Differential induction of cytotoxicity and apoptosis by influenza virus strains of differing virulence

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Two influenza viruses of differing virulence, clone 7a (virulent for humans and ferrets) and A/Fiji (attenuated for both species), induced differential levels of cytotoxicity (as measured by release of lactate dehydrogenase from cells) and apoptosis (as determined by altered nuclear morphology or flow cytometry) in MDCK and U-937 cells. Clone 7a induced more apoptosis and cytotoxicity than A/Fiji, despite the fact that its infectious virus yields were similar to or less than those for A/Fiji. This indicates a greater capacity of clone 7a to induce the two phenomena. Nevertheless the replication process was clearly essential, since UV-irradiated virus induced little or no apoptosis, and apoptosis occurred as a late event post-infection. The possible relevance of these findings to destruction of host cells by influenza virus in vivo is discussed.

Introduction

Influenza A virus, a major cause of morbidity and mortality in humans, is primarily a pathogen of the upper respiratory tract. Infection results in both respiratory effects (sneezing, hyperproduction of mucus, sore throat) and constitutional effects (fever, myalgia, anorexia) (Sweet & Smith, 1980). In humans and the ferret model, constitutional symptoms arise by the production of ‘endogenous pyrogen’ (probably pro-inflammatory, pyrogenic cytokines) following interaction between virus and inflammatory cells in the upper respiratory tract (URT), the primary site of virus replication (Sweet et al., 1979; Tinsley et al., 1987). In the ferret, influx of inflammatory cells into the URT begins approximately 24 h post-infection (p.i.) and correlates with the onset of fever (Sweet et al., 1979). The clinical effects of H3N2 influenza viruses in man are more severe than those of H1N1 viruses (Wright et al., 1980), and in ferrets a H3N2 strain (clone 7a) induced a significantly higher and longer fever than a H1N1 strain (A/Fiji) (Coates et al., 1986). Since both viruses replicated similarly in the URT and produced similar inflammatory responses, this indicated a greater fever-producing capacity of clone 7a than that of A/Fiji, an observation confirmed by direct injection of large quantities of both live and UV-inactivated viruses into ferrets (Coates et al., 1986). However, attempts to demonstrate a difference in the levels of pyrogenic cytokines induced from phagocytic cells by these viruses failed for both nasal inflammatory cells removed from infected febrile ferrets and incubated in vitro, and human peripheral blood leukocytes incubated with virus in vitro (Jakeman et al., 1991a; Tinsley et al., 1987). This raised the possibility that the differences in constitutional symptoms induced by these strains could arise from differential damage to the respiratory epithelium, resulting in the differential release of cytokines from the site of infection.

Influenza viruses destroy ciliated epithelial cells in the URT (Hers & Mulder, 1961). However, the mechanism involved has not been studied until recently, when induced programmed cell death (apoptosis) was demonstrated in epithelial cells and monocytes in vitro (Fesq et al., 1994; Hinshaw et al., 1994; Takizawa et al., 1993) and in virus pneumonia of mice (Mori et al., 1995).

Here we report the differential cytotoxicity and apoptosis produced by clone 7a and A/Fiji in two tissue culture systems.

