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Generation of Defective Virus After Infection of Newborn Rats with Reovirus

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When 2-day-old rats were inoculated subcutaneously with the R_2 strain of reovirus type 3 or with a class B (352) or class C (447) temperature-sensitive (*ts*) mutant, 5 to 10% of the animals died from acute encephalitis within 12 days. Approximately half of the survivors recovered rapidly and grew normally, but the remainder became runted. Two phases of infection are distinguished in the animals: an acute phase during which infectious virus reaches a maximum titer in brain and other tissues by 10 days p.i. and then rapidly decreases, and a succeeding chronic phase which involves runting of the rats and the slow disappearance of virus from their brains over a period of 2 months or so. Virus isolated from chronically infected brains generally retained the genetic character (*ts* or wild type) of the inoculated virus, but two exceptions to this are described. Defective virions lacking the L_1 segment of the viral genome (L_1 defectives) were generated in rat brains during the acute phase of infection. Defective virus was also generated during the chronic phase, but during this period defectives were found with multiple segments deleted from the genome in addition to L_1 defectives. In another type of experiment defective virus exerted a marked protective effect when inoculated intracerebrally with R_2 virus. In the absence of defectives all animals died, but in their presence 17 of 23 animals survived and 15 of 23 became runted and chronically infected. The formation and evolution of defective particles in the brains of these rats were similar to those found in rats chronically infected after subcutaneous inoculation of reovirus. We conclude that the formation of defective virus particles may play a role in the initiation and maintenance of chronic neurotropic infections with reovirus.

There is a rapidly growing body of evidence to show that an acute viral infection may be followed on occasion by a chronic phase during which slowly progressing degenerative disorders appear in one or another organ of the body. One of the best known examples is that of subacute sclerosing panencephalitis, which has been attributed to a measles-like agent that persists in the brain after recovery from acute measles infection during childhood (10). In some other chronic diseases of suspected viral etiology no virus has been detected in the affected tissues, and evidence that the disorders are virus-associated is largely indirect (11). How the chronic phase evolves from the acute viral infection is generally unknown, and very likely the mechanism of its development varies from one virus to another (14). Some recent reports suggest, however, that the formation of temperature-sensitive (*ts*) and defective virus during infection may play an important role in the evolution of chronic viral infections in animals. For example, *ts* mutations in measles virus (6) and vesicular stomatitis virus (VSV) (28) have been shown to lead to altered disease states in the brains of infected mice. Defective virions of VSV were found to exert a prophylactic effect when inoculated intracerebrally along with wild-type virus (2), and, moreover, defective virions of both VSV and rabies virus are formed in large amounts when the viruses are serially passaged in the brains of newborn mice (8). Reovirus can also induce chronic infections in animals, and in this work we have posed the question of whether defective virus might be involved in the development of the chronic disorder.

There is an extensive and detailed literature on reovirus infections in animal systems, especially in murine models (5, 9, 12, 13, 17, 26-30). In general, it would appear that a majority of newborn animals infected with reovirus die within 2 weeks. During this acute phase of the disease, virus spreads throughout the tissues of the animals, and a variety of symptoms are produced, including hepatitis, encephalitis, steatorrhea, and oily skin. The clinical syndrome varies markedly with type of virus, species of

animal, route of inoculation, and dose of virus. A small proportion of animals may survive the acute infection and go on to a more chronic illness in which they become runted. Recently Fields and Raine (3, 18, 19) have approached the problem of chronic infection by examining the neurovirulence of type 3 reovirus and its class B and C *ts* mutants (4) when inoculated intracerebrally into newborn rats. The wild-type virus induced an acute necrotizing encephalitis, with death occurring between 1 and 2 weeks. A small number of survivors recovered completely from the infection. On the other hand, the majority of animals survived intracerebral inoculation of the C mutant with no long-term effects. Survivors of the B mutant infection, however, slowly developed an illness whose major feature was brain degeneration resembling communicating hydrocephalus. Thus, single-gene differences in this virus have produced marked alterations in the nature of the disease produced.

Fields and Raine have interpreted their results mainly in terms of the phenotypic expressions of the *ts* mutants at temperatures close to nonpermissive in the brain, but the explanation could be more complex. Reovirus and its *ts* mutants readily generate defective virions when passaged in cell culture (15, 16, 20). Moreover, we have found (R. Ahmed and R. Y. Lau, unpublished observations) that persistently infected cultures of L cells can be readily obtained when the initial infection is carried out with *ts* mutants containing defective virus. Persistently infected lines are seldom produced if defective virus is not present during the initial infection, suggesting that defective virus can modify the virulence of the infectious moiety. Similar observations have been made with VSV and its defectives (7). For this reason we started the present work with the prime purpose of determining whether defective virus is formed in the brains of rats infected with reovirus. Newborn rats were infected subcutaneously with wild-type 3 reovirus and its B and C *ts* mutants. Defective virus was readily detected in the brains during both the acute and chronic phases of the infection and was still being formed by 60 days after the initial infection.

MATERIALS AND METHODS

Cells and virus. L cells were grown in suspension or as monolayers in Eagle minimal essential medium (MEM) supplemented with 5% fetal calf serum. The wild-type strain R_2 of reovirus type 3 and the *ts* mutants R_2B (352) and R_2C (447) were used (4, 22). All infections were carried out with purified virus suspensions in which no defective particles could be detected (21). Purified R_1d (L_1) defective virions were prepared as described (22).

Animals. Two-day-old Sprague-Dawley rats were inoculated with 0.01 ml of purified virus suspended in MEM, using a Hamilton 50-μl syringe. The amount of virus used and route of inoculation will be specified for each experiment. Animals were anesthetized with ether before the various required organs were removed. To label reovirus growing in the brains of newborn rats, 10 μCi of [^{3}H]uridine (25 Ci/mmol) was injected intracerebrally in 0.1 ml of MEM.

Extraction of virus from tissues. Tissue (brain, liver, lung, kidney, or heart) was weighed and homogenized in TMN buffer [0.25 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride, pH 8.0, and 0.01 M 2-mercaptoethanol] with an equal volume of Freon-113 (31). The aqueous phase was centrifuged at 25,000 rpm for 90 min in an SW27.1 rotor at 4°C, and the pellet containing the virus was resuspended by sonic oscillation in 1 ml of MEM. Virus was titrated by the plaque technique on monolayers of L cells (22). Blood was collected asceptically, approximately 1 ml/rat, diluted with 9 volumes of distilled water, and stored at −70°C until required for virus assay.

