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Defective Interfering Influenza Viruses and Host Cells: Establishment and Maintenance of Persistent Influenza Virus Infection in MDBK and HeLa Cells

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WSN (H0N1) influenza virus upon undiluted passages in different species of cells, namely, bovine kidney (MDBK), chicken embryo (CEF), and HeLa cells, produced a varying amount of defective interfering (DI) virus which correlated well with the ability of the species of cell to produce infectious virus. However, the nature of the influenza DI viral RNA produced from a single clonal stock was essentially identical in all three cell types, suggesting that these cells do not exert a great selective pressure in the amplification of specific DI viral RNAs either at early or late passages. DI viruses produced from one subtype (H0N1) could interfere with the replication of infectious viruses belonging to other subtypes (H1N1, H3N2). DI viral RNAs could also replicate with the helper function of other subtype viruses. The persistent infection of MDBK and HeLa cells could be initiated by coinfecting cells with both temperature-sensitive mutants (ts^-) and DI influenza viruses. Persistently infected cultures at early passages (up to passage 7) showed a cyclical pattern of cell lysis and virus production (crisis), whereas, at later passages (after passage 20), they produced little or no virus and were resistant to infection by homologous virus but not by heterologous virus. The majority of persistently infected cells, however, contained the complete viral genome since they expressed viral antigens and produced infectious centers. Selection of a slow-growing temperature-sensitive variant rather than the presence of DI virus or interferon appears to be critical in maintaining persistent influenza infection in these cells.

Over the last few years we have studied the properties of von Magnus influenza viruses (37) produced in MDBK cells (25a). Like other defective interfering (DI) viruses, these particles appear noninfectious, replicate in the presence of helper infectious virus, and interfere with the replication of helper virus. Furthermore, these influenza DI particles also possess, in addition to viral RNA (vRNA) segments, smaller RNA molecules (DI RNA) of vRNA origin which are absent in infectious virus preparation (6, 17, 25, 26). These DI RNA segments arise from specific vRNA segments (mostly from P genes) by internal deletion (7, 8, 25) and are responsible for the interfering property (18).

The production of DI viruses depends on the nature of the host cell. With vesicular stomatitis virus (VSV), Holland et al. (14), and more recently Kang et al. (19), have shown that the same clonal stock produces different DI viruses in different cell types, although the same DI virus is produced in a given cell type. Stark and Kennedy (21, 36) also reported a strong host influence in the production of Semliki Forest DI virus. The host range of influenza viruses varies widely (2, 16, 32-34). WSN virus (H0N1) has been shown to possess a differential growth potential in different host cells. For example, WSN virus produces largely infectious virus in MDBK cells, predominantly incomplete virus in chicken embryonic fibroblasts (CEF), and only abortive infection with little or no virus in HeLa cells (5). However, little information is available on the role of different species of host cells on the production of influenza DI viruses.

Since DI viruses appear to reduce the replication of infectious virus and thereby inhibit the cell killing effect of homologous lytic virus, they have been implicated in establishing and maintaining persistent infection both in vivo (15) and in cultured cells for a number of viruses, e.g., VSV (13), rabies virus (20), reovirus (1), measles virus (29), Japanese encephalitis virus (31), Sendai virus (30), lymphocytic choriomengitis virus (28, 39), Sindbis virus (38), and Semliki Forest virus (23). In addition, temperature-sensitive mutation and interferon have been shown to aid in establishing the persistently infected culture in vitro (1, 11, 41). Although the mechanism of viral persistency has been studied extensively

for a number of the viruses referenced above, very little information is available for influenza virus persistent infections either in nature or in cell culture.

In this paper we have investigated the role of host cells, e.g. bovine (MDBK), chicken (CEF), and human (HeLa) cells, on both the amount and the nature of the influenza DI virus produced in these cells. We have also analyzed the role of DI influenza virus in establishing and maintaining persistent infection of MDBK and HeLa cells.

MATERIALS AND METHODS

Cells and viruses. MDBK (bovine kidney), MDCK (canine kidney), and HeLa cells were grown and maintained in Eagle minimal medium containing 10% heat-inactivated fetal calf serum (26). Chicken fibroblast (CEF) cultures were prepared from 11-day-old embryonated eggs and grown in Eagle minimal medium containing 10% heat-inactivated fetal calf serum and 10% tryptose phosphate broth. A/WSN/33 (H0N1), A/USSR/90/77 (H1N1), A/PC/1/73 (H3N2), and A/Vic/3/75 (H3N2) influenza virus strains were used in these experiments. Clones were isolated after six successive plaque purifications, in MDBK for WSN virus and in MDCK cells for other viruses (26). Clonal stocks were grown in MDBK cells for WSN virus and in MDCK cells for other viruses as reported earlier (17). Both wild-type (ts^+) and a group II temperature-sensitive mutant (*ts*52) of WSN virus were used (26).

Production of influenza DI virus in different cells. Samples of clonal stocks of WSN virus prepared in MDBK cells were passed serially, undiluted, in MDBK, HeLa, or CEF cells to prepare DI virus (26). Clonal stocks of *ts*52 initially grown in MDBK cells contained no detectable amount of DI virus, as determined by infectious center reduction assay and by RNA analysis using polyacrylamide gel electrophoresis (17).

Long-term passage of DI virus. These experiments were initiated with DI virus produced at the undiluted passage 2 (p-2). Subsequently, at each passage, cells were infected with DI virus produced at the preceding passage as well as additional infectious stock virus. Briefly, MDBK, HeLa, and CEF cultures (150 cm^2) were infected with 5 ml of DI virus produced at the preceding passage, plus 0.5 ml of infectious virus (1 PFU/cell). Virus was harvested at 14 h postinfection by subjecting the cells and culture supernatant to two cycles of rapid freezing and thawing and was used as the source of DI virus inoculum for the next passage. This procedure of infection with DI and added infectious viruses was continued for up to 30 passages in respective cell lines. The multiplicity of infection of DI virus in these long-term passages varied from 2 to 20 defective interfering units (DIU) per cell at different passages.

Isolation and analysis of ^{32}P-labeled viral RNA. MDBK, MDCK, CEF, and HeLa cells (150-cm^2 flasks) were infected with a mixture of infectious virus (1 PFU/cell) and DI virus (2 DIU/cell). The labeled viral RNA was isolated (27) and analyzed by slab gel electrophoresis as described before (17). Electrophoresis was carried out either at 4°C or at room temperature for 21 h using 2.2% polyacrylamide–0.8% agarose gels containing 6 M urea, 0.036 M Tris-hydrochloride (pH 8.1), 0.03 M NaH_2PO_4, and 0.001 M EDTA as described by Floyd et al. (10).

