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Genetic Reassortment of Mammalian Reoviruses in Mice

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Reassortants between type 1 (Lang) and type 3 (Dearing) reoviruses were isolated from suckling mice infected perorally with an inoculum containing both type 1 and type 3 viruses. A total of five distinct reassortants (designated as E1 through E5) were isolated from animals during the course of the experiment. Two reassortants (E1 and E2) represented the majority of the reassortants isolated. The majority of genes of types E1 and E2 were derived from type 1 (Lang). However, E1 had an M2 gene and an S1 gene derived from type 3 (Dearing), while E2 had M2 and S2 genes derived from type 3 (Dearing). Thus, nonrandom reassortment between mammalian reoviruses can be demonstrated in vivo.

The ability of mammalian reoviruses to reassort their double-stranded RNA segments has been useful for defining the function of viral genes in pathogenesis. After mixed infection of cells with two different reoviruses, reassortants have been isolated which contain double-stranded RNA segments derived from each of the two parents (2, 16). In prior studies, reassortants were generated in tissue cultures and were then inoculated into mice to study the functions of the reovirus genes in vivo. Among the insights derived from these studies were the identification of distinct functions of the three viral outer capsid polypeptides. The viral hemagglutinin σ1 (encoded by the S1 segment) binds to cell surface receptors and is responsible for cell and tissue tropism (4, 20); the μ1C protein (encoded by the M2 segment) interacts with proteases and determines yield in differentiated tissues (3, 14); the σ3 protein (encoded by the S4 segment) inhibits host cell RNA and protein syntheses (1, 15).

In light of the experimental data involving reassortants generated in vitro, we wished to determine whether reassortment might take place in the host (in vivo). If this reassortment occurred, we wanted to determine whether certain viral genes were selected in particular organs and whether reassortants would have altered biologic properties. We report here that, after mixed infection of young mice with type 1 (T1) (Lang) and type 3 (T3) (Dearing), reassortants are generated in vivo, and different reassortants are isolated from selected organs.

It has been previously reported that T1 (Lang) and T3 (Dearing) differ in their growth and spread in 10-day-old and adult mice (11). It was, therefore, important to establish the ability of the two individual serotypes to infect 2-day-old animals under our experimental conditions. Since the natural portal of entry for reoviruses is through the gastrointestinal tract, we chose to inoculate the animals perorally into the stomach. Baby mice were infected with either the T1 or T3 parental serotype, and another group was infected with a mixed inoculum (MI) containing both T1 and T3.

Animals were injected with a total volume of 0.05 ml of viral solution mixed with blue dye (Durkee Famous Foods, Cleveland, Ohio). Only those animals exhibiting a blue stomach after inoculation were used. Animals inoculated with only T1 or T3 received 2×10^7 PFU of virus. Animals infected with an MI were inoculated with 10^7 T1 PFU plus 10^7 T3 PFU in a total volume of 0.05 ml. Animals were sacrificed on day 1 postinoculation (p.i.) and every other day thereafter through day 13.

The two groups of mice inoculated with parental viruses were used to determine the extent to which each virus would spread or grow in selected organs after peroral inoculation. The organs chosen for examination were the ileum (the primary site of replication), the liver (an organ through which virus passes after leaving the gastrointestinal tract), the spleen (a lymphoidal organ that collects virus spread by the blood), and the brain (an organ in which serotypes 1 and 3 exhibit different tropisms). The organs selected represented different sites of spread, drainage, and tissue tropism.

To measure the amount of virus in these organs after inoculation, two or three animals from each group were sacrificed on selected days p.i., their tissues were collected, and the titers of the virus were determined (11). Virus was recovered from ileum, spleen, liver, and brain of animals infected with either T1 or T3 or the MI. The amounts of virus recovered from the organs of the three groups are illustrated in Fig. 1.

