Passive Immunity Modulates Genetic Reassortment between Rotaviruses in Mixedly Infected Mice

JAMES L. GOMBOLD† AND ROBERT F. RAMIG*

Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030

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Genetic reassortment between simian rotavirus SA11 and rhesus rotavirus (RRV) occurs with high frequency following mixed infection of nonimmune suckling mice (J. L. Gombold and R. F. Ramig, *J. Virol.* 57:110-116, 1986). We examined the effects of passively acquired homotypic or heterotypic immunity on reassortment in vivo. Passively immune suckling mice obtained from dams immune to either serotype 3 simian rotavirus (SA11) or serotype 6 bovine rotavirus (NCDV) were infected orally with either SA11 or RRV or a mixture of SA11 and RRV (both serotype 3 viruses). At various times postinfection, signs of disease were noted and the intestines of individual mice were removed and homogenized for titration of infectious virus and isolation of progeny plaques. Electrophoresis of genomic RNA was used to identify reassortants among the viral progeny isolated from infected animals. No reassortants (<0.45%) were detected among 224 clones examined from mixedly infected, homotypically immune mice. Twenty-nine reassortants (10.66%) were identified among 272 progeny clones from mixedly infected, heterotypically immune mice. Thus, reassortment was reduced more than 50-fold by homotypic immunity and approximately threefold by heterotypic immunity compared with prior data obtained from mixed infections of nonimmune mice. In addition, reassortment between SA11 and RRV in nonimmune mice was shown to be dependent on the virus dose. Taken together, these results suggest that immune responses may modulate the frequency of reassortment by reducing the effective multiplicity of infection (by neutralization or other immune mechanisms), thereby preventing efficient mixed infection of enterocytes.

Reassortment of genome segments between related segmented-genome, double-stranded RNA viruses is a well-documented result of mixed infection in vitro (20). Many of the factors influencing reassortment in vitro have been defined (4, 18). The ability to generate reassortant viruses through in vitro crosses has provided a powerful tool for mapping temperature-sensitive mutations (5, 21), identifying determinants of type specificity (9, 11, 29), identifying determinants of viral virulence (12, 24), rescuing noncultivable viruses (7), and identifying genome segments encoding the unique phenotypes displayed by rotavirus variants (2).

Until recently, there has been little but circumstantial evidence to document reassortment among double-stranded RNA viruses in vivo (27, 28). However, there is now compelling evidence that several groups of double-stranded RNA viruses can indeed reassort genome RNAs during in vivo infections. Reassortment has been demonstrated among reoviruses and among rotaviruses following oral mixed infection of suckling mice (6, 30). Reassortment between bluetongue virus serotypes has been demonstrated in several host species, including sheep (23), cattle (26), and the vector species *Culicoides variipennis* (22).

In rotaviruses, little is known about reassortment in vivo except that it occurs at high frequency and that nonrandom segregation of genome segments appeared to occur following oral infection of mice (6). One obvious factor that could potentially affect reassortment among rotaviruses in vivo is the immune status of the host. Consequently, we examined the effects of homotypic and heterotypic passive immunity on reassortment in orally infected immune mice. Passive

immunity significantly reduced the frequency of reassortment compared with nonimmune controls. Further experiments suggested that the immune response prevents efficient mixed infection of enterocytes and reduces the opportunity for reassortment.

MATERIALS AND METHODS

Cells and viruses. MA104 cells were grown as monolayers in medium 199 supplemented with 5% fetal bovine serum as previously described (17). Simian rotavirus (SA11) and rhesus rotavirus (RRV) were from the laboratory stock collection and have already been described (6, 17). Bovine rotavirus (NCDV) was obtained from R. Wyatt (National Institutes of Health, Bethesda, Md.). The viruses were propagated in MA104 cell monolayers in 1.0 µg of trypsin per ml as previously described (17). All viruses were plaque purified three times and used at passage level 3. SA11 and RRV belong to serotype 3, and NCDV belongs to serotype 6 (3).

Mice. Multiparous pregnant CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were housed individually in Microisolator cages (Lab Products, Maywood, N.J.) as previously described (6). Female mice born to these dams were immunized (see below) and placed in separate cages when they reached breeding age. Following breeding, the male was removed and the pups born were left with the immune dam for the duration of the experiments to obtain passive immunity. Food (Wayne Pet Food, Chicago, Ill.) and water were provided ad libitum. Serum and milk samples were obtained from immune dams for use in plaque reduction neutralization assays. Sera taken from the tail vein or by puncture of the retroorbital capillary plexus were collected both before and after challenge of each litter.

