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# Intrinsic Interference Between Swine Influenza and Fowl Plague Virus

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With 2 Figures

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## Summary

Multiplication of swine influenza (SW) virus is inhibited by fowl plague virus (FPV) at the level of RNA synthesis when host cells are infected with both viruses at a high multiplicity of infection. Under these conditions reassortment between the two viruses cannot be detected. The inhibitory effect of FPV is highly reduced and recombinants between the two viruses could be obtained when the cells were superinfected with FPV 1—2 hours after infection with SW virus, or after simultaneous infection with a low multiplicity of infection. The phenomenon is compatible with the intrinsic interference.

## Introduction

Mammalian and avian influenza viruses can interact genetically at high frequency following mixed infection *in vitro* and *in vivo* [for review see Webster (15)]. The molecular basis of this interaction lies in the fact that the genome of influenza viruses is segmented, allowing reassortment of the individual genes to form "new" viruses [for review see Scholtissek (7)]. This implies that under appropriate conditions human pandemic viruses could arise by a similar process as suggested by Rasmussen (5) and Kilbourne (2).

A precondition for such a genetic interaction would be that influenza viruses of different origin must be capable of replicating in one and the same cell after double infection. In this paper we present evidence that this is not always the case. After simultaneous infection of cells with swine (SW) influenza and fowl plague virus (FPV) the multiplication of SW-virus is inhibited at the level of transcription. This phenomenon is in agreement with the intrinsic interference — an interferon-independent interference — as defined by Marcus and Carver (4) for NDV and other animal viruses (12, 6).

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## Materials and Methods

### *Virus Strains*

The following influenza A virus strains were used: PR8, A/PR/8/34 (H0N1); FM1, A/FM/1/47 (H1N1); Singapore, A/Singapore/1/57 (H2N2); FPV, A/FPV/Rostock/34 (Hav1N1); Dutch, A/FPV/Dutch/27 (Hav1Neq1); virus N, A/chicken/Germany/N/49 (Hav2Neq1); Equine 2, A/equine/Miami/1/63 (Heq2Neq2); SW, A/swine/1976/31 (Hsw1N1); SW/Wisc., A/swine/Wisconsin/1/67 (Hsw1N1); Duck/Alb., A/duck/Alberta/35/76 (Hsw1N1), and recombinant viruses containing the haemagglutinin of FPV and the neuraminidase of SW (FPV/SW) or the haemagglutinin of SW and the neuraminidase of FPV (SW/FPV). SW/FPV possesses the RNA segments 1, 3, 5, and 6 from FPV and the other RNA segments from SW-virus (SCHOLTISSEK, unpublished data). Furthermore, the following temperature-sensitive (*ts*) mutants of FPV were included: *ts* 3 with a *ts*-lesion in the RNA segment 1 (polymerase 1 gene) and *ts* 19 with a *ts*-lesion in the RNA segment 5 (nucleoprotein gene) (8, 11).

### *Tissue Culture and Virus Multiplication*

Primary chick embryo (CE) cells at a density of $5 \times 10^6$ in Petri dishes of 5 cm diameter were infected 24 hours after seeding. In some experiments MDCK cells were used. The cells were infected with an input multiplicity of 10 to 50 PFU/cell of each virus and overlayed with MEM with or without α-FPV haemagglutinin antiserum. The antibody content of the serum used was such that under the experimental conditions haemagglutination (HA) of the virus that carries the homologous haemagglutinin was completely inhibited. At the times after the infection, as indicated, virus yields were determined by the HA-test in medium plus cells after treatment by 3 cycles of freezing and thawing.

### *Production of Antigenic Hybrids*

Antigenic hybrids of SW virus and FPV were obtained by isolating recombinants after double infection of CE cells as described in the "Results". CE cells were kept after infection in MEM containing 5 μg/ml of trypsin. Mixed virus yields were diluted and plated on CE cells for plaque isolation. The agar overlay contained 10 μg/ml of trypsin (3). After 5 serial plaque passages the isolates were tested for their antigenic properties.

### *Biological Tests*

HA titration and HA inhibition tests were done according to standard procedures (1). Neuraminidase and neuraminidase inhibition assays using fetuin as a substrate were carried out as previously described (13). Plaque test was done as described (3) and the plaque number expressed as PFU/ml.

### *Antisera*

For most of the experiments an antiserum was used which was obtained by immunizing chickens with a recombinant virus containing the HA of FPV and the neuraminidase of virus N. In experiments with virus N an antiserum from chicken was used possessing α-FPV HA and α-Singapore (N2) neuraminidase. SW virus antiserum was used for inhibition of SW virus haemagglutinin.

