Inhibition of Influenza Virus Replication in Tissue Culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): Mechanism of Action

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SUMMARY

The neuraminidase inhibitor 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA) inhibits the multicycle replication of influenza viruses in tissue culture. Influenza virus grown in the presence of FANA contains neuraminic acid on its envelope which then serves as receptor for other virus particles causing extensive aggregation. Thus, FANA inhibits influenza virus replication by preventing the enzymatic removal of neuraminic acid from the virus envelope.

In a previous communication it was shown that the neuraminidase inhibitor 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA), an analogue of N-acetylneuraminic acid, reduces the yield of influenza and para-influenza viruses in multicycle replication (Palese et al. 1974a). Susceptibility to the effect of FANA was found to vary among different strains of influenza A viruses. Influenza A/WSN (H0N1) virus and recombinant viruses derived from this strain which contain the N1 neuraminidase showed plaque size reduction in tissue culture cells at a FANA concentration of approx. $10^{-6}$ M. On the other hand all viruses containing the N2 neuraminidase and various haemagglutinins required a 50 to 100 times higher concentration of FANA for plaque size reduction (Schulman & Palese, 1975). These results, and the observation that FANA does not inhibit the replication of other enveloped viruses which lack neuraminidase activity, such as measles and vesicular stomatitis virus, suggested to us that the antivirus effect of FANA is specifically mediated through its neuraminidase inhibiting activity (Kilbourne, Palese & Schulman, 1974; Palese et al. 1974a).

In our previous studies of temperature-sensitive influenza virus mutants with a defect in neuraminidase, we demonstrated that virions containing neuraminic acid residues were produced. These residues apparently serve as receptors for the virus haemagglutinin, resulting in the production of large aggregates, which are disaggregated by neuraminidase treatment. We have therefore proposed that the virus neuraminidase functions to produce particles which lack neuraminic acid, thereby avoiding aggregation, and that this function is essential for normal yields of progeny virions (Palese et al. 1974b). The present report describes studies designed to determine the precise mechanism by which the small mol. wt. neuraminidase inhibitor FANA inhibits influenza virus replication and also provides independent evidence demonstrating that neuraminidase functions to free influenza virions from neuraminic acid. The use of a chemical inhibitor of neuraminidase avoids possible difficulties occurring with temperature sensitive (ts) mutants, which may possess genetic defects, other than the defect in neuraminidase, which were not detected in our previous studies.

The synthetic neuraminidase inhibitor FANA (Meindl et al. 1974), first synthesized by Meindl & Tuppy (1973), and the influenza A/WSN virus and its mutant ts3, which possesses only 10 to 20% of the neuraminidase activity of the wild type (Sugiura, Tobita & Kilbourne 1972; Palese et al. 1974b) were used in the present studies. Fig. 1 shows the effect of FANA at a concentration of $6 \times 10^{-4}$ M on the growth of influenza WSN ts3 virus in MDBK
Fig. 1. MDBK cells were infected with influenza WSN ts3 virus at a multiplicity of 5 to 10 (Sugiura et al. 1972; Palese et al. 1974b). Maintenance medium was added 45 min p.i. at 33 °C and cultures were kept at the permissive temperature (33 °C). Half of the samples contained 6 × 10⁻⁴ M-FANA (●), the other half contained only maintenance medium (○). At the indicated times cultures were removed, cells were scraped off and maintenance medium cells and together were frozen and thawed twice. Half of each sample was treated with 200 units of *Vibrio cholerae* neuraminidase (Behringwerke, Marburg, Germany) for 15 min at 37 °C (solid lines). For each sample the haemagglutination (HA) and infectivity (p.f.u.) titre was determined (Sugiura et al. 1972; Palese et al. 1974b). *ts3* minus FANA: ○—○, without neuraminidase; ○—○, with neuraminidase. *ts3* plus FANA: ●—●, without neuraminidase; ●—●, with neuraminidase.

(bovine kidney) cells. At 12 h post infection (p.i.) there is a 64-fold reduction in the haemagglutination (HA) titre when virus is grown in the presence of 6 × 10⁻⁴ M-FANA, as compared with untreated controls (Fig. 1, broken lines).

It is known that infectious influenza and para-influenza viruses lack neuraminic acid on their envelope, presumably because of the virus neuraminidase (Klenk & Choppin, 1970; Klenk, Compans & Choppin, 1970). From earlier experiments we obtained evidence that virus neuraminidase activity is absent in cells infected with the *ts3* mutant at non-permissive temperature, and that particles found at non-permissive temperature contained neuraminic acid on the envelope. These residues served as receptors and led to extensive aggregation (Palese et al. 1974b). It was therefore likely that FANA inhibits virus replication by a similar mechanism, blocking the enzymatic removal of neuraminic acid from the virus envelope and producing sialylated particles. The neuraminic acid would then provide receptor sites for the haemagglutinin of other virus particles producing aggregation and this
would be expressed by a reduction in HA and infectivity titre. Support for this mechanism was obtained by the following experiment. After neuraminidase treatment of virus grown in the presence of FANA, the HA titre of this preparation increases significantly, indicating that virus particles were formed in the presence of FANA but were inactive in an HA test (Fig. 1, solid line). Most likely, neuraminic acid is removed from the virus surface by the neuraminidase treatment and the virus particles disaggregate, thereby markedly increasing the HA titre. It should be noted that FANA at a concentration of \(10^{-8}\) M has no effect on the haemagglutination titre of WSN virus when added during the haemagglutination test. This was also shown for other influenza A and B viruses (Meindl et al. 1974) and suggests that FANA does not possess a direct effect on the haemagglutinin.

