Role of neuraminidase in influenza virus-induced apoptosis

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The virulent influenza virus clone 7a produced a greater level of apoptosis in MDCK cells compared with the attenuated strain A/Fiji. In both cases, apoptosis could be partially blocked by treatment with three anti-neuraminidase compounds [4-amino- (GR121158A) and 4-guanidino- (GG167; Zanamivir) 2,3-dehydro-N-acetylneuraminic acid and 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA)] when they were given to cells during the virus attachment/entry phase, but not subsequent to this phase. In contrast, GG167, which does not enter cells, did not affect the numbers of infected cells and, in addition, acted late in the infection cycle to inhibit virus yields. Clone 7a neuraminidase was more active than A/Fiji neuraminidase when fetuin was used as the substrate. Similar differences in activity between the two viruses were seen when α-2,6 sialyl lactose was used as a substrate, but not with α-2,3 sialyl lactose. No sequence differences in the enzyme active site of the two neuraminidases were observed, indicating that differences in neuraminidase specificity and activity may be dictated by other residues. These results suggest that neuraminidase plays some role in the induction of apoptosis and that it acts prior to or during virus entry. However, apoptosis was considerably reduced when UV-irradiated virus, which retains >75% of its neuraminidase activity, was used. In addition, ammonium chloride, used to prevent virus entry, reduced virus-induced apoptosis. Amantadine, which inhibits virus uncoating, also inhibited apoptosis induced by the amantadine-sensitive strain A/Udorn/307/72 (H3N2), but not the amantadine-resistant clone 7a. Hence, one or more intracellular processes are also involved in influenza virus-induced apoptosis.

Introduction

Influenza virus induces apoptosis or programmed cell death in a number of cell types including HeLa, MDCK, Vero, monocytes and macrophages (Fesq et al., 1994; Govorkova et al., 1996; Hinshaw et al., 1994; Lowy & Dimitrov, 1997; Price et al., 1997 b; Takizawa et al., 1993). The mechanisms involved are unclear but, in HeLa cells, Fas is believed to be an important mediator (Takizawa et al., 1995). Some elements of virus replication are needed since β-propiolactone- or UV-inactivated viruses do not induce apoptosis and new protein synthesis is required (Takizawa et al., 1993). The viral neuraminidase is a candidate inducer of apoptosis because anti-neuraminidase antibody but not anti-haemagglutinin antibody inhibited the activation of transforming growth factor (TGF)-β (a known inducer of apoptosis) by influenza virus (Schultz-Cherry & Hinshaw, 1996). Another viral protein involved is NS1, as MDCK cells expressing NS1 under the control of a tetracycline repressor underwent apoptosis after removal of the tetracycline (Schultz-Cherry et al., 1998).

Recently, we have shown that two influenza viruses, A/Fiji/15899/83 (H1N1) (A/Fiji) and clone 7a (H3N2) of the A/Puerto Rico/8/34 (H1N1) × A/England/939/69 (H3N2) reassortant system induced different levels of apoptosis in two cell lines, MDCK and U-937 (Price et al., 1997 b). A/Fiji induced less apoptosis than clone 7a. Since the number of
infected cells and virus yields were similar for both viruses, the virus inducer of apoptosis may differ in identity or effectiveness for the two viruses.

In this paper we provide further evidence to support the role of influenza virus neuraminidase as an inducer of apoptosis by examining, in this system, compounds which specifically inhibit influenza virus neuraminidase activity. The three anti-neuraminidase compounds examined were 4-amino-(GR121158A) and 4-guanidino- (GG167) 2,3-dehydro-N-acetylenuraminic acid and 2,3-dehydro-2-deoxy-N-acetylenuraminic acid (DANA) (Ryan et al., 1995; Woods et al., 1993). We also report that strains A/Fiji and clone 7a differ in their neuraminidase activity and in the specificity of their active site. Also described are experiments with UV-irradiated virus and ammonium chloride or amantadine treatment of infected cells, which were suggested by the results with the anti-neuraminidase compounds.

Methods

Viruses and their titrations. The virus strains (A/Fiji and clone 7a), the production of virus stocks in fertile hen eggs and their titration in eggs and egg-bits have been described previously (Price et al., 1977b). Titration in Madin–Darby canine kidney (MDCK) cells followed standard protocols (von Itzstein et al., 1993). A/UDom/307/72 (H3N2), A/Singapore/1/57 (H2N2) and B/Victoria/102/85 were obtained from A. Douglas (National Institute for Medical Research, London, UK). Seed stocks and working stocks were prepared as described previously for other influenza viruses (Sweet et al., 1974).

