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Defective Influenza Viral Ribonucleoproteins Cause Interference

J. MICHAEL JANDA AND DEBI P. NAYAK*

Department of Microbiology and Immunology, University of California at Los Angeles School of Medicine, Los Angeles, California 90024

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Ribonucleoproteins (RNPs) isolated from infectious and defective interfering (DI) influenza virus (WSN) contained three major RNP peaks when analyzed in a glycerol gradient. Peak I RNP was predominant in infectious virus but was greatly reduced in DI virus preparations. Conversely, peak III RNP was elevated in DI virus, suggesting a large increase in DI RNA in this fraction. Labeled [^{32}P]RNA was isolated from each RNP region and analyzed by electrophoresis on polyacrylamide gels. Peak I RNP contained primarily the polymerase and some HA genes, peak II contained some HA gene but mostly the NP and NA genes, and peak III contained the M and NS genes. In addition, peak III RNP from DI virus also contained the characteristic DI RNA segments. Interference activity of RNP fractions isolated from infectious and DI virus was tested using infectious center reduction assay. RNP peaks (I, II, and III) from infectious virus did not show any interference activity, whereas the peak III DI RNP caused a reduction in the number of infectious centers as compared to controls. Similar interference was not demonstrable with peak I RNP of DI virus nor with any RNP fractions from infectious virus alone. The interference activity of RNP fractions was RNase sensitive, suggesting that the DI RNA contained in DI RNPs was the interfering agent, and dilution experiments supported the conclusion that a single DI RNP could cause interference. The interfering RNPs were heterogeneous, and the majority migrated slower than viral RNPs containing M and NS genes. These results suggest that DI RNP (or DI RNA) is also responsible for interference in segmented, negative-stranded viruses.

Interference mediated by defective interfering (DI) virus against homologous or closely related viral strains has been well established in a number of animal virus-host cell systems (12-16, 18, 20, 22). With influenza, von Magnus (26) originally described the formation of noninfectious virus by successive high-multiplicity undiluted passages of virus in embryonated eggs. Recently we and others have reported the presence of small deleted RNA segments (DI RNA) in DI influenza viral preparations (5, 17, 19, 20, 22). Furthermore, based on several lines of evidence (albeit indirect), we have postulated that, as in the case of other viruses, these DI RNA molecules are responsible for DI virus-mediated interference. First, DI RNA is present only in DI virus preparations and is absent in cloned infectious virus preparations. Second, during high-multiplicity passages of WSN virus in MDBK cells, DI RNAs appear in progeny virions and amplify in subsequent passages. The appearance and the amount of DI RNA correlate well with the amount of interference (defective interfering units [DIU] per milliliter). Finally, UV sensitivity data suggested that the target size of the interfering molecule is approximately one-fortieth that of the infectious viral genome, a size which closely approximates the size of the small RNA segments seen on gels (20). However, direct evidence that these DI RNA molecules cause interference is lacking. Because of the segmented nature of the genome, DI influenza virus, unlike other DI viruses, is likely to contain, in addition to the DI RNA segment(s), other viral RNA segments. Therefore, to obtain more direct evidence on the role of DI RNA in interference, we have in this report isolated and analyzed ribonucleoprotein complex (RNP) from infectious and DI virus preparations.

Six-times plaque-purified WSN (H0N1) viral clones were grown in MDBK cells and characterized as described previously (25). The characteristics of DI RNA from clone L used in these experiments have been reported earlier (6, 17, 20). Procedures for analyzing RNA profiles and assaying PFU, hemagglutinin units, and DIU

have been reported (17, 20). A number of clones used in these experiments essentially possessed similar properties, i.e., no visible DI RNA band, no measurable DIU, and PFU/hemagglutinin ratio of $>10^4$. Initially DI virus was produced by passaging serially undiluted virus three to four times (20). Subsequently, larger amounts of DI virus were obtained by coinfection of DI virus with infectious virus (17).