* Corresponding author.

† Present address: Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

Milk samples were collected from dams 2 to 3 days postpartum by the method of McBurney et al. (10).

Immunization of dams. Seven-day-old suckling female mice born to seronegative dams were immunized orally with either 3.7×10^6 PFU of SA11 or 2.0×10^6 PFU of NCDV as described previously (6). Intraperitoneal secondary vaccinations were given without adjuvant 3 and 5 weeks later as injections of 10^6 PFU of the same virus used for the primary oral immunization. Mice were bred after the second vaccination.

Infection of passively immune suckling mice. Seven-day-old suckling mice born to rotavirus-immune dams were inoculated orally with a mixture of SA11 and RRV containing 1.85×10^6 PFU of each virus (6). For single infections, the dose was 3.7×10^6 PFU. The viruses were not trypsin activated before inoculation. All litters were infected within 30 to 50 days of the final immunization of the dam. At various times postinfection (p.i.), the mice were observed for signs of disease and sacrificed. The intestines of individual animals were removed, placed in 1.0 ml of gelatin saline, and homogenized. Titration of infectious virus was performed at 37°C as described previously (6).

Plaque reduction neutralization assay. The virus neutralization titers of serum and milk samples collected from individual immune dams were determined. Both serum and milk samples were inactivated at 56°C for 30 min before the plaque reduction neutralization assay. Serum samples were diluted in phosphate-buffered saline, mixed with a constant amount of virus, and incubated at 37°C for 60 min. Titration of surviving virus was by standard plaque assay (6), except that following adsorption the monolayers were washed with phosphate-buffered saline before being overlaid with nutrient agar. Titers in serum and milk were expressed as the reciprocal of the highest dilution resulting in 80% reduction in plaques compared with controls (100 PFU).

Isolation and characterization of viral progeny. Progeny plaques from mixedly infected suckling mice were picked, grown to a high titer, labeled with $^{32}\text{P}_i$, and subjected to electrophoresis for electropherotype determination as described previously (6). Parental and reassortant clones were identified by comparing the mobility of each genome segment with those of parental markers run in the same gel.

RESULTS

Immune status of dams. Newborn mice are susceptible to rotavirus infection only during the first 14 days of life (19, 31). Therefore, this study required that experimental suckling mice receive immunity passively from immune dams. Dams were immunized as described in Materials and Methods and the virus neutralization titers of serum and milk were measured as indicators of the immune status of the suckling mice in which reassortment was ultimately examined.

Neutralization titers of serum and milk for both homotypic and heterotypic viruses were determined in a standard plaque reduction neutralization assay. Nonimmunized mouse serum (dam 323) failed to neutralize SA11, RRV, or NCDV at the lowest dilution tested (1:25; Table 1). Serum neutralization titers of dams vaccinated with SA11 varied between 50 and 800 when tested against either serotype 3 virus and, in general, did not increase following infection of the suckling pups. Some sera reacted at higher titer with RRV even though the mice were vaccinated with SA11 (e.g., S1 and S2). In contrast, sera obtained from animals vaccinated with NCDV failed to neutralize SA11 or RRV but

TABLE 1. Neutralization titers of sera from dams immunized against SA11 or NCDV

Serum (dam)	Vaccine virus	Prechallenge/postchallenge neutralization titers against ^a :		
		SA11	RRV	NCDV
S1	SA11	400/100	800/400	<25/<25
S2	SA11	100/400	200/800	<25/<25
S6	SA11	800/800	100/ND ^b	<25/ND
S8	SA11	200/200	50/50	<25/<25
N3	NCDV	<25/<25	<25/<25	>800/100
N4	NCDV	<25/<25	<25/<25	100/ND
N7	NCDV	<25/<25	<25/<25	400/50
N9	NCDV	<25/<25	<25/<25	ND/ND
323	Mock	<25/ND	<25/ND	<25/ND

^a Titers were determined by 80% plaque reduction.