Oligonucleotide mapping. ^{32}P-labeled infectious or DI viral RNA (approximately 5×10^6 to 20×10^6 cpm) was electrophoresed in a single lane of a slab gel as described earlier (8). The positions of viral and DI RNA segments were located from an autoradiograph obtained after 30 min to 1 h of exposure of Du Pont Cronex film. RNA was eluted from the corresponding gel segments by the procedure of Maxam and Gilbert (22). Labeled RNA segments were digested with RNase T_1 and analyzed by two-dimensional gel electrophoresis using a modified procedure of DeWachter and Fiers (9). The specific conditions of electrophoresis used for oligonucleotide mapping have been described previously (8).

Establishment of persistent infections in MDBK and HeLa cells. MDBK and HeLa cells were infected with either the infectious virus (1 PFU/cell) alone or a mixture of infectious (1 PFU/cell) and DI virus (p-2 for MDBK and p-2 for HeLa, 2 DIU/cell). At 14 h postinfection, infected cultures were washed thoroughly with phosphate-buffered saline (PBS) free of Ca^{2+} and Mg^{2+} (PBS^-) and overlaid with fresh medium which was replaced every day. Surviving cells eventually formed confluent monolayers of persistently infected (Pi) cells. These cells were subcultured twice a week at a ratio of 1:4. These Pi cells were termed MP1, MP2, and MP3 or HP1 and HP2 when they were of MDBK or of HeLa cell origin, respectively. Occasionally these Pi cells underwent crisis with extensive cytopathic effect and virus production, but they recovered after frequent changes of growth medium.

Challenge with infectious homologous and heterologous viruses. Pi cell cultures (MP1, MP2, MP3, HP1, and HP2) were infected with infectious virus (2 to 10 PFU/cell) at different passages. At 14 h postinfection the flasks were checked for cytopathic effect, and the supernatants were analyzed for PFU, DIU, and hemagglutination units (HAU) per milliliter as described earlier (17). For heterologous virus challenge, Pi cells were infected with Newcastle disease virus (2 to 10 PFU/cell) and analyzed for virus production.

Infectious center assay. Pi cells were trypsinized and added at different concentrations to fresh MDBK monolayers. After 1 h at 37°C, the agar overlay medium was added and kept either at 34°C or at 39°C; after 4 to 5 days, plaques were counted (17). DIU per milliliter were determined by using the infectious center reduction assay as described previously (17).

Indirect fluorescent-antibody staining. Uninfected cells (MDBK and HeLa), acutely infected MDBK and HeLa cells, and a number of Pi cells of different passages were grown on microscopic slides for 24 h and fixed with acetone in cold (−20°C) as described by Roux and Holland (30). Subsequently they were covered with a solution of anti-WSN rabbit serum (1:20 dilution in PBS containing 500 hemagglutination inhibition units per ml) for 30 min at room

temperature, washed thoroughly, and finally stained with fluorescein-labeled goat anti-rabbit 7S globulin (1:25 in PBS^-; GIBCO) for 30 min at room temperature. These labeled cells were further washed twice with PBS^- and examined under a microscope equipped with a UV light source.

To detect the presence of viral antigen on the cell surface, live cells were used. Cells were trypsinized and incubated for 2 to 3 h at 37°C with the original conditioned growth medium. These cells were then washed with PBS^- and incubated with anti-WSN antibody (1:20 in PBS) at 0°C for 30 min. Subsequently they were washed in PBS^- and stained with fluorescein-conjugated goat anti-rabbit 7S globulin for 30 min at 0°C. The suspended cells were washed twice further with PBS^- and examined for immunofluorescence.

RESULTS

Production of DI viruses in MDBK, CEF, and HeLa cells. To determine the effect of different species of host cells on the production of DI virus, infectious *ts*52 WSN clones, six times plaque purified, were used to prepare clonal stocks. Samples of clonal stock of *ts*52 (clone 4; 2.8×10^7 PFU/ml, 2,048 HAU/ml) were inoculated into MDBK and CEF (0.01 PFU/cell) and HeLa cells (1 PFU/cell). At low multiplicity (0.01 PFU/cell), Hela cells produced very little virus. Total virus was harvested from these infected cultures at 14 h postinfection and was called p-0. p-1 virus was produced in respective cells by using p-0 virus at 20 PFU/cell. Subsequently, 5 ml of undiluted virus preparation was passed serially for the production of DI virus in respective cells (26). Infectious virus (PFU per milliliter), DI virus (DIU per milliliter), and total virus production (HAU per milliliter) were assayed at different passages by the procedure described previously (17, 26).

DI virus was not detected at p-0 in MDBK, CEF, or HeLa cells. At p-1, MDBK cells produced 4×10^5 DIU/ml, whereas both CEF and HeLa cells produced 1×10^5 DIU/ml. Subsequently, in serially undiluted passages (p-3 to p-5), more DI virus was produced in all three types of cells. However, both CEF and HeLa cells produced only a moderate amount of DI virus (CEF, 2×10^5 DIU/ml at p-4; HeLa, 2.5×10^5 DIU/ml at p-3) when compared to the maximum production in MDBK cells (2.4×10^6 DIU/ml at p-5). The total virus particle yield, as determined by HAU, was reduced in all cells upon undiluted passages. As the production of DI virus increased, the overall yield of infectious virus decreased in all three species of cells. At p-4, HeLa cells contained a detectable hemagglutination titer (16 HAU/ml), although no DI or infectious virus could be detected. The production of hemagglutinating antigen at p-4 may represent an abortive replication of WSN virus in HeLa cells (e.g., intracytoplasmic inclusions [4]).

In experiments involving long-term passage of DI virus, cells were continuously coinfected with DI virus and additional infectious virus at each passage. The results (Fig. 1) show that the overall patterns of the production of DI virus and infectious virus, as well as the hemagglutinin titer, were essentially similar in all three types of cells. There was a gradual decrease of infectious virus yield, accompanied by an increase in DI virus production, irrespective of the nature of the cell. Again, the greatest amount of both infectious and DI virus was produced in MDBK cells as compared to that produced in CEF and HeLa cells. Finally, the DIU/PFU ratio showed a steady increase in all cells (from 0.125 in p-3 to 19 in p-10 for MDBK; from 0.005 in p-3 to 5.5 in p-10 for CEF; from 0.007 in p-3 to 11 in p-10 for HeLa cells). After passage 10, the same DIU/PFU ratio was maintained up to p-30 by continued coinfection with DI and infectious viruses (data not shown). Thus, it appears that after continued coinfection with infectious and DI viruses over many passages, an equilibrium in the production of infectious and DI viruses is established.

Nature of DI virus produced in MDBK, HeLa, and CEF cells. The nature of RNA of DI virus released by these cells at different passages was analyzed by polyacrylamide gel electrophoresis and oligonucleotide mapping. Figure 2 shows that the [^{32}P]RNA of purified virus at p-0 contained only eight vRNA segments without any detectable amount of DI virus in all three cell species. However, the virus at the undiluted p-3, at coinfected p-9, and at p-30 from all cell types contained a number of DI RNA segments in addition to the eight standard vRNA's, although present in varying ratios. Furthermore, we have rather consistently found here (Fig. 2 and 4), and previously (7, 8, 18, 26), that the molar ratio of M gene relative to other viral genes including the NS gene is increased in DI particles. This would suggest either that some of the DI particles contain only the M gene or that DI particles are polyploid in respect to the M gene. Another alternative, although unlikely, is the presence of a DI RNA comigrating with the M gene. At present we have no idea which, if any, of these possibilities is true.