### *Hybridization with [$^{32}$P]-Labeled Haemagglutinin Gene of Swine Influenza Virus*

SW virus was labeled *in vivo* with [$^{32}$P] as described (11). The vRNA was isolated from the purified virus, and the vRNA segments were separated by polyacrylamide gel electrophoresis in the presence of 6 M urea. The haemagglutinin gene (segment 4) was isolated and used for hybridization. The non-labeled cRNA was isolated 5 hours after single or double infection from the microsomal fraction of CE cells. The hybridization technique has been described elsewhere (11, 9). Aliquots of 10 μl [$^{32}$P]-vRNA were mixed with increasing amounts of non-labeled cRNA in a total volume of 110 μl 2 × SSC, heated overnight at 65° C, and digested at 20° C with 0.1 mg RNase for 20 minutes. The acid-precipitable radioactivity was recorded.

## Results

*Multiplication of Various Influenza A Viruses in Cells Doubly Infected with FPV*

Table 1 shows the results of double-infection of several influenza A viruses with FPV. It can be seen that FPV interferes to a certain extent after simultaneous infection of CE cells with all influenza viruses tested. However, interference is most distinct with viruses carrying the haemagglutinin of SW virus. This became evident by using a recombinant virus possessing the HA of SW and the neuraminidase of FPV. The recombinant is inhibited after simultaneous infection with fowl plague virus to the same extent as the wild type SW virus. SW virus was also inhibited by Dutch virus as well as by a recombinant virus carrying the haemagglutinin of FPV and the neuraminidase of SW virus (data not shown). In the following the inhibition of SW virus by FPV was studied in more detail.

*Table 1*

| Virus used for mixed infection with FPV | HA units of viruses added | |
|---|---|---|
| | 0 | 2 |
| $A_0$/PR 8 | 256<br>(1024) | 1024<br>(1024) |
| $A_1$/FM 1 | 256<br>(512) | 512<br>(512) |
| $A_2$/Singapore | 32<br>(64) | 64<br>(64) |
| Equine 2 | 64<br>(256) | 128<br>(256) |
| Virus N | 64<br>(256) | 128<br>(256) |
| SW | 2<br>(64) | 48<br>(64) |
| SW/Wisconsin | $<2$<br>(64) | 32<br>(64) |
| Duck/Alberta | 4<br>(128) | 64<br>(64) |
| SW/FPV | 2<br>(64) | 32<br>(64) |

CE cells were infected with the viruses listed either simultaneously (0) or 2 hours before infection with FPV (2) and overlayed with medium containing FPV-haemagglutinin antiserum. 10 hours after infection virus yield was determined by the HA-test in the presence of FPV-haemagglutinin antiserum. Controls in parentheses: HA units of the respective virus grown in the absence of FPV

If MDCK cells were used instead of CE cells, FPV also inhibited SW virus multiplication after simultaneous infection (data not shown). Thus the effect was not restricted to chicken cells.

When cells infected with SW virus were superinfected 2 hours later with FPV, no significant differences could be seen between singly and double infected cells concerning the yield of SW virus. The same was the case when another influenza

A virus was used for the first infection (Table 1). As shown in Fig. 1 FPV blocked the synthesis of SW virus only when added simultaneously with SW virus. The yield of SW virus haemagglutinin increased as the time period between SW virus infection and superinfection with the interfering FPV was extended until a minimum inhibitory effect was reached at 2 hours.

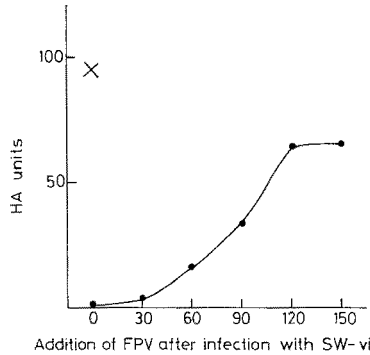


Fig. 1. Formation of SW virus haemagglutinin in the presence of FPV. FPV was added to SW virus-infected cells at the time indicated. After superinfection with FPV the cells were overlayed with medium containing FPV-HA antiserum. Assays were made 12 hours after infection. As control ( × ) the HA units without any addition of FPV were determined

*Antigenic Hybrids Between FPV and SW Virus*

More than 200 individual plaques obtained after simultaneous infection of CE cells with FPV and SW virus were cloned serially with 5 plaque passages and were tested for the composition of their surface antigens. The progeny of all these plaques possessed the viral glycoproteins of FPV and neither SW virus nor antigenic hybrids were obtained, although trypsin was present in the agar overlay (3). When, on the other hand, CE cells were infected with SW virus 2 hours prior to superinfection with FPV, antigenic hybrids were produced at a frequency of about 15 per cent with the haemagglutinin or the neuraminidase of FPV and the other corresponding antigen from SW virus. Similarly, when CE cells were infected with a mixture, both viruses with a total multiplicity of infection of 0.003, and the virus progeny was plaqued 40 hours later, antigenic hybrids of both kinds could be isolated. Under these conditions both viruses were synthesized as shown in Table 2.