Amplification test for detection of defective virus. In some experiments it was necessary to find whether defective virus was being generated in brain tissue, but the level of viral growth was too low to label it in vivo. To detect such defective virus an "amplification" technique was used. Virus was extracted from the brain and plaque titered. The virus was then concentrated by centrifugation and used to infect a monolayer of L cells at a multiplicity of infection of 10 PFU/cell. Adsorption was allowed for 1 h at 31°C, and then 5 ml of MEM containing 0.5 μg of actinomycin D per ml and 2% fetal calf serum was added and the plate was placed at 31°C. Progeny virus resulting from this L cell infection was labeled by adding 10 μCi of [^{3}H]uridine at 5 h postinfection (p.i.). After 40 h cells and medium were transferred to an SW27.1 nitrocellulose tube, and a known amount of ^{14}C-labeled purified virus was added as a marker. The mixture was subjected to sonic oscillation for three 30-s intervals, and the tubes were filled with 0.05 M Tris-chloride (pH 8.0) and centrifuged in an SW27.1 rotor at 25,000 rpm for 90 min at 4°C. The pellet was resuspended in 1 ml of 0.05 M Tris-chloride, pH 8.0. Recoveries of ^{14}C-labeled virus were 80 to 90%. A 0.4-ml quantity of the resulting suspension was made 0.2 M in CsCl, digested with chymotrypsin (100 μg/ml) layered onto a preformed gradient of CsCl (ρ = 1.35 to 1.46 g/ml), and centrifuged in an SW40 rotor at 37,000 rpm for 6 h at 4°C. Fractions were collected and assayed for radioactivity. The remaining 0.6 ml of viral suspension was made 0.3 M in NaCl, and the double-stranded (ds) RNA was extracted with 0.5% sodium dodecyl sulfate and phenol and analyzed by polyacrylamide gel electrophoresis. The tritiated RNA bands on the gel were detected by fluorography (1, 24).

Plaque reduction test for reoviral antibodies. Blood was taken from two rats at different times

after infection and allowed to clot. The serum was removed, heated to 50°C for 30 min, and diluted in 10-fold steps in MEM. To each dilution was added an equal volume of reovirus suspension in MEM containing 10^6 PFU of R_2 virus. The virus-serum mixtures were incubated at 37°C for 2 h, diluted, and assayed by the plaque technique. Virus suspensions containing no antibody were treated in the same way for controls. As a further control blood was withdrawn from uninfected rats 30 days of age and treated similarly to that from infected animals. Virus remaining after treatment with serum from infected rats was calculated as a percentage of the amount of virus added.

Electron microscopy of thin sections. Brains were excised divided at the midline and immediately immersed in Karnovsky fixative. While under the fixative, they were sliced with a razor blade into approximately 1-mm sections. Small pieces, approximately 1 mm^3, were cut out at random from different slices and fixed overnight at 4°C in Karnovsky fixative. The pieces of tissue were then washed several times in 0.18 M sodium cacodylate buffer, pH 7.4, postfixed for 1 h in 1.33% osmic acid buffered with S-collidine, block-stained for 0.5 h in saturated aqueous uranyl acetate, dehydrated in graded ethanol steps, and embedded in Vestopal W. Ultrathin sections were cut on an LKB Ultratome III with a diamond knife, and sections were mounted on copper grids with carbon-coated collodion support film, stained with lead citrate, and examined in a Phillips 300 electron microscope.

RESULTS

Preliminary experiments showed that intracerebral inoculation of newborn rats with 10^2 to 10^8 PFU of the R_2 strain of reovirus type 3 per rat resulted in death of all animals within 2 weeks, as already found by Raine and Fields (18). In agreement with previous work, it was also found that *ts* mutants of the R_2 strain (3, 19) and type 1 reovirus (17) were less virulent than R_2 virus itself by the intracerebral route, and animals frequently survived the acute phase of the infection. Nevertheless, inoculation of virus by the intracerebral route is an artificial means of producing a neurotropic infection and occurs rarely, if at all, in nature. In a naturally occurring infection, a viral population would normally be subjected to various selective pressures exerted by the host before the neurotropic component of the infection becomes manifest. To permit the host to react against the infecting virus, the subcutaneous route of infection was used in the majority of the succeeding experiments.

In summary, when 2-day-old rats were infected subcutaneously with 10^4 PFU or less of wild-type virus (R_2), no signs of illness developed. Higher doses, 10^7 to 10^8 PFU/rat, had no obvious effect when the rats were older than 5 days. However, when 2-day-old rats were infected subcutaneously with 10^7 to 10^8 PFU of virus, 5 to 10% developed acute encephalitis similar to that described for intracerebral inoculation (18) and died. Approximately 50% of the survivors became runted. It was easy to determine even during the first few days after infection which animals would be runted and to treat them as separate groups. Animals that appeared normal after infection gained weight at the same rate as uninfected controls, and those that became runted grew much more slowly, as shown in Fig. 1. At intervals during such an experiment, several animals were killed and the brains were examined for virus. Thirty days after subcutaneous infection of two-day-old rats with R_2 virus, no virus could be detected in any of the brains of rats that grew normally, and by 80 days p.i. none could be found in the brains of runted rats. Similar observations were made for newborn rats infected subcutaneously with R_2B (352) (4×10^7 PFU/rat) and R_2C (447) (2×10^7 PFU/rat) *ts* mutants of reovirus.

Appearance of reovirus in various tissues after subcutaneous infection of newborn rats. To determine the rate at which virus appeared in the various tissues, a group of 2-day-old rats was inoculated subcutaneously with 10^7 PFU of R_2 virus per rat. Every 2 days the virus was extracted from the tissues of a pair of animals, as described in Materials and Methods, and plaque titered. The results are shown in Fig. 2. Virus appeared rapidly in blood, brain, and

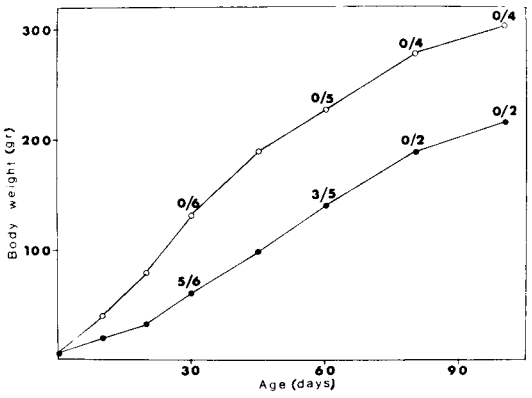

FIG. 1. *Rate of growth of rats that became runted through the subcutaneous inoculation of reovirus. Each 2-day-old rat received 10^7 PFU of R_2 virus. Symbols: (●) Runted rats; (○) uninfected rats and infected rats that grew normally. Each point on the curves represents the average weight of three to five rats. As explained in the text approximately 50% of the infected rats became runted, and the remainder grew as the controls. The fractions written above the points on the curves were obtained in a separate experiment and represent the number of rat brains in which virus was detected divided by the number of brains examined.*

