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Incomplete Influenza Virus: Partial Functional Complementation as Revealed by Hemadsorbing Cell Count Test

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In MDCK cells inoculated with an appropriate dilution of influenza virus, single hemadsorbing cells could be counted 8 h postinfection against a background of nonadsorbing cells. Standard virus preparation exhibited a linear relationship between the virus dilution and the number of hemadsorbing cells. With incomplete virus preparations obtained by passages of undiluted virus in chicken embryo, the dependence was nonlinear. A *ts* mutant (*ts*-29) of A/FPV/Weybridge (Hav1 Neq1) failed to convert MDCK cells into a hemadsorbing state at 42°C. The ability of *ts*-29 to produce hemadsorbing cells could be rescued by incomplete wild-type virus. The capacity of incomplete virus for this partial functional complementation was inactivated by UV irradiation with one-hit kinetics. The size of the target was estimated to be 5.5 times smaller than that of the virus genome. The results suggest that at least some of the influenza virus genes in defective interfering particles are functional.

The characteristics of defective interfering (DI) particles are usually ascribed to the presence of large deletions in their genomes. This seems to be a common pattern for a large variety of viruses (8). However, influenza DI particles may contain all eight genomic RNA segments as revealed by polyacrylamide gel electrophoresis (2, 3, 16). Their molar ratios are usually somewhat changed as compared with those in standard virus, but the difference is not large enough to account for the drastic loss of infectivity (2, 3, 16). Although additional small RNA segments have been detected (9, 15-18) and identified as deleted genomic segments (4, 15), their presence as such cannot explain the loss of infectivity.

A hypothesis explaining the properties of influenza DI particles has been recently advanced by Nayak et al. (16). The authors postulate that different DI particles lack different genomic segments. This accounts for the dramatic decrease of infectivity with a concomitant presence of all genomic segments in the whole DI virus RNA preparation. This hypothesis is attractive in several aspects: it allows us to explain the experimental data so far published and to make several predictions amenable to an experimental verification. The data on the nature of small additional RNA segments (4, 15) allow us to suggest that the lacking genomic segments are P1-P3 genes and that they are replaced by their deleted counterparts.

When cells are infected with such a heterogeneous DI population, it is reasonable to expect that a cell infected with two or more particles would compose the whole set of genomic segments. One might expect that at least some viral functions should be expressed in these cells so that they could be scored as immunofluorescent or hemadsorbing single cells. One might also suppose that a sufficient fraction of the DI population would be able to partially complement a *ts* defect, that is, to convert a *ts* mutant-infected cell at a restrictive temperature into an immunofluorescent or hemadsorbing state. The experiments presented in this report were designed so as to investigate whether influenza DI particles do exhibit these capacities.

MATERIALS AND METHODS

Viruses. Influenza strains A/WSN/33/HONI and A/FPV/Weybridge (Hav1 Neq1) were used. *ts*-29, a *ts* mutant of A/FPV/Weybridge was kindly provided by Y. Z. Ghendon. The viruses were grown in the allantoic cavity of 10-day-old chicken embryos, the temperature of incubation being 37°C for wild-type viruses and 36°C for *ts*-29. To obtain standard virus preparations, chicken embryos were inoculated with 100 50% egg infective doses of cloned virus and incubated for 24, 30, and 48 h for A/FPV/Weybridge, *ts*-29, and A/WSN/33, respectively. To obtain incomplete (von Magnus) virus, two passages of undiluted material were performed with growth periods of 8 and 24 h for A/FPV/Weybridge and A/WSN/33, respectively. The intervals of virus growth were designed to minimize the thermoinactivation of virus yield. All preparations were stored at 4°C and used within a week after the harvest. Hemagglutinating activity and infectivity

were determined immediately after the harvest and a week later. Plaque titrations were performed in chicken embryo cell cultures under trypsin-containing agar overlay (1).

Cells. MDCK cells were obtained from the Tissue Culture Department of the Institute of Virology as a suspension and grown in double minimal essential medium supplemented with 10% calf fetal serum in Carrel flasks (60 mm in diameter). Trypsinized primary chicken embryo cell cultures were grown in 0.5% lactalbumin hydrolysate in Hanks balanced salt solution with 10% bovine serum.

