Influenza A virus-induced apoptosis in bronchiolar epithelial (NCl-H292) cells limits pro-inflammatory cytokine release

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INtroduction

During influenza virus A infection, an inflammatory response occurs in airway epithelia and the inflammatory cytokines and chemokines released at this time have been studied. In vitro studies have concentrated on the infection of macrophage and epithelial cell lines. The cytokines interferon (IFN)-α/β, tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, and the mononuclear cell attractant chemokines macrophage inflammatory protein (MIP)-1α [CCL3; the new classification system for chemokines (Zlotnik and Yoshie, 2000) is used throughout this paper], MIP-1β (CCL4), monocytic chemoattractant protein (MCP)-1 (CCL2), MCP-3 (CCL7), interferon-inducible protein (IP)-10 (CXCL10) and regulated on activation, normal T cell-expressed and -secreted protein (RANTES, CCL5) have been found in influenza virus-infected cultures of human monocytes, rat alveolar cells and murine macrophages (Bussfeld et al., 1998; Hofmann et al., 1997; Matikainen et al., 2000; Nain et al., 1990). In contrast, IL-8 (CXCL8) production is suppressed in human monocytes infected with A/Puerto Rico/8/34 (A/PR/8/34) (Hofmann et al., 1997). In vivo, influenza virus infection of monocytes causes induction and enhanced immigration of mononuclear cells into infected tissue (Kaufmann et al., 2001) but the response to respiratory infection with virus is largely polymorphonuclear (Toms et al., 1977). It is possible that infection of epithelial cells promotes this response. In the normal healthy respiratory epithelium, immunologically competent cells make up less than 2% of the cell population (Danel et al., 1996). An increase of immunologically competent cells occurs after infection perhaps as a result of cytokines/chemokines secreted by epithelial cells. Cytokine/chemokine expression and release has been demonstrated in epithelial cells infected with influenza A virus in vitro, in particular for IL-6, CXCL8 and CCL5, (Choi and Jacoby, 1992; Arndt et al., 2002; Adachi et al., 1997; Matsukura et al., 1996, 1998) and possibly CCL2 (see Julkunen et al., 2000).

The inflammatory response in influenza contrasts with the observation that influenza virus infection results in cell death with the characteristics of apoptosis, hereafter referred to as apoptosis, which should act to prevent an inflammatory response (Raff, 1998). Apoptosis is induced by influenza virus infection in vitro in many cell types, e.g. HeLa and Madin–Darby canine kidney (MDCK) cells (Takizawa et al., 1993), lymphocytes (Hinshaw et al., 1994; Nichols et al., 2001), murine 3T3 fibroblasts (Balachandran et al., 2000), neutrophils (Colamussi et al., 1997; Matsukura et al., 1996). In vivo, influenza virus infection of mononuclear cells causes induction and enhanced immigration of monocytes into infected tissue (Kaufmann et al., 2001) but the response to respiratory infection with virus is largely polymorphonuclear (Toms et al., 1977). It is possible that infection of epithelial cells promotes this response. In the normal healthy respiratory epithelium, immunologically competent cells make up less than 2% of the cell population (Danel et al., 1996). An increase of immunologically competent cells occurs after infection perhaps as a result of cytokines/chemokines secreted by epithelial cells. Cytokine/chemokine expression and release has been demonstrated in epithelial cells infected with influenza A virus in vitro, in particular for IL-6, CXCL8 and CCL5, (Choi and Jacoby, 1992; Arndt et al., 2002; Adachi et al., 1997; Matsukura et al., 1996, 1998) and possibly CCL2 (see Julkunen et al., 2000).

