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

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Infection of cells with influenza A virus results in cell death with apoptotic characteristics. Apoptosis is regarded as a non-inflammatory process. However, during influenza an inflammatory response occurs in the airway epithelium. An examination of this apparent paradox was made using influenza A virus infection of human nasal and bronchiolar epithelial cells. Some cytokine genes (IL-18, CCL2 and CCL5) were expressed constitutively in nasal cells but no cytokine was released. In bronchiolar cells, IL-1$\beta$, IL-6 and CXCL8 expression was constitutive, whilst CCL2 and CCL5 expression was upregulated following influenza virus infection. IL-6, CXCL8 and CCL5 were released but IL-1$\beta$ and CCL2 were not. In bronchiolar cells, cell death was inhibited by the caspase-8 (Z-IETD-fmk) and pan-caspase (Z-VAD-fmk) inhibitors and these inhibitors enhanced expression of *CCL5* and increased the levels of the three secreted cytokines significantly. Thus, the amount of each cytokine released from bronchiolar cells is reduced during cell death, implying that the observed inflammatory response in influenza would be greater if cell death did not occur. Reduced cytokine release is also associated with fragmentation of the Golgi body, as the caspase inhibitors also rescued influenza A virus-induced fragmentation of the Golgi ribbon.

## 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)-$\alpha/\beta$, tumour necrosis factor (TNF)-$\alpha$, interleukin (IL)-1 and IL-6, and the mononuclear cell attractant chemokines macrophage inflammatory protein (MIP)-1$\alpha$ [CCL3; the new classification system for chemokines (Zlotnik and Yoshie, 2000) is used throughout this paper], MIP-1$\beta$ (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 priming 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.*,

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1999; Engelich *et al.*, 2001), and also *in vivo* in mice (Mori *et al.*, 1995; Technau-Ihling *et al.*, 2001) and chickens (Ito *et al.*, 2002). Apoptosis involves the upregulation of Fas (Takizawa *et al.*, 1995), which can be activated by the dsRNA-activated protein kinase R (PKR) and regulated by double-stranded RNA (Balachandran *et al.*, 1998; Tan and Katze, 1999). Fas ligand (FasL), which is upregulated on the cell surface following infection, may also play a role (Fujimoto *et al.*, 1998; Raff, 1998). Apoptosis can also be induced by the activation of latent transforming growth factor (TGF)-$\beta$ on the surface of virus-infected cells, and in the extracellular matrix. Influenza virus neuraminidase (NA) cleaves latent TGF-$\beta$ into its active form and influenza virus-induced apoptosis is reduced by neutralizing TGF-$\beta$ antibodies (Morris *et al.*, 2002; Schultz-Cherry & Hinshaw, 1996). In addition, influenza virus-induced apoptosis can be partially blocked by NA inhibitors, although it is likely that one or more intracellular processes are also involved in apoptotic induction (Morris *et al.*, 1999, 2002). In murine 3T3 cells and MDCK cells, Fas-activated apoptosis is due to influenza A virus infection triggering the recruitment of the Fas-associated death domain (FADD) and caspase-8, which activate downstream caspase proteolysis (Balachandran *et al.*, 2000; Takizawa *et al.*, 1999).

Despite the fact that apoptosis is considered to be an anti-inflammatory event, there is increasing evidence that the processes of apoptosis and inflammatory mediator release may be interlinked in infection. *Shigella* infection of mast cells releases IL-1$\alpha/\beta$ and CXCL8, and this is dependent on the cells undergoing apoptosis (Sansonetti *et al.*, 2000). Apoptosis activates the processing of intracellular stores of these cytokines by caspase-1 (also known as interleukin 1-converting enzyme or ICE) from their precursors to their active form (Zychlinsky *et al.*, 1994). This has also been shown for influenza virus infection of macrophages (Pirhonen *et al.*, 1999, 2001). Here, we investigated whether influenza A virus-induced apoptosis of a human nasal and bronchiolar cell line positively or negatively modulated cytokine release by these cells.