^b ND, Not done.

neutralized NCDV to a high titer. A limited number of milk samples from immunized dams were tested for the ability to neutralize SA11 and RRV. The titers of these samples were lower than the corresponding titers in serum. However, dams with titers of 200 or greater in serum also had significant titers (≥ 50) in milk (data not shown). Therefore, for immunized dams with titers of at least 200 in serum, a significant level of antibody in milk could also be assumed.

Kinetics of disease and viral growth in passively immune mice. Multiple litters of suckling mice passively immune to rotavirus serotype 3 (SA11) or serotype 6 (NCDV) were challenged orally with SA11 (serotype 3), RRV (serotype 3), or a mixture of SA11 and RRV. At regular intervals, mice were examined externally for diarrhea and killed. The appearance of the colon and the consistency of its contents were noted upon dissection. Mice passively immune to serotype 3 virus (homotypic immunity) generally showed minimal signs of disease regardless of the inoculum (Table 2). However, one mouse in one litter (S1) infected with the virus mixture did experience severe disease of short duration. S1 was the only dam in which the neutralizing titer dropped postchallenge. In contrast, the disease in animals passively immune to the serotype 6 virus (heterotypic im-

TABLE 2. Kinetics of disease in passively immune mice infected with SA11, RRV, or an SA11-RRV mixture

Time (h) p.i.	Range of disease symptoms in mice with ^a :					
	Homotypic immunity			Heterotypic immunity		
	SA11 ^b	RRV ^b	SA11-RRV ^c	SA11 ^b	RRV ^b	SA11-RRV ^c
0	-/-	-/-	-/-	-/-	-/-	-/-
4	-/-	-/-	-/-	-/-	-/-	-/-
8	-/-	-/-	-/-	-/-	-/-	-/-
12	-/-	-/-	-/-	-/-	-/-	-/-
24	-/-	-/-	+/+	+/+	-/-	++/-
48	+/+	+/+	++/-	+++/+	+/+	+++/++
72	+/+	+/+	+++/+	+++/+	+/+	+++/+
96	-/-	+/+	+/+	+/+	+/+	+++/+
120	-/-	+/+	+/+	+/+	+/+	+/+
144	-/-	-/-	-/-	+/+	-/-	+/+
168	-/-	-/-	-/-	-/-	-/-	-/-

^a Scale: -, no disease; +, semisolid feces; ++, liquid feces; +++, liquid feces with distended colon.

^b Three litters were studied.

^c Four litters were studied.

FIG. 1. Kinetics of viral growth in mice with passive homotypic immunity (left) and passive heterotypic immunity (right). Seven-day-old mice were inoculated orally with SA11 (■), RRV (◆), or a mixture of SA11 and RRV (▲) as described in Materials and Methods and indicated in each panel. At the indicated times p.i., mice were examined for signs of disease and sacrificed. Homogenates of individual intestines were plated on MA104 cell monolayers to determine the amount of infectious virus contained in the combined small and large intestines at each time. Mean values and ranges from three (SA11 or RRV) or four (SA11-RRV mixture) infected litters are shown. The limit of detection in these assays was 500 log_{2.7} PFU per intestine.

munity) was typically more severe and persisted longer than in mice with homotypic immunity (Table 2). Compared with results previously obtained with nonimmune mice (6), homotypic immunity generally provided protection against challenge, whereas only limited or no cross-protection was provided by heterotypic immunity.

Following examination of the intestinal contents, the combined large and small intestines of each mouse were removed and homogenized, and the virus titer was measured to determine viral growth kinetics. The mean titer of virus detected immediately following infection of serotype 3-immune mice was significantly less than the input and rapidly decreased to undetectable levels (Fig. 1; <500 PFU per intestine). The growth kinetics of virus in mice infected with the SA11-RRV mixture were similar to those in mice infected with either virus alone. The peak of virus replication observed at 12 to 24 h p.i. in nonimmune mice (6; Fig. 2) was absent in mice with homotypic immunity. Mice infected with RRV or the SA11-RRV mixture showed a marked increase in titer at 72 h p.i. An additional increase in titer was observed in RRV-infected mice at 144 h p.i. However, only the 72-h

peak was observed reproducibly. Although the basis of this late growth is not known, preliminary data suggest that this peak is RRV specific and occurs when mice are approximately 10 days old. It is also interesting that the plaque morphology of virus isolated from immune, RRV-infected mice at 72 h p.i. was not typical of RRV.

