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G (VP7) serotype-dependent preferential VP7 gene selection detected in the genetic background of simian rotavirus SA11

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Summary. We previously found the preferential selection of VP7 gene from a parent rotavirus strain SA11 with G serotype 3 (G3) in the sequential passages after mixed infection of simian rotavirus SA11 and SA11-human rotavirus single-VP7 gene-substitution reassortants with G1, G2, or G4 specificity. However, it has not been known whether or not VP7 genes derived from other strains with G3 specificity (G3-VP7 gene) are preferentially selected in the genetic background of SA11. To address this question, mixed infections followed by multiple passages were performed with a reassortant SA11-L2/KU-R1 (SKR1) (which possesses VP7 gene derived from G1 human rotavirus KU and other 10 genes of SA11 origin) and one of the five G3-rotaviruses, RRV, K9, YO, AK35, and S3. After the 10th passage, selection rates of SA11-L2/KU-R1 gene 9 (G1-VP7 gene) and gene 5 (NSP1 gene) reduced considerably (0 to 20.4%) in the clones obtained from all the coinfection experiments, while all or some of other segments were preferentially selected from SKR1 depending on the pairs of coinfection. When viral growth kinetics was examined, SKR1 exhibited better growth and reached a higher titer than any G3 viruses. Although the generated reassortants with VP7 gene and NSP1 gene derived from G3 viruses showed almost similar growth kinetics to that of SKR1 during the first 20 h of replication, the titers of these reassortants were higher than that of SKR1 after 36 h postinfection. The results obtained in this study suggested that G3-VP7 gene is functionally more adapted to the genetic background of SA11.

Introduction

Group A rotavirus, a member of the family *Reoviridae*, is widely distributed among mammalian and avian species, and recognized as an important pathogen causing diarreheal disease. Rotavirus virion consists of core containing 11 segmented double-stranded RNA (dsRNA), inner capsid composed of VP6, and outer capsid composed of VP4 and VP7 encoded by gene segment 4 and by

segment 7, 8, or 9 depending on the strain, respectively [4]. The two outer capsid proteins, VP7 and VP4, are involved in viral neutralization and define independent serotype specificities, G serotype and P serotype, respectively [4, 6, 22].

Due to the segmented nature of rotavirus genome, reassortment of viral genome segments occurs frequently both in vitro and in vivo during mixed infection of different rotaviruses in experimental conditions [5, 20, 23]. However, selection of RNA segments from parental strains is not considered to be random, since reassortants with specific genotypes (genome constellation) are preferentially generated or certain RNA segments from one parent virus are preferentially selected into reassortant clones [7, 12, 13, 24, 25].

Although little is known about the mechanism of preferential selection of certain gene segments, only explanation for this phenomenon is an increased infectivity of reassortants which possess certain foreign RNA segments in the genetic background of a parental virus [15, 27]. Indeed, it has been documented that a single RNA segment encoding VP4, VP7, or NSP1 derived from one parent strain could confer a replicative advantage on reassortants [2, 15, 21, 27].

However, preferential gene selection could be observed even when the preferentially generated reassortants have the same replication efficacy as that of parental strain [14, 15]. Our present study was planned to further characterize this type of preferential gene selection which had been detected in the genetic background of simian rotavirus SA11 [14].

We previously prepared single VP7 gene reassortants with G1, G2, and G4 in a genetic background of simian G3 rotavirus SA11 [11] and selection of VP7 gene was analysed in vitro. As a result, the gene encoding G3-VP7 from a parent virus SA11 was preferentially selected through multiple passages following the mixed infection of SA11 and single-VP7 gene-reassortants, while no difference in growth rate was observed among SA11 and reassortants [14, 16]. This result suggested that SA11-VP7 gene is more favorably assorted into the genetic background of SA11 than the VP7 gene derived from virus with G1, G2, or G4 specificity. However, it was not clear whether the preferential VP7 gene selection in SA11 background is either specific for SA11 gene or dependent on G serotype 3 specificity of the VP7. In order to clarify this point, we performed mixed infection experiments followed by multiple passages between a single-gene reassortant SA11-L2/KU-R1, having G1-VP7 gene in the genetic background of SA11, and five different G3 rotaviruses.

