1A) and VP7 3 (G3) for Wa × P. The designations of VP4 or P serotype noted above are based on a numbering system according to antigenic relationships as determined by neutralization assay (6). A numbering system based on genotype in which P1A and P1B are numbered 8 and 4, respectively, was described previously (4).

Primary AGMK cells were used for passage of the virus at reduced temperature during cold adaptation, plaque purification, and virus amplification. The established monkey kidney MA104 cell line was used for virus titration.

Cold adaptation. Incubation at suboptimal temperatures was carried out by submerging the infected roller tube cultures in a water bath maintained at the appropriate temperature. The water baths were kept in a cold room (4°C) in order to increase the temperature differential between the water bath and the environment. This allowed us to maintain temperatures within a ±0.1°C range. After 2 weeks, the infected cultures were frozen and thawed once and the lysate was passaged onto fresh primary AGMK cell cultures.

The suspensions of human rotavirus used to initiate the cold passages varied in titer from $10^{5.0}$ to $10^{6.8}$ PFU/ml in assays performed at 37°C. In contrast, each of the viruses was restricted in its ability to produce plaques at 30°C (Table 1). For this reason, 30°C was selected as the temperature of incubation for initial selection of *ca* mutants. It was possible to propagate each of the strains when the temperature of incubation was shifted from 37 to 30°C, except for Wa × DS-1, which required three passages at 32°C before the virus could be grown successfully at 30°C. After 10 serial passages at 30°C, the temperature was dropped to 28°C for the next 10 serial passages and subsequently to 26°C for 10 additional passages. After each low-temperature passage series was completed, the 10th-passage culture lysate was triply plaque purified in primary AGMK cells at the temperature of that passage series. Plaque-purified viruses were designated *ca* 30, *ca* 28, or *ca* 26 virus. These plaque-purified viruses were not used to initiate the subsequent lower-temperature passage but were used instead for characterization as representative clones of the passage series from which they were derived. Monkey kidney MA104 cells were used for analysis of efficiency of plaque formation at different temperatures. This assay was performed

TABLE 1. Selection of *ca* mutants of human rotavirus by serial biweekly passage at 30°C[a]

HRV strain[b]	Titer (log_{10} PFU/ml) of inoculum used to initiate passage series assayed at:		Titer (log_{10} PFU/ml) at indicated passage level assayed at 30°C									
	30°C	37°C	1	2	3	4	5	6	7	8	9	10
D	<1.0	5.0	<1.0	<1.0	2.8	4.2	4.6	4.6	ND[c]	4.7	5.3	5.3
DS-1	<1.0	6.8	<1.0	<1.0	2.8	2.9	4.2	4.7	ND	5.3	ND	5.0
Wa × DS-1	<1.0	5.4	<1.0	<1.0	2.7	2.8	4.4	4.8	ND	5.4	ND	ND
Wa × P	2.2	6.2	2.9	2.7	2.9	4.1	4.1	4.5	ND	5.3	ND	ND

[a] Wild-type parental virus (≈$10^{5.0}$ to $10^{6.8}$ PFU) was inoculated into AGMK roller tube cultures (containing ≈10^6 cells) which were incubated for 1 h at 30°C, after which the medium was decanted. Cultures were washed once with 1.5 ml of L-15 medium and refed with 1.5 ml of Eagle minimal essential medium with trypsin (0.5 μg/ml). Cultures were incubated at 30°C for 14 days in a water bath maintained at this temperature (±0.1°C) and were then frozen and thawed, and the lysate was used as an inoculum (0.3 ml) to initiate the next passage at 30°C. This procedure was repeated for each of the subsequent eight passages at 30°C. Lysates from the first six passages, as well as selected passages thereafter, were titrated by plaque assay in a single test in which MA104 cell monolayers were incubated at 30°C for 8 days.

[b] HRV, human rotavirus.

[c] ND, not done.