Methods

• Viruses. A/Fiji/15899/83 (H1N1) and the virulent clone 7a of the A/PR/8/34 × A/England/939/69 (H3N2) reassortment system (Beare & Hall, 1971) were grown for 48 h in the allantoic cavity of embryonated 10-day-old hen’s eggs. The viruses were harvested from these eggs, aliquoted and stored at −70 °C prior to use.
**Titration of viruses.** Previously, difficulties have been encountered in plaquing the two strains in MDCK cells (unpublished observations), thus egg-based assay systems were selected. Stock viruses were titrated in 10-day-old hen’s eggs as previously described (Sweet et al., 1974) and the 50% egg infectious dose (EID$_{50}$) was calculated according to the method of Thompson (1947). The many experimental samples were titrated in the more convenient allantois-on-shell (egg-bit) assay (Sweet et al., 1974). Assay plates were shaken at 37 °C for 72 h before reading. Titres were calculated as 50% egg-bit infectious doses (EBID$_{50}$). However, the egg-bit assay was less sensitive than the egg assay for both viruses, and A/Fiji replicated less well in egg bits than clone 7a. As in previous work (Coates et al., 1985), the results from the egg-bit assay were converted to EID$_{50}$ values with correction factors obtained as follows: ten replicate samples of clone 7a and A/Fiji grown in eggs or in MDCK cells (see below) were titrated in both egg bits and eggs (Coates et al., 1985) and the EID$_{50}$:EBID$_{50}$ ratios were calculated. The results were 1:5:5 (SE 1:6) and 1:8:3 (SE 2:1:4) respectively for egg-grown stocks of clone 7a and A/Fiji, and 1:4:2 (SE 0:9) and 1:113 (SE 32:2) respectively for clone 7a and A/Fiji grown in MDCK cells. In some experiments (see Results), samples from MDCK and U-937 cells were treated with trypsin (Sigma; 10 µg/ml) for 90 min prior to seeding into EBID$_{50}$ assay plates. Appropriate titrations showed that the EID$_{50}$:EBID$_{50}$ ratios were lower for these trypsin-treated samples, namely 1:1:4 (SE 1:5) for clone 7a and 1:1:4 (SE 5:1) for A/Fiji.

**Production of UV-inactivated virus.** Allantoic fluid harvested from 50 infected eggs was clarified from cell debris by low-speed centrifugation (10000 g, 30 min at 4 °C) and then spun at 80000 g for 90 min at 4 °C to pellet the virus. The pellets were soaked in TSE buffer (0:85% saline, 0:01 M Tris–HCl pH 7:4, 1:3 mM EDTA) overnight at 4 °C and 2 vols 57% sucrose (w/v) added to give a final concentration of 38% (w/v). The suspension was layered onto 38% sucrose (w/v) and spun at 80000 g for 60 min at 4 °C. The virus-containing supernatant was removed, diluted with TSE to give a sample of 30% sucrose (w/v) and spun overnight at 80000 g at 4 °C. Final virus pellets were resuspended in Dulbecco’s A PBS pH 7:3 and the protein concentration was determined using a Coomassie Plus protein assay kit (Pierce & Warriner, Chester, UK). The suspension was irradiated with UV light (250 nm) at a distance of 7:5 cm for 7 min (giving a light intensity of 120 µW/cm$^2$) in an open petri-dish on ice (Pickering et al., 1992). Inactivation was confirmed by double passage in 10-day-old eggs (Coates et al., 1986). Inactivated virus stocks were aliquoted and stored at −70 °C prior to use.

**Cell cultures.** Madin–Darby canine kidney (MDCK) epithelial cells and U-937 human histiocytic cells were used. MDCK cells were grown in Eagle’s minimum essential medium, supplemented with 10% newborn bovine serum (NBCS), 4 mM t-glutamine, 0:7% sodium bicarbonate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin. Cells were maintained in the above medium with NBCS concentration reduced to 2%. U-937 cells were grown in RPMI 1640 medium, supplemented with 10% NBCS, 4 mM t-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 2 mM sodium pyruvate (complete RPMI). All media components were obtained from Gibco. Both cell types were incubated at 37 °C in a 5% CO$_2$ atmosphere.

**Infection of cell cultures.** MDCK cells were seeded into either 96- or 48-well flat-bottomed plates and allowed to become confluent. Monolayers were washed twice with warmed PBS and the appropriate volume of virus suspension, diluted in PBS (50 µl or 100 µl for 96- and 48-well plates respectively) to give the required m.o.i., was added to each well. Controls contained only PBS. After 60 min at 37 °C, cells were washed three times in warmed PBS, and maintenance medium (200 µl and 300 µl for 96- and 48-well plates) was added.

