Interference following Mixed Infection of Reovirus Isolates Is Linked to the M2 Gene

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Following infection by pairs of reovirus isolates consisting of combinations of reovirus T1 Lang, T2 Jones, or T3 Dearing, we found that one of the isolates interfered with the yield of progeny RNA derived from the other parents. The most significant interference was produced by T2 Jones or T3 Dearing, when mixed with T1 Lang. Genetic analysis revealed that the presence of the M2 gene in the interfering parent (in the T1 Lang × T3 Dearing pair) was linked to interference. Studies on interference in infected cells indicated that interference occurs after adsorption and penetration.

Interference between mutant and wild-type animal viruses following mixed infections is well described, although the precise molecular mechanisms of interference remain obscure (for a review, see reference 23). Prior studies of reoviruses indicate that following mixed infection at restrictive temperatures, temperature-sensitive (ts) mutants of reovirus serotype T3 Dearing (T3D) interfere with the yield of wild-type T3D (2). Interestingly, the RNA+ ts mutants demonstrated interference while the RNA- mutants did not, suggesting that viral mRNAs or their protein products are important for the property of interference.

The genomes of reoviruses consist of 10 double-stranded RNA (dsRNA) segments enclosed in a double protein shell (for a review, see reference 18). The dsRNAs of the three mammalian reovirus serotypes have different electrophoretic mobilities, and this feature serves as a marker to identify the parental origin of each RNA segment and to characterize reassortants after mixed infections (15, 16, 19). In the present report we have extended the prior findings of interference of infectivity to show that interference of the yield of progeny RNAs derived from one of the two parental isolates occurred following mixed infections of different pairwise combinations of the three wild-type reovirus serotypes (T1 Lang [T1L], T2 Jones [T2J], and T3D) in the order: T2J = T3D > T1L. In addition, we found that the M2 gene is linked to the phenomenon of interference and that interference occurs after viral adsorption and penetration.

MATERIALS AND METHODS

Cells and viruses. Stock cultures of mouse L929 cells were grown in monolayers or in suspension cell culture in Joklik modified minimal essential medium (MEM) (Irvine Scientific, Santa Ana, Calif.) supplemented with 5% fetal bovine serum (HyClone Laboratories, Logan, Utah), 2 mM l-glutamine (Irvine Scientific), and 1% Fungi-Bact Solution (Irvine Scientific) (complete MEM). Second-passage L929 cell lysate stocks of doubly plaque-purified reoviruses T1L, T2J, T3D, T3 clone 9, T3 Abney, and T1L × T3D reassortants (laboratory stocks) were used for experiments.

Viral mixed infections. L929 cells were seeded into six-well multicluster plates at a density of 1.25 × 106 cells per well. After 24 h of incubation at 37°C, cells were infected by virus with a multiplicity of infection (MOI) of 10 or by a mixture of two viruses, both with MOIs of 10, if not specially indicated. Incubations continued for 43 h at 37°C, and infected cells then were lysed by three cycles of freeze-thawing. Nuclei and cell debris were spun down for 5 min at 1,000 × g, and supernatants were saved for experiments and kept at 4°C. The controls (infections by each virus alone) and mixed infections between T1L and T1L × T3D reassortants were prepared in the same way.

Virus purification. Growth of mixed viruses in preparative quantities on cell monolayers was as follows: L929 cells were seeded into T-75 flasks at a density of 9 × 106 cells per flask and after 24-h incubation at 37°C were infected by T1L plus T3D. Infected cells were incubated for 43 h at 37°C and then harvested by scraping. Cells were pelleted for 15 min at 500 × g, and all further steps of viral purification were as described previously (5). Virion particle concentrations were determined by measuring optical density (1 A260 unit = 2.1 × 1012 particles) (21).