The lower panel of Fig. 1 shows the infectivity titres (p.f.u.) of virus grown in the presence or absence of FANA. At 12 h p.i. there is a 100-fold reduction of infectivity in the sample grown in the presence of FANA compared with the control; treatment with bacterial neuraminidase causes a fourfold increase in infectivity. However, as discussed above, the increase in the haemagglutinin titre was significantly greater. The neuraminic acid-containing aggregated virus may possess a disproportionately higher infectivity because of the contribution of complementing aggregated non-infectious particles (Hirst & Pons, 1973). Therefore, bacterial neuraminidase treatment may cause a greater increase in haemagglutination than in infectivity titre.

Further evidence that influenza virions grown in the presence of a neuraminidase inhibitor contain neuraminic acid and form aggregates was obtained by electron microscopy. Fig. 2(a) shows wild type influenza WSN virus-infected MDBK cells incubated in the presence of \(5 \times 10^{-3}\) M-FANA. Virus particles are predominantly found as aggregates near the cell surface which are rarely seen when virus is grown without FANA. The morphology of virus particles formed in the presence of FANA is indistinguishable from that of control particles; the only difference seems to be the formation of aggregates by virus particles grown in the presence of FANA. More striking aggregates were found on cells infected with WSN ts3 virus and incubated in the presence of a lower concentration of \(6 \times 10^{-4}\) M-FANA (Fig. 2b). It should be noted that the concentration of FANA required to inhibit the ts3 mutant is about eightfold lower than that needed to inhibit wild type virus, which possesses a greater neuraminidase activity.

To determine whether virions present in such aggregates contain neuraminic acid residues, colloidal iron hydroxide staining was employed. Colloidal iron hydroxide binds to neuraminic acid in biological membranes, and appears as dense granules in electron micrographs (Klenk et al. 1970). A region of the surface of an MDBK cell infected with WSN ts3 virus in the presence of \(6 \times 10^{-4}\) M-FANA is shown in Fig. 2(c). Neuraminic acid is present on the envelope of the virions, as demonstrated by the electron dense granular material. In control experiments (Fig. 2d) virions are unstained, although iron granules are present on adjacent areas of cell membranes as described previously for cells infected with influenza virus (Klenk et al. 1970; Palese et al. 1974b) or a ts virus mutant defective in neuraminidase grown at permissive temperature (Palese et al. 1974b). Electron micrographic examination of cells infected in the presence of \(6 \times 10^{-4}\) M-FANA and treated with neuraminidase (12 h p.i.) revealed that all virus aggregates had been removed from the cell surface by the enzyme treatment (not shown here).

The available evidence suggests that FANA inhibits virus replication by inhibiting neuraminidase activity. The present results indicate that the neuraminic acid on virus particles grown in the presence of FANA results in the formation of aggregates. It was pointed out by Schulze (1975) that in vitro sialylation of influenza virus particles did not
Fig. 2. Influenza virions at the surface of infected MDBK cells. (a) Cells 12 h p.i. with WSN virions in the presence of $5 \times 10^{-3}$ M-FANA at 33 °C, showing virions in clusters and chains. Magnification $\times 50000$. (b) Cells infected with the $ts3$ mutant of influenza WSN virus at 33 °C and incubated in the presence of $6 \times 10^{-4}$ M-FANA for 12 h, with virions in aggregates covering a large region of the cell surface. Magnification $\times 40000$. (c) Colloidal iron hydroxide staining of a cluster of $ts3$ virions grown in the presence of $6 \times 10^{-4}$ M-FANA. Magnification $\times 55000$. (d) Colloidal iron hydroxide staining of the surface of a cell infected with WSN virus in the absence of FANA. The cell surface is stained, but budding virions (arrowed) are essentially unstained. Similar results were previously found for cells infected with $ts3$ and $ts11$ at the permissive temperature (Palese et al., 1974b). Magnification $\times 60000$. 

decrease but rather enhanced the infectivity. It should be noted, however, that it is difficult to compare \textit{in vitro} sialylation of mature virus particles with processes taking place in the cell when virus is grown in the presence of FANA. Sialylation of the particles might differ under these circumstances.

The experiments presented here, employing the potent neuraminidase inhibitor FANA, suggest that one of the functions of influenza virus neuraminidase is to produce virus particles which lack neuraminic acid, and that FANA and possibly other neuraminidase inhibitors influence virus replication by inhibiting this function.

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\textbf{REFERENCES}


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