The DI RNA segments produced at p-3, p-9, and p-30 in all cell types were essentially the same in nature. At least six distinct DI RNA segments were found. At p-3, DIa migrating faster than the NS gene was most conspicuous in all cell types (Fig. 2A). At p-30, DIa decreased and a smaller DI RNA (DIb) became more

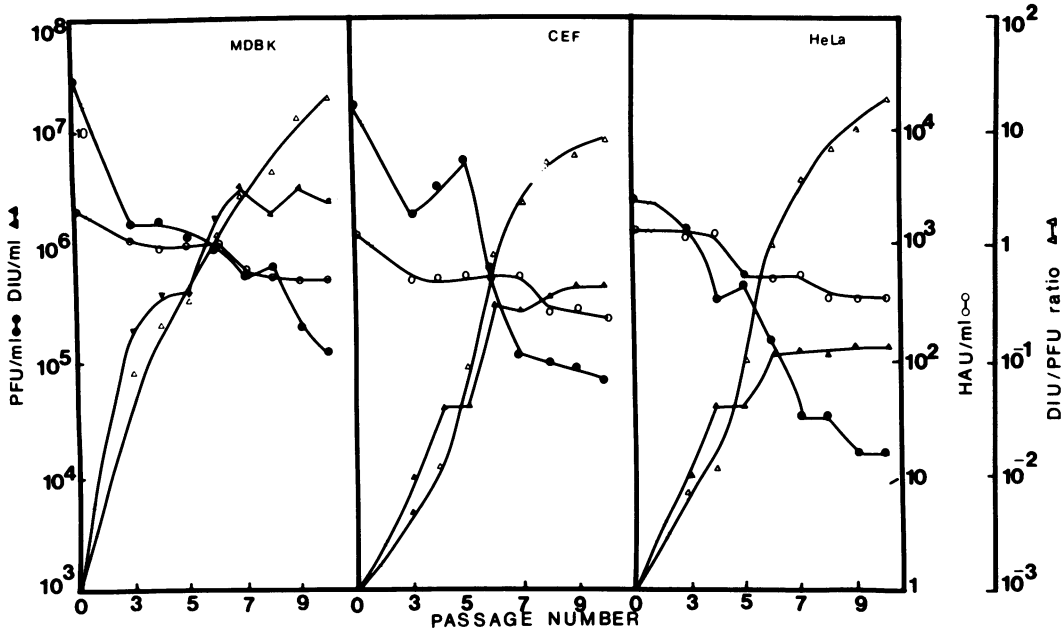

FIG. 1. *Analysis of total infectious virus (PFU per milliliter, ●), defective interfering virus (DIU per milliliter, ▲) and hemagglutinin (HAU per milliliter, ○) produced by the same clonal isolate (no. 4) in MDBK, CEF, and HeLa cells during continued coinfection using infectious virus and DI virus from preceding passages as described in the text. The corresponding DIU/PFU (△) ratio at each passage is also given under the same conditions for the three cell types.*

prominent in all three types of cells (Fig. 2B). At p-9, an intermediate result was obtained (data not shown). Thus it appears that after many passages the early DI virus is gradually replaced by a DI virus with a smaller DI RNA segment. Further sequence relationship among the DI RNAs was determined by oligonucleotide mapping (8). DIa isolated from MDBK cells contained spots characteristic of the P3 (polymerase) gene and therefore originates from the P3 gene (Fig. 3A and B). Similarly, DIa from HeLa cells and CEF cells contained spots identical to those present in DIa of MDBK cells and is, therefore, also of P3 origin (data not shown). Both DIb RNAs isolated from p-30 of MDBK and HeLa cells produced essentially identical oligonucleotide maps and are subsets of DIa (Fig. 3C). These results suggest that both DIa and DIb are interrelated and originate from the same P3 gene. These results also confirm our previous observation that the majority of influenza DI RNAs originate from one of the polymerase genes (8).

DI virus-mediated interference against different subtypes of influenza A viruses. Interference mediated by DI viruses appears to be highly specific and effective against homologous or closely related viruses but not against heterologous viruses. With VSV, heat-resistant (HR) DI particles of the Indiana serotype interfere poorly with New Jersey M serotype. However, DI New Jersey M virus interferes equally with M and Ogden virus (24). Since a large number of serotypes of influenza virus are available, we tested the interfering ability of WSN DI (H0N1) against USSR (H1N1), Port Chalmers (H3N2), and Victoria (H3N2) viruses. We determined the interfering titer of WSN DI virus against these viruses by using a direct infectious center reduction assay as described previously (17). The results (Table 1) show that essentially the same titer of interfering activity of WSN DI virus (DIU per milliliter) was obtained against different subtype A viruses. Additionally, in a separate experiment we found that the yield of different subtype viruses was reduced by coinfecting with WSN DI virus (data not shown). These results show that WSN DI virus is equally effective against all influenza A viruses. Preliminary data suggest that WSN DI is not effective against influenza B viruses.

To further determine whether WSN DI can replicate by using the helper function of other influenza A viruses, MDCK cells were coinfected with both DI and infectious viruses and labeled with $^{32}PO_4$ (8). Released virus was isolated and analyzed in polyacrylamide gels. Figure 4 shows that in spite of repeated cloning all three viruses contained visible DI RNA segments. These results confirm our earlier finding that most, if not