High titre stocks of purified virus. These were prepared as previously described (Price et al., 1997a).

UV-inactivation of viruses. Viruses purified as described above were inactivated by irradiation with UV light as described previously (Price et al., 1997b; Pickering et al., 1992).

Determination of the percentage of apoptotic and infected cells. The number of apoptotic cells were determined by acridine orange + propidium iodide staining as previously described (Price et al., 1997a). The number of apoptotic cells were determined by acridine orange cells. The number of apoptotic cells were determined by acridine orange + propidium iodide staining as previously described (Price et al., 1997a).

Effect of time of GG167 addition on its antiviral activity. MDCK cells in six-well tissue culture plates were washed twice with warmed PBS and inoculated with 300 µl A/Singapore/1/57 (H2N2) or B/Victoria/102/85 (m.o.i. 1 p.f.u. per cell) diluted in serum-free Eagle’s minimum essential medium (EMEM) containing 2 µg/ml TPCK-treated trypsin (Workington Biochemical). At 1, 2, 4, 6, 8, 10, 12, 15, 25 and 30 h post-infection (p.i.) the medium was removed and replaced with warmed EMEM containing 2 µg/ml TPCK-treated trypsin and 10 µM GG167. This concentration was chosen to give 99% inhibition of virus yield. Infected but otherwise untreated cell controls were included in each assay. After incubation at 37 °C in 5% CO2 for 48 h, 1 ml samples of supernatant medium were removed from each well and titrated for infectivity in MDCK cells.

Measurement of cellular uptake of GG167. Confluent MDCK cells in six-well tissue culture plates were washed in PBS and then inoculated with 1 p.f.u. per cell of A/Singapore/1/57 (H2N2) influenza virus. Control wells were mock-infected. After 1 h incubation at room temperature the plates were overlaid with 2 ml defined cell culture medium (DCCM-1, Biological Industries) supplemented with 2 µg/ml TPCK-treated trypsin, containing the required amount of radioactively labelled compound. Both 14C-labelled GG167 and 2-deoxy-D-[14C]-glucose, used as a control, were used at a final concentration of 5 µM. This represented 2-2 µCi GG167 and 1 µCi 2-deoxy-D-[1-14C]-glucose. The plates were incubated at 37 °C in 5% CO2 for 4 h, at which time the overlay medium was decanted and collected and the cells were washed six times in PBS before being detached by scraping, resuspended in PBS and disrupted by five freeze–thaw cycles. All samples were then centrifuged at 1000 r.p.m. in a bench centrifuge and the resultant pellet washed three times in PBS. The pellet was then treated with 0.5% SDS (w/v) for 30 min at room temperature. Samples (50 µl) were counted for radioactivity in 4 ml scintillation fluid in an LKB Rackbeta scintillation counter.

Assay of viral neuraminidase activity. The standard WHO assay using fetuin as substrate was used (Aminoff, 1961; Aymard-Henry et al., 1973) except that at the end of the assay 200 µl of the formed chromophore was added to a 96-well microtitre plate and absorbance (A) was read using a multiplate reader with a wavelength of 540 nm and a path length of 0.5 cm. Enzyme activities are expressed as µmol/l N-acetyl neuraminic acid (NANA) released per min, calculated from a standard curve of NANA concentration against A (y = 0.702x).

Examination of substrate specificity of clone 7a and A/Fiji. Sialyl lactoses with α-2,3 or α-2,6 linkages between the sialic acid residue and lactose were used as substrates. Purified influenza virus at a concentration of 2 µg viral protein/ml was added to a reaction mixture containing 32.5 mM 2-(N-morpholino)ethane sulphonic acid, 4 mM CaCl2, 1 mM MgSO4, 1 mM β-nicotinamide adenine dinucleotide (NAD+, from yeast; Sigma), 2.25 U/ml β-galactosidase (grade VIII purified from E. coli; Sigma), 10 U/ml glucose dehydrogenase (from Bacillus megaterium; Sigma) and 0.1 mM α-2,3 or α-2,6 sialyl lactose (Sigma) contained in a 96-well flat-bottomed microtitre plate in a final volume of 200 µl. The plate was incubated at 37 °C. During the reaction sialyl lactose is cleaved by neuraminidase to sialic acid and lactose, the latter being converted to galactose and glucose by β-galactosidase. Glucose is reduced by glucose dehydrogenase in the presence of NAD+ to give NADH which is read at 340 nm. Reactions were read at intervals in an ELISA plate reader. Enzyme activities are expressed as µmol/l glucose released per min, calculated from a standard curve of lactose concentration against A (y = 2.547x).