Materials and methods

Virus and mixed infection experiment

A single-gene reassortant rotavirus, SA11-L2/KU-R1 (SKR1) which was isolated previously, and five G3 rotavirus strains, simian rotavirus RRV, canine rotavirus K9, and human rotaviruses (HRVs) YO, AK35, and S3 were employed for mixed infection experiments. VP7 amino acid sequences of RRV, YO, and AK35 [8, 19] show higher degree of identity with SA11-VP7 (90.5–95.7%) as compared with KU-VP7 (81.9%–84.4%).

SKR1 and one of the G3 rotaviruses were simultaneously inoculated onto MA104 cell monolayer in 6 well plate (FALCON) at a multiplicity of infection (m.o.i.) of 5 plaque forming

units (PFU)/cell for each virus. Viruses were pretreated with acetylated trypsin (20 μg/ml) at 37 °C for 1 h and inoculated on MA104 cells after washing with Eagle's minimum essential medium (E-MEM). After addition of 1 ml maintenance medium containing acetylated trypsin (2 μg/ml), viruses were cultured until significant cytopathic effect was observed (2 or 3 days postinfection). The mixed culture fluid harvested was frozen and thawed 3 times, pretreated with acetylated trypsin and inoculated onto MA104 cell monolayer at an approximate m.o.i. of 5 PFU/cell. Sequential 10 or 20 passages of virus coinfection were performed in a similar way.

Isolation and characterization of virus clones

From mixed culture of the 3rd, 10th, and 20th passages, virus clones were obtained by randomly picking up plaques formed on CV-1 cells and were propagated in MA104 cells. Rotavirus dsRNA was extracted from 300 μl of each viral culture fluid, and separated by polyacrylamide gel electrophoresis (PAGE) followed by staining with silver, as described previously [10]. PAGE was performed also for dsRNAs extracted from mixed culture fluids, in order to roughly estimate the relative amount of VP7 gene from each parent virus.

Virus growth curve

Single-step growth curves were examined for SKR1, five G3 rotaviruses, and representative reassortant viruses generated after multiple passages, as described previously [1, 21]. Briefly, MA104 cell monolayer in 6 well plate (1.5×10^6 cells) was inoculated with high m.o.i. (5 PFU/cell) of each virus pretreated with acetylated trypsin (20 μg/ml) and incubated at 37 °C for 1 h. After washing cells with E-MEM, 1 ml of maintenance medium was added and the virus was cultured for each determined period of time. Virus titer was determined by a plaque assay using CV-1 cells.

Results

Rotavirus RNA profiles were examined for culture fluids obtained from coinfection and multiple passages. As shown in Fig. 1A, RNA segments from both coinfecting strains, in the pair of coinfections SKR1 × YO, SKR1 × AK35, and SKR1 × S3 could be detected in cultures at the 3rd passage. However, gene 5 (NSP1 gene) and gene 9 (VP7 gene) from SKR1 almost disappeared at the 10th passages and these segments were replaced by those from YO, AK35, and S3 strain, while most other segments were selected from SKR1. Similarly in coinfections of SKR1 × RRV, and SKR1 × K9, preferential selection of G3 rotavirus-gene 5 and VP7 genes (RRV: gene 7, K9: gene 8) was observed (Fig. 1B). Although in the mixed infection with SKR1 and K9, SKR1 VP7 gene was detected even at the 10th passage, this segment became undetectable at the 20th passage. At this stage, all the genes except for gene 5 and gene 9 were substantially derived from SKR1.

More than 25 and 50 clones were isolated from the 3rd and the 10th passages of each mixed culture, respectively. Frequencies of SKR1-RNA segments are shown in Table 1. In clones from both the 3rd and the 10th passages, RNA segments 1, 2, and 3 were selected mostly from SKR1 in all the coinfections. Similarly, the segment 4 was exclusively selected from SKR1 in the four