U-937 cells were pelleted and resuspended to 4 × 10$^6$ cells/ml in complete RPMI (without serum). One ml of this suspension was transferred to culture tubes and washed twice with serum-free complete RPMI (SF-cRPMI). One ml of virus suspension diluted to the required m.o.i. in SF-cRPMI was then added. After 60 min at 37 °C, cells were washed three times with 5 ml SF-cRPMI and resuspended in 2 ml SF-cRPMI prior to the addition of 2 ml complete RPMI to give a final concentration of 1 × 10$^6$ cells/ml in complete RPMI with 5% serum. Cells were then re-incubated for the required time.

When MDCK cells were treated with inactivated viruses, virus suspensions were diluted in PBS to give 100 and 10 pg viral protein per cell (this would be equivalent to approximately 10000 and 1000 EID$_{50}$ per cell respectively of infectious virus). Control cells had PBS added.

**Cytotoxicity assays.** Cytotoxicity assays were conducted with the Cytotox 96 (Promega), which enzymatically assays lactate dehydrogenase (LDH) released from damaged cells into the supernatant. Control samples were set up to correct for LDH in the medium (serum contains LDH at low levels) and for spontaneous release of LDH from uninfected cells. Maximum release of LDH from uninfected cells was measured after lysis of cells by adding 9% Triton X-100 to give a final concentration of 0:9% in the medium. To correct for this addition of lysis solution to the cell suspension, an equal volume of this solution was added to medium (volume correction control). For experiments with MDCK cells, supernatants were taken, diluted 1:2 and incubated with the assay substrate at room temperature for 15 min before the reaction was stopped by addition of 1 M acetic acid. For experiments with U-937 cells a similar procedure was used, but cells were sedimented by centrifugation, supernatants were assayed undiluted and the reaction was stopped after 30 min at room temperature. Percentage cytotoxicity values were calculated by subtraction of absorbance values at 492 nm into the equation: % Cytotoxicity = [(experimental − medium control) − (spontaneous − medium control)/(maximum release − volume correction control)] × 100.

**Determination of the percentage of apoptotic and infected cells by fluorescence microscopy.** Cell monolayers growing on 10 mm glass coverslips in 48-well plates were fixed for 10 min with either acid ethanol (1 part glacial acetic acid, 2 parts ethanol) for acridine orange staining or methanol for immunofluorescence. For acridine orange staining, fixed cells were washed twice in McIlvain’s buffer (71 mM Na$_2$HPO$_4$, 65 mM citric acid), stained for 10 min with 0:1% acridine orange in McIlvain’s buffer and rinsed twice more before mounting. For immunofluorescence, fixed monolayers were treated with propidium iodide (0:1 mg/ml) as a nuclear stain and washed in PBS. Then monoclonal antibody (1:250) to the nucleoprotein (NP) of the X-31 (H3N2) strain of influenza virus (kindly provided by A. Douglas, NIMR, Mill Hill, London, UK) was added and allowed to bind at 37 °C for 30 min before washing with PBS. Finally a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma; 1:100) was added and allowed to bind at 37 ºC for 30 min. After washing in PBS, the coverslips were removed, mounted in triethylenediamine–glycerol to reduce fading (Johnson et al., 1982) and examined by fluorescence microscopy. Fields were selected at random by moving the monolayer slightly out of focus when changing field, and at least 1000 cells were counted for each coverslip examined.

Apoptotic cells were identified by their characteristic nuclear morphology as revealed by the DNA-binding stains used, which showed small, brightly staining bodies (green in the case of acridine orange, red
Differential cytotoxicity and apoptosis produced by clone 7a and A/Fiji in MDCK cells

In preliminary experiments with inocula ranging from 100–0·1 EID₅₀ per cell and incubation times of 24, 36, 48 and 72 h, clone 7a was more cytotoxic for MDCK cells than was A/Fiji (Fig. 1). Only for an inoculum of 100 EID₅₀ per cell was the maximum toxicity for A/Fiji (ca. 40%) similar to clone 7a (ca. 45%), but it occurred later in the infection. For all other inocula, the maxima for A/Fiji were well below those for clone 7a, and at an inoculum of 0·1 EID₅₀ per cell A/Fiji showed no toxicity, in contrast to clone 7a, which was still appreciably toxic (ca. 20%).