Virus adsorption and uncoating. 35S-labeled T1L was grown in suspension cell culture for 70 h at 35°C in the presence of 66.6 μCi/ml of Tran[35S] label (ICN Biomedicals, Costa Mesa, Calif.) and purified as described previously (5). Cells in six-well multicluster plates were infected in 0.2-ml volume by (i) 35S-labeled T1L (MOI of 20), (ii) 35S-labeled T1L plus T2J (MOIs of 20 and 50, respectively), (iii) 35S-labeled T1L plus T3D (MOIs of 20 and 20, respectively). The same number of counts per minute of radiolabeled T1L was added (66,000 cpm [see Table 1]). Adsorption proceeded for 1 h at 4°C. Cells were extensively washed by complete MEM to remove unbound virus, and radioactivity was counted. The percentage of adsorption was estimated as (cpm of cell-bound virus/cpm of added virus) × 100%, where cpm is counts per minute. For uncoating experiments, 1.5-ml portions of complete MEM were added to infected cells and incubation was continued at 37°C for 2 or 4 h. Cells were washed by ice-cold phosphate-buffered saline and lysed in 0.2 ml of the same buffer containing 2 mM phenylmethylsulfonylfluoride and 0.5% Nonidet P-40 on ice for 15 min. The lysates were spun down for 10 min at 1,000 × g, and supernatants were saved for sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE).

Plaque assays. Viral titer determinations were done as described previously (5). Plaques of T1L and T3D were counted on day 6 or 7, and plaques of T2J were counted on day...
10. In mixed infections between T2J and T1L (or T3D), all plaques were counted on day 10. Plaque assays were performed in triplicate.

Electropherotypic analysis of the dsRNAs. Two-hundred microliters of viral lysates was mixed with 40 μl of 10% SDS and 160 μl of TEN buffer (10 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 100 mM NaCl) and incubated for 12 min at 50°C. The nucleic acids were extracted by the addition of an equal volume of phenol-chloroform, precipitated by 2 volumes of ethanol, and dissolved in 20 μl of water. Samples (10 μl) were electrophoresed per lane. After discontinuous SDS–10% PAGE (10), gels were stained in an ethidium bromide solution (0.5 μg/ml) and photographed in UV light on Polaroid film. The same procedure was applied for dsRNAs from purified viruses except that 4 μg of total dsRNAs per lane was used. If needed, gels or films were scanned with a laser densitometer-integrator (Ultrascan XL; LKB Instruments, Inc., Bromma, Sweden). The order of the gels from the top of the gel to the bottom has been previously shown to be L1, L2, L3, M2, M1, M3, S1, S2, S3, and S4, except for (i) poor resolution of T3D M2 and M1 dsRNAs (a doublet), (ii) reversed mobilities of T1L and T2J S3 and S4 dsRNAs, and (iii) reversed mobilities of T2J M1 and M2 dsRNAs (19) (see Fig. 1, lanes 1, 2, and 5).

RESULTS

Interference following mixed infections of pairs of certain reovirus isolates. Following pairwise crosses between certain ts mutants and ss* reovirus at 39°C, the yield of infectious wild-type reovirus decreases (20). To see if interference occurs between pairs of viruses that are not temperature sensitive, we chose to determine if one parent could reduce the yield of progeny genomic dsRNA produced by the other member of the mixed infection pair. Ten reovirus dsRNA segments have been previously shown to be resolved by SDS–10% PAGE with Tris-glycine buffer (15). The corresponding dsRNAs from three reovirus serotypes have different electrophoretic mobilities (15), allowing us to evaluate whether interference occurs following mixed infections derived from T1L, T2J, T3D, and other T3 clones (T3 clone 9 and T3 Abney).

In order to determine whether mixed infections result in differential inhibition among reovirus isolates, we analyzed five pairwise crosses: T1L and T3D, T1L and T3 clone 9, T1L and T3 Abney, T1L and T2J, and T3D and T2J. Pairwise infections with equal MOIs (both MOIs 10) were performed on cell monolayers for 43 h at 37°C (see Materials and Methods). To determine whether interference was occurring in the progeny of doubly infected cells, we compared yields of viral dsRNAs from single and mixed infections. Electropherotypic analysis of the dsRNAs from infected cell lysates revealed that the yield of T1L RNA was strongly inhibited following mixed infections with T3D (Fig. 1, lane 6) and T2J (Fig. 1, lane 9), was less inhibited by T3 clone 9 (Fig. 1, lane 7), and was not inhibited by T3 Abney (Fig. 1, lane 8). These results indicate that there is a difference in the capacities of different isolates to interfere with T1L RNA synthesis.