Infection and hemadsorption procedures. Immediately after the formation of confluent monolayers, MDCK cultures were washed twice with minimal essential medium and inoculated with serial dilutions of influenza virus. After 1 h of adsorption at room temperature, the cells were washed three times, 10 ml of double minimal essential medium was added, and the cultures were incubated at 37 or 42°C. At the end of incubation, the cells were washed three times with cold phosphate-buffered saline, overlaid with 4 ml of a 4% suspension of guinea pig erythrocytes (RBC) in phosphate-buffered saline and held at 4°C with periodic stirring. Then the RBC fraction was removed, and the monolayers were rinsed three times with phosphate-buffered saline lacking Mg^{2+} and Ca^{2+} and examined under a light microscope at low magnification. Hemadsorbing cells were counted in no less than 10 fields of vision.

UV irradiation. Virus-containing allantoic fluid was clarified by centrifugation at 6,000 rpm for 20 min, diluted 1:10 in phosphate-buffered saline, sonicated three times for 20 sec, with 1-min intervals, at 4°C, and irradiated in a layer 2-mm thick in a petri dish at 4°C with permanent rocking.

Virus purification, RNA extraction, and polyacrylamide gel electrophoresis. Virus was purified by centrifugation in potassium tartrate solutions as described by Kingsbury (11) with slight modifications (10). RNA was extracted with the use of the sodium dodecyl sulfate-pronase-phenol method (12). Polyacrylamide gel electrophoresis analysis was performed in tubes as described by Floyd et al. (6) (omitting agarose). The gels were stained with ethidium bromide and photographed in UV light.

RESULTS

Characteristics of incomplete virus preparations. Incomplete virus preparations obtained by two passages of undiluted material as described by von Magnus (13) (see above) usually had a PFU-to-hemagglutinating activity ratio 2 to 3 lg lower than standard preparations. RNA extracted from purified incomplete virus contained additional small RNA segments (Fig. 1).

Hemadsorbing cell count titration of standard and incomplete virus. MDCK cells at 8 h postinfection acquired an ability to absorb guinea pig RBC firmly at 4°C. At a high multiplicity of infection, the whole cell sheet was covered with RBC, whereas, at higher dilutions, single hemadsorbing cells could be counted against the background of nonadsorbing cells (Fig. 2). The number of hemadsorbing cells (HadC) per flask could be calculated with the following formula: $x = a \cdot S/s$, where x is the number of HadC per field of vision, S is the area of the cell sheet, and s is the area of the field of vision. The mean number of HadC decreased linearly with dilution when standard virus was titrated. The titer determined by the HadC count for standard virus preparations was usu-

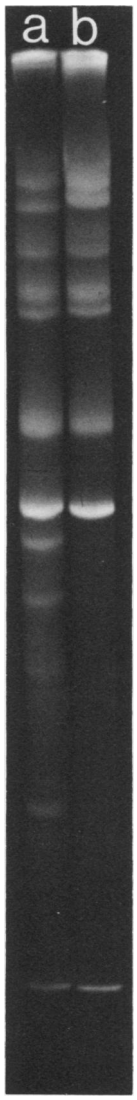

FIG. 1. *Polyacrylamide gel electrophoresis analysis of RNA extracted from standard (b) and incomplete (a) A/WSN/33 influenza virus. Acrylamide concentration, 2.8%; 6 mA per tube; 8 h.*