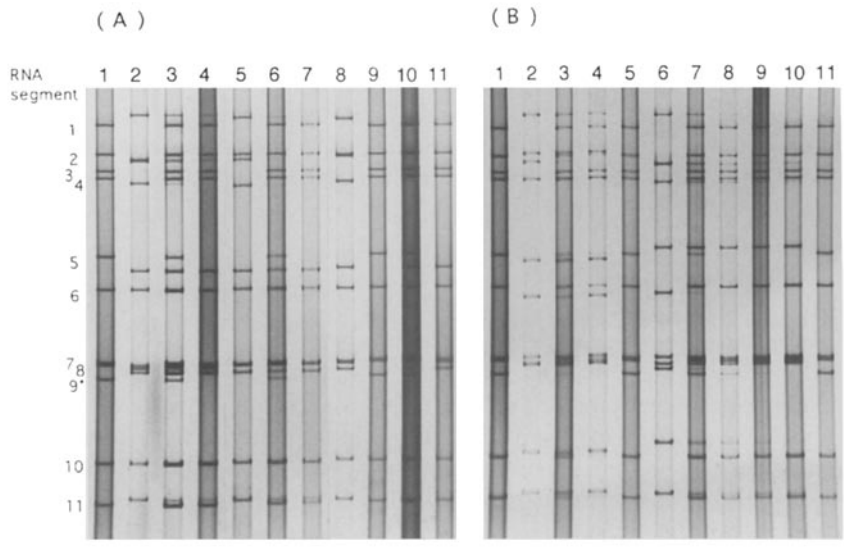

Fig. 1. RNA profiles from coinfected cultures after multiple passages. **A** *1, 9* SKR1; *2* YO; *3, 4* 3rd and 10th passages of coinfection SKR1 × YO, respectively; *5* AK35; *6, 7* 3rd and 10th passages of coinfection SKR1 × AK35, respectively; *8* S3; *10, 11* 3rd and 10th passages of coinfection SKR1 × S3, respectively. **B** *1. 5, 11* SKR1; *2* RRV; *3, 4* 3rd and 10th passages of coinfection SKR1 × RRV, respectively; *6* K9; *7–10*, 3rd, 10th, 15th, and 20th passages of coinfection SKR1 × K9, respectively. RNA segments 1 to 11 are indicated on the left. VP7 gene of SKR1 (gene 9) is marked with an asterisk

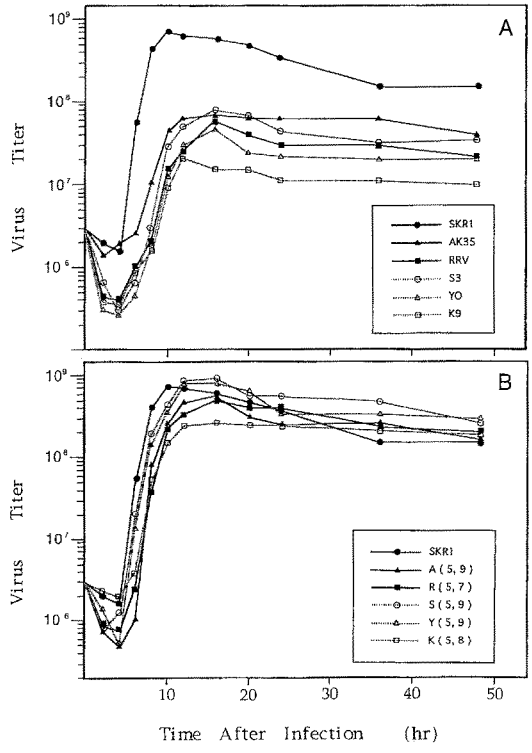

Fig. 2. Single-step growth curves of SKR1 and G3 rotaviruses (**A**) and representative reassortants isolated after multiple passages of each mixed infection (**B**). Virus titers are expressed as PFU/ml. Genotypes of reassortants shown in **B** are identical to those described in Table 2

Table 1. Frequencies (%) of SKR1 gene segments in the plaque-derived clones obtained from passages 3 (P3), 10 (P10), and 20 (P20) progeny of co-infection between the reassortant SKR1 and G serotype 3-rotaviruses YO, AK35, S3, RRV, and K9