Quantitative analysis of the dsRNAs produced by single and mixed infections (Fig. 1 and 2) was performed by laser densitometry of ethidium bromide-stained gels, and the ratios of the progeny dsRNAs were tabulated (Table 1). The amount of T1L S1 dsRNA in the yield of the T1L and T3D cross was 4.1 times less than the amount of T3D S1 dsRNA (the percentage of T1L S1 dsRNA was 100/5.1 = 19.6) and the reduction of the RNA of the other nine genes was even greater. It should be noted that (i) yields of genomic dsRNA of T1L and T3D in single infections were similar to each other (compare lanes 1 and 2 in Fig. 1; compare lanes 1 and 2 in Fig. 2); (ii) T3D and T2J (which separately inhibited T1L) did not significantly influence the yield of each other in the mixed infection T3D plus T2J (Fig. 1, lane 10). The quantification of dsRNA therefore allowed us to determine that the order of inhibition of different reovirus isolates of different serotype by each other was T2J = T3D > T1L.

In addition to differences between isolates of different serotypes, the capacities of different reovirus serotype 3 clones to inhibit the yield of T1L dsRNA varied. T3D caused the most inhibition, T3 clone 9 caused less inhibition, and T3 Abney

![FIG. 1. Electropherotypes of the parental viruses and progeny of mixed infections. SDS–10% PAGE (ethidium bromide staining) of dsRNAs prepared from unpurified viral lysates (see Materials and Methods). Abbreviations: Mix(1+3), T1L and T3D; Mix(1+3 clone 9), T1L and T3 clone 9; Mix(1+3 Abney), T1L and T3 Abney; Mix(1+2), T1L and T2J; Mix(2+3), T2J and T3D. L, M, and S to the right of the gel designate the three size groups of dsRNAs: large (L1, L2, and L3), medium (M1, M2, and M3) and small (S1, S2, S3, and S4), respectively.](http://jvi.asm.org/)

![FIG. 2. Quantitation of interference. SDS–10% PAGE (ethidium bromide staining) of dsRNAs extracted from CsCl-purified viruses. Single infections of T1L (lane 1) and T3D (lane 2) were done. Mixed viruses were grown on cell monolayers (43 h at 37°C): specifically, mixture of T1L and T3D at MOIs of 10 for both viruses (lane 4) and MOIs of 21 and 7, respectively (lane 5). Mixed viruses T1L and T3D at MOIs of 10 for both (lane 3) was grown in suspension cell culture for 48 h at 35°C. L, M, and S are explained in the legend to Fig. 1. The gel was scanned with a laser densitometer-integrator (see Materials and Methods) to quantify the ratios of progeny dsRNAs following mixed infections.](http://jvi.asm.org/)
showed no inhibition. Thus, interference is not serotype specific.

As noted above, the measurement of dsRNA in infected cells allowed us to show that certain isolates inhibited the yield of progeny dsRNA. However, it did not allow us to determine directly whether the yield of infectious virus is similarly inhibited. Since T2J does not replicate as well as TIL or T3D does (yield of this virus is at least 10 times less than TIL and T3D) (18), we were also able to determine that in addition to the interference with the yield of progeny dsRNA, the yield of infectious TIL virus following mixed infection of T2J and TIL was 10 times less than that of TIL single infection (data not shown). Unfortunately, a comparison of this type was not possible for other mixed infections, since the other viruses studied have approximately equal yields.

Features of interference. To see if interference was due to a soluble cellular factor or a viral component, we asked whether it was necessary to purify virus in order to detect interference. We checked the ratios of yields of corresponding TIL and T3D dsRNAs in crude versus pure (CsCl-purified) mixed viral preparations. The ratios were the same (Fig. 1, lane 6; Fig. 2, lane 4), indicating that purification of virus was not necessary to obtain interference and that the property of interference was a property of the virion or its products itself.

To further determine whether virion particles or their products were responsible directly for interference, we analyzed the role of MOI in interference. We increased the MOI ratio of TIL over T3D in mixed infection threefold (MOIs of 21 and 7, respectively, versus 10 for both). The results showed a proportional threefold reduction of T3D/TIL dsRNA ratios (compare lanes 4 and 5 in Fig. 2; Table 1). Thus, the ratio of MOIs of different viruses directly affects the extent of interference.

To see if the conditions of growth of the cells affect interference, we asked whether the level of interference depended on whether cells were cultured in suspension or monolayer. The level of TIL inhibition by T3D increased approximately 3.5 times when mixed infection with T3D was performed in suspension cell culture (growth for 48 h at 35°C) versus viral growth in cell monolayers (compare lanes 3 and 4 in Fig. 2; Table 1). Thus, although interference occurs during growth in both cell monolayers and suspension cell cultures, the extent and presumably the efficiency of interference varied with the nature of cell growth.