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Infection of cells for measurement of infection, apoptosis and cytotoxicity. RPMI-2650 and NCI-H292 cells were seeded on 9 mm coverslips in 48-well flat-bottomed plates using 0.3 ml diluted cell suspension, or directly into the wells of 6-well plates using 3 ml diluted cell suspension, and allowed to grow to confluence overnight. Monolayers were then washed twice in PBS, virus (5 EID₅₀ per cell) in PBS was added and the plates were incubated at 37°C for 1 h. PBS was added to unoinoculated control wells. After incubation, virus was removed, the monolayers were washed three times with PBS and the plates incubated at 37°C in 5% CO₂ in a humidified atmosphere with 0.3 ml of maintenance medium until the time point for analysis.

Measurement of numbers of infected cells. The number of infected RPMI-2650 and NCI-H292 cells was determined as described previously (Price et al., 1997) using the influenza virus A/X31 (H3N2) nucleoprotein (NP) mouse monoclonal antibody NP147 (kindly provided by A. Douglas, NIMR, UK) as the primary antibody and goat anti-mouse IgG-FITC conjugate (Sigma) as the secondary antibody. Propidium iodide (PI; Sigma) (1 μg ml⁻¹ in PBS) was used as the nuclear stain. Coverslips were mounted on glass slides using DABCO/glycerol anti-fade mountant. Infected cells were identified by the presence of green fluorescence from the FITC molecule within them.

Measurement of apoptosis. Apoptosis was quantified using the TUNEL (TdT-mediated dUTP nick-end labelling) method (Boehringer Mannheim), following the manufacturer’s instructions, which preferentially labels DNA strand breaks in apoptotic cells with the dye tetramethyl rhodamine (TMR). The nuclear counterstain used was DAPI (Boehringer Mannheim) and the percentage of apoptotic cells was determined by counting the number of DAPI-positive (whole cell population) and TUNEL-TMR-positive (apoptotic) cells under an UV microscope.

Cytotoxicity assay. Cytotoxicity assays of infected cells were conducted using the Cytotox 96 kit (Promega) as described previously (Price et al., 1997).

Calculation of total % apoptotic and infected cells. The percentage of dead cells as determined by the cytotoxicity assay was combined with the percentage of apoptotic cells (determined by TUNEL) on the monolayer to give the total percentage of apoptotic cells, i.e. those that had lysed and detached from the monolayer plus those that were apoptotic but remained on the monolayer, as described previously (Mohsin et al., 2002). Similarly, the percentage of cells found dead by the cytotoxicity assay was combined with the percentage of infected cells (determined by fluorescence microscopy) on the monolayer to give the total percentage of infected cells.

Inhibition of apoptosis using specific inhibitors. Cell monolayers were treated with pan-caspase (Z-VAD-fmk), caspase-8 (Z-IE TD-fmk), caspase-9 (Z-LEHD-fmk) inhibitors (all from R and D Systems, Abingdon, UK) or the reactive oxygen species (ROS) inhibitor butylated hydroxyanisole (BHA) (Sigma) 12 h pre-inoculation with virus and immediately after incubation with virus at a concentration of 100 μM, previously shown to be the optimum concentration. The inhibitor was replenished 24 h post-infection (p.i.) in assays that went on longer than this time.

Measurement and quantification of Golgi fragmentation. To show Golgi body and endoplasmic reticulum (ER) morphology, NCI-H292 cells were fixed in methanol at −20°C for 5 min. Cells were then incubated for 30 min at room temperature with primary rabbit anti-human GM130 (Golgi) or anti-human PDI (protein disulfide isomerase, ER) (1:300 in PBS/0.5% BSA) (kindly provided by Dr. M. Lowe, University of Manchester) antibodies. Secondary antibody visualization of the primary antibodies was carried out using goat anti-rabbit IgG Molecular Probes Alexa fluor 568 at a 1:300 dilution. Coverslips were rinsed with PBS, washed 3 times with PBS, and mounted with DABCO/glycerol anti-fade mountant. Infected cells were identified by the presence of green fluorescence from the FITC molecule within them.
1:1000 dilution (2 μg ml⁻¹) (Cambridge Biosciences). Nuclear counterstain DAPI (1 μg ml⁻¹ in methanol) was used to determine apoptosis by morphology and cell nucleus localization. This protocol was used in conjunction for staining for infection, as above.