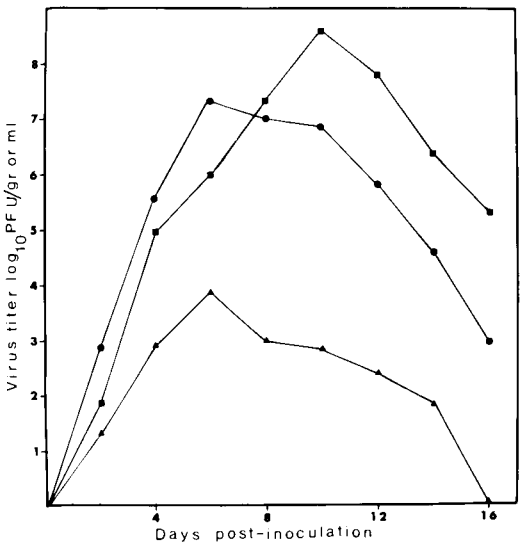

FIG. 2. *Appearance of reovirus in organs of rats infected with reovirus. Two-day-old rats were injected subcutaneously with 10^7 PFU of R_2 virus per rat. Each point represents the average results for the organs of two rats. Virus isolated from the organs was assayed by plaque titer on L cell monolayers. Symbols: (■) Brain; (●) liver; (▲) blood.*

liver (and lungs, results not shown) and reached a maximum between 6 and 10 days. Viral concentrations were highest in brain, reaching 4×10^8 PFU of tissue per g by 10 days after infection. After reaching a peak in each tissue, the viral concentrations rapidly decreased during the next several days. Similar experiments were carried out with the two *ts* mutants of reovirus, B (352) and C (447). Both mutants followed the same general course and multiplied to approximately the same maximum titers as R_2 virus in the various tissues. Moreover, both mutants retained their *ts* characters during this period. Death occurred during the first 10 to 14 days for the 5 to 10% of the animals that died as a result of these infections. This will be referred to as the acute phase of the infection. After this acute phase some 50% of the animals recovered rapidly and completely as described in the preceding section. However, the remaining animals became runted, and virus persisted in the brains of these animals for at least 2 months. The runted animals will be referred to as chronically infected.

Formation of defective virions during the acute phase of infection. Since virus multiplied to a high titer in the brains of acutely infected baby rats, it was of interest to determine whether defective virus was being generated during this stage of infection. This question was examined in rats infected by two routes, intracerebrally and subcutaneously.

In the first experiment 2-day-old rats were inoculated intracerebrally with 10^7 PFU of R_2 virus per rat. At the time of infection or 6 days later, 10 μCi of [^{3}H]uridine was injected intracerebrally to label the progeny virus. All animals were either dead or moribund by 8 days p.i. At this time the brains were removed from moribund animals, and the virus was extracted from them, mixed with ^{14}C-labeled, purified R_2 virions, digested with chymotrypsin to convert the virus to cores, and sedimented in a gradient of CsCl. The results are shown in Fig. 3. Most of the virus formed during the 8-day period was standard virus, since the majority of ^{3}H-labeled cores banded at the characteristic density of 1.43 g/ml (Fig. 3a). However, the small trailing peak at ρ = 1.415 g/ml represents defective virus that had been generated during the 8-day period of viral multiplication, approximately 10% of the total viral population; about 50% of the virus formed between 6 to 8 days after infection was defective (Fig. 3b). Analysis of the double-stranded RNA of these defective virions by polyacrylamide gel electrophoresis showed that they lacked the L_1 segment of the viral genome. Thus defective virions were generated in the brains of rats and accumulated most rapidly during the latter stages of this acute infection.

Next, after the subcutaneous inoculation the brains of acutely infected rats were examined for the formation of defective virions. It was found that viral multiplication under these circumstances was not sufficiently extensive to permit the viral populations to be radioactively labeled in vivo. The nature of these populations was determined through recourse to the amplification technique described in Materials and Methods. Virus was extracted from the brains of rats at different times during the acute phase of the infection. The virus from each brain was concentrated and used to infect a monolayer of L cells. A multiplicity of infection of 10 PFU/cell was used to ensure that every cell would receive an infectious particle and thus complement the growth of defectives in any co-infected cells. Virus was labeled during its growth, and the resulting progeny from each L cell monolayer were examined for defective virus by sedimentation on CsCl gradients after chymotrypsin digestion. Results of such experiments after infection of baby rats with R_2 virus and with B (352) and C (447) *ts* mutants are shown in Fig. 4. Defective virions whose cores banded at 1.415 g/ml were generated in all three infections, and in the infection with the C mutant the small core peak at ρ = 1.40 suggested that some

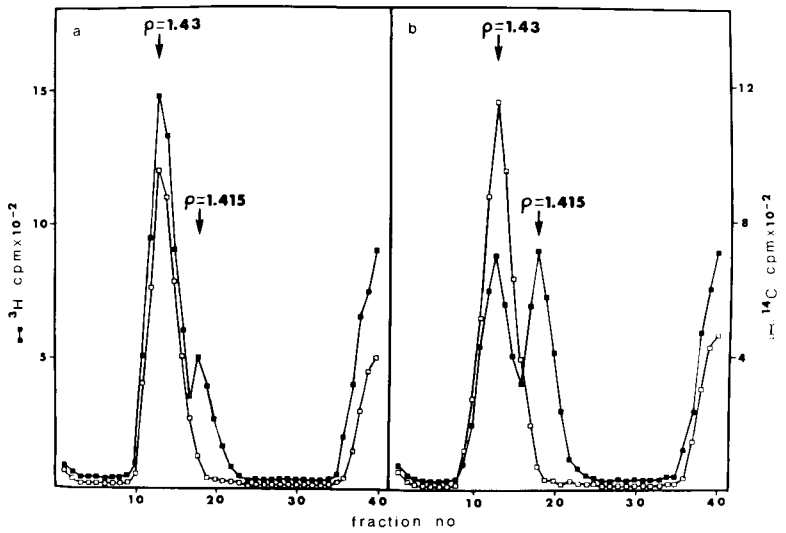

FIG. 3. *Isopycnic centrifugation in CsCl of ^{3}H-labeled virus obtained from the brains of rats at the acute stage of infection after intracerebral inoculation. Two-day-old rats were inoulated intracerebrally with 10^7 PFU of R_2 virus, and the brains were removed at 8 days p.i. The isolated ^{3}H-labeled virus was mixed with ^{14}C-labeled purified R_2 virus, the mixture was digested with chymotrypsin, and the resulting cores were analyzed by sedimentation through a preformed gradient of CsCl. Symbols: (□) ^{14}C-labeled marker cores; (■) ^{3}H-labeled cores. (a) Each rat received 10 μCi of [^{3}H]uridine at the time of infection; (b) each rat received 10 μCi of [^{3}H]uridine intracerebrally 6 days p.i.*

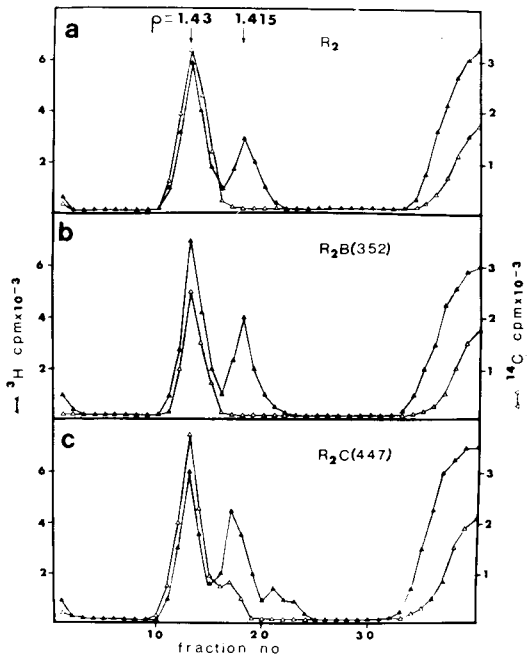

FIG. 4. *Isopycnic centrifugation in CsCl of amplified virus obtained from the brains of rats at the acute stage of infection after subcutaneous inoculation of 2-day-old rats. Virus was isolated from the brains of rats, concentrated, and then amplified by a single passage in L cells during which the progeny was labeled with [^{3}H]uridine. Purified marker virus labeled with ^{14}C was added to the L cell lysate, and the virus was concentrated, digested with chymotrypsin, and centrifuged in a CsCl gradient. (a) Animals were infected with R_2 virus, and virus was isolated from the brain 9 days p.i. (b) Animals were infected with R_2B (352) mutant, and virus was isolated from the brain at 10 days p.i. (c) Animals were infected with R_2C (447) mutant, and virus was isolated from the brain 11 days p.i. Symbols: (▲) ^{3}H-labeled virus from rat brain; (△) ^{14}C-labeled marker virus. To amplify the virus from brains infected with the two mutants the infected L cells were incubated at 31°C. Marker virus for the gradient analyses was prepared by infecting L cells with a portion of the same virus used to infect each group of animals, labeling the progeny with [^{14}C]uridine during its growth (at 31°C for R_2B and R_2C) and then purifying it.*

defective virions lacking more than one segment were being formed.