*Requirement of Active FPV for the Inhibition of SW Virus*

FPV inactivated with UV light to a survival level of $5 \times 10^3$ PFU/ml was compared with active virus ($3 \times 10^8$ PFU/ml) in its capacity to prevent SW virus multiplication. The results presented in Table 3 show that infectivity of FPV is essential to inhibit the growth of SW virus.

When CE cells were infected with a mixture of SW virus and the *ts* 3 mutant of FPV, the latter with a *ts*-defect affecting RNA replication (8), SW virus growth was not affected at the non-permissive temperature (41° C). On the other hand, *ts* 19, with a *ts*-defect in virus maturation, inhibited SW virus multiplication (Table 4).

Table 2. *Growth of SW virus after mixed infection of CE cells with FPV at low multiplicity*

| Virus used for infection | HA units determined in the presence of antiserum against | | |
|---|---|---|---|
| | FPV | SW | Without antiserum |
| FPV | <2 | 32 | 64 |
| SW | 16 | <2 | 16 |
| FPV+SW | 16 | 16 | 64 |

CE cells were mixedly infected with FPV and SW virus with a multiplicity of 0.003 and incubated in the presence of 10 μg trypsin pro ml culture medium for 24 hours. The HA-test was done in the presence of FPV- or SW virus-antiserum

Table 3. *Simultaneous infection of SW virus with UV-light-inactivated FPV*

| | FPV antiserum added | HA units |
|---|---|---|
| SW | — | 128 |
| | + | 256 |
| FPV | — | 128 |
| | + | <2 |
| FPV (UV) | — | <2 |
| | + | <2 |
| SW+FPV | — | 128 |
| | + | <2 |
| SW+FPV (UV) | — | 128 |
| | + | 256 |

CE cells were infected with SW virus and UV-light-inactivated FPV simultaneously and incubated for 10 hours in the absence (—) or presence (+) of FPV-haemagglutinin antiserum

Table 4. *Growth of SW virus in the presence of* ts-*mutants of FPV*

| | FPV-antiserum added | HA units after infection at | |
|---|---|---|---|
| | | 33° C | 41° C |
| SW | — | 64 | 32 |
| | + | 64 | 32 |
| *ts* 3 | — | 64 | <2 |
| | + | <2 | <2 |
| SW+*ts* 3 (0) | — | 128 | 16 |
| | + | <2 | 16 |
| SW+*ts* 3 (2) | — | 128 | 16 |
| | + | 32 | 16 |
| *ts* 19 | — | 64 | 16 |
| | + | <2 | <2 |
| SW+*ts* 19 (0) | — | 64 | 16 |
| | + | <2 | <2 |
| SW+*ts* 19 (2) | — | 64 | 16 |
| | + | 32 | 16 |

CE cells were infected with SW virus simultaneously (0) with or 2 hours (2) before addition of *ts* 3 or *ts* 19 of FPV and incubated for 16 hours at 33° or 41° C in the absence (—) or presence (+) of FPV-haemagglutinin antiserum

*Synthesis of Complementary RNA (cRNA) in Singly and Doubly Infected Cells*

After hybridization of [$^{32}$P]-labeled RNA segment 4 (haemagglutinin gene) of swine influenza virus with a surplus of non-labeled cRNA of FPV the RNase protection is only 30 per cent. Therefore, it is possible to estimate the amount of SW virus cRNA in doubly infected cells by saturation hybridization. For this purpose $10^9$ chick embryo cells were either infected concomitantly with SW virus and FPV, or preinfected with SW virus and 2 hours later superinfected with FPV, or they were infected singly with either SW virus or FPV. The cRNA isolated 5 hours later from the microsomes was dissolved in 1 ml 2×SSC, each. Increasing amounts were used for hybridization with aliquots of [$^{32}$P]-labeled vRNA segment 4 of the SW virus. As can be seen in Fig. 2, about 10 times more