Sequence of clone 7a and A/Fiji neuraminidases. Virion RNA, extracted from purified virus using phenol: diethyl ether extraction
followed by ethanol precipitation, was reverse transcribed with avian myeloblastosis virus reverse transcriptase using primers for the 3’ end of segment 6 (neuraminidase) of A/Chile/1/83 (H1N1) (GCGAGGTT-TAAAAATGACTCCAAAAT) or A/Northern Territories/60/68 (H3N2) (GCGAGGTAAAAATGAATCCAAAAT) for A/Fiji and clone 7a, respectively (Scheurer et al., 1988; Bentley & Brownlee, 1982). Double-stranded (ds) DNA was then produced using Tag polymerase and primers specific for the 5’ end of the neuraminidase gene; CAACGACTACTTGTCATAGTTGA and CTAATAATTGCACAAGTATATAG for A/Fiji and clone 7a, respectively (Scheurer et al., 1988; Bentley & Brownlee, 1982). Segment 6 from clone 7a and A/Fiji was amplified by PCR as described (Adeyfa et al., 1994). Following purification using the Wizard PCR prep DNA purification system (Promega) or Microspin S-400HR columns (Pharmacia Biotech), the PCR products were either sequenced directly or following cloning into a PCR 2.1 vector and INVAF-competent cells using the original TA cloning kit version F (Invitrogen). DNA was then sequenced using an ABI PRISM automated gene sequencer with the PE Applied Biosystems four-dye technology.

**Treatement of infected cells with ammonium chloride.** MDCK cells were seeded into 48-well flat-bottomed plates containing 10 mm coverslips and allowed to become confluent. Monolayers were washed twice in warmed PBS and growth medium containing 20 mM NH$_4$Cl was added. After 60 min incubation at 37 °C, the medium was removed and PBS containing 10 EID$_{50}$ virus per cell and 20 mM NH$_4$Cl added. After 60 min at 37 °C, cells were washed three times in warmed PBS, maintenance medium containing 20 mM NH$_4$Cl was added and the cells were incubated at 37 °C for a further 23 h.

**Treatement of infected cells with amantadine.** MDCK cells were seeded into 48-well flat-bottomed plates containing 10 mm coverslips and allowed to become confluent. Monolayers were washed twice with warmed PBS and 100 µl virus suspension, diluted in PBS to give 10 EID$_{50}$ per cell and containing 0.3, 0.4 mM or 10 µM amantadine hydrochloride, was added. After 60 min at 37 °C, cells were washed three times in warmed PBS, and maintenance medium containing the same concentration of amantadine was added. The cells were then incubated at 37 °C for a further 23 h.

**Results**

**Differential apoptosis produced by strains A/Fiji and clone 7a**

Previous results on clone 7a and A/Fiji (Price et al., 1997b) were confirmed using an inoculum of 10 EID$_{50}$ per cell. In two experiments using three replicates (n = 3) per sample the levels of apoptosis 24 h after infection were: 89-0% (SD 5-8) and 97.6% (SD 0.3) for clone 7a; 10-4% (SD 0.4) and 9-4% (SD 2.5) for A/Fiji; and 0-3% (SD 0.3) and 0-5% (SD 0.3) for controls.

**The inhibitory effect of GR121158A or GG167 on apoptosis induced by clone 7a and A/Fiji**

MDCK cells infected with 10 EID$_{50}$ per cell of clone 7a or A/Fiji were treated with 500 µg/ml GR121158A or GG167. The drug was added at the time of addition of virus, and throughout the subsequent incubation until the cells were examined for apoptosis by nuclear staining at 24 h p.i. (i.e. throughout the 24 h incubation as shown in Table 1); the latter was found to be the optimal time-point for sampling in preliminary experiments. Uninfected control cells were also examined in the presence and absence of the drug. The percentage of cells exhibiting apoptosis following infection with clone 7a was significantly decreased by treatment with GR121158A or GG167. Similarly, the smaller percentage of apoptotic cells produced by A/Fiji was also reduced by treatment with the drugs. With both viruses apoptosis was not completely abrogated by the anti-neuraminidase compounds even though the neuraminidase activity of both viruses was completely inhibited by 200 µg/ml GR121158A [enzyme activity of 5 × 10$^6$ EID$_{50}$ clone 7a in the presence and absence of GR121158A was 0-08 (SD 0.03; n = 2) and 1-29 (SD 0.40; n = 2)] µmol/l NANA released per min, respectively, and for 5 × 10$^6$ EID$_{50}$ A/Fiji it was 0-04 (SD 0.01; n = 2) and 0-4 (SD 0.06; n = 2), respectively] or GG167 [enzyme activity for clone 7a in the presence and absence of GG167 was 0-05 (SD 0.03; n = 2) and 1-29 (SD 0.4; n = 2)] µmol/l NANA released per min, respectively, and for A/Fiji it was 0-04 (SD 0.01; n = 2) and 0-4 (SD 0.06; n = 2), respectively].