Thus, the defective virus found at the acute stage of infection was similar to that most readily generated by serial passage of reovirus in cell culture (15, 16). Although we could estimate that about 10% of the viral population isolated after intracerebral inoculation was defective, it is impossible to give any such quantitative result for the population derived after subcutaneous infection because of the amplification step involved.

Multiplication of virus during the persist-

ent stage of infection. Runted animals that survived the acute phase of the infection from subcutaneous inoculation were examined periodically for the presence of virus. A representative set of results is shown in Table 1. There was considerable variation in the amounts of virus in the brains within any one group, and in a few animals no virus could be detected. As the time after infection increased, the amounts of virus in the brains decreased, and, in fact, no virus could be detected in any animals examined at 80 and 100 days p.i. Considerably less virus was found in the liver than in the brain at all times. By 30 days p.i. no virus could be detected in lung and blood.

In general the *ts* properties of the virus isolated during the persistent infection were the same as that used in the original infection. There were two exceptions. Thirty days after infection with R_2 virus a *ts* mutant was isolated from the brain of one animal (third row, Table 1), and this mutant will be designated R_2X. It will be shown later that this is a class A *ts* mutant. Virus from the liver of the same animal was not *ts*, suggesting that the mutant originated during growth of virus in the brain. In addition, one animal infected with the R_2B mutant had ts^+ virus in both brain and liver after 30 days (Table 1).

To determine whether defective virions were generated during the chronic stage of infection, virus isolated from brains 30 days after subcutaneous inoculation was concentrated and amplified on monolayers of L cells, and cores from the resulting progeny were analyzed by sedimentation on CsCl gradients. Results on the virus from three individual brains are shown in Fig. 5. In each case the viral population contained defective virus, and similar observations were made with the virus from all brains examined at this stage. It should be noted that in Fig. 5a an analysis has been included for a rat persistently infected with R_2X virus. The result with this mutant was similar to that found in rats persistently infected with R_2 virus 30 days p.i. The defective viral populations in these

TABLE 1. *Persistence of virus in tissue of runted rats inoculated subcutaneously with reovirus at 2 days of age*

Infecting virus	Days after infections	Yield of virus[a]			
		PFU/brain titrated at:		PFU/liver titrated at:	
		31°C	39°C	31°C	39°C
R_2^3, 10^7 PFU/rat	30	9×10^3	6×10^3	NV[b]	NV
		6×10^6	8×10^5	3×10^2	1×10^2
		8×10^7	7×10^4	6×10^4	3×10^4
		2×10^7	1×10^7		
		7×10^7	3×10^7		
		NV	NV	NV	NV
	60	8×10^2	5×10^2	NV	NV
		4×10^4	4×10^5	10^4	2×10^4
		3×10^5	1×10^5		
		2×10^4	2×10^4		
		NV	NV	NV	NV
R_2^3B (352), 4×10^7 PFU/rat	30	8×10^7	4×10^4	3×10^5	5×10^2
		9×10^7	7×10^7	1×10^5	5×10^4
		3×10^7	1×10^4		
		8×10^6	3×10^3		
		NV	NV		
	60	2×10^4	80	NV	NV
		3×10^5	2×10^2	1×10^3	NV
		1×10^3	NV		
		NV	NV		
R_2^3C (447), 4×10^7 PFU/rat	30	5×10^5	8×10^2	NV	NV
		7×10^7	1×10^5	9×10^4	80
		2×10^6	7×10^2		
		NV	NV		
	60	7×10^5	2×10^2	NV	NV
		5×10^3	NV		
		NV	NV		

[a] Each row represents titrations on the tissue of a single infected rat.
[b] The tissue was assayed, and no virus (NV) was detectable.

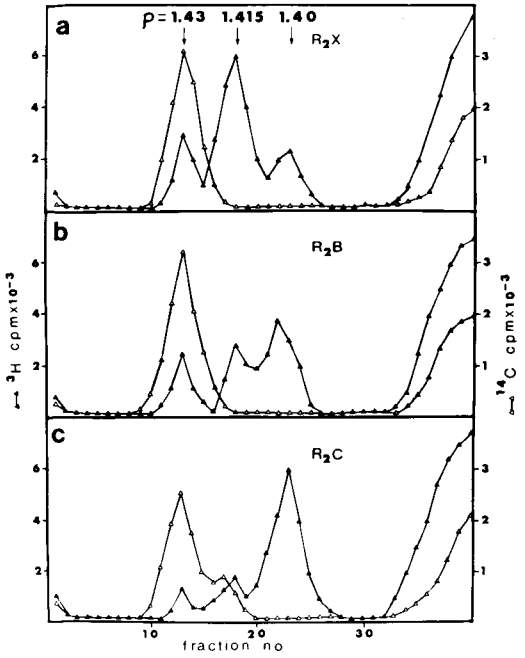

FIG. 5. *Isopycnic centrifugation in CsCl of amplified virus obtained from the brains of rats persistently infected 30 days after subcutaneous inoculation. Procedure was similar to that described in the legend to Fig. 4. (a) Animals infected with R_2X virus. (b) Animals infected with R_2B (352) mutant. (c) Animals infected with R_2C (447) mutant. Symbols: (▲) ^3H-labeled virus obtained from rat brain; (△) ^{14}C-labeled marker virus.*

persistently infected brains differed from acute-phase defectives in that in each persistent infection a considerable fraction of defective cores appeared at ρ = 1.40 g/ml in addition to that at 1.415 g/ml. Polyacrylamide gel electrophoretic analysis of the double-stranded RNA isolated from cores with ρ = 1.40 g/ml showed that segments L_1 and L_3 of the viral genome were missing, and these defectives therefore arose through multiple deletions from the original infectious viral genome.