	SKR1 × YO		SKR1 × AK35		SKR1 × S3		SKR1 × RRV		SKR1 × K9		
	P3	P10	P3	P10	P3	P10	P3	P10	P3	P10	P20
Segment											
1	96.2	93.2	100	100	100	100	82.1	98.1	60	93.2	100
2	100	98.3	100	98.4	100	100	78.6	87	90	83.1	96.9
3	88.5	93.2	100	98.4	96.9	95.2	92.9	100	86.7	100	100
4	100	100	100	100	100	100	–[a]	–	100	100	100
5	50	0	45.2	1.6	53.1	1.6	32.1	20.4	13.3	0	0
6	88.5	91.5	74.2	39.1	100	95.2	82.1	94.4	96.7	100	100
7	69.2	40.7	71	10.9	78.1	40.3	67.9	1.9	86.7	98.3	96.9
8	84.6	64.4	–	–	–	–	82.1	90.7	86.7	100	100
9	7.7	0	22.6	3.1	34.4	0	17.9	13	46.7	20.3	0
10	–	–	–	–	–	–	64.3	20.4	100	98.3	100
11	92.3	64.4	93.5	65.6	–	–	75	85.2	80	78	71.9
No. of clones	26	59	31	64	32	62	28	54	30	59	32

[a] The parental origin of the segment in the clones could not be determined

Table 2. Genotypes of the plaque-derived clones obtained from passage 3, 10, and 20 progeny of each co-infection

Cross	SKR1 × YO				SKR1 × AK35				SKR1 × S3			
Passage no.	3		10		3		10		3		10	
Genotype[a] (%)	Y9	(19.2)	Y5,9	(20.3)	A9	(35.5)	A5,9	(28.1)	S0[b]	(18.8)	S5,9	(90.3)
	Y5,9	(19.2)	Y5,7,9	(11.9)	A5,9	(19.4)	A5,6,9	(34.4)	S5	(18.8)		
	Y7,9	(15.4)	Y5,7,9	(11.9)	A5,6,9	(16.1)	A5,6,9,11	(23.4)	S9	(28.1)		
	Y5,8,9	(11.5)	Y5,7,8,9	(15.3)					S5,9	(31.3)		
No. of clones	26		59		31		64		32		62	
No. of genotype	12		19		8		7		5		3	

Cross	SKR1 × RRV				SKR1 × K9						
Passage no.	3		10		3		10		20		
Genotype[a] (%)	R5,7	(17.9)	R5,8,10	(11.1)	K5	(13.3)	K5	(13.6)	K5,8	(65.6)	
			R7,8,10	(13.0)	K5,8	(10)	K5,8	(47.5)	K5,8,11	(28.1)	
			R5,7,8,10	(35.2)			K5,8,11	(13.6)			
No. of clones	28		54		30		59		32		
No. of genotype	23		20		21		11		4		

[a] Genotypes which appeared more than 10% of total clones examined are shown, and expressed as the segment number of the parental virus other than SKR1 (Y:YO, A:AK35, S:S3, R:RRV, and K:K9). In differentiation of genotypes, electrophoretically indistinguishable segments were not taken into account. VP7 gene is represented by either of ninth segment (YO, AK35, and S3), seventh segment (RRV), or eighth segment (K9)

[b] This genotype did not contain S3-segments which were distinguishable from those of SKR1 in PAGE

coinfections. Preferential selection of SKR1-gene 6 was detected in all the coinfections except for a coinfection SKR1 × AK35. Judging from the selection rates in the 10th passage, an increase in the selection rates of SKR1-genes 7 and 10, and SKR1-genes 8 and 11 was observed in a coinfection of SKR1 × K9, and in those of SKR1 × RRV and SKR1 × K9, respectively. In contrast, reduced selection rates of SKR1-gene 5 (NSP1 gene) and gene 9 (VP7 gene) were commonly found in all coinfections. Selection rates of SKR1-gene 9 ranged from 7.7% to 46.7% at passage 3, and they decreased considerably at passage 10 (0% to 20.3%).

Assorted segment constitutions (genotypes) of the viruses yielded after coinfections were also analysed. Although some segments were not distinguishable between two coinfecting viruses, two genotypes were predominantly observed in all coinfections; one with only VP7 gene (gene 7, 8, or 9) from G3 parent rotavirus, and the other containing only gene 5 and VP7 gene from G3 rotavirus (Table 2). However, in coinfection SKR1 × RRV, a number of genotypes was detected with low frequencies.