In the ileum, T1 was initially recovered at high titers on day 3 (10^6 PFU/ml) and rapidly decreased after day 3 (Fig. 1A). T3 virus never reached high titers (a maximum of 10^4 PFU/ml) but was recovered from the ileum until day 9 p.i. Virus titers from the ileum of MI-infected animals maintained levels of between 10^5 and 10^6 PFU of virus from days 1 through 7. On day 13, 10^3 PFU of virus was still recovered from MI animals. In the spleen and liver, T3 was present in lower amounts in T3-inoculated animals than in the same organs from T1- and MI-infected animals (Fig. 1B and 1C). MI-infected animals had slightly higher virus titers in the spleen and liver, as well as in the ileum (see above), at the end of the experiment. In the brain, the titers of T1 and MI were similar, and T3 reached titers equal to those of T1 by day 11, remaining at higher levels at the termination of the experiment on day 13 (Fig. 1D). Since animals infected with MI often had enhanced yields of virus compared with those from animals inoculated with T1 or T3 alone, it was likely that reassortants were present in such samples.

To determine whether reassortant viruses were present in the MI-infected animals, sonicated and diluted tissue samples of organs collected from MI-infected animals were titrated on L-cell monolayers (10). Clones were isolated from plates having one to five plaques, ensuring that reassortment did not take place during isolation of virus. Virus from the resulting plaques was amplified, and its electropherotype

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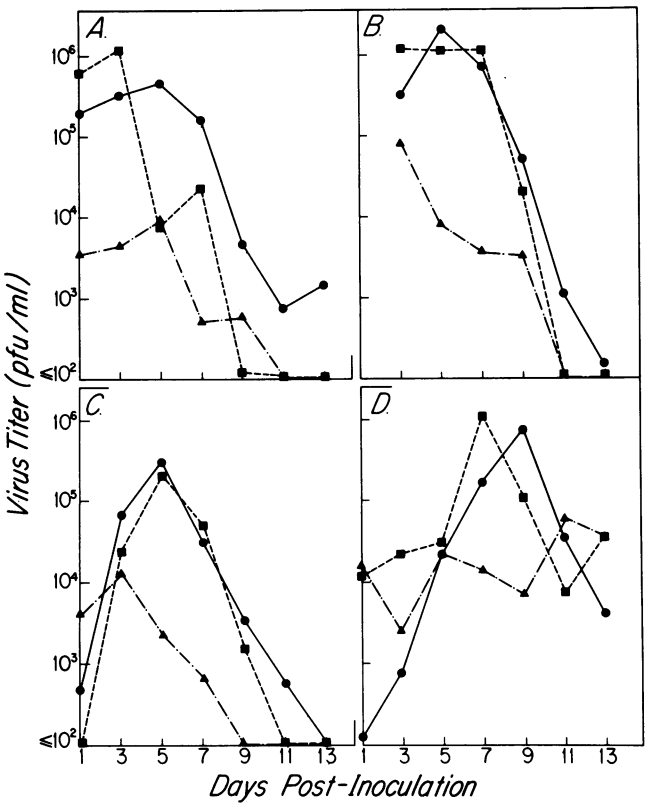

FIG. 1. Growth of T1, T3, or T1 plus T3 in NIH Swiss suckling mice after peroral inoculation. Mice were perorally inoculated with 2×10^7 PFU of T1 (■), 2×10^7 PFU of T3 (▲), and 1×10^7 PFU of T1 plus 1×10^7 PFU of T3 (●). Pairs of mice were sacrificed at the indicated times, and organs were removed and titrated for infectious virus by plaque assay. (A) Ileum. (B) Liver. (C) Spleen. (D) Brain.

was determined by polyacrylamide gel electrophoresis (10–13, 15). Reassortant viruses were identified by comparing the migrations on polyacrylamide gel electrophoresis of the double-stranded RNAs from viral clones isolated from tissues of MI-infected animals with those of parental T1 and T3 viruses (13). Viruses were scored as reassortants if their genomes consisted of RNA segments derived from both T1 and T3 RNA parents.

The RNAs of 1,276 plaque-purified clones were examined from samples collected on days 1 through 13 p.i. (Tables 1 and 2). Before day 7, T1 was the only virus isolated from the four tissues examined (ileum, spleen, liver, and brain). At later times (days 9, 11, and 13), T3 virus was also isolated from brain, ileum, or both. T3 was not isolated from spleen or liver samples. Beginning on day 7, reassortants were isolated, and 121 reassortant clones were isolated from a total sample of 1,276 clones examined.