The results in Table 1 also show that adding the compounds for only the attachment period was as effective as having them present throughout the incubation period.

**Lack of inhibition of apoptosis when anti-neuraminidase compounds were added after the period of virus attachment**

Table 1 also shows the results of adding the anti-neuraminidase compounds after virus attachment had taken place. The lack of inhibitory effect on apoptosis clearly indicates that the initial 1 h period is the crucial phase for the action of the compounds.

**Inhibition of influenza virus-induced apoptosis by DANA**

Both 4-guanidino-2,3-dehydro-N-acetylmuramic acid (GG167) and 4-amino-2,3-dehydro-N-acetylmuramic acid (GR121158A) contain basic residues and could thus act as weak bases (like NH$_4$Cl, see later) to inhibit apoptosis. This is unlikely due to their different optimal times of action (see above and later) and further evidence that this was not the case is provided using DANA (2,3-dehydro-2-deoxy-N-acetyl-

**Effect of time of addition of GG167 on its antiviral activity**

As expected from previously published studies on the mode of action of viral neuraminidase (Liu et al., 1995; Palese
Table 1. Levels of apoptosis in MDCK cells 24 h p.i. with 10 EID$_{50}$ per cell of clone 7a or A/Fiji and treated with the anti-neuraminidase compounds GR121158A, GG167 or DANA during and after initial attachment of virus

Differences between tests comparing samples with and without treatments were not significant except for the 23 h after the attachment period [99.7% (SD 0.11; n = 3) and 99.6% (SD 0.6; n = 3) cells were infected in the absence and presence of compound, respectively]. This suggested that the drugs had little or no effect on virus attachment, entry, RNA replication or protein synthesis. This was confirmed in a study which examined the effect of time of addition of compound on its antiviral activity. When GG167 was added at early times post-infection (1 and 2 h p.i.) it almost completely inhibited virus replication, whilst addition between 6 and 12 h p.i. considerably reduced virus yields (Table 2). In contrast, when compound was added 15 h p.i. or later, yields were similar to control cultures. These results confirm previous observations that neuraminidase inhibitors act late in the infection cycle.

Lack of entry of GG167 into uninfected or infected cells

The majority [99.7% (SD 0.28; n = 2) and 99.6% (SD 0.28; n = 2), respectively] of total input radioactivity associated with the $^{14}$C-labelled GG167 added to influenza virus-infected or uninfected cells was recoverable in the medium or subsequent washes. Similarly, little radioactivity was found in the cell pellets [0.78% (SD 0.18; n = 2) and 0.78% (SD 0.04; n = 2), respectively] of infected and uninfected cells. In contrast, 5.49% (SD 1.43; n = 2) and 8.10% (SD 0.8; n = 2) total input $^{14}$C-labelled glucose was found in infected and uninfected cells, respectively, while yields from supernatants and washes were 91.5% (SD 4.9; n = 2) and 95% (SD 2.5;

Table 2. Effect of time of addition of GG167 on the yields of influenza virus A/Singapore/1/57 (H2N2) and B/Victoria/102/85 from MDCK cells

![Table 2. Effect of time of addition of GG167 on the yields of influenza virus A/Singapore/1/57 (H2N2) and B/Victoria/102/85 from MDCK cells]
n = 2), respectively. These results suggest that GG167 does not enter cells in significant quantity.

The neuraminidase activity of clone 7a is greater than that of A/Fiji when fetuin is used as a substrate

The neuraminidase activities of the two viruses were significantly different (P < 0.05). At equal infectivities of 5 × 10^6 EID_{50} the enzyme activities were 0.25 (SD 0.03; n = 2) and 0.03 (SD 0.01; n = 2) µmol/L NANA released per min for clone 7a and A/Fiji, respectively, while at equal protein concentrations of 0.25 µg virus the activities were 2:1 (SD 0.04; n = 2) and 1:2 (SD 0.01; n = 2) µmol/L NANA released per min for clone 7a and A/Fiji.