Defective virus at late stages of persistent infection. Whereas infectious virus could be found in the brains of a large majority of rats 60 days after infection, none was found at 80 or 100 days p.i. Nevertheless, the possibility was considered that defective virus might still be present at these later times. Brains from six rats were obtained 100 days after subcutaneous infection with R_2B virus, each was subjected to the Freon extraction procedure, and the extracts were concentrated by centrifugation. Each pellet was resuspended and titrated for infectious virus, and a portion was added to a monolayer of L cells along with 10 PFU of purified R_2B (352) virus per cell. It was expected that if any defective virus was present in the Freon extracts it would be amplified by co-infecting the cells with infectious virus. Viral progeny from the L cell infection was labeled with [^{3}H]uridine during its growth, partially purified, subjected to chymotrypsin digestion, and centrifuged to CsCl gradient sedimentation. The results of one such test are shown in Fig. 6a along with the requisite controls in the two lower panels (Fig. 6b and 6c). It is clear from this test that defective virions were present in the brain of the persistently infected rat, although no infectious virus could be detected. Two of the six rat brains examined in the exper-

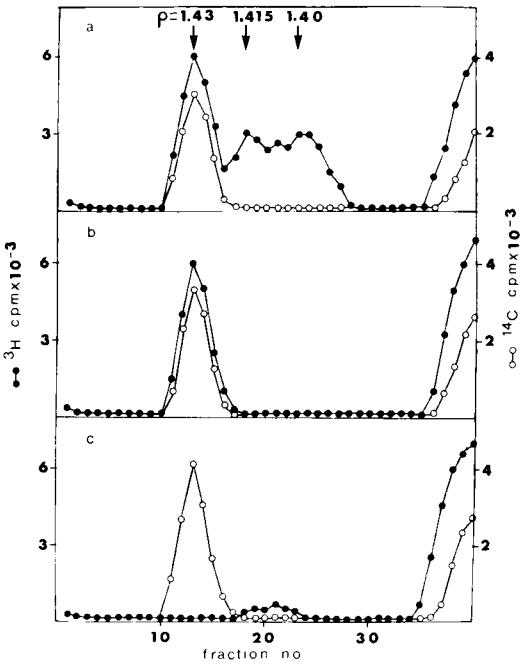

FIG. 6. *Isopycnic centrifugation in CsCl of amplified virus obtained from the brains of persistently infected rats 100 days after subcutaneous inoculation of R_2B virus. (a) A monolayer of L cells was co-infected with 10 PFU of R_2B (352) virus per cell and 0.2 ml of a concentrated extract from a rat brain 100 days after subcutaneous infection of a 2-day-old rat with 4 × 10^7 PFU of R_2B (352) virus. (b) A monolayer of L cells was co-infected with 10 PFU of R_2B (352) virus per cell and 0.2 ml of a concentrated extract from the brain of a 100-day-old uninfected rat. (c) A monolayer of L cells was infected with 0.2 ml of the same concentrated extract of a rat brain used in (a). Symbols: (○) [^{14}C]uridine-labeled cores of purified R_2B (352) virus used as a sedimentation marker in all three gradients; (●) [^{3}H]uridine-labeled cores obtained from the progeny of the cell co-infection.*

iment gave this same result, whereas no defectives were found in the other four brains. Apparently defective virus can be maintained at later stages in the brains of some persistently infected animals, even in the absence of detectable infectious virus.

Electron microscopy of brains from persistently infected rats. Thin sections were prepared from the brains of a number of rats 30 days after subcutaneous infection with R_2 virus, and it was easy to detect reovirus in the cytoplasm of neurons. Representative results are shown in Fig. 7. Figure 7a shows two viral factories close to the nuclear membrane. A different viral factory at higher magnification is shown in Fig. 7b, and the majority of the particles lack the double-stranded RNA genome, a common observation at this stage of infection. It was very difficult to find viral particles by this technique at 60 days p.i., although they were seen in some specimens.

Appearance of reoviral antibodies in the blood of infected rats. One reaction of the host to viral infection is through the production of humoral antibodies. To determine when this reaction might become a significant factor in influencing the course of infection, antibody levels were determined at intervals in the blood of rats inoculated subcutaneously with R_2 virus at 2 days of age. Antibody against reovirus was readily detected 30 and 60 days p.i. but not at 10 days (Fig. 8). Neutralizing antibody does not appear to play any significant role during the acute phase of the infection at least.

Effect of defective reovirus on the neurovirulence of wild-type virions. As shown in the preceding sections, defective virus rapidly appeared in the brains of infected rats even during the acute phase of infection. In an attempt to determine whether defective virions might modify the virulence of infectious virus, 2-day-old rats were inoculated intracerebrally with R_2 virus (10^4 PFU/rat) along with purified defective virus R_2d (L_1) (10^7 particles/rat) (22). All 26 animals infected with the R_2 virus alone died between 6 and 12 days p.i., and all 17 animals survived the inoculation of defective virions alone. During co-infection 6 out of 23 animals died of acute disease, and 15 of the remaining animals became runted. Thus the defective virions afforded considerable protection in the early phase of the intracerebral infection.

To examine the nature of the viral populations in the infected animals, virus was extracted from brains of moribund animals 10 days, and of runted animals 30 days, after the co-infection with defective and infectious virus. The virus from each brain was amplified on a monolayer of L cells, converted to cores, and analyzed by isopycnic centrifugation in a CsCl gradient. The results from two such amplified populations are shown in Fig. 9. Virus recovered from the brain during the acute phase of infection contained defective virus corresponding to R_2d (L_1) virions whose cores band at 1.415 g/ml. Virus obtained from runted animals 30 days p.i. contained, in addition, a second and relatively large population of defectives whose cores banded at 1.40 g/ml and which resulted from multiple deletions from the viral genome. This changing pattern of defectives from the acute to the persistent stage of infection is similar to that found when rats were infected subcutaneously with defective-free reovirus.

Effect of defective virus on the growth of wild-type virus in cell culture. It is well known for several virus-cell systems that defective virus can interfere more or less strongly with the development of infectious virus in co-infected cells. The following experiment was done to find whether such interference occurred between wild-type reovirus and defective virus in cell cultures. R_2d (L_1) virus was used, being the only clearly defined pure population of defective reovirus so far obtained (22). Cultures of L cells were co-infected with R_2 virus and defective virus under different conditions, and the growth curve of reovirus was determined in each case with the results shown in Fig. 10. When cells were infected simultaneously with infectious and defective particles in the ratio of 1/2, the yield of infectious virus was reduced by 0.3 logs. The yield was reduced by a further log if the cells were superinfected with infectious virus 4 h after the adsorption of defectives. These defective particles, therefore, definitely interfere with the growth of infectious virus, although the effect is not as marked as it is in some other viral systems.

Genetic characterization of a *ts* mutant isolated from a rat brain persistently infected by R_2 virus. A *ts* mutant designated R_2X was isolated from the brain of one rat that had been infected subcutaneously 30 days before with R_2 virus (Table 1). To determine whether this might be a naturally selected mutant or, perhaps, an inadvertant laboratory contamination, the mutant R_2X was tested by recombination and complementation with a number of known mutants in order to classify it genetically. The infectious center technique used for the genetic tests has been fully described (23). In summary, it consists of co-infecting a culture of L cells with two mutants, each at a multiplicity of infection of 10 PFU/cell. These cells are