Growth kinetics of SA11 alone or together with RRV in mice passively immune to the serotype 6 virus more closely approximated the growth kinetics of virus in nonimmune animals (6; Fig. 2). Virus titers closely approximated the input immediately after infection, declined rapidly during the initial 12 h following infection, and then increased slightly by 24 h p.i. (Fig. 1). The virus decreased gradually to undetectable levels over the subsequent 2 to 3 days. A peak of replication was observed again at 72 h p.i. in mixedly infected mice. In contrast to the rapid clearance of virus in SA11-infected or mixedly infected animals, growth of RRV in the presence of heterotypic immunity appeared to be augmented, resulting in detectable amounts of virus as late as 5 days p.i., whereas RRV was undetectable by 2 days p.i.

FIG. 2. Dose response of viral growth in 7-day-old mice inoculated with various doses of an SA11-RRV mixture. Single litters of nonimmune mice ($\blacktriangle$) were inoculated orally with a mixture containing 5×10^6 (A), 1×10^6 (B), 5×10^5 (C), or 1×10^5 (D) PFU of virus. At the indicated times, mice were examined for disease and sacrificed. Intestinal homogenates were plated on MA104 cell monolayers to measure the amount of infectious virus present. Plotted for comparison are the growth kinetics of an SA11-RRV mixture (3.7×10^6 total PFU) in nonimmune mice ($\square$) or mice with passive heterotypic immunity ($\diamond$) inoculated with 3.7×10^6 PFU of the virus mixture. The limit of detection was $500 \log_{2.7}$ PFU per intestine.

in seronegative animals (6). The basis of this potentiation of RRV growth is unknown.

Isolation and characterization of progeny virus clones from passively immune mice. Progeny virus clones were obtained from plaques of intestinal homogenates from mixedly infected, passively immune mice at high dilutions. The electropherotypes of the progeny clones were determined by polyacrylamide gel electrophoresis of viral genomic RNA (Fig. 3).

Virus was isolated from four litters of serotype 3-immune animals that had been sacrificed between 12 and 36 h p.i. No reassortant viruses were identified among the 224 progeny screened, indicating that reassortants represented less than 0.45% of the yield of the mixed infection of homotypically immune mice (Table 3). The reduction in reassortment was statistically significant ($P < 0.05$) at all times compared with reassortment in seronegative mice (6). The SA11 and RRV parental electropherotypes were isolated at equal overall frequencies. However, the frequencies of isolation were not equal at all times; SA11 was most prominent early during infection, and RRV was prominent late.

Serotype 6-immune animals had intestinal virus titers sufficient to allow isolation of progeny plaques as late as 48 h p.i. From a sample of 272 clones obtained from four independent litters of heterotypically immune mice, 29 reassortants (10.66%) were identified (Table 4). Reassortants were isolated at similar overall frequencies at all times examined, although extensive variation was observed between individual litters sampled at any time. Statistical analysis revealed that, in comparison with reassortment in seronegative mice (6), reassortment was not significantly reduced at 12 and 24 h p.i. However, at 48 h p.i., reassort-

ment was significantly reduced ($P < 0.05$), as was the overall reassortment for the experiment. Parental SA11 was isolated at the greatest overall frequency (86%), while parental RRV represented only a minor fraction of the yield.

Twenty-five different electropherotypes were identified among the 29 reassortant clones isolated from heterotypically immune mice (data not shown). None of the electropherotypes were represented by more than three clones, and 22 electropherotypes were represented only once. All genome segments reassorted themselves, but the small sample size precluded a statistical analysis of the randomness of reassortment. The overall percentage of SA11 segments in the reassortant population was 69%. However, the SA11 contribution of individual genome segments ranged from 41% (segment 8) to 90% (segment 3; data not shown).

Dose-response experiments. The simplest explanation for the reduction in the frequency of reassortment observed was that the effective multiplicity of infection was reduced by the passive immune response. This would result in infection of fewer enterocytes by both parental viruses and decrease the opportunity for reassortment. This hypothesis was supported by the large loss of infectivity noted immediately following inoculation (Fig. 1). To test this hypothesis, litters of 7-day-old mice born to seronegative dams were infected with systematically decreasing doses of the SA11-RRV mixture. Infection of nonimmune mice with either 5×10^5 (Fig. 2C) or 1×10^6 (Fig. 2B) PFU per animal resulted in viral growth kinetics that approximated those seen in heterotypically immune mice (Fig. 1). We wished to determine whether the frequency of reassortment resulting from mixed infection with these reduced doses was similar to the frequency observed in the presence of heterotypic immunity.