The different specificities of clone 7a and A/Fiji neuraminidases

The results in Fig. 1 show that although the neuraminidases of clone 7a and A/Fiji show similar activity when α-2,3 sialyl lactose was used as a substrate, the clone 7a preparation was considerably more active than that of A/Fiji when α-2,6 sialyl lactose was used as a substrate. Neuraminidase activities for 2 µg of clone 7a and A/Fiji using α-2,3 sialyl lactose as substrate were 0.86 (SD 0.09) and 0.82 (SD 0.07) µmol/L lactose released per min, respectively, and for α-2,6 sialyl lactose were 0.7 (SD 0.03) and 0.45 (SD 0.03) µmol/L lactose released per min. The neuraminidase activity of clone 7a using α-2,6 sialyl lactose as substrate was significantly greater than that for A/Fiji (P < 0.01), whereas the comparison with α-2,3 sialyl lactose as substrate was not significantly different (P > 0.05).

Amino acid sequences of clone 7a and A/Fiji neuraminidases

As with other viruses of the N1 and N2 serotypes, the amino acid compositions of the two neuraminidases showed 50–96% similarity and 42–46% identity. However, the amino acids comprising the enzyme active site were conserved in both neuraminidases (Fig. 2). A number of cysteine residues likely to be involved in the disulphide bridge formation are boxed; potential glycosylation sites are underlined (Varghese et al., 1983). Vertical lines denote amino acid identity and colons refer to conservative changes.

UV-inactivated viruses induce little apoptosis

When both clone 7a and A/Fiji were UV-inactivated, the amount of apoptosis was markedly decreased 24 h post-
inoculation. With inocula of 10 or 100 pg per cell (≈ 1000 and 10,000 EID<sub>50</sub> infectious virus per cell, respectively) of clone 7a, levels of apoptosis were only 10.3% (SD 4.3%; n = 3) and 2.9% (SD 1.4%; n = 3), respectively; levels for A/Fiji were barely discernible [2.2% (SD 1.1%; n = 3) and 0% (SD 0; n = 3), respectively]. No nucleoprotein synthesis occurred in these cells, as judged by monoclonal antibody staining (data not shown). Both haemagglutinating (data not shown) and neuraminidase activities of UV-irradiated virus [1:67 (SD 0.27; n = 2) and 1:23 (SD 0.06; n = 2) μmol/l NANA released per min for clone 7a and A/Fiji, respectively] were not significantly reduced (P > 0.05) compared to unirradiated virus [1:69 (SD 0.04; n = 2) and 1:26 (SD 0.01; n = 2) μmol/l NANA released per min for clone 7a and A/Fiji, respectively].

### Effect of ammonium chloride on virus-induced apoptosis

The above results suggested that virus replication was important for the induction of apoptosis by influenza virus. To examine this further, cells were treated with NH₄Cl, which is known to prevent virus entry into cells (Jakeman et al., 1991). Virus-infected MDCK cells were treated with 20 mM NH₄Cl continuously from 60 min prior to virus inoculation until examined for apoptosis. As in previous experiments, clone 7a induced significantly more apoptosis than A/Fiji (P < 0.05) in untreated cells (Table 3). However, induction of apoptosis was severely reduced with both viruses if NH₄Cl was included in the medium throughout the experimental period (i.e. 25 h; 1 h pretreatment + 1 h virus attachment period + subsequent 23 h incubation; see Methods) (Table 3). The percentage of infected cells decreased from 99.6% (SD 0.6; n = 3) to 21.8% (SD 6.21; n = 3) after treatment of clone 7a-infected cells with NH₄Cl for 25 h. In contrast, the neuraminidase activity of clone 7a virions increased slightly but significantly (P < 0.05) from 1:23 (SD 0.02) to 1:35 (SD 0.02) μmol/l NANA released per min in the presence of 20 mM NH₄Cl, whereas that of A/Fiji did not change significantly (P > 0.05) [0.42 (SD 0.02) and 0.34 (SD 0.02) in the presence and absence of NH₄Cl, respectively]. Gradually decreasing the time of exposure to NH₄Cl abrogated its effect on reduction of virus-induced apoptosis, although exposure to NH₄Cl at any stage did affect the percentage of apoptotic cells (Table 3). Treatment of infected cells for 1 h only with NH₄Cl also reduced the percentage of cells infected significantly (P < 0.05), but only slightly, to 94.7% (SD 1.2; n = 3), whereas treatment for the 23 h post-attachment period had no effect (99.0% SD 0.5; n = 3). These results suggest that inhibition or reduction of virus entry reduces apoptosis. While treatment with NH₄Cl after the virus attachment period did reduce apoptosis, suggesting that NH₄Cl may be anti-apoptotic, it is unlikely that this explains the considerably greater reduction in virus-induced apoptosis seen when infected cells were exposed for a further 1 h during virus attachment.