RNA extraction and semi-quantitative RT-PCR for cytokine/chemokine mRNA. RPMI-2650 and NCI-H292 cells were grown in 6-well plates and, when confluent, infected at an m.o.i. of 5 EID₅₀ per cell or mock-infected with PBS. At the relevant time point p.i./stimulation, 1-5 ml of the maintenance medium was removed to an Eppendorf tube for assay by ELISA (see section below) and the rest discarded. Total RNA extraction was performed by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) using Tri-Reagent (Sigma). RT-PCR was performed as follows. All reagents were from Promega unless stated otherwise. A 20 μl reverse transcription (RT) reaction was set up on ice in a 0-5 ml Eppendorf using 10 μg of RNA chlaurte, 1 μg oligo(dT)₁₅mer primer and made up to 11 μl with DEPC-treated distilled H₂O. This solution was spun briefly for 5 s at 8500 g, boiled for 2 min and replaced on ice. Added to this was 1 x RT buffer (50 mM KCl, 10 mM MgCl₂, 50 mM DTT, 0-5 mM spermidine and 50 mM Tris/HCl, pH 8-3), 0-5 μM of each dNTP (MBI Fermentas), 20 units of RNAse and 5 units of avian myeloblastosis virus reverse transcriptase (AMV RT). The RT reaction was performed on a Mastercycler gradient thermocycler (Eppendorf) and run at 25 °C for 10 min, 42 °C for 1 h and 99 °C for 10 min and placed on ice or stored at −20 °C until used. Negative RT controls, which lacked either AMV RT or sample RNA, were treated similarly. PCR was performed on 5 μl of RT cDNA product in a 50 μl reaction containing forward and reverse (sense and anti-sense) primers (25 pM) (synthesized by MWG Biotech), 1 × PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl) (Gibco-BRL), 1-5 mM MgCl₂, 0-5 mM of each dNTP, made up to 49-5 μl with DEPC-treated distilled H₂O and 2-5 units of Thermus aquaticus YT1 (Taq) DNA polymerase (Gibco-BRL). Negative RT and RNA controls were subject to the same conditions and negative PCR controls were run with 5 μl distilled H₂O instead of cDNA. The thermal cycling program was performed on the same machine as the RT using the following program: initial denaturation at 95 °C for 1 min then x cycles (see primer references) of 95 °C for 45 s, annealing (see primer references) for 45 s, extension at 75 °C for 45 s; then 4 °C until ready for visualization. Primers and conditions used for each set were published previously. The primer sequences and information were obtained from the following references: β-actin, IFN-γ, IL-1β, IL-6, CXCL8, TGF-β1, TNF-α (Nilsen et al., 1998), IL-18 (Tomita et al., 2001), IFN-β (Rommi et al., 1995), CCL5 (Matsukura et al., 1998) and CCL2 (Beck et al., 1999). PCR products were run on 100 ml 1 or 2 % agarose/1 % TBE gels containing 1 μg of ethidium bromide for visualization on an ultraviolet transilluminator. They were run as 10 ml samples with 1 × loading dye (2 μl of 6 × loading dye), alongside a 100 bp DNA ladder (New England Biosciences) at 100 V for approximately 1 h.

Cytokine ELISA analysis. ELISA for cytokines IL-1β, IL-6, CXCL8, TGF-β and TNF-α were performed using the manufacturer’s instructions. Transfected cells were grown in the presence of gentamicin (G418) until clonal colonies had grown. These were trypsinized off the wells and grown separately in 75 cm² tissue culture flasks. Once confluent the cells were trypsinized and seeded on 9 mm glass coverslips in 48-well plates. The cells were stained for anti-GM130 as above and analysed by fluorescence microscopy for co-localization of GFP and Alexa fluor 568 fluorescence indicating insertion of the expressed protein into the Golgi body. Once this had been confirmed cells were maintained as per untransfected NCI-H292.