## METHODS

**Cells and viruses.** The influenza virus clone 7a (H3N2) of the reassortant virus A/Puerto Rico/8/34 (H1N1) × A/England/939/69 (H3N2) was grown in 10-day old embryonated hens' eggs as described previously (Price *et al.*, 1997). The human nasal septum squamous carcinoma cell line RPMI-2650 (ATCC) was grown in 90 % Eagle's minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0·225 % sodium bicarbonate, 100 IU penicillin ml$^{-1}$, 100 μg streptomycin ml$^{-1}$, 1 mM sodium pyruvate (Sigma) and 10 % foetal calf serum (FCS) (BioWhittaker). The human muco-epidermoid bronchiolar carcinoma cell line NCI-H292 (ATCC) was grown in 90 % RPMI-1640 medium supplemented with 2 mM L-glutamine, 0·225 % sodium bicarbonate, 100 IU penicillin ml$^{-1}$, 100 μg streptomycin ml$^{-1}$ (Life Technologies), 3·6 M glucose (Fisher Scientific), 10 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma). MDCK-NBL2 cells were grown in 90 % EMEM supplemented as above. Post-infection, cells were maintained in 98 % modified medium with 2 % serum.

**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 $EID_{50}$ per cell) in PBS was added and the plates were incubated at 37 °C for 1 h. PBS was added to uninoculated control wells. After incubation, virus was removed, the monolayers were washed three times with PBS and the plates incubated at 37 °C in 5 % $CO_2$ 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 $NP_{147}$ (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-IETD-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

1:1000 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 with 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 $EID_{50}$ 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 eluate, 1 μg $oligo(dT)_{15mer}$ primer and made up to 11 μl with DEPC-treated distilled $H_2O$. This solution was spun briefly for 5 s at 8500 ***g***, boiled for 2 min and replaced on ice. Added to this was 1× RT buffer (50 mM KCl, 10 mM $MgCl_2$, 50 mM DTT, 0·5 mM spermidine and 50 mM Tris/HCl, pH 8·3), 0·5 mM of each dNTP (MBI Fermentas), 20 units of RNAsin 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 $MgCl_2$, 0·5 mM of each dNTP, made up to 49·5 μl with DEPC-treated distilled $H_2O$ 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 $H_2O$ 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 as 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 (Ronni *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 μl 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, CCL2 and CCL5 (R and D systems, Abingdon, UK) or IL-18 (MBL, distributed by R and D Systems) were carried out by following the manufacturer's instructions. Samples were diluted up to 1000× or 5× for IL-6 or CXCL8 assays respectively. The plates were read on a microplate reader at 450 nm, and again at 540 nm for correction by subtraction of the reading at 540 nm from that at 450 nm. Standard curves were constructed on GRAPHPAD prism software and sample concentration was determined by linear regression analysis.

**Generation of NCI-H292 GRASP65-GFP stable cell lines.** NCI-H292 cells grown in 6-well plates were transfected with wild-type or caspase-resistant rat GRASP65–pEGFP-N2 constructs, kindly provided by Dr. M. Lowe, University of Manchester, using LipofectAMINE PLUS (Invitrogen/Life Technologies) according to the manufacturer's instructions. Transfected cells were grown in the presence of geneticin (G418) until clonal colonies had grown. These were trypsinized off the wells and grown separately in 75 $cm^2$ 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 uninfected 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