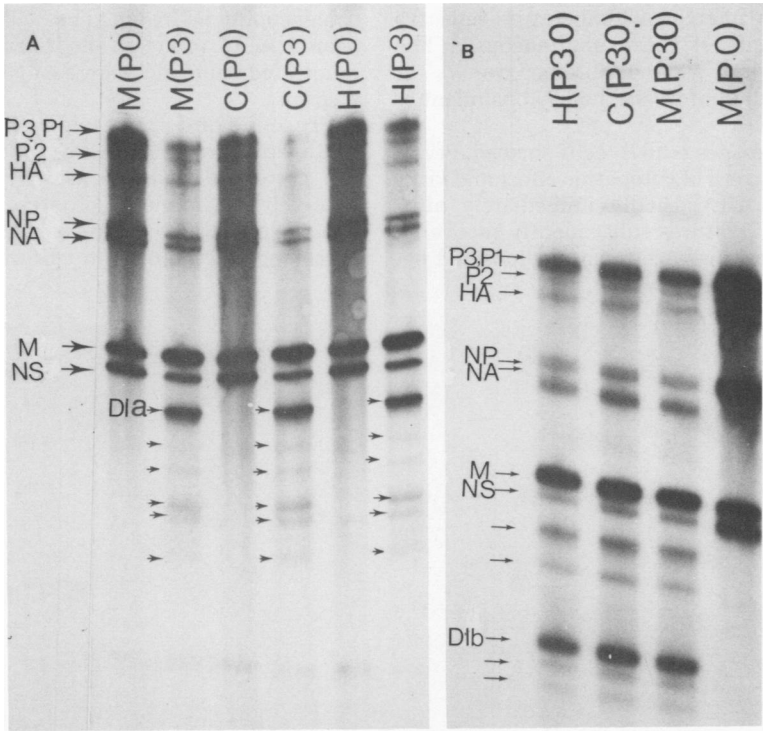

FIG. 2. *Analysis of [^{32}P]RNA of standard virus at p-0 and DI viruses produced at p-3 (A) and p-30 (B) during continued coinfection in MDBK, CEF, and HeLa cells using the same clonal stock virus. Cells were infected with a mixture of standard virus (1 PFU/cell) and DI virus (2 DIU/cell). ^{32}P-labeled RNA was isolated from purified virus and analyzed by polyacrylamide gel electrophoresis for 21 h at 4°C using 180 V (17). Arrows indicate the positions of some of the DI RNA segments. DIa and DIb appear to be the most prominent DI RNAs in all three types of cells at p-3 and p-30, respectively. M(P0), M(P3), M(P30), C(P0), C(P3), C(P30), H(P0), H(P3), H(P30) represent MDBK, CEF, and HeLa cells at p-0, p-3, and p-30, respectively.*

all, wild-type viruses contain DI viruses. Whether these visible DI RNA segments are newly formed during plaque isolation and production of clonal stock or are pre-existing contaminants is being currently investigated. Our gel analyses showed that WSN DI RNA can replicate efficiently in the presence of H1N1 and H3N2 helper viruses and can interfere with the replication of vRNA segments. Analyses of RNA by polyacrylamide gel electrophoresis at two temperatures (room temperature and 4°C) clearly showed that the majority of vRNA segments, as expected, are predominantly of helper virus origin (Fig. 4). A reduction of specific vRNA segments of challenge virus as reported previously (5, 17, 25) was also observed (e.g., band 2 from top is missing in USSR × DI, lane 6, Fig. 4). When the helper virus also contained DI RNA segments, both WSN DI RNA and helper virus DI RNA could replicate in coinfected cells. Similarly, DI virus made from Port Chalmers (H3N2) virus could also interfere with WSN, USSR, and Victoria subtypes, and Port Chalmers DI RNA could replicate with the helper function of these viruses (data not shown). These results suggest that WSN DI virus can interfere with as well as replicate in the presence of other subtypes of influenza A viruses.

Establishment of Pi cultures. Since persistent infection of cultured cells with many lytic viruses has been established, it was of interest to determine whether influenza virus can also establish persistent infection and to analyze the conditions that favor the establishment of persistent infection.

No Pi cultures of MDBK (Pi MDBK) and HeLa cells (Pi HeLa) could be established when cells were infected with either wild-type (ts^+) virus (0.1 to 1 PFU/cell) or temperature-sensitive mutant alone (0.1 to 1 PFU of *ts*52 per cell). Pi cultures could not be established even when cells were coinfected with ts^+ and DI virus. On the other hand, Pi MDBK (MP1, MP2, and MP3) and Pi HeLa cell (HP1 and HP2) cultures could be routinely obtained by infecting cells

with both an infectious temperature-sensitive (*ts*52) virus and DI virus simultaneously. The source of DI virus, whether obtained from ts^+ or ts^- mutants, did not affect the establishment of Pi cultures.

At early passages (<p-7), cells showed a typical cyclical pattern of cytopathic effect and virus production (crisis) usually immediately after subculture. Pi cultures subsequently recovered upon daily washing with fresh medium and became confluent again. These Pi cultures appeared more rounded and refractile than the uninfected cells and were easily dispersed with trypsin.

Presence and expression of viral genome in Pi cultures. Pi cultures, once established, can grow and multiply as well as produce a relatively small amount of virus. There are two likely explanations for these results: (i) a small percentage of the cells in cultures are infected