### Additive action of GG167 and ammonium chloride

The above results suggested that more than one mechanism contributed to influenza virus-induced apoptosis; GG167 acted...
induced apoptosis (data not shown). Similar results were also
of A
Puerto Rico
when both inhibitors were present (Table 4).

The percentage of apoptotic cells was calculated by staining for
apoptosis (as descibed in Methods). Standard deviations
from the mean of three replicates in Expt 1 and six replicates in Expt 2
are shown; at least 1000 cells were counted for each replicate.

**Table 4. Levels of apoptosis in MDCK cells 24 h p.i. with 10 EID_{50} per cell of clone 7a treated with ammonium chloride and/or GG167**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Percentage of cells showing apoptosis (± SD)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH₃Cl</td>
</tr>
<tr>
<td>Uninfected</td>
<td>—</td>
</tr>
<tr>
<td>Infected</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>—</td>
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<tr>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

* Cells were treated with (+) or without (−) 500 µg/ml GG167 and/or 20 mM NH₃Cl throughout the 24 h incubation period in both Expt 1 and Expt 2.
† The percentage of apoptotic cells was calculated by staining for nuclear morphology (as described in Methods). Standard deviations from the mean of three replicates in Expt 1 and six replicates in Expt 2 are shown; at least 1000 cells were counted for each replicate.

To reduce apoptosis at the virus attachment/entry stage, whilst
NH₃Cl and UV-inactivation inhibited apoptosis by preventing
virus replication. Support for this was provided in clone 7a cells
treated with both NH₃Cl and GG167, where an additive or
synergistic effect was obtained (Table 4). Clone 7a-infected
cells exhibited 78–83% apoptosis, this being reduced to
42–51% and 13–31%, respectively, by treatment with GG167
or NH₃Cl. A further reduction to only 2–3% apoptosis was observed when both inhibitors were present (Table 4).

**Effect of amantadine chloride on virus-induced apoptosis**

Amantadine inhibits uncoating of human influenza viruses
but, as shown in Table 5, it had little effect on apoptosis
induced by either clone 7a or A/Fiji. However, virus yields
were unaffected by treatment with amantadine, even at doses
of 10 µM (data not shown) and the percentage of infected cells
was only reduced from 99.6 (SD 0.7; n = 3) to 86.0% (SD 2.2;
\( n = 3 \)). Higher doses of amantadine could not be used as they
induced apoptosis (data not shown). Similar results were also
obtained with an inoculum of 1 EID_{50} per cell.

It is likely that clone 7a was not inhibited by amantadine as
it contains the M2 gene of A/Puerto Rico/8/34 (H1N1)
(Matsuyama et al., 1980), which is resistant to amantadine
(Wharton et al., 1994). The results suggest that the M2 protein
of A/Fiji may also be resistant. An experiment with 1 EID_{50} per
cell of A/Udorn/307/72 (H3N2) showed that this
amantadine-sensitive virus induced 16.2 (SD 4.1) % apoptosis,
which was reduced to 3.1 (SD 0.4) % on treatment with
0.3 mM amantadine. Again similar results were obtained with
an inoculum of 10 EID_{50} per cell. Thus, inhibition of virus
uncoating with amantadine also reduces apoptosis.

**Discussion**

It is clear from the above results that neuraminidase is
involved in induction of apoptosis by influenza viruses. First,
three anti-neuraminidase compounds partially abrogate
apoptosis during infection of MDCK cells without affecting
numbers of infected cells. Second, the differential abilities of
clonet 7a and A/Fiji to induce apoptosis is reflected in
differences in the amounts of neuraminidase activity in the two
strains, and differences in the specificity of the two enzymes.