FIG. 3. Electrophoretic analysis of virus isolated from immune mice mixedly infected with SA11 and RRV. Viral clones were prepared, labeled with $^{32}\text{P}_i$, and resolved in 8% gels as described in Materials and Methods. Lanes 1 to 9 contained progeny clones isolated from heterologously immune litter N7. The clones in lanes 3, 5, and 7 were reassortants. Lanes SA11 and RRV contained parental viruses as markers. The distance from segments 1 to 11 in SA11 was 23.5 cm in the original gel.

Therefore, virus was isolated from animals that had been infected with 5×10^5 or 1×10^6 PFU and sacrificed between 12 and 48 h p.i. Only 2 (1.4%) of the 147 clones from mice infected with a dose of 5×10^5 PFU were reassortants (Table 5). No reassortants (<1%) were identified among 100 clones from mice infected with a dose of 1×10^6 PFU (Table 5). At both doses, the frequency of reassortants was significantly less than that observed in heterotypically immune mice.

DISCUSSION

This study of the effects of immunity on rotavirus reassortment in vivo made use of two serotype 3 animal rotaviruses, SA11 and RRV, and suckling mice with passively acquired homotypic or heterotypic immunity. SA11 and RRV were chosen for study because significant data on

TABLE 3. Summary of electropherotypes of clones derived in vivo from homotypically immune mice mixedly infected with SA11 and RRV

Animal	Time (h) p.i.	No. of clones examined	No. (%) of clones with the following electropherotype ^a :		
			SA11	RRV	Reassortant
S1-A4	12	19	15 (79)	4 (21)	0
S2-A4	12	19	16 (84)	3 (16)	0
S6-A4	12	18	11 (61)	7 (39)	0
S8-A4	12	19	4 (21)	15 (79)	0
S1-A5	24	38	38 (100)	0 (0)	0
S6-A5	24	33	24 (73)	9 (27)	0
S8-A6	36	78	1 (1)	77 (99)	0

^a Reassortants represented <0.45% of the population.

reassortment of these viruses in seronegative mice exist, providing a comparative baseline (6). Passively immune suckling mice were used for two reasons. (i) Newborn mice are susceptible to rotavirus infection only during the first 14 days of life (19, 31), precluding the use of an active immunization model. (ii) Significant data exist demonstrating protection from rotavirus-induced disease by passive immunization and demonstrating homologous passive protection following oral immunization (13, 14, 25).

The studies reported here can be briefly summarized as follows. (i) Homologous passive immunization generally provided protection from disease and prevented significant viral replication in mice infected with SA11, RRV, or a mixture of the two viruses compared with previous experiments performed with seronegative mice (6). Reassortment between SA11 and RRV could not be detected in the presence of passively acquired homotypic immunity. (ii) Heterotypic passive immunization (to rotavirus serotype 6) generally provided partial but incomplete protection from disease. In addition, it significantly reduced but did not totally block viral replication in mice infected with SA11, RRV, or a mixture of the two viruses compared with previous experiments performed with seronegative mice (6). Reassortment occurred in the presence of the heterotypic immune response at a frequency of 10.66%. (iii) Both homotypic and heterotypic passive immunity reduced the frequency of reassortant progeny isolated from mixedly infected mice. With homotypic immunity, reassortment was significantly reduced (>50-fold) relative to the frequency observed in nonimmune mice (6). Surprisingly, heterotypic

TABLE 4. Summary of electropherotypes of clones derived in vivo from heterotypically immune mice mixedly infected with SA11 and RRV

Animal	Time (h) p.i.	No. of clones examined	No. (%) of clones with the following electropherotype:		
			SA11	RRV	Reassortant
N3-A4	12	20	19 (95)	1 (5)	0 (0)
N4-A4	12	18	14 (78)	1 (5)	3 (17)
N7-A4	12	35	27 (77)	1 (3)	7 (20)
N9-A3	12	19	18 (95)	0 (0)	1 (5)
N3-A5	24	20	18 (90)	0 (0)	2 (10)
N4-A5	24	20	20 (100)	0 (0)	0 (0)
N7-A5	24	44	41 (93)	1 (2)	2 (5)
N9-A4	24	18	10 (56)	0 (0)	8 (44)
N3-A6	48	32	27 (84)	5 (16)	0 (0)
N7-A6	48	27	21 (78)	0 (0)	6 (22)
N9-A5	48	19	19 (100)	0 (0)	0 (0)