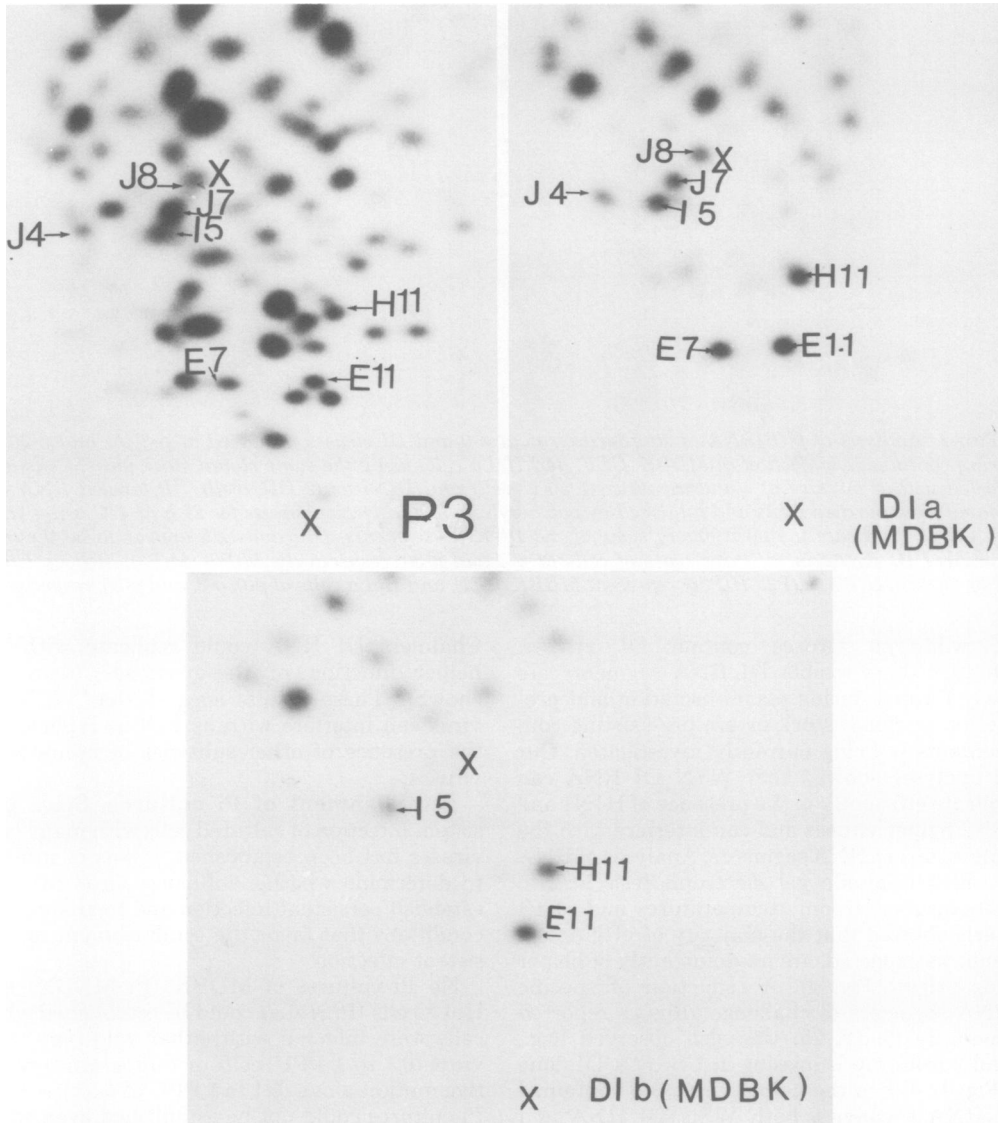

FIG. 3. *Oligonucleotide maps of P3, DIa, and DIb RNAs. Individual [^{32}P]RNA segments were isolated, digested with T_1 ribonuclease, and analyzed by two-dimensional gel electrophoresis (8). Detailed oligonucleotide analyses of individual genes have been reported previously (17, 25a). Only the spots common among P3, DIa, and DIb are identified. ×'s (bottom and top) represent xylene cyanol and bromophenol blue, respectively.*

and are producing virus at a given time or (ii) the majority, if not all, of the cells are infected and contain viral genome, but either some or all of them are producing small amounts of virus. A number of experiments described below support the latter explanation. First, the Pi cultures (MP1, MP2, MP3, and HP1, HP2) were resistant to superinfection by homologous but not heterologous viruses. Both early- (<p-16) and late-passage (>p-30) Pi MDBK and Pi HeLa cells showed little cytopathic effect (<15%) after superinfection with WSN virus. Control MDBK cells, when infected with either *ts*52 or *ts*$^+$ WSN virus, produced 2,048 HAU/ml and 300 × 10^6 PFU/ml, whereas Pi MDBK cells (MP2 and MP3) produced only 32 HAU/ml and 3 × 10^3 to 5 × 10^3 PFU/ml upon superinfection (Table 2). Similar results were obtained with HP1 and HP2 cells as compared to normal HeLa cells. However, when cells were challenged with a heterologous virus (Newcastle disease virus), both normal cells and Pi cells were equally sensitive. Over 80% of cells showed cytopathic effect within 24 h after infection, and high HAU (4,096 to 8,194 HAU/ml in MDBK; 512 to 1,024 HAU/ml in HeLa cells) and infectious virus titers (50 × 10^6 to 70 × 10^6 PFU/ml for MDBK and 3 × 10^5 to 5 × 10^5 PFU/ml for HeLa cells) were obtained in both normal and Pi cultures.

Second, the expression of viral antigens in Pi cells was examined by immunofluorescence and electron microscopy. Accordingly, MP2, MP3, HP1, and HP2 cultures were examined for the expression of viral antigens by indirect immunofluorescence using antisera against WSN virus. Approximately 70 to 80% of the cells showed a positive reaction. Both acetone-fixed (Fig. 5) and live cells (Fig. 6) demonstrated the presence of viral antigen in the cytoplasm and cellular membrane of infected cells. To further confirm the expression of viral genes, thin sections of both acutely infected and Pi cells were studied by transmission electron microscopy. Results confirmed that although no virus was released in the supernatant, budding virus particles were present on the membrane of Pi cells (data not shown). Finally, infectious center assay further demonstrated the presence of the viral genome in Pi cells. Pi MDBK and Pi HeLa cells, when plated on monolayers of uninfected MDBK cells, showed a 70 to 80% plating efficiency as compared to the 100% plating efficiency of acutely infected MDBK and HeLa cells (Table 3). This slightly lower plating efficiency of Pi cells can be due to either DI virus present in some Pi cells

TABLE 1. *Titer of WSN DI virus measured against challenge infectious viruses of different A subtypes*[a]

Challenge virus	DIU/ml
WSN (H0N1)	16.5 × 10^6
Port Chalmers (H3N2)	7.5 × 10^6
USSR (H1N1)	10.5 × 10^6
Victoria (H3N2)	7.8 × 10^6

[a] Titer of WSN DI virus was determined by infectious center reduction assay against different A subtype viruses. WSN DI virus was prepared by high-multiplicity passages as discussed previously (26). MDCK cells were infected with different dilutions of WSN DI virus and superinfected with 4 PFU/cell of challenge viruses of different subtypes. Cells were trypsinized and plated on monolayers of MDCK cells for infectious center assay. Reduction of infectious centers of each challenge virus at different dilutions of WSN DI virus was determined and the number of DIU of WSN DI virus per milliliter was calculated in comparison with each challenge virus as described previously (17). Clonal stocks of infectious WSN (H0N1), Port Chalmers (H3N2), Victoria (H3N2), and USSR (H1N1) virus preparations contained 30 × 10^6 PFU/ml and 1,024 HAU/ml, 15 × 10^6 PFU/ml and 512 HAU/ml, 17 × 10^6 PFU/ml and 1,024 HAU/ml, and 10 × 10^6 PFU/ml and 512 HAU/ml, respectively.

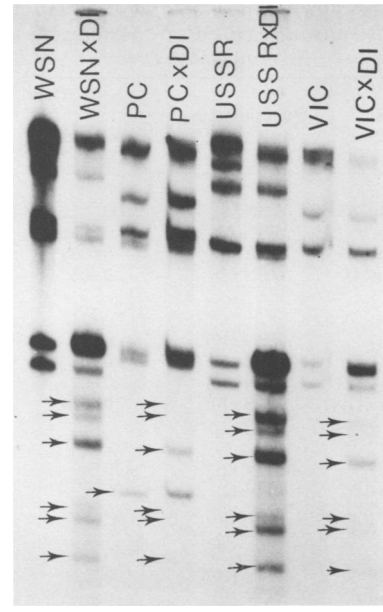

FIG. 4. *Replication of WSN DI RNA with the helper function of other influenza A subtypes: USSR (H1N1), Port Chalmers (H3N2), and Victoria (H3N2). WSN DI was prepared from a six-times-cloned virus by three consecutive passages. Subsequently, cells were either infected with standard virus or coinfected with both standard virus and WSN (p-3) DI virus.* ^{32}*P-labeled viral RNA was isolated and analyzed by polyacrylamide gel electrophoresis as described in Fig. 2. Arrows show a number of prominent DI RNA segments. In spite of repeated cloning, standard Port Chalmers and Victoria viruses also contain DI RNA segments.*

TABLE 2. *Virus production after superinfection of persistently infected cells by homologous and heterologous viruses*[a]