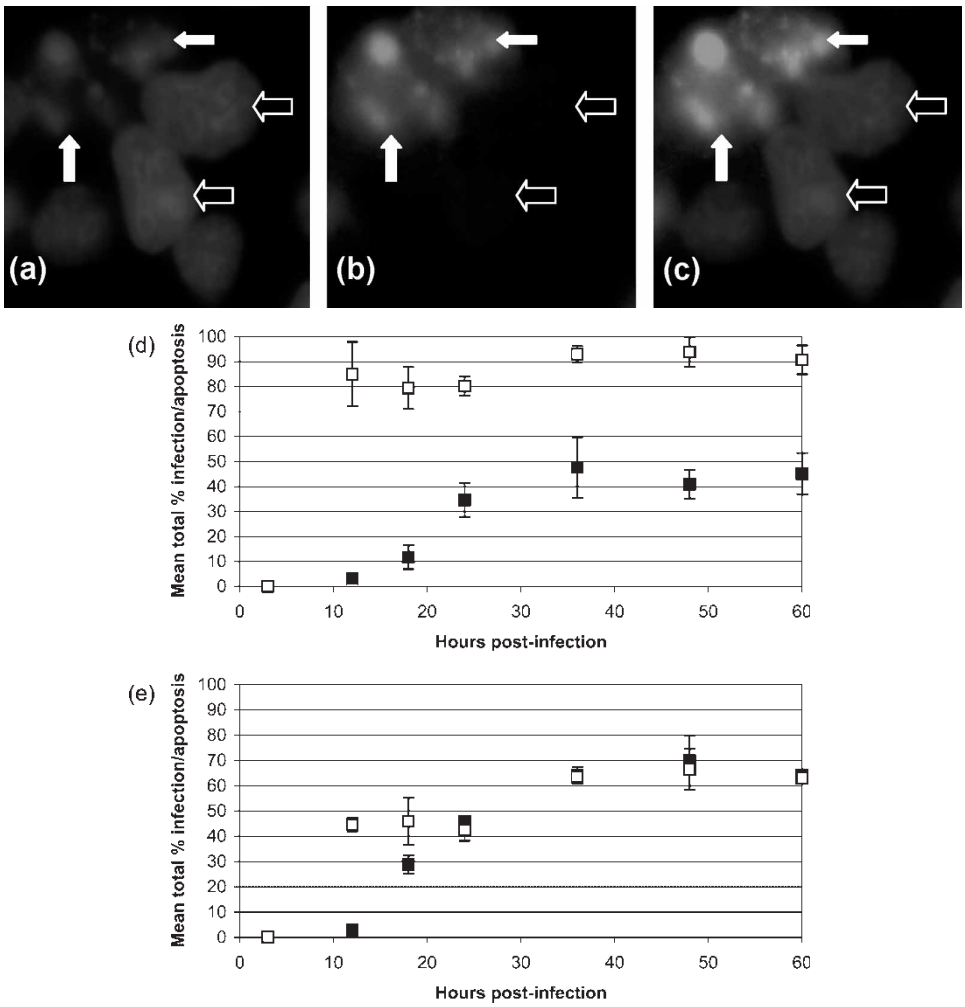


**Fig. 1.** Levels of infection and apoptosis in human respiratory epithelial cell lines were determined by immunostaining techniques using anti-NP + IgG-FITC staining together with a TUNEL-based assay and DAPI staining. Both TUNEL labelling and nuclear morphology as revealed by DAPI staining were used as markers for apoptosis. (a) DAPI staining, (b) TUNEL labelling and (c) merged image of nasal epithelial cells infected with clone 7a. The black-filled arrows indicate the position of non-apoptotic cells whilst the white-filled arrows indicate the position of apoptotic cells. Nasal (d) or bronchiolar (e) epithelial cells were either uninfected or infected with 5 $EID_{50}$ of clone 7a influenza A virus and quantified by counting at various times p.i. for levels of infection (□), determined by the cellular presence of anti-NP + IgG-FITC staining, and apoptosis (■) by TUNEL labelling and DAPI staining. Cell death was also measured by lactate dehydrogenase release using the cytotoxicity assay, allowing calculation of mean total % infection/apoptosis as described in Methods. Results shown are the means (± SD) from a single representative experiment of three, with four replicates in each experiment. More than 500 cells were counted in each replicate.

of any cytokine (IL-1$\beta$, IL-6, CXCL8, IL-18, CCL2, CCL5, data not shown). Similarly, IL-1$\beta$, 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 ($P<0{\cdot}02$) 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 % ($P<0{\cdot}01$), whilst the caspase-8 inhibitor (Z-IETD-fmk) reduced apoptosis by 67 % ($P<0{\cdot}01$) (Fig. 4a). The caspase-9 (Z-LEHD-fmk) and ROS BHA inhibitors had little or no effect (Fig. 4a).