The experiments were then extended to examine apoptosis as well as cytotoxicity, and the percentage of infected cells and virus yield were also measured. Fig. 2 shows how apoptotic cells were recognized and Table 1 summarizes the results of four experiments using an inoculum of 10 EID₅₀ per cell examined at 24 h p.i. The percentage cytotoxicity varied between experiments; in these experiments from about 14–30% for clone 7a and 3–15% for A/Fiji, but in each experiment the difference between clone 7a and A/Fiji was statistically significant (P ≤ 0·01). The percentage of apoptotic cells also varied between experiments from about 27–40% for clone 7a and between 11% and 22% for A/Fiji in the experiments cited in Table 1. Again, differences between the viruses were significant (P ≤ 0·05) in each experiment. Variation in the levels of cytotoxicity and apoptosis induced by clone 7a and A/Fiji was apparent between experiments, but within each experiment clone 7a induced significantly more cytotoxicity and apoptosis than A/Fiji. The reasons for this are unknown, but could include variations in susceptibility between passage number of cells, cell density at infection or LDH content of the serum used. Fragmentation of chromosomal DNA is a characteristic feature of apoptosis, and ‘apoptotic ladders’ were detectable in DNA extracted from adherent clone 7a-infected cells at 12 h p.i., but not in either A/Fiji-infected or control cells, or culture supernatants at this time (not shown). At 24 h p.i. and subsequent times, laddering was seen in apoptotic cells in culture supernatants (Fig. 3) and adherent cells infected with either virus, and at 48 h p.i. in control cells to some extent. The DNA fragments were found to be on average 189 bp multimers, which is in agreement with...
other reports. The percentages of infected cells were high and not significantly different for the two viruses. Finally, and importantly, the yields of both viruses were not significantly different, except in experiment 2 when the yield for A/Fiji was greater than for clone 7a. It is important to note that these experiments were essentially carried out under single-cycle conditions with an inoculum that infected over 90% of the cells. Further (multicycle) replication would be minimal, as liberated virus would be largely non-infectious for MDCK cells since trypsin was not included in the growth medium of these cultures.

The differences between the viruses might have reflected different optimum times for sampling, and thus the experiments described above were repeated, but samples were taken at various times p.i. The results of a typical experiment are shown in Table 2. Mean percentage cytotoxicities increased with time from about 3% at 2 h p.i. to about 70% at 36 h p.i. for clone 7a and from about 1–36% for A/Fiji. Whilst again percentage cytotoxicities differed between experiments, at all time-points except 6 h p.i. the mean percentage cytotoxicity induced by clone 7a was greater than that induced by A/Fiji, but only in one case (24 h p.i.) was this significant. Apoptosis was not evident until 12 h p.i., then the mean percentages of apoptotic cells increased with time, and those for clone 7a were significantly higher than for A/Fiji at all time-points. Although the mean percentage of cells infected with A/Fiji was lower than that for clone 7a (80% compared to 100%), this was not significant in most cases. Clone 7a-infected cells showed very bright fluorescence, but cells infected with A/Fiji gave less intense staining, with a more speckled pattern than the diffuse staining seen with clone 7a (Fig. 2). Although NP was detectable in the cytoplasm earlier for clone 7a-infected cells...
Table 1. Levels of cytotoxicity and apoptosis in MDCK cells 24 h p.i. with 10 EID₅₀ per cell of clone 7a or A/Fiji

Clone 7a did not significantly differ from A/Fiji, except a, P < 0.05; b, P < 0.02; c, P < 0.01; and d, P < 0.001. Other figures are not significantly different between strains.