Cells (passage no.)	Superinfecting virus (MOI[b])	Yield	
		PFU/ml ($\times 10^6$)	HAU/ml
MDBK			
Control	WSN (2)	300	2,048
MP3 (p-16)	WSN (2)	0.005	32
MP3 (p-30)	WSN (2)	0.0035	32
MP3 (p-44)	WSN (2)	0.003	32
HeLa			
Control	WSN (10)	4.0	1,024
HP2 (p-14)	WSN (10)	0.003	16
HP2 (p-29)	WSN (10)	0.002	16
HP2 (p-41)	WSN (10)	0.0015	16
MDBK			
Control	NDV (2)	70	8,192
MP3 (p-16)	NDV (2)	50	4,096
MP3 (p-30)	NDV (2)	60	4,096
HeLa			
Control	NDV (10)	0.5	1,024
HP2 (p-14)	NDV (10)	0.4	1,024
HP2 (p-29)	NDV (10)	0.3	512

[a] Normal MDBK and HeLa cells and a number of persistently infected cells at different passages were superinfected with WSN virus or Newcastle disease virus (NDV) for 14 h as described in the text. The supernatants were then harvested and analyzed for HAU as well as PFU (for WSN virus we used MDBK monolayers at 34°C, and for Newcastle disease virus we used CEF at 37°C).

[b] MOI, Multiplicity of infection.

preventing plaque formation (17) or some cells not producing any infectious virus at all. These above experiments suggest that the virus genome is present and expressed (at least partly) in the majority of Pi cells, although complete infectious particles are not produced.

Nature of virus expressed by the Pi cultures. As discussed above, little or no detectable infectious virus was released by the Pi cultures (Table 4), although Pi cells produced infectious centers on monolayers of MDBK cells (Table 3). To determine the nature of the virus released by the established Pi cells, a number of experiments were done. In one, persistently infected cells were trypsinized and plated on monolayers of MDBK cells for infectious center assay at 34°C (permissive) and 39°C (nonpermissive temperature). Results show that little or no wild-type (ts^+) virus was released from Pi cultures (Table 3). This was not surprising since Pi cultures could be established only with ts^- mutants, and we did not detect any wild-type revertants in Pi cultures. In the second type of experiment, the virus released by Pi cells was amplified by cocultivating with monolayers of MDBK cells. Again, virus was released only at 34°C and not at 39°C. Additionally, the viruses released at 34°C were also ts^- mutants and did not form plaques at 39°C. Viruses isolated from Pi cultures formed smaller plaques even at 34°C than did the original clone 4 of *ts*52 virus. Additionally, the clones isolated from Pi cultures produced a lower yield at 34°C.

Role of ts^- mutants in establishing and maintaining persistent cultures. Our data suggest that both DI virus and ts^- mutants are needed in establishing Pi cultures with influenza virus. Two types of experiments were performed to further evaluate the role of *ts* mutants in both establishing and maintaining the persistent cultures. First, MDBK and HeLa cells were infected with the ts^- mutant alone and incubated either at permissive (34°C) and semipermissive (37°C) or at restrictive (39°C) temperatures. We found that over 99% of the cells were destroyed in 3 days either at 34 or 37°C, whereas only 40 to 50% of cells were killed at the restrictive (39°C) temperature, using the ts^- virus alone. However, eventually all cells underwent lysis, and no Pi cultures were obtained at either permissive or restrictive temperatures. In the second type of experiment, Pi cultures (MP2, MP3, HP2, HP3) that were established by coinfection with ts^- mutant and DI virus and maintained at 37°C were shifted to either 34 or 39°C. After shift down, these Pi cultures demonstrated neither crisis nor any increased amounts of infectious virus, as compared to Pi cultures kept at 37°C. Nor was there any observed change when the Pi cultures were incubated at 39°C.

Absence of detectable interferon in Pi cultures. Culture supernatants from Pi MDBK and Pi HeLa cells were used for preparing exogenous interferon and were assayed for interferon by plaque reduction assay in homologous cells with VSV (3). There was no detectable amount of interferon in either Pi MDBK or Pi HeLa cells. Additionally, both of these Pi cultures, although resistant to homologous infection, were sensitive to heterologous infection, namely Newcastle disease virus (Table 2) and VSV (data not shown). These two experiments suggest that neither exogenous nor endogenous interferon was a major factor in the maintenance of persistent influenza virus infection of HeLa and MDBK cells.

Role of DI virus in the maintenance of Pi cultures. Since DI particles have been implicated in persistent infection, a number of experiments were done to determine whether DI viruses were present in persistently infected cultures. Accordingly, the released virus was amplified and labeled by plating Pi cells on the monolayer of MDBK cells and incubating in the presence of $^{32}PO_4$ medium. [^{32}P]vRNA was analyzed in polyacrylamide agarose gels (26). The