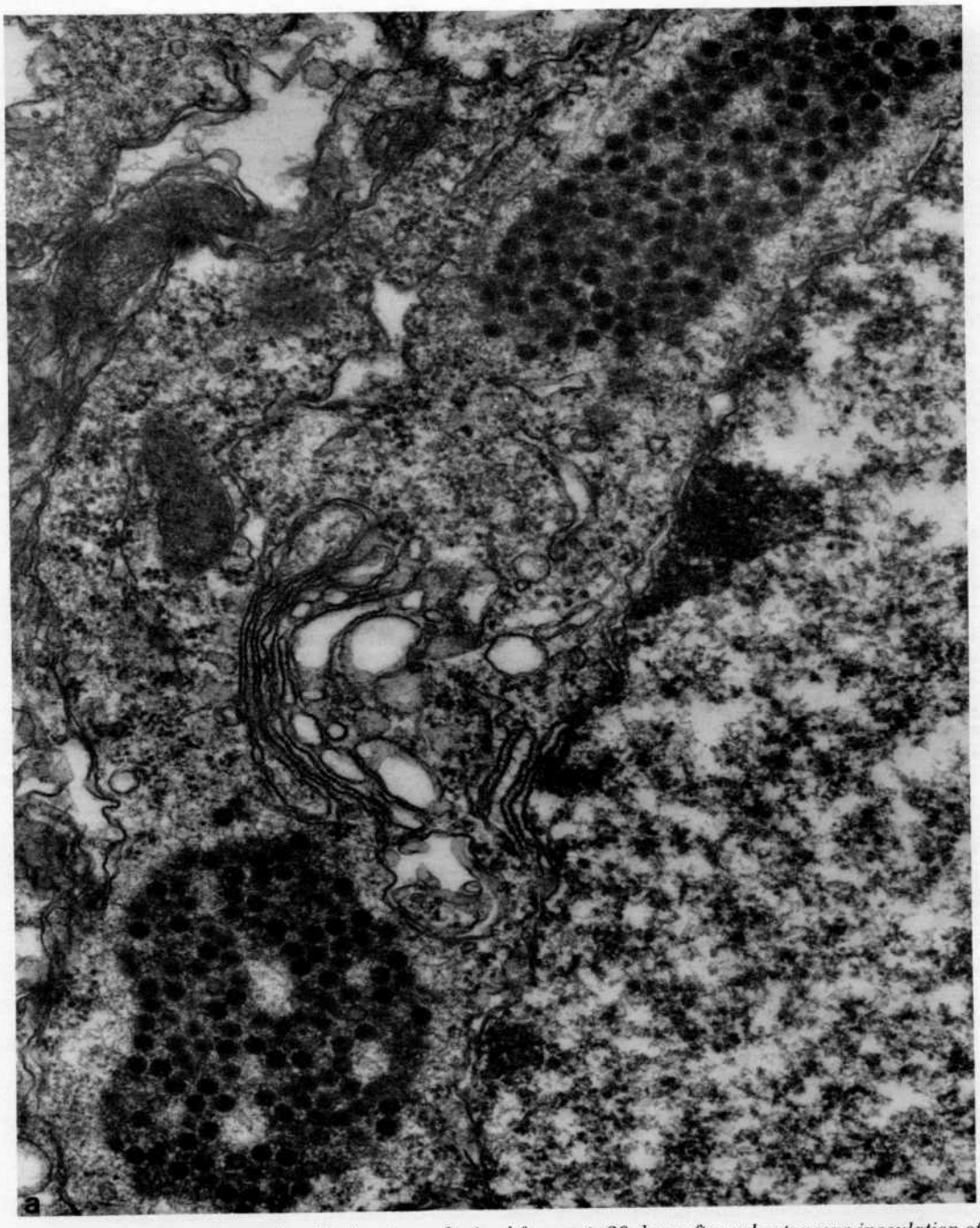

FIG. 7. *Electron micrographs of brain tissue obtained from rats 30 days after subcutaneous inoculation of 10^7 PFU of R_2 virus. Preparation of the tissue is described in Materials and Methods. (a) × 39,600; (b) × 284,000.*

then plated as infectious centers at 39°C, and the resulting plaques are scored and compared to the number of plaques obtained from similar treatment of cultures infected with each mutant alone. A complementation level has been defined (23), and if this complementation level is found to be greater than unity, complementation has occurred between the mutants. To determine whether recombination occurs between the mutants, a known number of cells

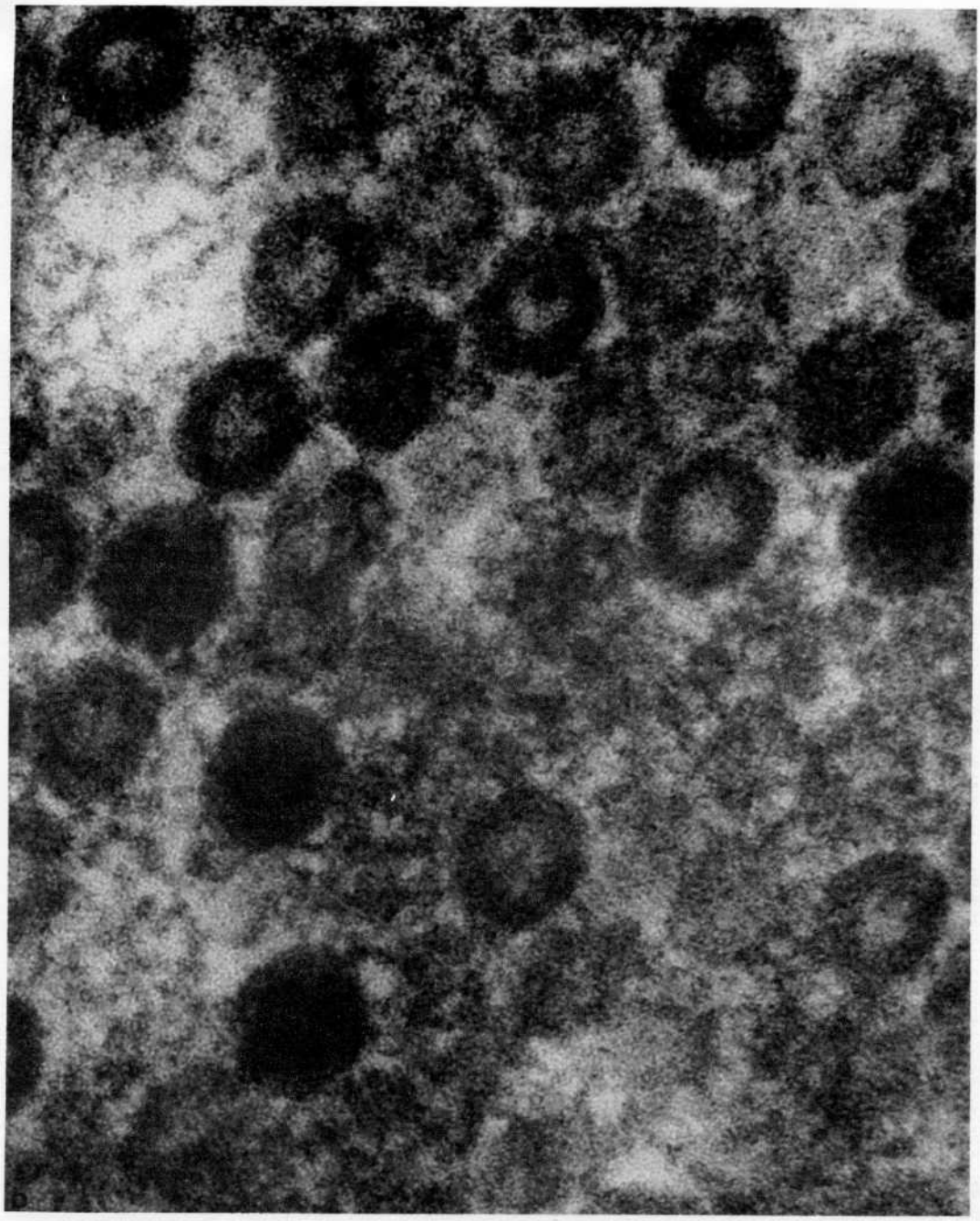

FIG. 7b

from the mixedly infected culture is plated as infectious centers at 31°C. After 30 h to permit some intracellular viral growth and any consequent recombination, the plates are raised to 39°C, and any plaques resulting from ts^+ recombinants are scored. In general, recombination between two mutants has been found to occur in all mixedly infected cells or not at all (23). The results with R_2X are shown in Table 2. R_2X did not recombine with or complement three known R_2A mutants but did so with prototype mutants from the other six known classes of *ts*