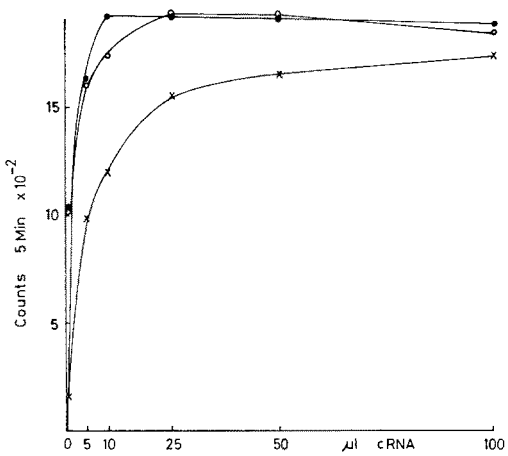


Fig. 2. Saturation hybridization of [$^{32}$P]-labeled segment 4 of the swine influenza virus with cRNA of singly or doubly infected cells. Increasing amounts of cRNA isolated 5 hours after infection, either singly with swine influenza virus, or with swine virus and FPV concomitantly or with swine virus and superinfected 2 hours later with FPV, were hybridized with 10 µl aliquots of [$^{32}$P]-labeled segment 4 of swine influenza virus in a total volume of 110 µl 2×SSC. The RNase-protected radioactivity is recorded in dependence of the amount of cRNA used. The values obtained with cRNA of FPV were substracted. ⊙ cells were infected with swine virus alone; o swine virus-infected cells were superinfected with FPV 2 hours later; × cells were infected concomitantly with swine virus plus FPV

cRNA of doubly infected cells are needed than of cells singly infected with SW virus in order to obtain the same degree of RNase protection. There is no significant difference between the cRNA of cells either singly infected with the SW virus or superinfected 2 hours later with FPV. This indicates that in doubly infected cells synthesis of cRNA of the SW virus is highly suppressed, while there is no inhibition when the SW virus-infected cells were superinfected with FPV 2 hours later.

## Discussion

It has been shown that after simultaneous infection of chicken embryo cells with FPV and SW virus only FPV multiplies to normal yields while the production of SW virus is highly suppressed. The suppression of SW virus was found to depend upon the integrity of the genome of the interfering virus, since UV light-inactivated FPV had no inhibitory effect. Furthermore, when cells were doubly infected with SW virus and *ts*-mutants of FPV it became evident that only those mutants with a late defect (*ts* 19) were able to interfere, but not those with an early defect (*ts* 3). These data demonstrate that interference does not become effective at the level of adsorption and penetration, but at a step in virus multiplication immediately thereafter, inhibiting SW virus RNA synthesis.

If cells were infected with SW virus and superinfected with FPV later than one hour thereafter, the suppressing effect of FPV is greatly diminished. Because of these considerations the phenomenon described is in agreement with intrinsic interference and it demonstrates that it does not only occur between viruses of different groups, but also within one and the same virus group.

Intrinsic interference was found to be strongest with all virus strains carrying the haemagglutinin Hsw1 and was negligible with all other influenza A virus subtypes tested. Even a virus recombinant possessing RNA segments 1, 3, 5, and 6 of FPV and the others of the SW-virus was suppressed in its multiplication by FPV. Further studies with recombinants with different gene constellations should settle the question of which genes are involved in the phenomenon and whether the HA-gene alone is important in this context.

As a consequence of intrinsic interference, no recombinants could be isolated from cells infected simultaneously with high multiplicities of FPV and SW virus. This observation does not contradict the results obtained by WEBSTER *et al.* (14), who were able to isolate recombinants from swine doubly infected with these two viruses. It might be assumed that *in vivo* most permissive cells are not infected at the same time with both viruses. These *in vivo* conditions have been mimicked experimentally by infecting cultured cells with both viruses at low multiplicity. Here both viruses have been synthesized to normal yields and recombinants were obtained as well. These results demonstrate that the failure to obtain recombinants between FPV and SW virus after simultaneous infection with high multiplicity is not due to incompatibility of such newly reassorted viruses to grow on the host cells used, as found with certain recombinants between FPV and the influenza virus strain Hong Kong (H3N2) (10).

One may conclude from the results presented that intrinsic interference might indirectly affect reassortment of the influenza virus genome *in vitro* and the isolation of respective recombinants by inhibiting the RNA synthesis of the SW virus. However, it is highly improbable that intrinsic interference has any significance in the formation of influenza virus recombinants under natural conditions.

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