TABLE 2. Cold-adapted mutants of human rotavirus: efficiency of plaque formation at various temperatures

HRV strain[a]	Temp (°C) at which HRV propagated[b]	Plaque titer at indicated temp (log_{10} PFU/ml)							Phenotype (°C)[c]	
		26°C	28°C	30°C	36°C	37°C	38°C	39°C	*ca*	*ts*
D	37 (wt)	<1	<1	<1	5.1	5.0	5.2	5.1	NA[d]	NA
	30	1.2	2.2	6.0	6.3	6.0	<1	<1	30	38
	28	5.8	5.7	6.1	<1	<1	<1	<1	<26	36
	26	5.2	5.3	5.4	<1	<1	<1	<1	<26	36
DS-1	37 (wt)	<1	<1	<1	6.9	6.8	6.9	6.8	NA	NA
	30	1.6	4.2	6.8	6.9	6.7	5.5	<1	30	39
	28	3.9	5.5	5.5	5.6	2.2	<1	<1	26	37
	26	6.1	6.0	6.2	5.8	1.2	<1	<1	<26	37
Wa × DS-1	37 (wt)	<1	<1	<1	5.2	5.4	5.0	5.1	NA	NA
	30	<1	3.1	5.5	5.5	5.6	4.4	<1	30	39
	28	3.4	5.9	6.3	6.0	1.3	<1	<1	28	37
	26	4.6	4.4	4.1	4.3	1.2	<1	<1	<26	37
Wa × P	37 (wt)	<1	<1	2.2	6.2	6.2	6.3	6.4	NA	NA
	30	<1	4.0	5.7	6.3	6.4	3.2	<1	30	38
	28	4.2	6.1	6.0	6.3	6.0	<1	<1	28	38
	26	4.9	5.2	5.0	5.1	1.2	<1	<1	<26	37

[a] HRV, human rotavirus.
[b] HRV propagated at the indicated temperature for 10 passages, after which a clonal population was derived by three successive plaque-to-plaque passages. Uncloned virus was used to initiate the next serial passage series at a lower temperature. Efficiency of plaque formation assays were performed with triply plaque-purified virus. wt, wild type.
[c] *ca*, lowest temperature at which plaque titer is reduced less than 10^2 times the highest titer attained. *ts*, highest temperature at which plaque titer is reduced 10^2 or more times the highest titer attained.
[d] NA, not applicable.

with six-well culture plates that were inoculated with decimal dilutions of virus and then incubated for 8 days at 26 or 28°C, 6 days at 30°C, or 5 days at 36, 37, 38, or 39°C.

Virus that produced plaques at 30°C was not detected during the first two serial passages of D, DS-1, or Wa × DS-1 at 30°C (Table 1). The first three serial passages of Wa × P yielded a low titer of virus that produced plaques at 30°C; the amount of such virus recovered after three cold passages did not differ significantly from that present in the inoculum used to initiate the passage series.

The first evidence for selection of *ca* mutants of D, DS-1, and Wa × DS-1 was observed during the third serial passage at 30°C. A low titer of virus that produced plaques at 30°C was detected in each instance. After an additional passage (D) or two additional passages (DS-1 and Wa × DS-1) the titer of virus producing plaques at 30°C increased, suggesting that one or more new mutations had been selected. A further increase in 30°C plaque-forming virus occurred at the eighth (DS-1 and Wa × DS-1) or ninth (D) cold passage, suggesting that at least one additional mutation had been selected. Evidence for selection of one or more *ca* mutations in the Wa × P virus was not observed until the fourth cold passage. A subsequent increase in titer of 30°C plaque-forming virus was seen at the eighth passage. In every instance, the virus that was cloned biologically after 10 serial passages at 30°C was a mutant that exhibited both the *ca* and *ts* phenotypes (Table 2).