Comparison of growth curves of parent viruses employed for coinfections indicated that SKR1 grew better and reached a higher titer than did any other parental G3 rotaviruses (Fig. 2A). Growth curves were also compared among SKR1 and representative reassortant clones with predominant genotype, having gene 5 and VP7 gene from G3 rotaviruses and other segments of SKR1 origin. Growth kinetics were similar among SKR1 and most of the reassortants during the first 20 h of replication including maturation phase as shown in Fig. 2B, except that maximum titer of one reassortant (genotype K (5, 8)) was approximately 0.3–0.6 $\log_{10}$ lower than that of SKR1 and other reassortants. However, after 36 h postinfection, the titer of the reassortants with G3-VP7 gene was approximately 0.04–0.6 $\log_{10}$ higher than that of SKR1.

Discussion

We previously observed that compared with G1-, G2-, and G4-VP7 gene, SA11(G3)-VP7 gene was preferentially introduced into the genetic background of SA11. Furthermore, our present study indicated that irrespective of the viral origin, G3-VP7 gene was selected more preferentially than G1-VP7 gene in SA11 genetic background except for gene 5.

Xu and Woode [27] also observed the preferential selection of VP7 gene from bovine rotavirus B223 in coinfection and multiple passage with different rotaviruses including HRV 69M strain. They showed that a single-B223 VP7 gene introduced in the genetic background of HRV 69M conferred a replication advantage to the reassortant, presenting a hypothesis that the substituted B223-VP7 modified the efficacy of the VP4 function as the cell attachment protein.

Similarly in our present study, the preferential selection of VP7 gene derived from G3-roravirus strains may have been caused by difference in replication efficacy of SKR1 and generated reassortants since the titer of reassortants

between SKR1 and G3 viruses was approximately 0.04–0.5 $\log_{10}$ higher than that of SKR1 around 40–48 h postinfection, when mixed culture fluid was harvested for subsequent virus inoculation (Fig. 2B).

Chen et al. [2] described a hypothesis of "fit" between VP4 and VP7 which is related to expression of viral phenotypes. Using single-VP4 gene-substitution reassortants, they observed that SA11-Cl3 gained replication advantage when VP4 of SA11-4F was introduced, but such enhancement was not found in reassortants containing VP4 from SA11-4F in B223 background. According to their hypothesis, VP4 from SA11-4F has a good fit with VP7 from SA11-Cl3(G3), but has a poor fit with B223-VP7(G10). The presence of such functional fit between VP4 and VP7 may be supported by other findings, i.e., alteration of antigenicity on outer capsid proteins caused by the presence of heterologous VP4 and VP7 [3, 17, 26, 27]. An involvement of such functional fit between VP7 and VP4 in the stage of virion formation in the cells may be conceivable also in our study. Except for SA11 × RRV, VP4 gene (gene 4) was completely (100%) selected from SKR1 in all the mixed infections and in any passage level. The SA11-VP4 might have conferred growth efficiency on virus clones yielded, resulting in predominant selection, on the analogy of previous observation from a reassortant between SA11 and an HRV [21]. Hence it is suggested that the VP4 from SA11 has better fit with G3-VP7 than VP7 from other G serotypes. Such assumption might be favored by the findings that simian rotavirus strain SA11 has VP7 with G3 specificity and that VP7 with G1, G2, or G4 has not been found in wild rotavirus strains having VP4 genotype identical to SA11 [4, 22].

It was also remarkable that gene 5 was selected exclusively from G3 rotaviruses in all the coinfections. Similarly, we previously observed lower selection rates of SA11-gene 5 compared with other SA11 segments in reassortants which were yielded from coinfection with SA11 and HRVs with G1, G2, G4 and G9 [11]. The non-random gene 5 selection has been described in several studies on rotavirus reassortment [5, 11, 12]. Rotavirus gene 5 product, the nonstructural protein NSP1, possesses a zinc finger motif which is putative RNA binding structure, and is involved in packaging of RNAs [9, 18]. Considering observations which indicated association between gene 5 selection and host cell species or animals used for coinfections [5, 7, 13], it appears that the gene 5 selection is related to efficiency in the function of NSP1 in the given host cells.

Our present study suggested that G3-VP7 is more adapted to the genetic background of SA11 than G1-VP7, and difference in such functional adaptability seems to be one of the mechanisms of preferential gene selection during reassortant formation. Further analysis on molecular basis for such genetic compatibility of RNA segments will contribute to understand the evolution of rotavirus genome.

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