<table>
<thead>
<tr>
<th>Expt</th>
<th>% Cytotoxicity (±sd)*</th>
<th>% Cells showing apoptosis (±sd)†</th>
<th>% Cells infected (±sd)‡</th>
<th>Virus yield (log₁₀ EID₅₀ per ml ±sd)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a Fiji</td>
<td>7a Fiji Control</td>
<td>7a Fiji Control</td>
<td>7a Fiji</td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>17±3.7±2.9</td>
<td>10±3.4</td>
<td>ND</td>
<td>5±4±0.4</td>
</tr>
<tr>
<td>Expt 2</td>
<td>13±4±2.9</td>
<td>3±0.2</td>
<td>ND</td>
<td>5±4±0.4</td>
</tr>
<tr>
<td>Expt 3</td>
<td>29±4±6±1.9</td>
<td>21±9±0.0</td>
<td>0±0.1</td>
<td>5±3±0.4</td>
</tr>
<tr>
<td>Expt 4</td>
<td>28±3±4.9±3.9</td>
<td>17±8±0.9</td>
<td>0±0.1</td>
<td>5±3±0.4</td>
</tr>
</tbody>
</table>

* LDH release as a percentage of that obtained from fully lysed cells (see Methods).
† Percentage of apoptotic cells was calculated by examination of nuclear morphology (as described in Methods), by staining with acidine orange in experiments 1 and 2, and propidium iodide in experiments 3 and 4. At least 1000 cells were counted for each sample.
‡ Percentage of infected cells was calculated by counting cells staining positive for NP by immunofluorescence (see Methods). At least 1000 cells were counted for each sample.
§ EID₅₀ per ml estimated from EBID₅₀ measurement (see Methods) on virus not treated with trypsin.
ND, Not determined.

Fig. 3. DNA laddering induced by influenza virus in MDCK cells. DNA extracted from control and infected (10 EID₅₀ per cell) cell supernatants 24 h p.i. run on a 1.8% agarose gel and visualized with ethidium bromide. Samples of 15 µl DNA extracted from supernatants of 1 x 10⁶ cells were loaded onto each lane. This is representative of the DNA content from cells released into the medium following different treatments. Lane A, marker (1 kb ladder; Gibco); lane B, clone 7a-infected; lane C, A/Fiji-infected; lane D, control cells; lane E, high molecular mass marker (uncut bacteriophage JDNA; Gibco). Note the increased intensity of bands from clone 7a-infected cell supernatants compared to A/Fiji-infected supernatants, indicating more extensive apoptosis.

than for A/Fiji-infected cells (6 h p.i. vs 12 h p.i.), cells infected with either virus had NP present in the nucleus at 6 h p.i. The presence of NP in the nucleus would be masked by staining DNA with propidium iodide for the detection of apoptosis. Thus, the apparently lower number of infected cells with A/Fiji did not account for the observed differences in cytotoxicity or apoptosis. With regard to virus yield, where significant differences were observed, yields for A/Fiji were greater than those for clone 7a. The apparently high virus yield 2 h p.i. probably represents eluted attached virus that was not removed by washing. In these experiments, residual inoculum was not neutralized by antibody and remaining attached virus was not removed by neuraminidase treatment, as these procedures may have affected the induction of apoptosis. Nevertheless, it is clear that virus yields increase significantly at time-points after 2 h. Interestingly, yields were similar only after applying the EBID₅₀:EID₅₀ conversion factors (see Methods) to correct for the poor ability of A/Fiji to replicate in egg bits. Further protease treatment of virus with trypsin prior to assay in egg bits enhanced A/Fiji titres by about 1 log₁₀ whereas similar treatment of clone 7a enhanced titres by only about 0.2 log₁₀ suggesting that under single-cycle conditions A/Fiji produces as many virus particles as clone 7a, but these particles are largely non-infectious, with uncleaved haemagglutinin.

When both viruses were inactivated by UV irradiation, treatment of MDCK cells with 100 and 10 pg/cell of viral protein (corresponding to 10000 and 1000 EID₅₀ per cell of infectious virus) for 12, 24 and 48 h produced only low levels (< 10%) of cytotoxicity and apoptosis, and any differences in production of apoptosis between the viruses were not apparent (two experiments, results not shown).