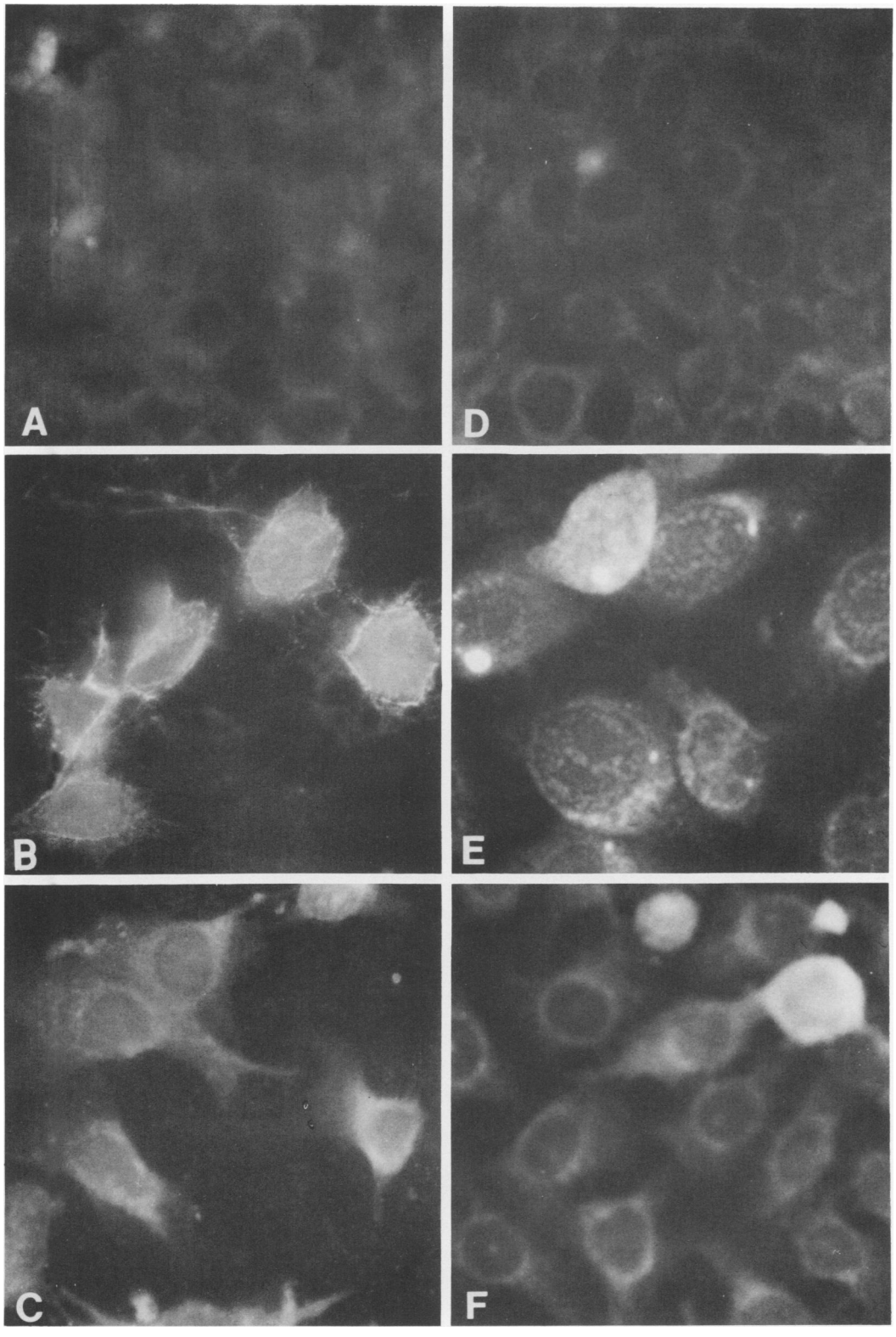

FIG. 5. *Presence of viral antigen in persistently infected MDBK (MP3) and HeLa (HP2) after acetone fixation. Cultures (24 h old) grown on cover slips were fixed in acetone and examined for viral antigens by indirect immunofluorescence as described in the text. (A) Uninfected MDBK; (B) acutely infected MDBK; (C) persistently infected MDBK, MP3; (D) uninfected HeLa; (E) acutely infected HeLa; (F) persistently infected HeLa, HP2.*

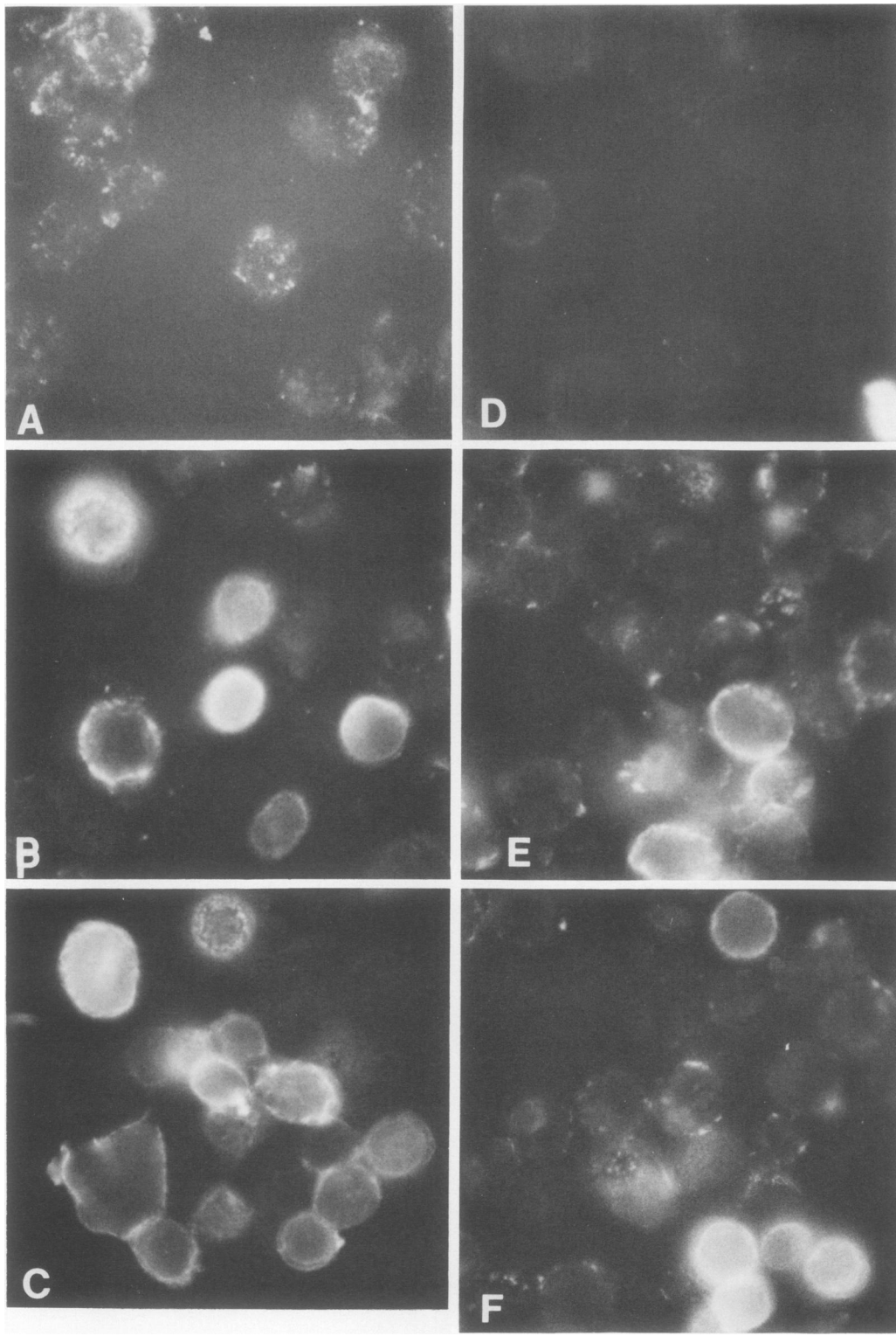

FIG. 6. *The presence of viral antigen on the membranes of persistently infected cells. Live cells were examined for the presence of viral antigen by indirect immunofluorescence as described in the text. (A) Uninfected MDBK; (B) acutely infected MDBK; (C) persistently infected MDBK, MP3; (D) uninfected HeLa; (E) acutely infected HeLa; (F) persistently infected HeLa, HP2.*

TABLE 3. *Infectious center assay of Pi cells*[a]

Cells (passage no.)	No. of infective centers per 100 cells	
	34°C	39°C
MDBK × *ts*52[b]	100	1
MP1 (p-16)	70	0
MP2 (p-17)	75	0
MP3 (p-17)	71	0
MP1 (p-30)	55	0
MP2 (p-31)	50	0
MP3 (p-35)	60	0
HeLa × *ts*52[b]	70	0
HP1 (p-14)	90	0
HP2 (p-16)	80	0
HP1 (p-41)	78	0
HP2 (p-43)	70	0

[a] Results were calculated from duplicate assays using 400, 200, and 100 trypsinized cells. Pi cells of different passages were trypsinized, plated on fresh MDBK monolayers (5-cm plates) in different numbers (400, 200, and 100 cells per plate), and incubated for 1 h at 37°C after the addition of 5 ml of agar overlay. These plates were then incubated at 34 and 39°C, and plaques were counted on days 3 and 4.