TABLE 5. Summary of electropherotypes of clones from seronegative mice mixedly infected with reduced doses of SA11 and RRV

Dose and animal ^a	Time (h) p.i.	No. of clones	No. (%) of clones with the following electropherotype:		
			SA11	RRV	Reassortant
5.0 × 10 ⁵					
L323-A4	12	49	43 (88)	4 (8)	2 (4)
L323-A5	24	49	49 (100)	0 (0)	0 (0)
L323-A6	48	49	27 (22)	22 (45)	0 (0)
1.0 × 10 ⁶					
L308-A4	12	50	50 (100)	0 (0)	0 (0)
L308-A5	24	50	50 (100)	0 (0)	0 (0)

^a Designations for litters (e.g., L323) and animals (e.g., A4) are combined.

immunity also affected reassortment, causing roughly a threefold reduction in the frequency of reassortants compared with nonimmune mice (6). (iv) Mixed infection of nonimmune mice with reduced doses of SA11 and RRV suggested that the reduction of reassortment in homotypically immune mice could be accounted for by neutralization of input virus in the gastrointestinal tract, preventing efficient mixed infection of enterocytes. However, simple type-specific neutralization did not appear to explain the reduction in the frequency of reassortant progeny observed in heterotypically immune mice, since these mice had no detectable neutralizing antibody to the serotype 3 viruses used.

Protection from rotavirus-induced disease by passive immunity has been well documented in the murine model system. Protection from disease has been noted for both homotypic and heterotypic immunity following parenteral immunization, whereas oral immunization afforded protection against only homotypic challenge (13). Passive immunity appears to be mediated by both immunoglobulin A and immunoglobulin G fractions of colostrum and milk, with immunoglobulin A being more potent (14). In addition, protection closely correlates with the *in vitro* neutralizing titer of the maternal serum to the challenge virus (13), and passive transfer of monoclonal antibodies has shown that neutralizing monoclonal antibodies protect from subsequent challenge, whereas nonneutralizing monoclonal antibodies do not (16). In the experiments reported here, the dams initially were immunized orally and received parenteral secondary immunizations. The serum titers present in the immune dams most closely approximated those obtained by others using oral primary immunization with oral secondary immunizations (13). As in the studies of others (13), we generally found nearly complete protection from homotypic challenge and weak partial protection from heterotypic challenge. However, single animals in two litters (S1 and S6) of homotypically immune mice showed severe disease when challenged with the SA11-RRV mixture. Similar increased severity of disease was observed in mixed infections of seronegative mice (6). The S1 litter was the only case in which the antibody titer in the serum of the dam dropped after challenge, suggesting that the titer of passively transferred antibody fell below protective levels during infection of this litter. However, a similar increase in disease severity seen in heterotypically immune mice challenged with the SA11-RRV mixture suggests that neutralizing antibody titers alone cannot explain the increased symptoms seen in some infections. The mechanism for increased severity of disease in mixedly infected immune or nonimmune animals is not

understood. However, this phenomenon clearly does not require the generation of viable reassortants, since no reassortants were isolated from litter S1, in which the phenomenon was clearly observed. The isolation of plaque morphology variants of RRV from RRV-infected immune mice suggests that the increased severity of disease noted in some mixed infections may be associated with variation of the RRV parent in the absence of reassortment. Finally, we demonstrated that homotypic passive immunity severely reduced viral replication in infected mice, while heterotypic passive immunity reduced viral replication only slightly. In these experiments, virus titers at p.i. peaks considered to represent viral replication did not exceed the input titers, suggesting that they could represent the activation of previously noninfectious virus present in the inoculum. Support for the notion that these peaks represent viral replication rather than activation comes from (i) infections at very low doses which show replication significantly above the input titers at these times (19) and (ii) the finding that reassortants can be isolated at these times, a phenomenon requiring viral replication (6; this work).