Statistical analysis. The Student’s t-test and 2-way analysis of variance (ANOVA) with replicates was used to analyse data.

RESULTS

Levels of infection and apoptosis in nasal and bronchiolar cells infected with clone 7a

Fig. 1(a–c) illustrates apoptosis in influenza virus-infected nasal cells. Infection of nasal cells was first observed 12 h p.i. (Fig. 1d). Most (85 %) of the cells inoculated with virus were infected at 12 h p.i. and this increased little with time (maximum 95 % 36–60 h p.i.). Apoptosis was barely detectable at 12 h p.i. but subsequently began to increase, reaching a maximum of ~50 % 36–60 h p.i. (Fig. 1d). Interestingly, not all infected cells became apoptotic. Similarly, infection of bronchiolar cells was first observed 12 h p.i. and the level of infection (~45 %) did not markedly increase with time (maximum ~65 % at 48 h p.i.) (Fig. 1e). In nasal cells 50 % of infected cells became apoptotic whilst in bronchiolar cells 100 % of infected cells became apoptotic.

Cytokine response to influenza A virus infection of epithelial cells

The expression and release of several pro-inflammatory cytokines by nasal and bronchiolar cells after clone 7a infection was examined using RT-PCR and ELISA. The level of expression of β-actin was used as an internal standard for RT-PCR. The cytokines/chemokines expressed in the nasal epithelial cell line (IL-18, CCL2, CCL5) were expressed constitutively (Fig. 2a). mRNA for IL-1β, IL-6, CXCL8, TGF-β and TNF-α could not be detected in infected or uninfected nasal cells (data not shown).

IL-1β, IL-6 and CXCL8 were expressed constitutively in uninfected bronchiolar cells, whilst CCL2 and CCL5 were not expressed (Fig. 2b). In addition, CCL2 and CCL5 expression was induced at 6 and 24 h p.i. respectively. The expression of IL-1β, IL-6 and CXCL8 mRNA was not increased above their constitutive levels as observed in uninfected cells (Fig. 2b). Expression of IL-18, TNF-α and TGF-β could not be detected in infected or clone 7a-infected cells (data not shown).

Supernatants of mock-infected and infected cells from both the human nasal and human bronchiolar cell lines were examined for released cytokine by ELISA. Mock-infected cells and infected nasal cells did not release detectable levels of IL-1β, IL-6 and CXCL8. In addition, expression of IL-18, TNF-α and TGF-β could not be detected in infected or clone 7a-infected cells.
of any cytokine (IL-1β, IL-6, CXCL8, IL-18, CCL2, CCL5, data not shown). Similarly, IL-1β, IL-18 and CCL2 were not released by either uninfected or infected bronchiolar cells. However, IL-6, CXCL8 and CCL5 were released in significant amounts at late time points p.i. (Fig. 3). Release of IL-6 was first detected 24 h p.i. with large amounts, approximately 60 ng ml⁻¹, present in cell culture supernatants by 48 h p.i. (Fig. 3a). CXCL8 release appears to occur later, between 24 h and 48 h p.i., although there is a large amount present by this time, approximately 20 ng ml⁻¹ (Fig. 3b). CCL5 release began between 6 and 24 h p.i. and increased significantly (P<0.02) at 48 h p.i. to approximately 1200 pg ml⁻¹ (Fig. 3c).