Five different electropherotypes of reassortants (designated as E1 through E5) isolated from mouse organs were present among the total of 121 reassortant clones (Table 1). The majority of the isolates had either the E1 or E2 electropherotype. The distribution of the reassortant isolates found in individual tissues and their times of isolation are shown in Table 2. The E2 isolates were found mainly on day 7, with fewer on day 9. Isolates of E2 were found from all four organs, although there was only a single isolate from spleen tissue. E1 was isolated at later times, appearing first on day 11, but appearing in larger numbers by day 13. Most E1 isolates were from ileum or brain tissue, and there were no isolates from spleen tissue. E3, E4, and E5 were each single isolates. The E3 and E4 clones were isolated from the ileum of a single animal sacrificed on day 11 p.i. The E5 isolate was from the liver of an animal sacrificed on day 9 p.i.

In this study, we analyzed virus clones isolated from selected organs of animals that were simultaneously infected with reoviruses T1 and T3 and found that reovirus serotypes reassorted genome segments in the infected animal. The reassortants isolated did not represent the spectrum of electropherotypes typically found when reoviruses reassort in vitro (3). Instead, there was selection for two reassortants, E1 and E2. In both reassortants most of the double-stranded RNA segments were derived from the T1 parent. However, the M2 and S1 segments of E1 and the S1 and S2 segments of E2 were derived from T3. These two isolates represent 97% of the reassortants that were recovered. Since both were isolated from more than one organ and from different mice on different days, the data strongly suggest that host factors were playing an important role in the selection of E1 and E2. The three other reassortants were represented by single isolates and were thus more difficult to interpret.

Earlier studies showed that the reovirus T1 (Lang) serotype was consistently recovered in higher titers than was reovirus T3 (Dearing) after peroral inoculation (10). Thus, it was not surprising that the majority of genes found in the reassortants were derived from T1 since it was expected that there would be a larger pool of T1 genes in the infected animals.

The fact that the M2 gene in the reassortants isolated in vivo was derived from the T3 (Dearing) parent was surprising since the T1 M2 gene conferred a growth advantage in prior experiments. Previous studies of 2-day-old inoculated animals indicated that reassortants generated in vitro which contained an M2 gene of T3 (Dearing) did not grow in intestinal tissues as well as did those which contained an M2 gene derived from T1 (Lang). However, viruses containing mutant M2 genes of T3 origin were capable of growing to a higher titer in intestinal tissue (14). Such mutants were frequently found in mutagenized virus stocks (14). Thus, it is possible that the M2 of the in vivo reassortants is an altered T3 gene whose selection conferred a growth advantage over either parental strain. Which host factors may be influencing such a selection have not been defined.

The finding of the S1 gene of T3 in reassortants isolated from the brain is not surprising. Viruses having the T3 S1 grow well in neurons, while viruses containing the T1 S1 gene do not (20). The latter are restricted to growth in ependymal cells, a population of cells found in much lower numbers than are neurons in the central nervous system. Thus, the tropism of T3 to neurons would confer a selective growth advantage in the brain.

The finding of in vivo reassortment under laboratory conditions suggests that reassortment among mammalian reoviruses may also occur in nature. However, Gaillard and Joklik, based on RNA hybridization of three laboratory strains [T1 (Lang), T2 (Jones), and T3 (Dearing)], have suggested that the three serotypes have evolved independently (6). This would imply that in vivo reassortment, at least between serotypes 1, 2, and 3, plays an insignificant role in the evolution of reoviruses in nature. In contrast to the hybridization analysis, prior studies from our laboratory with a large number of field isolates collected from various species suggest that in vivo reassortment is taking place. This conclusion is based (i) on the high degree of heterogeneity of electropherotypes among various isolates (9) and (ii)

TABLE 1. Derivation of viral RNA segments from tissue samples of MI-infected mice