In all experiments, purified virus isolated from sucrose density gradients was used for RNP isolation. A number of published procedures (4, 7, 8, 10, 11, 23, 24) used for RNP isolation essentially yielded similar RNP profiles. However, since Rochovansky (23, 24) showed that RNP isolated by her procedure was biologically active and free from contaminating virus, we adopted a similar procedure, unless otherwise mentioned, to isolate RNP for the experiments reported here.

Profiles of viral RNP from infectious virus (clone T) and DI virus (clone L) are shown in Fig. 1. Three peaks of RNP were consistently observed. The largest peak (I) was predominant in infectious virus, whereas in DI virus the smallest RNP peak (III) was prominent. The labeled RNA in each fraction was RNase sensitive (<2% RNase resistance), indicating the ribonucleoprotein nature of the complex. Any intact virus will be pelleted to the bottom of the gradient. When peaks from ^{32}P-labeled viral RNP were analyzed for an RNA profile in agarose polyacrylamide gels (Fig. 2), we found that peak I contained genes of polymerases (V1, -2, -3) and some of hemagglutination (HA) (V4). Similarly, peak II contained predominantly nucleoprotein (NP) and neuraminidase (NA) (V5, V6) and some HA genes, and peak III contained matrix protein (M) and nonstructural (NS) genes. In DI viral RNP, peak III also contained all DI RNA segments. Since peak I and peak III were essentially free from contamination by each other (as determined by RNA analysis), they were used to test and localize the interfering property of RNP complexes. Results from two of these experiments (Table 1) show that peak I did not interfere with infectious virus, whereas peak III caused a 40 to 70% reduction as tested by infectious center assay (17). RNase treatment abolished the interfering ability of peak III RNP, suggesting that the RNA of RNP complex was responsible for the interference. Since peak III also contained M and NS genes in addition to the DI RNA, we carried out two types of experiments to determine the role of DI RNP as opposed to that of M and NS genes in interference. In the first set of experiments the position of the interfering gene was located by assaying every two pooled fractions of the peak III region. Interfering activity had a heterogeneous profile, most of which did not coincide with peak III (Fig. 3). This would be expected, since DI-L contained four DI RNA bands, three of which migrated faster than V8 (NS gene) (Fig. 1). Finally, data in Table 2 and Table 3 show that RNP from peaks I, II, and III obtained from an infectious viral preparation failed to interfere significantly even when increasing concentrations of peak III RNP were used (Table 3).

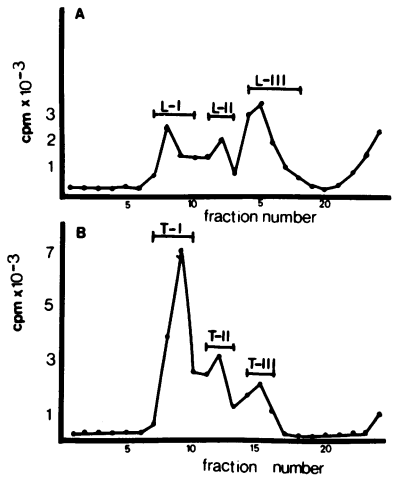

FIG. 1. *Sedimentation profile of ^{32}P-labeled influenza viral RNP. Infectious virus (clone T) or DI virus (clone L) was obtained according to the procedures described previously (20). The purified virus band was isolated in a sucrose density gradient and used for RNP isolation. RNP from infectious (T) and DI (L) viruses was isolated (23) and analyzed in glycerol gradients (15 to 30%) containing 0.05 M NaCl and 0.05 M Tris-hydrochloride (pH 7.5) for 5 h and 40 min at 39,000 rpm in an SW40 rotor. RNP profiles of such DI viral RNP (clone L) (A) and infectious viral RNP (clone T) (B) are shown. RNP profiles from infectious L clone and other infectious clones are essentially the same as in (B). Intact virus will pellet to the bottom of the gradient.*

To determine whether a cooperative effect among the DI RNP(s) was required for the interfering activity, DIU were assayed using varying amounts of RNP. The amount of DIU was directly proportional to the amount of RNP used, suggesting that cooperative effect among DI RNP complexes was not needed to produce interfering activity and that a single DI RNP may cause interference (Fig. 4).