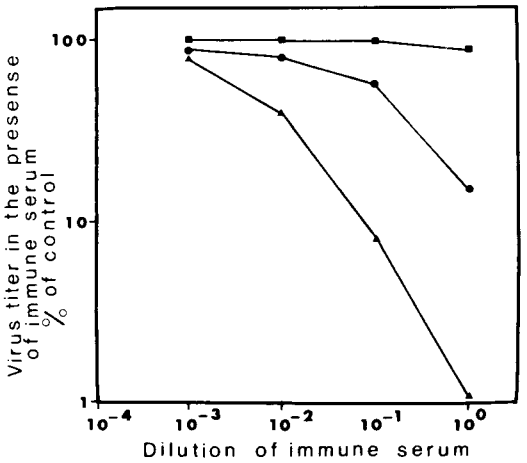

FIG. 8. *Plaque reduction test for the appearance of reovirus antibody in the blood of rats inoculated subcutaneously at 2 days of age with 10^7 PFU of R_2 virus. Symbols: (■) 10 days after infection; (●) 30 days after infection; (▲) 60 days after infection.*

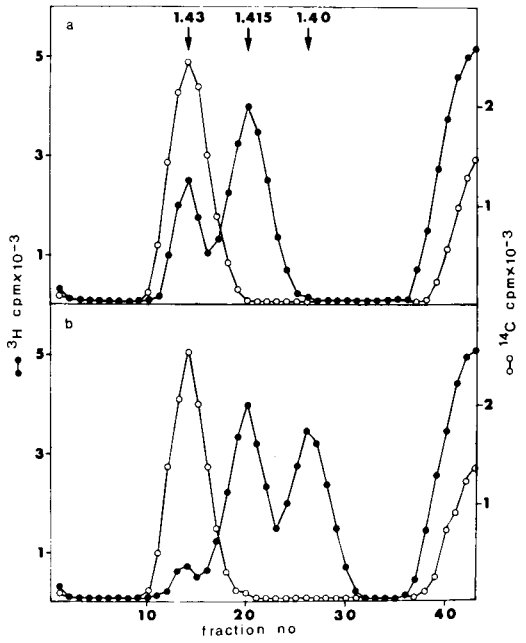

FIG. 9. *Isopycnic centrifugation in CsCl of amplified virus obtained from the brains of rats inoculdted intracerebrally at 2 days of age with R_2 virus (10^4 PFU/rat) and R_1d (L_1) virus (10^7 particles/rat). Procedure was the same as that described in the legend to Fig. 4. (a) Virus obtained from the brain of a moribund rat 8 days p.i. (b) Virus obtained from the brain of a persistently infected, runted rat 30 days p.i. Symbols: (○) Cores derived from purified, ^{14}C-labeled R_2 marker virus; (●) cores derived from [^{3}H]uridine-labeled virus derived by amplification in L cell monolayers.*

mutants. R_2X is therefore a class A mutant, and since no other experiments were being carried out with A mutants in the laboratory at the time, R_2X must have originated in the rat that had been infected with the wild-type virus.

Persistent infection with R_2X virus. To get some information on the in vivo behavior of R_2X virus, a large group of 2-day-old rats was inoculated intracerebrally with doses ranging up to 10^8 PFU/rat. All rats (11/11) infected with 10^7 PFU or more developed acute encephalitis and died by 10 days p.i. With a dose of 10^4 PFU/rat, 13/13 rats survived the infection, and 8 of them became runted. Virus isolated from the brains of these runted rats 30 days p.i. retained its *ts* character and was associated with defec-

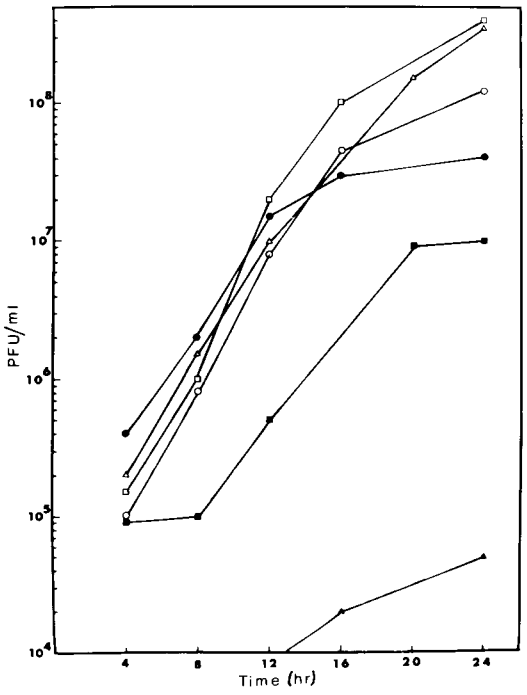

FIG. 10. *Effect of defective reovirus on the growth of wild-type virus in L cells. Suspension cultures containing 5 × 10^6 L cells/ml were infected with R_2 virus and/or purified R_1d (L_1) virions (22). Adsorption was permitted at 20°C for 1 h, and the cultures were diluted 10-fold with MEM-2% fetal calf serum and incubated at 37°C. Triplicate 1-ml samples were removed from each culture at intervals, frozen and thawed three times, subjected to sonic oscillation, and plaque titered. Symbols: (○) 10 PFU of R_2 virus per cell and 1,000 R_1d (L_1) particles/cell adsorbed simultaneously; (□) 10 PFU of R_2 virus per cell; (△) 10 PFU/cell of R_2 virus and (4 h later) 1,000 R_1d (L_1) particles/cell; (●) 10 PFU/cell of R_2 virus and 10,000 R_1d (L_1) particles adsorbed simultaneously; (■) 1,000 R_1d (L_1) particles/cell and (4 h later) 10 PFU of R_2 virus per cell; (▲) 1,000 R_1d (L_1) virions/cell.*

TABLE 2. *Recombination and complementation between ts mutants of reovirus and the R_2X mutant determined by infectious center assay (23)*

Infection[a]	% Infected cells giving plaques at 31°C[b]	% Infected cells giving ts^+ recombinants[c]	% Infected cells giving plaques at 39°C	CL[d]
X × A (201)	72	2	0.37	0.77
X × A (340)	81	1	0.46	0.88
X × A (376)	76	2	0.48	0.87
X × B (352)	69	72	1.1	2.5
X × C (447)	73	75	0.95	2.3
X × D (585)	74	79	1.1	2.0
X × E (320)	71	69	0.98	1.9
X × F (556)	78	76	1.4	3.2
X × G (453)	79	80	1.2	2.5
X	72	2	0.30	
A (201)	75	1	0.18	
A (340)	67	1	0.22	
A (376)	73	0	0.25	
B (352)	82	1	0.14	
C (447)	74	0	0.10	
D (585)	75	0	0.25	
E (320)	78	2	0.22	
F (556)	71	0	0.14	
G (453)	76	0	0.17	

[a] L cells were infected at a multiplicity of infection of 10 PFU/cell for each mutant.