In each instance, subsequent passage at 28°C selected for a mutant that was more cold adapted than the corresponding 30°C-derived mutant which produced plaques efficiently at 30°C but not at lower temperatures. In contrast, the clonal 28°C-derived mutants produced plaques efficiently at 26°C (D and DS-1) or 28°C (Wa × DS-1 and Wa × P). Three of the 28°C-derived mutants also exhibited an increase in temperature sensitivity (D, DS-1, and Wa × DS-1) compared with that of the corresponding 30°C-derived mutant. Finally, three of the clonal mutants derived at 26°C (DS-1, Wa × DS-1, and Wa × P) were more cold adapted than the corresponding 28°C-derived mutants. In the case of the *ca* 26 Wa × P mutant, a greater degree of temperature sensitivity was noted compared with the corresponding *ca* 28 mutant.

When tested by enzyme-linked immunosorbent assay, the various *ca*, *ts* mutants of D, DS-1, Wa × DS-1, or Wa × P

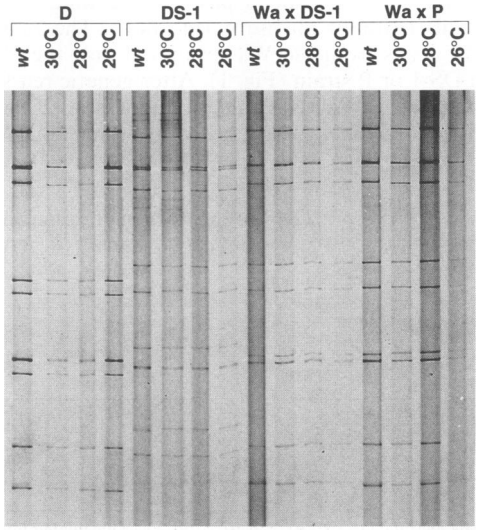

FIG. 2. Electropherotype of wild-type (wt), parental human rotaviruses D, DS-1, Wa × DS-1, and Wa × P and their mutants selected by successive serial passage at the suboptimal temperatures of 30, 28, and 26°C. Conditions of electrophoresis were as described in the Fig. 1 legend.

exhibited the same serotype-specific reactivity with VP7 monoclonal antibodies as the parental rotaviruses from which they were derived (data not shown) (8, 20, 23, 24).

In addition, the identity of the mutants recovered after selection at the suboptimal temperature of 30, 28, or 26°C was confirmed by gel electrophoresis of viral RNAs (Fig. 2). The genotype (i.e., electropherotype) of each mutant appeared to be indistinguishable from that of its wild-type, parental virus. On the other hand, the genotype of each of the parental viruses was distinctive and easily differentiated from those of the other parental viruses.

Our findings suggest that during the total selection procedure each of the human rotaviruses sustained a minimum of four to five mutations that contributed to the *ca* and/or *ts* phenotype. The acquisition of this number of mutations should enhance the genetic stability of the *ca* and *ts* phenotypes of the recovered mutants.

During the course of the three-phase low-temperature selection procedure, 12 mutants were recovered. Collectively, the mutants exhibited a relatively wide spectrum of growth restriction (as measured by efficiency of plaque formation) within the temperature range of 36 to 39°C. This degree of diversity should increase the likelihood of identifying one or more candidate live rotavirus vaccine strains that exhibit the desired level of attenuation, immunogenicity, and protective efficacy. Experience with *ts* mutants of influenza A virus suggests that promising live rotavirus vaccine strains might exhibit a 37 to 38°C shutoff temperature (21). However, this is only an extrapolation from influenza A virus to rotavirus that may not be valid. If this is so, 36 and 39°C shutoff mutants are also available to spread the net wider to increase the probability of identifying the desired vaccine strains. In any case, the ultimate identification of candidate vaccine mutants will require clinical evaluation in susceptible humans. It is possible that passage in a permissive animal host (i.e., gnotobiotic calf) or in permissive cell cultures prior to cold adaptation selected for attenuated rotavirus mutants; however, there is no evidence from prior studies to support this possibility. Even if the starting viruses are partially attenuated, it might be possible to document an additional decrease in virulence caused by acquisition of the *ca* and *ts* phenotypes.

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