The efficiency of interfering activity of DI RNP relative to that of intact DI virus was calculated. We found that in preparations 1 and 2 (Table 1) the ratios of DIU in RNP to that in virus were 1×10^{-3} and 2×10^{-3}, respectively,

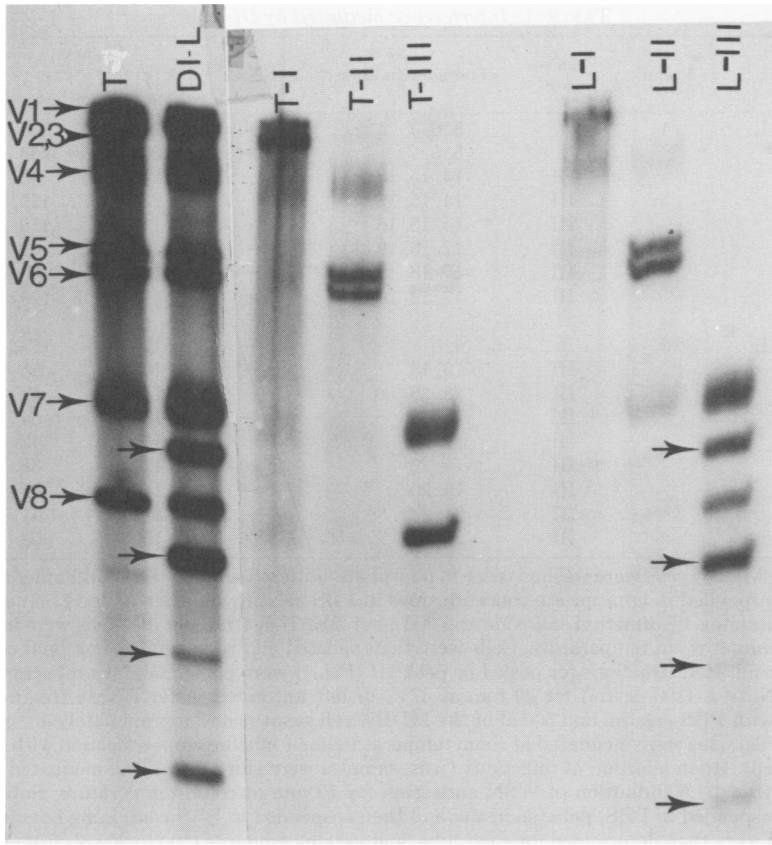

FIG. 2. *RNA profile of infectious and DI influenza viral RNP peaks. Peaks as shown by bars in Fig. 1 were pooled. RNA was extracted and analyzed by electrophoresis in slab gels containing polyacrylamide (2.1%) and agar (0.6%) as described previously (17). Total RNA from infectious (T) and DI (L) virus is shown in the first two lanes from the left; RNAs from individual RNP peaks (I, II, III; Fig. 1) of infectious virus (T) and DI-L are shown in the next six lanes. Arrows indicate the positions of four major DI RNA bands in DI-L.*

i.e., the RNP from 500 to 1,000 DIU of intact virus was required for 1 DIU of RNP. These values were based on 70% recovery of RNP in glycerol gradients (we obtained 60 to 80% recovery of RNP in different experiments).

Since DI influenza virus cannot be physically separated from infectious virus, the exact genomic content of DI influenza virus cannot be determined. Additionally, since a number of DI RNA segments are produced even when DI virus is generated from a single clone (17), it is not possible to determine whether a DI virus particle contains one or multiple copies of DI RNA. Furthermore, expecting that the average genomic content of DI virus is 60 to 80% of the infectious virus (1), DI virus is likely to contain some of the viral genomic segments in addition to one or more DI RNA segments. In this respect, DI influenza virus is different from other nonsegmented DI viruses, which contain only the DI genome. For these reasons it has been rather difficult to show the role of DI RNA segments, as opposed to that of viral RNA segments, in interference. In this report using analysis of RNP complexes, we have shown that DI RNP, as expected, sediments more slowly than most viral RNPs. Viral RNP complexes (peak I, II, or III) do not cause interference, which is exhibited by the DI RNP complexes, and the interfering activity of DI RNP is destroyed completely after RNase treatment. The possibility that modified M or NS genes in DI virus (peak III) rather than DI RNA are responsible for interference is unlikely because the data in Fig. 3 show that small DI RNP molecules sedimenting around fraction 25 are relatively free from M and NS genes (sedimenting around fraction 15) but still can cause interference. Furthermore, the specific activity of interference by RNP (i.e., percent interference per counts per minute in RNP) peaks around fraction 25 (Fig. 3). Thus, our data suggest that the RNA in the DI RNP