The results also show that the action of neuraminidase in
promoting apoptosis occurs at, or shortly before, virus binding
and entry to the cells (Table 1). This is rather surprising as a
considerable body of evidence suggests that the role of the
viral neuraminidase during infection is not in virus attachment,
entry, replication, assembly or budding, but in preventing
aggregation of virus particles at the cell surface to allow further
infection of uninfected cells (Liu et al., 1995; Palese & Comans,
1976; Palese et al., 1974). When neuraminidase activity was
abrogated by antibody (Comans et al., 1969), inhibitors such as
FANA (2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic

**Table 5. Levels of apoptosis in MDCK cells 24 h p.i. with 10 EID_{50} per cell of clone 7a or A/Fiji treated with various doses of amantadine hydrochloride**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Treatment*</th>
<th>Clone 7a</th>
<th>A/Fiji</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 mM</td>
<td>—</td>
<td>75.4±9.8</td>
<td>67.7±3.3</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>+</td>
<td>74.6±8.9</td>
<td>59.1±3.3</td>
<td>2.7±0.8</td>
<td></td>
</tr>
<tr>
<td>0.4 mM</td>
<td>—</td>
<td>95.3±2.5</td>
<td>45.3±1.8</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td>+</td>
<td>75.8±8.2</td>
<td>38.2±2.1</td>
<td>3.0±0.7</td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>—</td>
<td>63.5±1.5</td>
<td>3.8±2.1</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>+</td>
<td>68.1±1.8</td>
<td>3.7±0.3</td>
<td>1.7±0.2</td>
<td></td>
</tr>
</tbody>
</table>

* Cells were treated with (+) or without (−) amantadine hydrochloride throughout the 24 h incubation period.
† The percentage of apoptotic cells was calculated by staining for nuclear morphology (as described in Methods). Standard deviations from the mean of six replicates for 0·3 mM and three replicates for 0·4 mM and 10 µM are shown; at least 1000 cells were counted for each replicate.
acid) (Palese & Compans, 1976) and GG167 (this communication), or temperature-sensitive mutation (Palese et al., 1974), virus particles attached to each other and aggregated at the cell surface. A neuraminidase-deficient virus behaved similarly (Liu et al., 1995). In our studies on the timing of the antiviral effect of the compounds on virus replication, the compounds acted late in the infection cycle and did not affect virus replication as judged by numbers of infected cells; also, the experiments were performed under single cycle conditions, where further rounds of replication were not applicable. In addition, use of radiolabelled GG167 showed that it did not enter cells and that it must therefore act extracellularly. In the case of its inhibition of virus replication this would be newly emerged virus particles on the cell surface. The implication of this is that the target for neuraminidase inhibition of apoptosis must also be on the cell surface. A candidate for this target is TGF-β. This is synthesized as a latent precursor present on cell surfaces. Active TGF-β is released from the C terminus of the latent form following proteolytic cleavage by a furin-like protease (Blanchetta et al., 1997). This activation probably occurs at the cell surface where the pro-TGF-β segments are retained and active TGF-β is released into the medium (Gitelman & Dernyck, 1994). Latent TGF-β has three glycosylation sites (Brunner et al., 1988), and removal of oligosaccharides may facilitate proteolytic cleavage or enhance the release of TGF-β. This is conceptually similar to cleavage of influenza haemagglutinin into the fusion-competent HA1 and HA2 domains following removal of oligosaccharide side chains around the cleavage site (Kawaoka, 1991). Recent studies have shown that TGF-β may be activated by influenza virus neuraminidase and, once active, is capable of inducing apoptosis in MDCK cells (Schultz-Cherry & Hinshaw, 1996).

The differences in neuraminidase activities of clone 7a and A/Fiji are interesting. It is possible that different quantities of neuraminidase are incorporated into virions of clone 7a and A/Fiji, but this does not explain the difference in substrate specificity observed between the two strains. A/Fiji has lower enzyme activity than clone 7a for fetuin and a similar activity to clone 7a against α-2,3 sialyl lactose, which is surprising because fetuin contains α-2,3 sialyl linkages. Clone 7a neuraminidase is clearly more active than that of A/Fiji for α-2,6 sialyl linkages (Fig. 1). The different activity of the neuraminidase from A/Fiji for the α-2,3 sialyl linkages of the smaller molecular weight sialyl lactose and the larger molecular weight glycoprotein fetuin do not reflect neuraminidase ‘stalk’ length as these are similar in both neuraminidases (Table 3). Viruses with ‘stubby stalks’ have been shown to elute more slowly from erythrocytes (Els et al., 1985). The amino acid residues comprising the enzyme active site are also conserved in both neuraminidases, but there are differences in the number of cysteine residues (two are missing in A/Fiji N1) and asparagine residues, which could affect the conformation of the active site and contribute to neuraminidase activity and specificity. These changes are conserved in other N1 neuraminidases. We are currently examining other N1 viruses for enzyme activity and specificity and for ability to induce apoptosis.