Differential cytotoxicity and apoptosis produced by clone 7a and A/Fiji in U-937 cells

Using flow cytometry cell cycle analysis protocols, it was possible to discriminate between apoptotic and normal cells.
Table 2. Levels of cytotoxicity and apoptosis in MDCK cells at various times p.i. with 10 EID$_{50}$ per cell of clone 7a or A/Fiji

Differences between clone 7a and A/Fiji were not significant, except $a$, $P < 0.05$; $b$, $P < 0.01$; and $c$, $P < 0.001$.

<table>
<thead>
<tr>
<th>Time (h p.i.)</th>
<th>% Cytotoxicity (±sd)*</th>
<th>% Cells showing apoptosis (±sd)†</th>
<th>% Cells infected (±sd)‡</th>
<th>Virus yield (log$<em>{10}$EID$</em>{50}$ per ml ±sd)$§$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.1 ± 3.7</td>
<td>0.7 ± 2.8</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>6</td>
<td>3.8 ± 7.8</td>
<td>5.6 ± 2.0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>12</td>
<td>11.7 ± 6.5</td>
<td>7.1 ± 4.8</td>
<td>48.3 ± 2.1$^c$</td>
<td>1.7 ± 1.7</td>
</tr>
<tr>
<td>24</td>
<td>6.3 ± 3.7$^a$</td>
<td>30.9 ± 6.5</td>
<td>97.8 ± 1.5$^c$</td>
<td>30.5 ± 6.3</td>
</tr>
<tr>
<td>36</td>
<td>6.9 ± 3.4</td>
<td>36.3 ± 27.6</td>
<td>97.4 ± 3.0$^c$</td>
<td>35.8 ± 13.5</td>
</tr>
</tbody>
</table>

* LDH release as a percentage of that obtained from fully lysed cells (see Methods).
† Percentage of apoptotic cells was calculated by examination of nuclear morphology (as described in Methods), by staining with propidium iodide. At least 1000 cells were counted for each sample.
‡ Percentage of infected cells was calculated by counting cells staining positive for NP by immunofluorescence (see Methods). At least 1000 cells were counted for each sample.
§ EID$_{50}$ per ml estimated from EBID$_{50}$ (with trypsin added) measurements (see Methods).

Fig. 4. Flow cytometric analysis of apoptosis in U-937 cells. U-937 cells infected with 10 EID$_{50}$ per cell of clone 7a (solid line) or A/Fiji (dashed line) influenza viruses and control cells (shaded) were taken at 24 h p.i., fixed and stained with 7-AAD prior to cell cycle analysis. The first marker (M1) denotes the apoptotic (sub-G$_1$) cell population with reduced cellular DNA content as determined by reduced 7-AAD binding (see Methods), whilst M2 shows the non-apoptotic population. Note the increased sub-G$_1$ peak with both viruses (clone 7a > A/Fiji) in comparison to control cells.

The profiles for U-937 cells infected with clone 7a and A/Fiji are shown in Fig. 4. The results of an experiment in which cytotoxicity and apoptosis were measured at various times p.i. is shown in Table 3; these results are typical of three such experiments. Only low levels of apoptosis were detected at 2 and 12 h p.i., but at 24 h p.i. around 30% of clone 7a-infected cells were undergoing apoptosis, in comparison to about 10% of A/Fiji-infected cells. By 48 h p.i. these figures had risen again to about 40% and 20% for clone 7a and A/Fiji respectively. Cytotoxicity levels were lower than for MDCK cells, with no significant cytotoxicity being seen until 48 h p.i., when that induced by clone 7a was significantly greater than that induced by A/Fiji (Table 3). The virus yields were low, and not significantly different between the two strains. It is possible that the virus yields at all time-points represent residual inoculum, since it is known that influenza virus is poorly productive in monocytic cells (Rodgers & Mims, 1982), and virus was found to be stable in culture medium at 37 °C over this time period (data not shown).