TABLE 1. *Interference mediated by DI RNP*[a]

Sample	Prepn[b]	Peak region	Fractions contained[c]	RNase	Plaques		% Interference
					Expected	Obtained	
ts-52 × DI-L	1	I	5, 6, 7	−	225	264	0
		I	5, 6, 7	+	264	266	0
		III	14, 15, 16	−	115	83	27.8
		III	14, 15, 16	−	230	129	43.9
		III	14, 15, 16	+	110	110	0
		III	17, 18, 19, 20	−	90	41	54.4
		III	17, 18, 19, 20	−	180	103	42.7
		III	17, 18, 19, 20	+	170	179	0
ts-52 × DI-L	2	I	4, 5	−	126	117	7.1
		III	15, 16	−	108	41	62.0
		III	15, 16	−	144	47	67.3
		III	17, 18	−	119	54	54.6
		III	17, 18	−	160	42	60.0
		III	19, 20	−	111	38	65.7
		III	19, 20	−	148	45	69.5
		III	21, 22	−	112	41	64.2
		III	21, 22	−	150	53	64.6

[a] Trypsinized MDBK cells were washed twice in phosphate-buffered saline (PBS) containing 1% gelatin. Cells were counted, suspended in appropriate concentrations in PBS-gelatin, and then added to an equal volume of PBS-gelatin containing 4% dimethyl sulfoxide and 300 μg of DEAE-dextran per ml. Cells were incubated in this medium for 15 min at room temperature. Cells were then pelleted and resuspended to a final concentration of 2×10^6 cells per ml. RNP fractions for peak I or peak III (Fig. 1) were pooled, and 0.4-ml samples were either treated with RNase A (100 μg/ml) for 20 min at 37°C or left untreated. After RNase treatment, both were diluted to 1 ml with PBS-gelatin, and 500 μl of the MDBK cell suspension (approximately 10^6 cells) was added to each sample. Samples were incubated at room temperature for 1 h before superinfection with infectious virus (*ts*-52, 2 PFU/cell). Upon addition of infectious virus, samples were shifted to 37°C, incubated for 45 min and then treated with a 1:1,000 dilution of WSN antiserum for 10 min at room temperature. Subsequently, cells were pelleted, suspended in PBS, pelleted again, and then suspended in PBS containing bovine albumin (1%). Final cell counts were then determined for each tube, and varying amounts (100 to 300 μl) from the appropriate dilution (usually 1:1,000) were plated onto confluent monolayers of MDBK cells for determining infectious centers (11). Cells were incubated at 37°C for 1 h before being overlaid with an agar overlay medium. Plates were then incubated for 4 days at 37°C. Plaques were counted on both days 3 and 4. Data represent plaque counts on day 4. The results represent an average of duplicate samples. However, essentially identical results (i.e., percent interference) were obtained using 0.1-ml or 0.3-ml samples. RNP fractions were isolated from a gradient similar to that shown in Fig. 1, and peaks were monitored using [^{3}H]uridine-labeled RNP. "Expected plaques" indicates the number of infected cells plated on MDBK monolayers.

[b] Two different preparations of *ts*-52 × DI-L were used. Preparation 1 contained RNP derived from approximately 1.2×10^5 hemagglutinin units and 5.5×10^9 DIU (total). Preparation 2 contained approximately 2.0×10^5 hemagglutinin units and 9.2×10^9 DIU (total).

[c] Numerically indicated glycerol gradient fractions were pooled (Fig. 1). Each sample tested contained an equal sample of each fraction in a 0.4-ml volume.