**Effect of caspase inhibitors on apoptosis**

In bronchiolar cells the pan-caspase inhibitor (Z-VAD-fmk), which inhibits caspases-1, -3, -6 and -7, had a marked effect, reducing apoptosis by up to 75% (P<0.01), whilst the caspase-8 inhibitor (Z-IETD-fmk) reduced apoptosis by 67% (P<0.01) (Fig. 4a). The caspase-9 (Z-LEHD-fmk) and ROS BHA inhibitors had little or no effect (Fig. 4a).
In contrast, in nasal cells the greatest amount of inhibition (~40%) occurred with BHA (Fig. 4b). No inhibition was observed with Z-LEHD-fmk while Z-IETD-fmk and Z-VAD-fmk reduced apoptosis only by ~20% and ~25%, respectively (Fig. 4b).

The caspase inhibitors were dissolved in DMSO and BHA in ethanol, both of which can inhibit the generation of ROS, but infected cells treated with DMSO or ethanol at the same dilution as used in the inhibitor-treated samples had no effect on influenza virus-induced apoptosis (Fig. 4a and b). Interestingly, in both cell lines, Z-VAD-fmk reduced spontaneous apoptosis and so did Z-IETD-fmk in uninfected bronchiolar cells (Fig. 4a and b), indicating that these
caspases are involved in spontaneous apoptosis. The level of infection in both types of cells remained unaffected by any of these treatments and thus the reduction in apoptosis could not be attributed to a reduction in infection (data not shown).

**Effect of caspase inhibitors on pro-inflammatory cytokine release and expression**

Treatment of infected cells with the caspase-8 inhibitor Z-IETD-fmk and the downstream pan-caspase inhibitor Z-VAD-fmk did not increase viral titres (data not shown), despite inhibiting the induction of apoptosis, which is at variance with the hypothesis that virus-induced apoptosis is an antiviral event (Fig. 4b). Therefore, these inhibitors were used to examine their effect on cytokine release (Fig. 5a). Treatment of influenza virus-infected bronchiolar cells with Z-VAD-fmk, significantly increased the release of IL-6, CXCL8 and CCL5 compared to clone 7a alone (P<0.01 in each case) (Fig. 5a). Z-IETD-fmk also increased cytokine/chemokine release (P<0.02 in each case). BHA had no significant effect. Thus, virus-induced apoptosis limits the amount of pro-inflammatory cytokines released by infected bronchiolar epithelial cells.

IL-6 and CXCL8 mRNA expression remained at constitutive levels following treatment with caspase inhibitors (Fig. 5b). However, CCL5 mRNA expression, which was barely detectable in untreated infected cells (Fig. 5b), appeared to be greater in the presence of the inhibitor and expression was prolonged presumably because of increased cell survival. Z-VAD-fmk was better able to rescue mRNA expression than was Z-IETD-fmk.
Influenza A virus-induced apoptosis is associated with fragmentation of the Golgi ribbon

While the increased release of CCL5 correlated with increased CCL5 mRNA levels (Fig. 5b), the increased levels of IL-6 and CXCL8 released did not, as mRNA was expressed at similar levels in treated or untreated infected cells. A possible explanation for this is that secretory organelle morphology may be disrupted during apoptosis. Recently it has been shown that the Golgi ribbon fragments during apoptosis due to caspase-3 dependent cleavage of the stacking protein GRASP65 (Lane et al., 2002) and caspase-2 dependent cleavage of Golgin-160 (Mancini et al., 2000). Fragmentation of the Golgi ribbon may prevent secretion of mature cytokines by the cell during infection-induced apoptosis. In uninfected cells the ER can be seen to be in a juxtanuclear and cytoplasmic network (Fig. 6a), whilst the Golgi is a defined ribbon-like structure (Fig. 6b). In infected cells undergoing apoptosis, the structure of the ER remains intact, as shown in Fig. 6c, while the Golgi ribbon is fragmented (Fig. 6d). This suggests that the disruption of secretory organelle morphology during apoptosis may limit the release of cytokines.