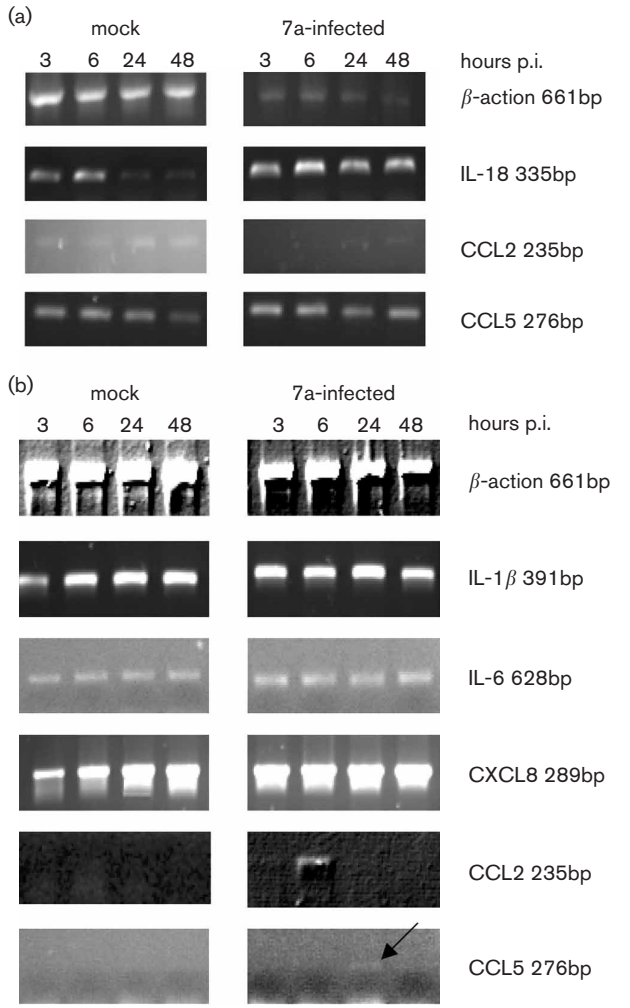


**Fig. 2.** Cytokine expression after influenza A virus infection of human nasal and bronchiolar epithelial cells. Nasal (a) or bronchiolar (b) cells were mock-infected with PBS (mock) or infected (7a) with 5 $EID_{50}$/cell of clone 7a. Total mRNA was extracted from cells at 3, 6, 24 and 48 h p.i. and amplified by RT-PCR using specific primers. All RT-PCR products were resolved through 0·7 % agarose containing ethidium bromide and visualized under UV light. $\beta$-actin was used as the internal standard. Representative results of three independent experiments for each cytokine are shown. The band for CCL5 mRNA, although faint, was observed in all three experiments and is indicated with an arrow.