TABLE 2. *Effect of RNP peaks from infectious virus on interference*[a]

RNP peak region	Plaques		% Interference
	Predicted	Obtained	
I	55	55	0
I	83	79	4.8
II	54	52	3.8
II	72	70	2.7
III	62	65	0
III	86	79	8.1

[a] Clone T; 2.5×10^5 hemagglutinin units from infectious virus were used for RNP preparation. Peaks were isolated as shown in Fig. 1, and 0.4-ml samples were used for interference assay by infectious center reduction.

TABLE 3. *Effect of increasing concentration of peak III RNP from infectious virus on interference*[a]

Peak region	Amt of RNP (μl)	Relative RNP concn	% Interference[b]
III	400	1×	4.0
III	800	2×	9.5, 0, 0
III	1,200	3×	0, 0, 0

[a] Sample: Clone T.

[b] RNP isolated from Table 2 was used. Each value (percent interference) represents duplicate samples determined by infectious center reduction assay.

complex is responsible for the interference. Although small RNA molecules that are not required for viral replication have been observed

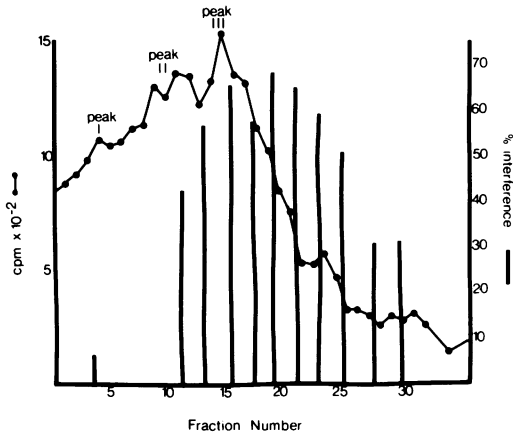

FIG. 3. *Analysis of DI RNP fraction for interference activity. Ten flasks of MDBK monolayers (150 cm^2) were coinfected with DI-L and infectious virus (17, 20). Purified virus band was obtained from a sucrose density gradient. Approximately 2 × 10^5 hemagglutinin units were used for RNP preparation. RNP was analyzed in a glycerol gradient (15 to 35%) containing 0.05 M NaCl-0.05 M Tris-hydrochloride (pH 7.8) in an SW27 rotor at 20,000 rpm for 17 h. The profile of RNP was obtained from the [^{3}H]uridine-labeled RNP in the preparation. Selected fractions were assayed for interference activity by infectious center reduction assay as described in Table 1. Bars (■) show the percent interference. Longer centrifugation, in an attempt to further separate the DI RNPs from peak III viral RNP, resulted in a reduced resolution among peak I, II, and III RNPs compared to that seen in Fig. 1.*

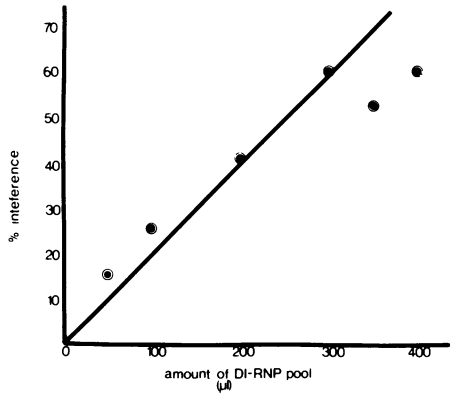

FIG. 4. *Lack of cooperative effect among DI RNPs for causing interference. RNP was pooled from peak III fraction of DI RNP (Fig. 3). Different amounts of RNP samples were used and made to 1 ml with PBS-gelatin. Interference activity in each sample was determined and calculated as described in Table 1. Percent interference when plotted against the amount of RNP used shows a linear relationship.*

in many influenza viral preparations by a number of workers (3, 5, 9, 19, 20, 22) and have been suspected to be the agent for interference (20), this report indicates a direct involvement of DI RNP (and DI RNA) in DI-mediated influenza viral interference.