Fig. 6. Secretory organelle morphology in human bronchiolar epithelial cells during influenza A virus-induced apoptosis. Fluorescence micrographs depicting the morphological state of the ER (a) and Golgi ribbon (b) in uninfected healthy control cells and of the ER (c) and Golgi ribbon (d) in cells undergoing apoptosis due to clone 7a-infection. Human bronchiolar epithelial cells were fixed and stained using primary rabbit anti-human GM130 (Golgi) and PDI (ER) antibodies. These were visualised using secondary anti-rabbit IgG-FITC conjugates and DAPI nuclear counterstain 18 h p.i. (i) = DAPI nuclear stain, (ii) = staining for infection (anti-NP147), (iii) = staining for secretory organelle, (iv) = merge, and arrows indicate the position of cells undergoing apoptosis as identified by DNA condensation.
stable until late apoptosis (secondary lysis) when the cell begins to decompartmentalize and collapse (Fig. 6c). Unlike the ER, the Golgi ribbon fragmented away from its juxtanuclear position in small, scattered pieces (Fig. 6d). Golgi ribbon fragmentation could be rescued by Z-VAD-fmk and to a lesser extent by Z-IETD-fmk (Fig. 7), although some fragmentation was seen, representing a more abundant intermediate stage of mixed ribbon and scattered fragments (data not shown as any scattering was considered indicative of fragmentation due to active caspases). However, many more cells were visible on the caspase-inhibited monolayers and therefore many more with a complete or near complete Golgi ribbon, when compared with clone 7a-infected cells (data not shown), indicating inhibition of caspases rescues cleavage of this particular secretory organelle. In contrast, Z-LEHD-fmk, the caspase-9 inhibitor that could not abrogate apoptosis in this system, was also unable to rescue Golgi morphology. Thus, we have shown that the Golgi body is a major target of caspases in influenza A virus-induced apoptosis, acting possibly to halt secretion of pro-inflammatory cytokines as well as other secreted proteins.

Caspase-resistant GRASP65 protects IL-6 and CXCL-8 secretion

The Golgi protein GRASP65 has been shown to be important for higher order structure of the Golgi cisternae and is cleaved by caspase-3 during apoptosis. This also occurs during influenza A virus-induced apoptosis in

![Fig. 7. Quantification of Golgi ribbon fragmentation in infected human bronchiolar cells with or without treatment with caspase inhibitors.](image_url)

(a) Fluorescence micrographs depicting the morphological state of the Golgi ribbon in uninfected healthy control cells (i) and cells undergoing apoptosis due to clone 7a-infection (ii). These morphological states were used to define cells with healthy or apoptotic Golgi ribbon morphology for quantification. (b) Quantification of the mean percentage of cells with scattered Golgi ribbon in uninfected cells (mock), 7a-infected cells (7a) and 7a-infected cells treated with the caspase inhibitors Z-VAD-fmk (VAD), Z-IETD-fmk (IETD) or Z-LEHD-fmk (LEHD). Fluorescence microscopy quantification was carried out by counting more than 200 cells per coverslip and three coverslips were counted per treatment (n = 3). All counts were done blind. Coverslip areas to be counted were chosen at random by moving the field of view out of focus and then settling on a new area of the monolayer. Results show the means (± SD) of one representative of three independent experiments.

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human bronchiolar epithelial cells (Fig. 6d) and is rescued by pan-caspase and caspase-8 inhibitors (Fig. 7). To assess whether this cleavage was responsible for perturbation of IL-6 and CXCL8 release the bronchiolar cells (NCI-H292) were stably transfected with wild-type (wtH292) or caspase-resistant GRASP65 (3ΔH292) in an EGFP vector. The caspase-resistant GRASP65 has mutations at three sites normally cleaved by caspase-3 that prevent this occurring (Lane et al., 2002). Double staining with antibody to Golgi and localization of GFP expressed as a fusion protein with GRASP65 indicated that both wtH292 and GRASP65 (3ΔH292) localized to the Golgi (data not shown). Approximately 70–80% of the cells expressed GFP, indicating that they were stably transfected. After infection of the cells, supernatants were collected at 48 h p.i. and analysed for CCL5, IL-6 and CXCL8 content.