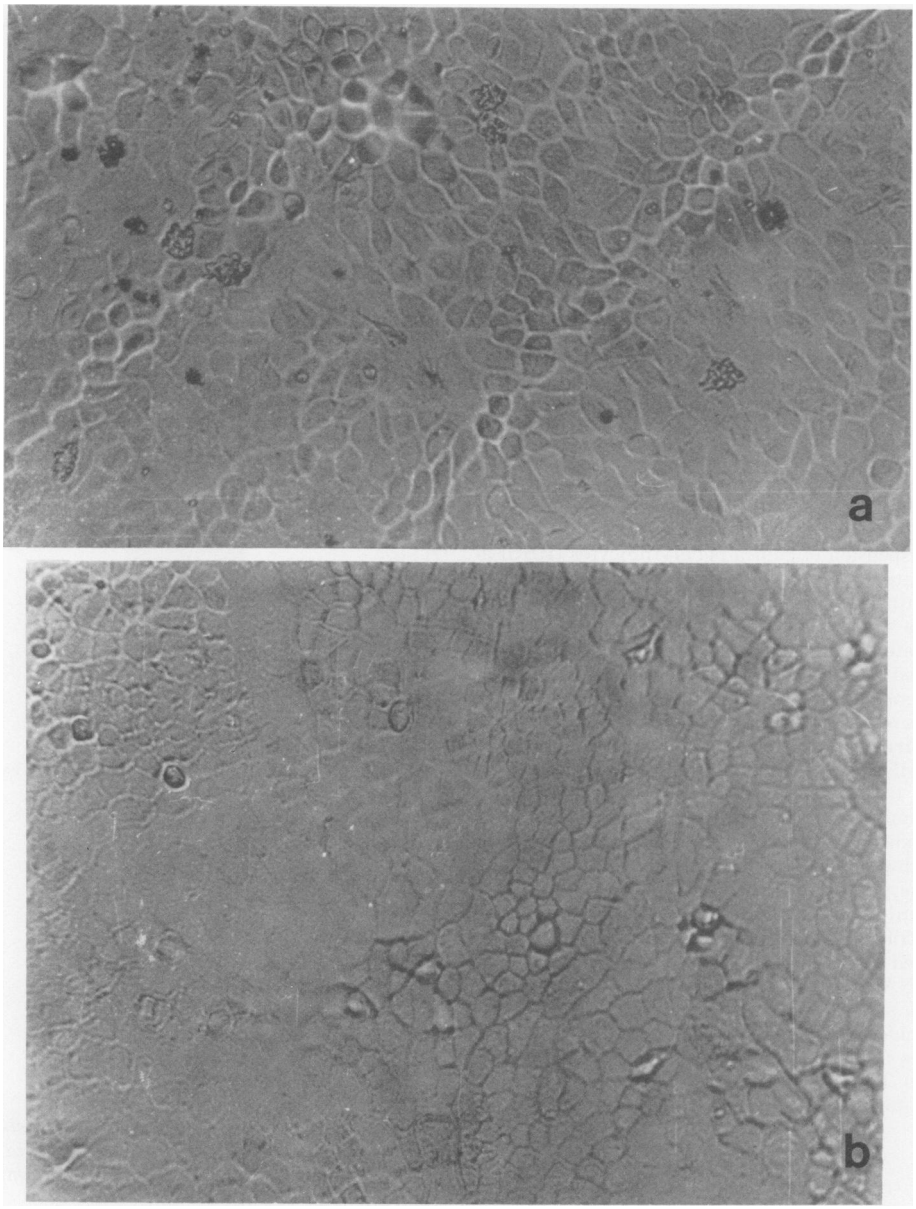

FIG. 2. *Hemadsorbing cells in MDCK monolayer 8 h postinfection. (a) Multiplicity of infection of 0.01 PFU/cell, A/WSN/33; (b) mock-infected cells.*

ally slightly higher than the titer determined by plaque titration in chicken embryo cell cultures.

When the same procedure was applied to incomplete virus, we usually could not obtain a linear relationship between the virus dilution and the number of HadC. With the increase of incomplete virus concentration, the number of HadC increased rapidly, so that the hemagglutinating activity content of the inoculum producing a confluent hemadsorption was only 3 to 10 times higher than that for standard virus. Since the PFU-to-hemagglutinating activity ratio is 100 to 1,000 times lower in incomplete virus than in standard preparations, this effect cannot be ascribed to the admixture of infectious particles in incomplete virus. An example of both standard and incomplete virus HadC count titration is presented in Table 1.

Coinfection of MDCK cells with *ts*-29 and incomplete wild-type virus. The stock of *ts*-

TABLE 1. *Dose-response relationships between the concentration of standard and incomplete A/WSN/33 influenza virus and the number of hemadsorbing cells*

Prepn	PFU/ml	Hemagglutinating activity units	Hemadsorbing cells at the following dilutions:							
			10^{-1}	$10^{-1.5}$	10^{-2}	$10^{-2.5}$	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$
Standard	1.4×10^8	512		C[a]	AC[b]	50%[c]	>300[d]	~200	68.4	20.3
Incomplete	7.5×10^5	256	C	AC	>300	128	16.5	3.5		

[a] C, Complete hemadsorption (the monolayer completely covered with RBC).
[b] AC, Almost complete hemadsorption.
[c] 50%, Half of the area is covered with RBC.
[d] Mean number of hemadsorbing cells per field of vision.