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LITERATURE CITED

1. **Ada, G. L., and B. T. Perry.** 1955. Infectivity and nucleic acid content of influenza virus. Nature (London) **175:** 209-210.
2. **Choppin, P. W.** 1969. Virus in a continuous cell line: high yield of infective virus from cells inoculated at high multiplicity. Virology **39:**130-134.
3. **Choppin, P. W., and M. W. Pons.** 1970. The RNAs of incomplete influenza virus grown in MDBK and HeLa cells. Virology **42:**603-610.
4. **Compans, R. W., J. Content, and P. H. Duesberg.** 1972. Structure of the ribonucleoprotein of influenza virus. J. Virol. **10:**795-800.
5. **Crumpton, W. M., N. J. Dimmock, P. D. Minor, and R. J. Avery.** 1978. The RNAs of defective-interfering influenza virus. Virology **90:**370-373.
6. **Davis, A. R., and D. P. Nayak.** 1979. Sequence relationships among defective interfering influenza viral RNAs. Proc. Natl. Acad. Sci. U.S.A. **76:**3092-3096.
7. **Duesberg, P. H.** 1969. The RNAs of influenza virus. Proc. Natl. Acad. Sci. U.S.A. **59:**390-397.
8. **Duesberg, P. H.** 1969. Distinct subunits of the ribonucleoprotein of influenza virus. J. Mol. Biol. **42:**485-499.
9. **Hay, A. J., B. Lomniczi, A. R. Bellamy, and J. J. Skehel.** 1977. Transcription of the influenza virus genome. Virology **83:**337-355.
10. **Hirst, G. K., and M. Pons.** 1972. Biological activity in ribonucleoprotein fractions of influenza virus. Virology **47:**546-549.
11. **Hirst, G. K., and M. W. Pons.** 1973. Mechanism of influenza recombination II. Virus aggregation and its effect on plaque formation by so-called noninfective virus. Virology **56:**620-631.
12. **Holland, J. J., L. P. Villarreal, and M. Breindl.** 1976. Factors involved in the generation and replication of rhabdovirus defective T particles. J. Virol. **17:**805-815.
13. **Huang, A. S.** 1973. Defective interfering viruses. Annu. Rev. Microbiol. **27:**101-117.
14. **Huang, A. S.** 1977. Viral pathogenesis and molecular biology. Bacteriol. Rev. **41:**811-821.
15. **Huang, A. S., and D. Baltimore.** 1970. Defective viral particles and viral disease processes. Nature (London) **226:**325-327.
16. **Huang, A. S., and D. Baltimore.** 1978. Defective interfering animal viruses, p. 73-116. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Publishing Corp., New York.
17. **Janda, J. M., A. R. Davis, D. P. Nayak, and B. K. De.** 1979. Diversity and origin of defective interfering influenza virus particles. Virology **95:**48-58.
18. **Leppert, M., L. Kort, and D. Kolakofsky.** 1977. Further characterization of sendai virus DI-RNAs: a model for their generation. Cell **12:**539-552.
19. **Nakajima, K., M. Ueda, and A. Sugiura.** 1979. Origin of small RNA in von Magnus particles of influenza virus. J. Virol. **29:**1142-1148.
20. **Nayak, D. P., K. Tobita, J. M. Janda, A. R. Davis, and B. K. De.** 1978. Homologous interference mediated by defective interfering influenza virus derived from a temperature-sensitive mutant of influenza virus. J. Virol. **28:**375-386.

21. **Palese, P.** 1977. The genes of influenza virus. Cell **10**:1–10.
22. **Palese, P., and J. Schulman.** Differences in the RNA patterns of influenza A virus. J. Virol. **17**:876–884.
23. **Rochovansky, O. M.** 1976. RNA synthesis by ribonucleoprotein-polymerase complexes isolated from influenza virus. Virology **73**:327–338.
24. **Rochovansky, O. M., and G. K. Hirst.** 1976. Infectivity and marker rescue activity of influenza virus ribonucleoprotein-polymerase complexes. Virology **73**:339–349.
25. **Sugiura, A., M. Ueda, K. Tobita, and C. Enomoto.** 1975. Further isolation and characterization of temperature-sensitive mutant of influenza virus. Virology **65**: 363–375.
26. **von Magnus, P.** 1954. Incomplete forms of influenza virus. Adv. Virus Res. **2**:59–78.