[b] Plating efficiency of infected cells at 31°C.

[c] Recombination is determined in the X × B cross, for example, as follows: % cells giving ts^+ recombinants (X × B) − % cells giving ts^+ recombinants (X alone) − % cells giving ts^+ recombinants (B alone) = 72 − 2 − 1 = 69%. This is equivalent to the plating efficiency of co-infected cells at 31°C (column 2) and shows that each co-infected cell gave rise to ts^+ recombinants.

[d] Complementation level (CL) = % of infected cells giving plaques in a cross (X × Y)/(% of X-infected cells giving plaques + % of Y-infected cells giving plaques).

tive virions as shown in Fig. 5. This A class mutant therefore behaves similarly to the B and C mutants in initiating persistent neurotropic infection and giving rise to defective virus in the course of infection.

DISCUSSION

When wild-type reovirus is inoculated intracerebrally into suckling rats under our conditions, all animals die from acute encephalitis during the first 8 to 10 days, and there is extensive formation of defective virus. A similar situation was observed with VSV by Holland and Villarreal in newborn mice (8), although two serial high-multiplicity intracerebral passages were required to generate defective interfering particles in their case. These results serve to demonstrate that defective virions can multiply readily when either virus grows extensively in the brain, but it is difficult to place further significance on them for two reasons. First, a high multiplicity of infection by the intracerebral route is an artificial means of initiating infection and essentially uses the brain as an in vivo cell culture system. Since defective reovirus can be rapidly generated by serial passage in vitro (15, 16, 20) it would be expected to arise in the brains of newborn animals under the conditions used. Second, although no defective virus could be detected in the reovirus inoculum, we cannot be certain it was defective-free. Any defective virus present in the inoculum would probably be amplified during multiplication of the infectious moiety in the brain cells.

By using the subcutaneous route for inoculation, the host's defense mechanisms are permitted to react against the infection in a more normal way. Also, a few defective particles in the inoculum might be "screened out," and if defective virions are later found in a particular tissue or organ, they are much more likely to have originated there during viral multiplication. The subcutaneous route provided a further experimental advantage in that most animals survived the infection. About half the survivors were runted, chronically infected, and went on to show the gross signs of progressive brain degeneration described by Raine and Fields (18, 19), and this was the condition in which we were primarily interested. It is interesting that in using this means of inoculation we have not observed the altered pathogenicity of the *ts* mutants found by Raine and Fields, although there is no ready explanation for the difference between our two systems.

The main results can be summarized as follows. After infection with wild-type virus, B or C class *ts* mutants, virus multiplied rapidly in the brain and other tissues and reached a peak by 10 days p.i., and the viral titer then rapidly declined. During this acute phase of the infection the formation of defective virus could be readily demonstrated, and the defective population contained almost exclusively virions with the L_1 segment of the genome deleted. As the chronic phase of the disease progressed, infectious virus disappeared from the brain and could no longer be found by 80 days after infection. During this phase the nature of the defective viral population changed. By 30 days p.i. a fraction of the virions contained multiple deletions in addition to those with only the L_1 deletion. In fact, in two of six brains examined 100 days p.i. (Fig. 6) a mixed population of defective virions was found even though no infectious virus could be detected. This latter observation bears further study, since it raises the important possibility that a population containing a mixture of defective virions with various genomic segments deleted might be able to multiply through mutual complementation even in the absence of infectious virus. Such a situation

could help to explain long-term degenerative effects that result from viral infections but which occur long after the infectious virus has disappeared from the host.

During the course of our experiments gross changes in the nature of the infectious virus were detected on only two occasions. Virus isolated from the brain of one animal 30 days after infection with wild-type virus was shown to be an A class *ts* mutant, although virus isolated from the liver of the same animals was still wild type. A ts^+ revertant was isolated from the brain and liver of a second animal 30 days after infection with a B class *ts* mutant. In the remaining cases the virus isolated from infected animals apparently retained the genetic character of the inoculated virus. Perhaps the nature of the viral populations derived from chronically infected animals, particularly after wild-type virus infection, should be reexamined in more detail. There is increasing evidence that the selection of *ts* mutants may contribute to the maintenance of viral populations in persistently infected cell cultures (32), and we have shown that complex genetic interactions can occur between *ts* mutants and deletion mutants of reovirus (21, 22). If a slow drift were occurring in the genetic nature of the infectious viral populations in our experiments, it might have gone undetected.

Although defective virus is clearly formed in the brains during the acute and chronic phases of reovirus infection, the question is whether the defective virus plays any role in initiating and maintaining the chronic infection. There are two pieces of evidence presented here to suggest that defective virus can modify the virulence of infectious virus and initiate the chronic phase. First, R_1d (L_1) virions can interfere with the multiplication of wild-type virus growing in co-infected L cells (Fig. 10). Second, R_1d (L_1) virions exerted a powerful prophylactic effect when inoculated intracerebrally with wild-type virus into newborn rats. More important, the majority of surviving rats went on to be chronically infected. The virus isolated from the brains of these rats 30 days p.i. contained a mixture of defective virions similar to that found in chronically infected rats infected by the subcutaneous route with wild-type virus. Moreover, similar populations of defectives can be readily produced by repeated serial passage of wild-type virus in L cells or isolated from persistently infected L cell cultures (R. Ahmed and R. Y. Lau, unpublished observations). These defectives interfere much more markedly with the multiplication of infectious reovirus in cell culture than do R_1d (L_1) virions alone.

On the strength of these results we might suppose that during the acute phase of infection defective virions with a deleted L_1 segment are generated, and as they increase in concentration they interfere with the growth of the infectious virus. The interference is sufficiently great to ensure complete recovery of many animals, but in upwards of half of them infectious virus and defectives come into equilibrium with each other in the brains and the chronic phase of the disease is initiated. During the succeeding weeks virus and defectives multiply slowly in concert, and a new population of defectives containing a variety of deletions evolves. Among these latter defectives are some that exert a much more powerful interfering effect on the growth of the infectious moiety than the L_1 defectives, to the extent that infectious virus can no longer be detected by 80 days p.i. Possibly some of the defectives can continue to multiply slowly after disappearance of infectious virus through mutual complementation between genomes carrying different deletions.

In this scheme the onus in controlling the course of the neurotropic infection has been placed on the formation and influence of defective virions, and the picture has undoubtedly been much oversimplified. In fact, the generation of defective virions must be only one of a complexity of factors exerting selective pressures on the evolving viral population (14). It is quite impossible as yet to assess the relative importance of defective virus in the progress of the disease, but further studies on the reovirus-rat model utilizing the well-defined mutants of the virus should give more insight into the problem.

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