Both IL-6 (Fig. 8a) and CXCL8 (Fig. 8b) levels were significantly higher in supernatants of 3ΔH292 than wtH292 or untransfected H292 cells (P<0.02 and P<0.01 respectively). However, levels were also increased in wtH292 compared to H292 cells, suggesting that integration of more copies of the gene causes more protein to be expressed which counteracts caspase cleavage, or that the transfection process itself stimulates some cytokine release. CCL5 levels were not protected and indeed its secretion significantly decreased in both wtH292 and 3ΔH292 cells compared to untransfected H292 cells (P<0.02). It appears that transcription is more important for the CCL5 response and suggests that its production is uncoupled from the IL-6 and CXCL8 response.

**DISCUSSION**

Influenza virus induces apoptosis in human respiratory cells of nasal and bronchiolar origin. However, apoptosis is generally regarded as a mechanism that limits the inflammatory response as apoptotic cells are phagocytosed and destroyed before they liberate their cellular contents. This contrasts with observations in humans and ferrets that influenza is associated with a profound inflammatory response in the respiratory airways (Toms et al., 1977). This general conclusion that apoptosis is anti-inflammatory has been questioned recently for *Shigella* infection (Zychlinsky et al., 1994; Zychlinsky & Sansonetti, 1997a, b). In this case, caspase-1 (ICE) is the critical regulator and its activation leads to the processing of intracellular stores of IL-1 and IL-18. Thus, infection of an immune cell may lead to a pro-inflammatory response despite the cell undergoing apoptosis. Interestingly caspase-1 deficient mice develop normally, indicating that this caspase may be intrinsic to defence against infection and is not required in developmental apoptosis (Sansonetti et al., 2000). Similarly, influenza A virus infected macrophages/monocytes, known to undergo apoptosis, also produce IL-1 and IL-18 in this manner (Julkunen et al., 2000, 2001; Matikainen et al., 2000; Pirhonen et al., 1999, 2001).

Could this happen with influenza virus-infected respiratory epithelial cells? In response to influenza A virus infection epithelial cells including bronchiolar and lung cells produce a limited number of cytokines such as the type I interferons (IFN-α/β) and IL-6 and chemokines such as CCL5, CCL2 and CXCL8 (Adachi et al., 1997; Julkunen et al., 2000, 2001; Matsukura et al., 1996). We have confirmed the release of IL-6, CXCL8 and CCL5 in this study but have also shown that CCL2 mRNA is expressed but the cytokine is not released in detectable quantities. However, most importantly, inhibition of caspase activation and hence apoptosis...
causes an increase in the release of pro-inflammatory cytokines. Thus, influenza A virus-induced apoptosis does act to limit the pro-inflammatory cytokine response, but not by the accepted mechanism. However, induction of apoptosis is insufficient to repress the pro-inflammatory response to infection, once engaged. In contrast, no cytokines were released from nasal cells.

The results obtained here are important in the context of the innate immune response during influenza. Influenza A virus infection and replication occurs primarily in the upper respiratory tract of humans where the respiratory epithelial cell is the dominant cell type present when compared to intraepithelial leukocytes (Danel et al., 1996), so virus infection and the specific response will occur primarily in these cells. However, nasal cells apparently do not release pro-inflammatory cytokines and thus it is unclear how the inflammatory response is initiated at this site. Either the nasal cell line examined here is not a good model for the in vivo situation or the small number of phagocytes present in nasal tissue perform the function. Studies with other nasal cell lines or primary nasal tissue are required to clarify this. The limiting of the inflammatory response in the narrow lower airways may be extremely important for the pathogenesis of the virus as this could have profound effects on respiration. Blockage of the narrow airways of the upper respiratory tract of neonatal ferrets following clone 7a infection can be lethal whereas infection with a less virulent virus, which induced less of an inflammatory response, was not lethal (Collie et al., 1980; Husseini et al., 1983). In another scenario, a virus that induces less apoptosis may cause indirect tissue damage due to elevated pro-inflammatory cytokine release. Conversely, if a consequence of virus-induced apoptosis in the lower respiratory tract is a reduction in the release of pro-inflammatory cytokines, and therefore a reduction in the immune response, then the virus may spread further down the respiratory tree to hinder oxygen exchange and allow fluid leakage in to the lungs, resulting in pneumonia. These are important points that warrant further study.