The M2 gene is linked to the property of interference. In order to analyze which T3D genes are involved in interference, we coinfect ed cells with a panel of 21 TIL × T3D reassortants (1) with TIL (MOIs of 10 for both viruses). The electropherotypes of these reassortants are shown in Table 2. The ratios of the corresponding dsRNAs of T3D and TIL in the yield served as markers of interference. Electropherotypic analysis of the progeny of mixed infections allowed us to divide these reassortants into two classes: (i) EB15, EB31, EB88, EB96, EB120, EB121, and EB124 did not inhibit the yield of TIL dsRNAs (Fig. 3 and Table 2); (ii) the remainder of the reassortants (listed in Table 2) inhibited TIL dsRNAs yields similar to that of T3D (illustrative examples include EB62 and EB133 shown in Fig. 3, lanes 3 and 9, respectively). A summary of these results is shown in Table 2. All the reassortants which did not inhibit the yield of TIL dsRNAs contained the M2 gene of T3D origin. Reassortants which inhibited TIL in the same manner as the T3D parent did contained the M2 gene of T3D origin with a single exception (reassortant EB133). Moreover, a monoreassortant EB88, containing a single TIL M2 gene, showed no interference (Fig. 3, lane 7; Table 2). These data strongly indicated that the T3D M2 gene was linked to the phenomenon of interference of TIL by T3D. The other nine reovirus genes were not involved in interference (Table 2).

Interference and early steps of infection: adsorption and uncoating. In order to determine if interference was due to an early event of infection (viral adsorption and uncoating),
radiolabeled TIL virions were adsorbed at 4°C for 1 h to L929 cells (MOI of 20) alone and in combination with unlabeled T3D (MOI of 20) or T2J (MOI of 50). After the infected cells had been thoroughly washed, the radioactivity of cell-bound virus was determined. The percentage of adsorption was calculated as described in Materials and Methods. We found no difference between the efficiencies of T1L adsorption in the absence or presence of T3D or T2J (Table 3). Therefore, adsorption is not involved in interference.

To see if interference was occurring by blocking viral uncoating, infected cells were incubated for 2 or 4 h at 37°C, allowing viral uncoating to proceed. In these experiments, uncoating during 2 and 4 h proceeded as determined by the proteolytic digestion of the μ1C protein, leading to the formation of the 8 fragment and degradation of α3 protein, as has been previously described (20). The pattern of T1L uncoating was not altered whether T1L was alone or mixed with T3D and T2J (Fig. 4). Thus, the two early steps in viral infection, adsorption and uncoating, are not involved in the phenomenon of interference, indicating that interference occurs later.

**DISCUSSION**

In this report we have shown that laboratory isolates of T3D and T2J reovirus interfered with the yield of T1L dsRNA and that T2J interfered with the infectious yield of T1L following mixed infection. These results extend the finding of transdominant interference following coinfec tions of ts mutants with the wild type to coinfections between wild-type strains and each other. In addition, we have found that the M2 gene of the T3D strains is linked to interference.

Prior studies on viral interference have largely focussed on interactions between deletion mutants or ts mutants and their wild-type coinfecting strain (7-9, 11, 17, 23, 24). In order to do an analysis with reassortants, we used “wild-type” stocks of prototype isolates. The results of the present study showed that interference can occur following infection of so-called wild-type strains. It is unclear why “wild-type” stocks should interfere with another virus. There are at least two possibilities to explain this property. One is that what we call “wild type” is in fact a mutant. This would not be surprising, since all isolates have in fact been passaged in cell culture prior to its use as a prototype isolate. The reovirus T3 Dearing “wild type” has been passaged for several years since its isolation in the 1960’s. Thus, the definition of “wild type” largely refers to its growth as an isolate that has not “intentionally” been mutagenized or otherwise selected as a mutant virus. The passage of viruses in cell culture, however, clearly can select changes (mutations) that cause the virus to differ from the original virus in nature. In fact, the high rate of mutation of RNA viruses provides an opportunity for mutations to appear in putative wild-type stocks very rapidly (3, 6). Thus, interference may be a general property of a number of “wild-type” viruses that have been passaged in cell culture. A second possibility relates to the fact that viruses that are separated in nature are constantly evolving and may change in ways that lead to interference. For example, if the process of interference involves the interaction of viral proteins that are coassembling (as for example, if the dual infection leads to phenotypically mixed progeny virions), the independent evolution of capsid proteins from each isolate could lead to enough differences in the protein structure of one virus that protein-protein interactions between the two parents might, as a result of modified conformations, not allow normal assembly (see below). Thus, while virion assembly might be quite permissive for individual virions, the combinations of proteins from two parents could be inefficient or even lethal. It