29 had an infectious titer of 5×10^8 PFU/ml when titrated at 36°C and 5×10^2 PFU/ml when titrated at 42°C. MDCK cells infected with *ts*-29 at a multiplicity of infection of 2 to 4 PFU/cell and incubated for 8 h at 37°C produced a complete confluent hemadsorption, whereas there was no hemadsorption in the cultures incubated at 42°C. At a multiplicity of infection of 20 PFU/cell, a weak, scattered hemadsorption was observed at 42°C.

MDCK cells were infected with *ts*-29 at a multiplicity of infection of 2 to 4 PFU/cell, superinfected immediately after adsorption with serial dilutions of standard or incomplete wild-type virus, and incubated at 42°C. In parallel flasks the wild-type virus preparations were titrated without *ts*-29 (Table 2). The preinfection with *ts*-29 increased slightly the number of HadC produced by standard virus at 42°C, whereas the amount of HadC produced by incomplete virus increased dramatically. The result was obtained consistently with different stocks of standard and incomplete virus. The data suggest that in incomplete preparations the amount of virus particles able to complement *ts*-29 in its ability to produce hemadsorption at 42°C is almost equal to the amount of infectious virus particles in standard virus per hemagglutinating activity unit.

UV inactivation of the ability of standard and incomplete virus for the functional complementation of *ts*-29. Standard and incomplete virus preparations were subjected to UV irradiation and used for superinfection of *ts* mutant-infected MDCK cells. The ability of both preparations to form HadC in *ts*-29-infected cells at 42°C was inactivated at a much slower rate than their infectivity. The slope of the UV inactivation curve was the same for standard and incomplete virus (Fig. 3). The target size of the ability to complement *ts*-29-induced hemadsorption is about 5.5 times smaller than that of infectivity; that is, the size of the component responsible for the functional complementation is 5.5 times smaller than that of the influenza virus genome.

TABLE 2. *Functional complementation of ts-29 with standard and incomplete wild-type virus as revealed by hemadsorbing cell count*[a]

Inoculum	Wild-type viruses counted at the following dilutions:			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Standard[b]	C[c]	C	~200	21.5
ts-29 + standard	C	C	50%[d]	128
Incomplete[e]	C	83.2	1.3	0
ts-29 + incomplete	C	50%	200	39

[a] For the characteristics of *ts*-29 stock and the conditions of inoculation, see text.
[b] Standard virus contained 1×10^8 PFU/ml and 256 hemagglutinating activity units.
[c] C, Complete hemadsorption.
[d] 50%, Half of the area is covered with RBC.
[e] Incomplete virus contained 4.8×10^4 PFU/ml and 128 hemagglutinating activity units.

DISCUSSION

Von Magnus influenza virus preparations were never reported to be able to undergo multiplicity reactivation. However, a cell producing mostly DI particles, or synthesizing viral components without virion maturation, would not be scored as an infectious center; therefore, multiplicity reactivation would not be registered. On the other hand, such cells may be scored with the use of such methods as immunofluorescent cell count or the counting of hemadsorbing cells used in these studies. This approach enabled us to reveal a nonlinear response to the increase of incomplete virus concentration. The phenomenon may be considered to be due to a kind of partial self-complementation of DI particles. This may be possible if different single particles lack different genomic segments. In cells infected with *ts*-29 and incubated at 42°C, the ability of incomplete virus to convert the cells into a hemadsorbing state almost equalled that of standard virus (Table 2). These data are in agreement with the hypothesis advanced by Nayak et al. (16) suggesting that, whereas the whole population of DI particles composes the whole set of genomic RNA segments, every single particle