Parent or reassortant	Parental origin of gene:										No. isolated (% of total)[a]
	Outer capsid			Core					Nonstructural		
	S1	M2	S4	L1	L2	L3	M1	S2	M3	S3	
Parents											
T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	1,041 (82)
T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	114 (8.9)
Reassortants											
E1	T3	T3	T1	T1	T1	T1	T1	T1	T1	T1	64 (5.0)
E2	T1	T3	T1	T1	T1	T1	T1	T3	T1	T1	54 (4.2)
E3	T3	T3	T3	T3	T3	T3	T1	T1	T3	T3	1 (0.08)
E4	T3	T1	T3	T1	T1	T1	T1	T3	T1	T3	1 (0.08)
E5	T1	T1	T1	T3	T3	T3	T1	T1	T1	T1	1 (0.08)

[a] Total number of plaques analyzed was 1,276.

on our analysis of tryptic peptides of the outer capsid proteins. For example, σ3 proteins of some T1 and T3 isolates appear to be closely related (7). In fact, tryptic peptide digests of the σ3 proteins can be grouped independently of the serotype of the isolates (7). These data are difficult to reconcile with independent evolution of reovirus serotypes 1, 2, and 3. Reassortment in nature is a likely explanation for these results.

Examples of naturally occurring reassortants of segmented viruses come from studies of influenza viruses. Both in vitro and in vivo reassortment of influenza viruses have been extensively documented (8, 19). Evidence indicating the presence of naturally occurring reassortants of other segmented RNA viruses has also been reported. Oligonucleotide fingerprint studies of the bluetongue virus (17) and bunyavirus (18) isolates indicate that these viruses exist as naturally occurring reassortants. Reassortment of the bluetongue virus is of particular interest since orbiviruses are in the *Reoviridae* family. In addition, reassortment of rotaviruses, another member of the *Reoviridae*, has been suggested to explain the high degree of electrophoretic heterogeneity of rotavirus isolates (5).

An important question derived from these findings is whether the progeny reassortant viruses selected in vivo differ from the parental viruses in their capacity to grow and cause disease in the infected mice. Preliminary studies indicate that both E1 and E2 grow to high titers and are more pathogenic than either T1 (Lang) or T3 (Dearing) after

TABLE 2. Presence of T1, T3, and reassortants in mouse organs

Day p.i.	Mouse no.	Total no. of plaques picked per mouse	No. of plaques of clone type:															
			Ileum				Spleen				Liver				Brain			
			T1	T3	E1	E2	T1	T3	E1	E2	T1	T3	E1	E2	T1	T3	E1	E2
1	1	12	12	0	0	0	NU[a]	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU
	2	20	20	0	0	0	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU
3	1	57	57	0	0	0	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU
	2	42	42	0	0	0	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU	NU
5	1	155	39	0	0	0	40	0	0	0	26	0	0	0	50	0	0	0
	2	109	18	0	0	0	27	0	0	0	58	0	0	0	6	0	0	0
7	1	78	16	0	0	20	NU	NU	NU	NU	18	0	0	11	2	0	0	11
	2	74	39	0	0	5	NU	NU	NU	NU	19	0	0	2	9	0	0	0
	3	84	21	0	0	0	22	0	0	0	25	0	0	1	15	0	0	0
9	1	170	42	0	0	0	24	0	0	1	32	0	0	1[b]	67	2	0	1
	2	146	49	0	0	1	18	0	0	0	33	0	0	0	33	12	0	0
11	1	128	ND[c]	ND	ND	ND	24	0	0	0	54	0	5	0	45	0	0	0
	2	99	26	17	3[d]	0	ND	ND	ND	ND	5	0	0	0	8	40	0	0
13	1	44	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	43	1	0
	2	55	0	0	27	0	ND	ND	ND	ND	ND	ND	ND	ND	0	0	28	0

[a] NU, Not used to isolate virus.
[b] E5 reassortant detected as a single isolate.
[c] ND, No virus detected in these organs.
[d] E3 and E4 reassortants each detected as single isolates.

peroral inoculation. Further studies are in progress to study the significance of these findings.