In light of the effects of passive immunity on homotypic and heterotypic viral replication, the effects of immunity on reassortment were predictable. Homotypic passive immunity resulted in rapid loss of infectivity of the inoculum, presumably by neutralization, and effectively blocked the formation of reassortant progeny. Experiments with reduced virus doses in seronegative mice suggested that this effect was simply a result of a lower efficiency of mixed infection of enterocytes. It is interesting that homotypic immunity resulted in an equal overall distribution of parental electropherotypes, whereas the SA11 electropherotype had dominated the progeny from infections of seronegative mice (6). However, the isolation of parental electropherotypes from homotypically immune animals was not equal before 36 h p.i. At early times (12 and 24 h p.i.), SA11 predominated among the yield, accounting for 74% of the progeny. Thus, it is clear that the overall equal isolation of SA11 and RRV from these mice was due to the preponderance of RRV isolated from the one sample taken at 36 h p.i. The excess of RRV isolates from this animal, as well as from its sibling (S8-A4; Table 3), might have been expected, since serum from dam S8 neutralized RRV less well than SA11. Also interesting was the sharp peak of replication seen at 72 h p.i. in passively immune mice infected with RRV or the SA11-RRV mixture. This increase in RRV titer somewhat parallels the increase in RRV isolates from mixedly infected animals. The significance of this late peak of RRV replication in mice is unclear. In other studies of mixedly infected mice, progeny virus isolated during the late peak in animals mixedly infected with RRV and another virus contained only virus with an RRV electropherotype. The virus isolated from samples taken at this time made plaques with atypical morphology (small, indistinct plaques rather than larger, clear, sharp-edged plaques for the parental RRV), suggesting that the peak represents replication of variants of RRV selected during growth in the mouse. These putative RRV variants were not further characterized.

Heterotypic passive immunity resulted in a threefold reduction of reassortment relative to that seen in seronegative mice (6). The basis of this reduction in reassortment is not understood. Neutralization titers of maternal sera did not predict neutralization of virus in the inoculum, and relatively little loss of infectivity was noted immediately following inoculation. The kinetics of viral growth, as well as the predominance of the SA11 parental electropherotype among

the progeny, was similar to that of mixed infections of seronegative mice (6). If the reduction in reassortment were based on immune selection against progeny reassortants by a cross-reactive heterotypic antibody, we would have expected to see nonrandom segregation of genes encoding neutralization antigens among reassortants. This was not found, and the only gene that appeared to be preferentially derived from one parent (segment 3) is not known to encode a neutralization antigen. In addition, this segment was also preferentially derived from the same parent in reassortants isolated from seronegative mice (6).

It therefore seems unlikely that the effect of heterotypic immunity on reassortment was due to virus neutralization by a cross-reactive antibody. Aggregation of the virus in the inocula would reduce the apparent multiplicity of infection and might be expected to reduce the frequency of reassortment. However, this would occur only if the aggregates were homogeneous, composed of only SA11 or RRV. Heterogeneous aggregates composed of both parental viruses would ensure mixed infection of single cells. The probability of heterogeneous aggregates seems good, since the parental viruses were present in equal proportions. Furthermore, if a heterotypic antibody caused viral aggregation *in vivo*, we should have observed neutralizationlike activity in the heterotypic sera in neutralization tests. Therefore, aggregation does not appear to adequately explain the effects of heterotypic immunity on reassortment.

Specific cell-mediated immunity could explain these findings. Cytotoxic T lymphocytes or antibody-dependent cell cytotoxicity could result in lysis of infected enterocytes, limiting the duration of infection and the extent of reassortment. Indeed, cross-reactive cytotoxic T lymphocytes have recently been characterized following oral infection of mice with rotavirus (15), and functional immune cells have been shown to be transferred during lactation in mice (8). However, a role for cross-reactive cytotoxic T lymphocytes in protection from challenge and reducing viral growth and reassortment in mixedly infected animals remains to be demonstrated. Recently, partial protection from rotavirus infection, mediated by colostral mononuclear cells, was demonstrated in bovines (1).

In summary, these results demonstrate that passively acquired homotypic and heterotypic immunity modulates reassortment during mixed infections of suckling mice. Reduced reassortment would be a desirable side effect of vaccination programs, because it would block or reduce the rate of reassortment-mediated evolution of the virus population.

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