The levels of anti-neuraminidase compounds used in these experiments (1·51, 1·71 and 1·72 mM for GG167, GR121158A and DANA, respectively) were sufficient to eliminate all neuraminidase activity of the inoculum (Gubareva et al., 1996; this communication). Indeed, the levels were well above their IC₅₀ values (the concentration required to reduce plaque formation in MDCK cells by 50%) which are 0·014 μM and 1·5 μM for GG167 and GR121158A, respectively, for A/Singapore/1/57 (H2N2) (von Itzstein et al., 1993). However, some apoptosis still occurred despite this neutralization of neuraminidase activity (Table 1). Therefore, neuraminidase activation of TGF-β cannot be the sole mechanism for apoptosis induced by influenza virus. An intracellular mechanism probably also contributes to apoptosis as inhibition of virus replication inhibited apoptosis. Thus, UV-inactivated virus did not induce apoptosis and treatment of virus-infected cells with NH₄Cl abrogated apoptosis (Table 4). Also, amantadine inhibited apoptosis induced by A/Udorn/307/72; it inhibited clone 7a-induced apoptosis only poorly, presumably as this virus contains the M2 gene from the amantadine-resistant A/Puerto Rico/8/34 parent (Wharton et al., 1994). It is possible that some early aspect of replication is also needed for neuraminidase to manifest its apoptotic activity because UV-inactivated virus retained much, if not all, of its neuraminidase activity, but showed little if any ability to induce apoptosis. It thus seems likely that more than one mechanism, and hence virus component, is contributing since NH₄Cl and GG167 were at least additive and possibly synergistic when added together to virus-infected cells (Table 5).

The nature of this second pro-apoptotic pathway is unclear. One possibility is that it may involve the interferon-induced, dsRNA-activated protein kinase (PKR) (Takizawa et al., 1995, 1996). PKR activation follows its interaction with dsRNA and results in a cascade of effects including activation of transcription factors NF-κB and NF-IL6 (Jacobs & Langland, 1996). NF-IL6 regulates the pro-apoptotic transmembrane protein Fas (Wada et al., 1995), a member of the tumour necrosis factor/nerve growth factor receptor family that induces the cell to become apoptotic when cross-linked by its ligand (FasL). PKR inhibitors prevent expression of Fas (Takizawa et al., 1995) and cells transfected with inactive mutant PKR resist influenza virus-induced apoptosis (Takizawa et al., 1996). In contradiction to this, recent preliminary evidence suggests that viral NS1 alone can induce apoptosis yet it inhibits PKR activation (Lu et al., 1995) and a mutation in the RNA-binding domain of NS1 abrogates its ability to induce apoptosis (Schultz-Cherry et al., 1998).

Elucidation of the pathways of influenza virus-induced apoptosis is not merely of academic interest. These pathways may be involved in stimulation of the constitutional symptoms of influenza as well as damage to the respiratory tract (which
may promote secondary bacterial infections. Cytokine release from infected cells during influenza virus-induced apoptosis, brought about either directly (TGF-β activation by neuraminidase) or indirectly (via de novo gene transcription following NF-κB and NF-IL6 activation by PKR) is likely to be critical in the severity of symptoms and the effectiveness of the host inflammatory response. For example, NF-κB regulates interferon-β, interleukin (IL)-1β, IL-6, IL-8, IL-1 receptor antagonist (IL-1ra), macrophage inflammatory protein 1α and β, and tumour necrosis factor expression (Baldwin, 1996; Lenardo & Baltimore, 1989; McDonald et al., 1997), whilst NF-IL6 also regulates IL-6, IL-8 and granulocyte- and granulocyte/macrophage colony stimulating factors (Jamaluddin et al., 1996; Wada et al., 1995), all of which have important regulatory roles in the inflammatory response. In addition, active IL-1β (a potent pro-inflammatory cytokine and endogenous pyrogen) is released from pro-IL-1β by IL-1β-converting enzyme (ICE), which is an intracellular pro tease known to be activated during apoptosis (Kumar, 1995). Also, once activated and bound to its receptors, TGF-β from infected cells may trigger transcription of further genes involved in the inflammatory response (Knaus & Lodish, 1994). Overall, this suggests that the symptoms of influenza virus infection may be ameliorated by the use of treatments which inhibit virus-induced apoptosis, for example the anti-neuraminidase compounds described in this paper.

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References


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