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LITERATURE CITED

1. **Ahmed, R., and B. N. Fields.** 1982. Role of the S4 gene in the establishment of persistent reovirus infection in L cells. Cell **28**:605–612.
2. **Cross, R. K., and B. N. Fields.** 1976. Use of an aberrant polypeptide as a marker in three-factor crosses: further evidence for independent reassortment as the mechanism of recombination between temperature-sensitive mutants of reovirus type 3. Virology **74**:345–362.
3. **Drayna, D., and B. N. Fields.** 1982. Genetic studies on the mechanism of chemical and physical inactivation of reovirus. J. Gen. Virol. **63**:149–159.
4. **Fields, B. N., and M. I. Greene.** 1982. Genetic and molecular mechanisms of viral pathogenesis: implications for prevention and treatment. Nature (London) **300**:19–23.
5. **Flores, J., I. Perez, L. White, M. Perez, A. R. Kalica, R. Marquina, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1982. Genetic relatedness among human rotaviruses as determined by RNA hybridization. Inf. Immun. **37**:648–655.
6. **Gaillard, R. K., Jr., and W. K. Joklik.** 1982. Quantitation of the relatedness of reovirus serotypes 1, 2, 3 at the gene level. Virology **123**:152–164.
7. **Gentsch, J. R., and B. N. Fields.** 1984. Genetic diversity in natural populations of mammalian reoviruses: tryptic peptide analysis of outer capsid polypeptides of murine, bovine, and human type 1 and 3 reovirus strains. J. Virol. **49**:641–651.
8. **Hinshaw, V. S., W. J. Bean, R. G. Webster, and G. Sriram.** 1980. Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology **102**:412–419.
9. **Hrdy, D. B., L. Rosen, and B. N. Fields.** 1979. Polymorphism of the migration of double-stranded RNA genome segments of reovirus isolates from humans, cattle, and mice. J. Virol. **31**:104–111.
10. **Hrdy, D. B., D. H. Rubin, and B. N. Fields.** 1982. Molecular basis of reovirus neurovirulence: role of the M2 gene in avirulence. Proc. Natl. Acad. Sci. USA **79**:1298–1302.
11. **Kauffman, R. S., J. L. Wolf, R. Finberg, J. R. Trier, and B. N. Fields.** 1983. The σ1 protein determines the extent of spread of reovirus from the gastrointestinal tract of mice. Virology **124**:403–410.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Ramig, R. F., R. K. Cross, and B. N. Fields.** 1977. Genome RNAs and polypeptides of reovirus serotypes 1, 2, and 3. J. Virol. **22**:726–733.
14. **Rubin, D. H., and B. N. Fields.** 1980. Molecular basis of reovirus virulence: role of the M2 gene. J. Exp. Med. **152**:853–868.
15. **Sharpe, A. H., and B. N. Fields.** 1982. Reovirus inhibition of cellular RNA and protein synthesis: role of the S4 gene. Virology **122**:381–391.
16. **Sharpe, A. H., R. F. Ramig, T. A. Mustoe, and B. N. Fields.** 1978. A genetic map of reovirus. I. Correlation of genome RNAs between serotypes 1, 2, and 3. Virology **84**:63–74.
17. **Sugiyama, K., D. H. L. Bishop, and P. Roy.** 1981. Analyses of the genomes of bluetongue viruses recovered in the United States. I. Oligonucleotide fingerprint studies that indicate the existence of naturally occurring reassortant BTV isolates. Virology **114**:210–217.
18. **Ushijima, H., C. M. Clerx-Van Haaster, and D. H. L. Bishop.** 1981. Analyses of Patois bunyaviruses: evidence and existence of immune precipitable and nonprecipitable nonvirion proteins induced in bunyavirus-infected cells. Virology **110**:318–332.
19. **Webster, R. G., W. G. Laver, G. M. Air, and G. C. Schild.** 1982. Molecular mechanisms of variation in influenza viruses. Nature (London) **296**:115–121.
20. **Weiner, H. L., M. L. Powers, and B. N. Fields.** 1980. Absolute linkage of virulence and central nervous system cell tropism of reoviruses to viral hemagglutinin. J. Infect. Dis. **141**:609–616.