Discussion

Although apoptosis induced by influenza virus has been described in many cell types, such as HeLa cells (Takizawa et al., 1993), human monocytes (Fesq et al., 1994), avian lymphocytes (Hinshaw et al., 1994), murine macrophages (Lowy & Dimitrov, 1995) and Vero cells (Govorkova et al., 1996), to our knowledge this is the first report of differential induction of apoptosis by different influenza virus strains. It is clear that for MDCK cells clone 7a is more cytotoxic and induces more apoptosis than A/Fiji (Fig. 1, Tables 1 and 2). Furthermore, these differences appear not to be due to greater overall replication of clone 7a compared to A/Fiji, because yields of clone 7a were either similar to or less than those of A/Fiji (Tables 1 and 2). Nevertheless, elements of the replication process must occur for these effects to become manifest, because UV-inactivated virus had barely any cytotoxic or apoptotic effect, and apoptosis occurred as a late event p.i. The experiments with U-937 cells were on a smaller scale than those using MDCK cells. Both cytotoxicity and apoptosis
Table 3. Levels of cytotoxicity, apoptosis and infectious virus yields in U-937 cells at various times p.i. with 10 EID₅₀ per cell of clone 7a or A/Fiji

Differences between clone 7a and A/Fiji were not significant, except a, P < 0.001.

<table>
<thead>
<tr>
<th>Time (h p.i.)</th>
<th>7a Fiji</th>
<th>7a Fiji Control</th>
<th>Virus yield (log₁₀ EID₅₀ per ml ± SD) ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>−0.2 ± 0.7 0.1 ± 0.4</td>
<td>1.8 1.2</td>
<td>4.4 5.8 ± 0.3 5.3 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>0.2 ± 0.5 0.7 ± 0.6</td>
<td>0.7 2.2</td>
<td>0.5 5.7 ± 0.1 5.5 ± 0.4</td>
</tr>
<tr>
<td>24</td>
<td>−1.1 ± 1.8 −0.2 ± 1.3</td>
<td>2.64 9.9</td>
<td>3.2 5.7 ± 0.1 5.7 ± 0.1</td>
</tr>
<tr>
<td>48</td>
<td>17.0 ± 0.8 a 6.6 ± 1.7</td>
<td>40.7 18.0</td>
<td>5.2 4.7 ± 0.6 4.8 ± 0.1</td>
</tr>
</tbody>
</table>

* LDH release as a percentage of that obtained from fully lysed cells (see Methods).
† Percentage of apoptotic cells was determined by flow cytometry (see Methods). At least 10000 cells were counted for each sample.
‡ EID₅₀ per ml estimated from EBID₅₀ (with trypsin added) measurements (see Methods).

were less evident than for the experiments with MDCK cells, but the pattern of clone 7a being more active than A/Fiji without its yield being greater than that of A/Fiji was the same. The induction of a lower yield of clone 7a from cells showing greater apoptosis fits with the suggestion that apoptosis may be a cellular mechanism induced to limit virus replication (Martz & Howell, 1989; Teodoro & Branton, 1997). However, influenza virus yields from MDCK cells transfected with bcl-2, an inhibitor of apoptosis, were reduced suggesting that apoptosis was required for maximal yield (Olsen et al., 1996).

What component or product of influenza virus induces apoptosis? The non-structural protein (NS1) of influenza virus A/Turkey/Ontario/7732/66 (H5N9) has some homology to a region of the Fas antigen, a pro-apoptotic protein. Also, co-expression of bcl-2 alters localization of NS1 and NP from the nucleus to the cytoplasm, as well as alteration of the haemagglutinin glycosylation pattern (Hinshaw et al., 1994). Both NS1 and NP are produced early in infection and are candidates for triggering apoptosis. The fact that UV-inactivated virus had little apoptotic activity suggests that a virus-induced product rather than a virion component is involved. In the present work, the differential induction of apoptosis by clone 7a and A/Fiji may reflect differential distribution of viral proteins within subcellular compartments in infected cells. NP was distributed throughout the cytoplasm as well as in the nucleus of clone 7a-infected cells, whereas it was largely restricted to the nucleus of A/Fiji-infected cells until later in infection. Clone 7a derives its matrix and PB2 genes from A/PR/8/34 (H1N1), but all other genes are derived from A/England/939/69 (H3N2) (Matsuyama et al., 1980). Hence, it may be of interest to examine other A/England × PR/8 reassortants, especially as preliminary results show that PR/8 induces an intermediate level of apoptosis compared to that of clone 7a and A/Fiji (unpublished data).