Although inhibition of infected cells undergoing apoptosis may simply buy more time for the cells to release cytokines, how is it that apoptotic cells hinder the release of cytokines? The downstream targets of caspasas are many. As in staurosporine-treated HeLa cells (Lane et al., 2002), the Golgi ribbon fragments during influenza A virus-induced, caspase-8 dependent apoptosis. One protein cleaved by the host cell caspasas is the Golgi stacking protein GRASP65 (Lane et al., 2002). Another is Golgin-160 (Mancini et al., 2000), although its function is unknown. The loss of higher order Golgi structure can be rescued significantly, but not completely, by expression of a caspase-resistant mutant, GRASP65 (Lane et al., 2002). The Golgi is an important organelle through which cytokines must pass during the process of secretion. Why it is necessary to fragment the Golgi body during ligand/chemically stimulated apoptosis is not known (Lane et al., 2002) but in the case of influenza virus, it may be to disrupt the secretion of pro-inflammatory mediators, the production of which is stimulated by the infection. Another virus that limits pro-inflammatory cytokine secretion via its 3A protein is poliovirus (Dodd et al., 2002). However, this is due to an inhibition of ER-to-Golgi traffic and not through apoptosis (Dodd et al., 2002; Doedens et al., 1997). Thus, viruses have evolved different methods of suppressing the pro-inflammatory innate response.

The decrease in CCL5 mRNA production during influenza A virus-induced apoptosis is reversed in the presence of caspase inhibitors indicating that apoptosis may be affecting transcription, possibly through PKR phosphorylation of eIF-2α. However, in bronchiolar cells CCL5 production is regulated by the p38 MAPK/ERK/JNK pathway, and possibly by ROS as secondary messengers (Kujime et al., 2000). The same pathway has also been implicated in influenza A virus-induced apoptosis (Lin et al., 2001). How this pathway mediates an apoptotic response at the same time as CCL5 production, which is reduced by the apoptotic response, is unknown and awaits further research.

It is, therefore, important to identify the virion components that induce apoptosis as the pathway activated appears to vary little between cell lines. NA can induce apoptosis by facilitating the release of TGF-β (Schultz-Cherry and Hinshaw, 1996), but replication dependent events are also required as UV inactivated virus that retains NA activity has reduced capacity to cause apoptosis (Morris et al., 1999). The M1 protein also interacts with caspase-8 but it is not clear whether this is as an inhibitor or activator (Timofeeva et al., 2001; Zhirnov et al., 2002b). The NS1 protein has also been associated with an anti-apoptotic effect using a virus with a deleted ns1 gene (Zhirnov et al., 2002a), possibly relating to its PKR inhibitory activity (Lu et al., 1995; Tan and Katze, 1998, 1999). Recently a new protein was found to be encoded by a second reading frame in the PB1 protein of influenza A virus (Chen et al., 2001). This protein, PB1-F2, induces apoptosis by a cytochrome c-dependent method, particularly in T cells. How it causes apoptosis and why it is T cell specific has yet to be elucidated.

What is clear is that there is mounting evidence to show that influenza viruses induce apoptosis and, from this study, that apoptosis may regulate the cytokine response in virus infection. Studies that reveal how the virus induces and modulates these responses should help to clarify the complex relationship between them and their relevance to the pathogenesis of the virus in vivo, and in particular that of virulent strains with pandemic potential.

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