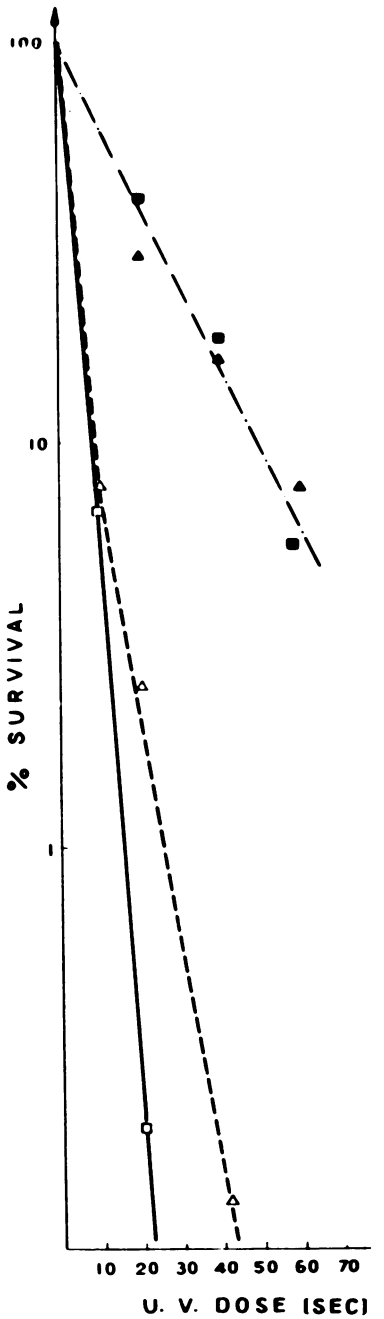

FIG. 3. *UV inactivation of the ability for functional complementation of ts-29 by standard and incomplete wild-type virus as revealed by HadC count.* ▲△, *Standard virus;* ■□, *incomplete virus;* □△, *PFU titration;* ■▲, *HadC titration.*

lacks different segments; probably, every particle lacks one of the P1–P3 genes (4). Such heterogeneous particles would be expected to complement one another partially; that is, the cell infected with two or several particles would be expected to perform virus-specific synthesis (including RNA replication), even if it would not produce infectious virus. A sufficient part of such a population would be able to complement *ts* mutants with respect to the production of virus-specific components.

In our experiments on the UV inactivation, it is noteworthy that the ability of both standard and incomplete virus to complement *ts*-29 followed a one-hit inactivation curve (Fig. 3). This indicates that the predominant part of the particles responsible for the complementation (both in standard and incomplete virus) contains one copy of the genomic segment corresponding to the gene carrying the *ts* lesion in *ts*-29 (segment 1 coding for P3 [6a]). This may indicate that, although the population of DI particles is heterogeneous with respect to the set of viral genes they compose, their assembly is not a random process. This, however, needs further verification with a variety of *ts* mutants. We obtained recently a similar one-hit curve with *ts*-43: the *ts* lesion of this mutant has been preliminarily located in the NP gene (Y. Z. Ghendon, personal communication).

The slope of the inactivation curve shown in Fig. 3 indicates that the target size is ~5.5 times smaller than the size of the influenza genome. This agrees well with the ratio of the size of segment 1 to the genome size as determined by polyacrylamide gel electrophoresis (5).

The unequivocal interpretation of our data is hampered by the fact that influenza virus preparations usually contain noninfectious particles capable of multiplicity reactivation (7) and marker rescue (14). They may be represented by partially inactivated or randomly incomplete virions. One cannot disregard the possibility that the effects observed in our experiments were due (at least partially) to such particles. In fact, the slight increase of the number of hemadsorbing cells in the culture infected with *ts*-29 and standard virus, as compared to their number in the culture infected with standard virus alone (Table 2), may be due to the noninfectious virions which are capable of reactivating. However, it seems doubtful that the drastic increase obtained with incomplete virus may be ascribed to such particles. In this case they would have to dominate in the population. This seems doubtful, since the standard virus particles seem to be replaced by particles containing DI segments (Fig. 1) and not by randomly incomplete or partially inactivated virions.

Taking all this into consideration, it seems warranted to suggest that the partial functional complementation exerted by incomplete virus

and registered with the use of hemadsorbing cell count is due to influenza DI particles. This would mean that the genes they compose are functional. Besides, our data suggest that at least some of the genes are represented by no more than one copy in any single DI particle.

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