Is the cytotoxicity detected by release of LDH connected with apoptosis? Probably. The release of LDH is consistent with the observation that influenza virus enhances the leakiness of cell membranes in conjunction with bacterial toxins (Jakeman et al., 1991b) and may indicate that influenza viruses are intrinsically harmful to cell membranes either on entry into the cell or budding from it. Apoptosis results in the formation of apoptotic bodies surrounded by an intact plasma membrane (Cohen et al., 1992), but later the membranes surrounding apoptotic bodies may degenerate (Savill et al., 1993), and this would result in the release of LDH. This is the most likely source of the LDH detected in our in vitro cytotoxicity studies. This is supported by the observation that cytotoxicity generally increased significantly after apoptosis had begun. In vivo, apoptotic bodies would probably be engulfed by professional phagocytes before membrane breakdown occurred.

LDH release has been suggested as a marker for replication in the assessment of antiviral compounds active against influenza virus (Watanabe et al., 1995). Our findings suggest caution in drawing this conclusion, because for our two strains the levels of replication (which were essentially similar) cannot be correlated with LDH release (which was clearly far greater for clone 7a). In a similar vein, the cytopathic effect (CPE) of influenza virus is sometimes used as a marker for virus replication, for example in TCID₅₀ determinations. But in this work, production of apoptosis (which is basically responsible for CPE) was different for the two strains for similar levels of replication.

Differential induction of apoptosis between strains has been seen with Thelers’s murine encephalomyelitis virus (Jelachich & Lipton, 1996) and reovirus (Tyler et al., 1995). In
each case the more virulent strain also induced more apoptosis, and in the latter case this was associated with reduced replication (Rodgers et al., 1997). For reovirus, production of pro-inflammatory cytokines also correlated with capacity to induce apoptosis (Farone et al., 1996). Since previous work has shown that clone 7a is more virulent for ferrets than A/Fiji and more rapidly induces a greater and longer fever (Smith & Sweet, 1988), the effects observed in this work may well have some connection with pathogenicity. Apoptosis may, for example, trigger the release of active pro-inflammatory or pyrogenic cytokines. Several cellular enzymes are activated during apoptosis, including interleukin (IL)-1β converting enzyme (ICE), which cleaves pro-IL-1β into active IL-1β (Kumar, 1995). IL-1β is secreted by inflammatory cells and is a pyrogen which induces fever in several animal species (Dinarello, 1992). The fact that cytotoxic effects and apoptosis have been shown in a human histiocytic lymphoma cell line (U-937) is important because cytokines are readily inducible in such cells, and genes involved in both apoptosis and cytokine production are inducible by the same transcriptional regulators (e.g. NF-IL-6 and NF-κB) (Siebenlist et al., 1994; Wada et al., 1995). Two strains which differ in their ability to induce apoptosis may be useful tools in the search for the viral component or cellular products involved in these pathways.

We should like to thank Mr Graham Davies, Department of Anatomy for assistance with flow cytometry and Claire Grattan and Parveen Akhtar for assistance with inactivated viruses and the LDH assay respectively. G.E.P. was supported by Glaxo-Wellcome Research and Development, whilst C.G. and P.A. were supported by Wellcome Trust and SGM vacation stidentships.

References


Influenza virus cytotoxicity and apoptosis


Received 24 March 1997; Accepted 6 June 1997