[b] Control MDBK and HeLa cells were infected with infectious *ts*52 virus (4 PFU/cell) for 1 h at 37°C, trypsinized, and assayed by the same procedure.

results show that only standard vRNA segments could be detected, with no appreciable amount of specific DI RNA segment in the released virus. This was surprising because DI virus was used in establishing the persistent infection. The lack of DI RNA cannot be due to the inability of DI virus to replicate in either of these cells, since DI viruses can be produced and amplified in both of these cells.

To further analyze the presence of DI virus in Pi cultures, culture supernatant obtained after amplification was analyzed for interference by determining the reduction of virus yield and the reduction of infectious center after coinfection with infectious virus (17). Although the virus released from Pi cultures had a lower PFU/HAU ratio, it did not possess any interfering activity against homologous virus either in the yield reduction (Table 4) or in the infectious center reduction assay (data not shown). This would suggest that the virus released by Pi cultures was defective but noninterfering. We are in the process of further characterization of this virus.

DISCUSSION

Effects of host on DI virus production. The production and amplification of DI virus depend partly on the parental infectious virus and partly on the host cells (14, 19). WSN virus replicates most efficiently in MDBK cells and least efficiently in HeLa cells (5). Experiments reported here show that both infectious and DI viruses are produced most in MDBK cells and least in HeLa cells. CEF cells produce an intermediate amount of both infectious and DI viruses. Such a result would suggest that the steps required in the replication for influenza DI virus are essentially the same as those needed for the replication of infectious virus.

The nature of DI RNA(s) generated in all three species of cells from a single clonal stock appears identical by polyacrylamide gel electrophoresis and oligonucleotide spot analysis. The results, although apparently different, are not totally inconsistent with those observed for VSV (14, 19). The host factor(s) influencing the nature of VSV DI virus does not appear to be absolutely selective because (i) some of the DI viruses produced in different species of cells appear identical and (ii) any predominant DI virus, irrespective of its source of origin, can replicate in a given cell. Our results suggest that the nature of influenza DI virus is primarily determined by those that are already present in the clonal stock and that MDBK, CEF, and HeLa cells do not exert a great selective pressure in the amplification of influenza DI virus.

DI virus-mediated interference among different subtypes of influenza viruses. DI virus prepared from WSN virus (H0N1) can effectively interfere with the replication of other influenza A viruses, namely H1N1 and H3N2 viruses. Equally, WSN DI RNAs can replicate

TABLE 4. *Analysis of virus particles produced by Pi cells*

Expt no.	HAU/ml	PFU/ml	PFU/HAU ratio
1. MP3 (p-20) supernatant[a]	<2	0	
2. HP2 (p-21) supernatant[a]	<2	0	
3. MP3 (p-20) trypsinized cells[b]	256	20×10^4	0.78×10^3
4. HP2 (p-21) trypsinized cells[b]	512	70×10^4	1.3×10^3
5. MP3 supernatant (expt 3) × *ts*52[c]	512	15×10^6	3.0×10^4
6. HP2 supernatant (expt 4) × *ts*52[c]	512	30×10^6	6×10^4
7. *ts*52 alone[d]	1,024	90×10^6	8.8×10^4

[a] Supernatants of MP3 (p-20) and HP2 (p-21) were analyzed for HAU and PFU at 34°C.

[b] MP3 and HP2 cells were trypsinized, and 5×10^6 cells were added to the monolayer of fresh MDBK cells and incubated at 34°C for 48 h. Supernatants were harvested and analyzed for HAU and PFU at 34°C.

[c] Monolayers of MDBK cells (150 cm^2) were coinfected with 5 ml of the supernatant (experiments 3 and 4) and infectious *ts*52 virus (1 PFU/cell). The virus yield was harvested at 48 h and 34°C and assayed for HAU and PFU.

[d] MDBK cells were infected with *ts*52 virus alone (1 PFU/cell) for 48 h at 34°C.

in the presence of infectious virus of other subtypes. These results suggest that the gene products required for the replication of DI RNAs, as well as those for the interference of vRNA's, can function equally well among the viral subtypes. These results are not surprising since genetic reassortment experiments among different subtypes suggest that the genes among different influenza A subtypes can be exchanged freely and that gene products of one subtype are functional with other subtypes. Our results extend the idea of functional genetic exchange among the viral subtypes to the DI virus-mediated interference.

Establishment and maintenance of persistent influenza infection. The results reported here show that influenza viruses under certain conditions can establish persistent infection of MDBK and HeLa cells in culture. The presence of both DI virus and ts^- mutants appears to be critical in establishing the initial condition favorable for persistent infection. As yet we have been unable to obtain Pi cultures by using either ts^+ virus alone, ts^- virus alone, or ts^+ virus with DI virus. A similar initial requirement of both DI virus and ts^- mutant has been reported for VSV and BHK-21 cells (13), whereas DI virus in the presence of ts^+ virus has been shown to establish persistent infection of a number of other cell-virus systems (23, 30, 38). Whether different ratios of DI virus to ts^+ virus or other conditions such as interferon treatment could help in establishing persistent influenza infection in cell cultures is being currently investigated.

Persistent influenza infection in MDBK and HeLa cells appears not to be maintained at the population level (i.e., a small fraction of cells being infected at a given time) since the majority of cells contain and express viral antigen(s). Nor does interferon appear to play a significant role in the maintenance of persistent infection of influenza virus in these cells. Our results suggest that the selection of virus variants with greatly reduced growth capacity at 37°C without killing host cells is probably required for maintaining persistent influenza infection. Repeated crises observed in the early passages would assist in the selection of a virus population with a growth behavior compatible to cellular growth. The virus population obtained from stable Pi cultures produced small plaques and a reduced yield at 34°C compared to the original clone 4 of *ts*52. Whether these virus clones isolated from Pi cultures would be less cytolytic and establish Pi infection more easily, and probably without the help of DI viruses, is being studied currently.

Although DI virus appears to be critical in the initial phase of establishing persistent infection, we were unable to detect either the DI virus or the DI RNA in released virus from Pi cultures. During the initial phase of infection, DI virus is needed to suppress the cytopathic effect of infectious virus and to help the survival of host cells during crisis. In addition, DI viruses may accelerate the selection of an appropriate variant of infectious virus (12, 35). However, once the variant is selected and a stable cell-virus association is established, DI virus may not be needed and thus may be eliminated from Pi cultures. The presence of viral antigens and defective budding particles on the cell membrane would block the receptors for superinfecting homologous infectious virus but not for heterologous virus.

In nature as well as in cell culture there appears to be a continuous evolution of influenza viruses (40). DI viruses, which appear to occur commonly in influenza virus replication (17), may further aid in the selection of variants and thus help in the evolution of the virus and the creation of diversity among the virus population.

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