**TABLE 3. T1L adsorption in the presence of T2J and T3D**

<table>
<thead>
<tr>
<th>Virus infection</th>
<th>Cell-bound 35S-labeled T1L (cpm)</th>
<th>Adsorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S-labeled T1L</td>
<td>15,200</td>
<td>23.0</td>
</tr>
<tr>
<td>35S-labeled T1L + T2J</td>
<td>16,300</td>
<td>24.7</td>
</tr>
<tr>
<td>35S-labeled T1L + T3D</td>
<td>13,900</td>
<td>21.1</td>
</tr>
</tbody>
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* Radiolabeled T1L (35S-labeled T1L) (66,000 cpm) was added to each cell culture. This experiment was done in triplicate, and values varied no more than ±9.5% from the values shown.

**FIG. 3.** Electropherotypes of progeny of the mixed infections of T1L and reassortants T1L × T3D. SDS–10% PAGE (ethidium bromide staining) of dsRNAs prepared from unpurified viral lysates (see Materials and Methods). T1L (lane 4), T3D (lane 5), and the mixed infection of T1L and T3D (lane 6) are the controls. Mixed infections between T1L and T1L × T3D reassortants: EB15 (lane 1), EB31 (lane 2), EB62 (lane 3), EB88 (lane 7), EB121 (lane 8), and EB133 (lane 9). L, M, and S are explained in the legend to Fig. 1.

**FIG. 4.** Uncoating of T1L in the presence of T2J and T3D. SDS–10% PAGE autoradiogram. Virions were disrupted by 3 min of boiling in sample buffer. CsCl-purified 35S-labeled T1L (lane 1) was adsorbed to L929 cells for 1 h at 4°C, and infected cells were incubated at 37°C for 0, 2, or 4 h (see Materials and Methods): 35S-labeled T1L alone (lanes 2, 3, and 4, respectively), 35S-labeled T1L plus unlabeled T2J (lanes 5, 6, and 7, respectively), and 35S-labeled T1L plus unlabeled T3D (lanes 8, 9, and 10, respectively). Reovirus proteins are indicated on the right side; (λ), a degradation product of α3 protein during uncoating; F, bromphenol blue dye front.
is noteworthy that if the two independent isolates are evolving separately in nature, the interfering strains would have a natural advantage following coinfections in the field and thus, might be expected to exist in nature under conditions where mixed infections are found. In fact, many reovirus isolates are found as mixed infections in nature.

Mapping the capacity to interfere with the M2 gene provides a possible clue as to the mechanism of interference. The M2 gene product (μl) is a protein that has a variety of properties that indicate a role in reovirus interaction with membranes. μl is a myristoylated protein (14) that contains highly conserved amphipathic α helices and also has a small highly basic fragment (Φ) (13). μl is genetically linked to the property of chromium 51 release from prelabeled cells (12) and may be involved in allowing channels to form in lipid bilayers (22). Thus, although one possible site of interference might have involved interference with the capacity of input virions to interact with cell membranes, the fact that adsorption and penetration are not blocked by the interfering virus indicates that interference must be occurring at a later step, most likely viral assembly (possibly via phenotypic mixing of wild-type and mutant proteins) or a later event (such as release). It is known that μl forms a critical protein network involved in assembling the virion outer shell (4), and thus may inhibit assembly. Current experiments in the laboratory indicate that phenotypic mixing occurs following mixed infection (unpublished data).

Whether phenotypically mixed particles containing proteins derived from both parents are responsible for interference remains to be determined. All the studies in this report indicate that interference is directly related to the nature of the input virions in a MOI-dependent manner, consistent with viral assembly as the site of interference.

The fact that EB133 was an exception to the M2 gene mapping is at present not understood. It is possible that silent mutations have occurred in this reassortant that have altered its behavior.

It is noteworthy that interference between viral mutants and wild-type virus is a general phenomenon for most, if not all, viruses (for a review, see reference 23), yet the mechanisms remain poorly understood. The fact that interference can be generated between wild-type viruses suggest that it may be a more widespread phenomenon. Also the fact that a specific viral protein, μl, appears to play a key role in interference should lead to more precise understanding of how interference occurs.

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