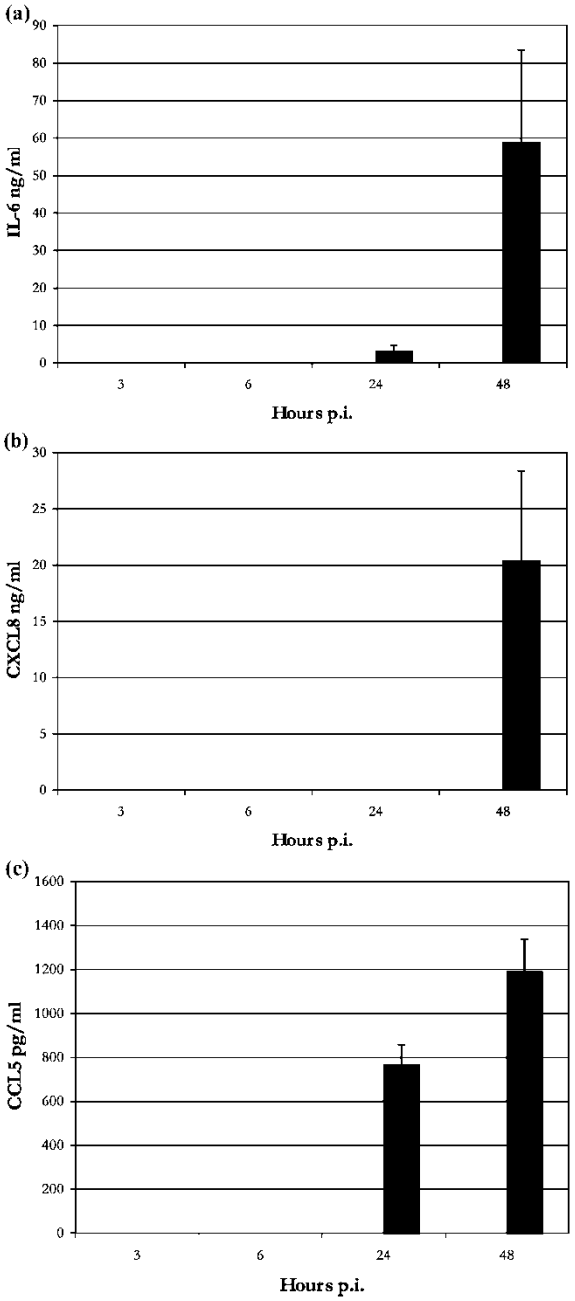


**Fig. 3.** Cytokine secretion from influenza A virus-infected human bronchiolar cells. The supernatants of human bronchiolar cells (NCI-H292) mock-infected with PBS or infected with 5 $EID_{50}$ per cell of clone 7a (■) were collected and analysed for IL-6 (a), CXCL8 (b) or CCL5 (c) content by ELISA. Results are the means (±SD) of one representative of three independent experiments. No cytokines were detected in mock-infected cells.

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

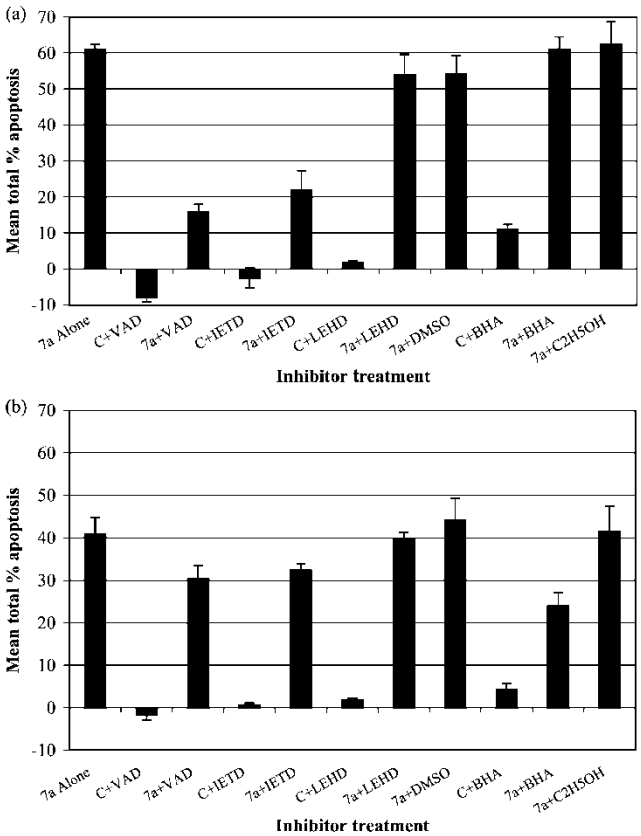


**Fig. 4.** Inhibition of apoptosis in human respiratory epithelial cells. Inhibitors of caspases-1, -3, -6 and -7 (Z-VAD-fmk), caspase-8 (Z-IETD-fmk), caspase-9 (Z-LEHD-fmk) and ROS (BHA) were added to the medium pre- and post-infection with clone 7a (7a) at 100 μM. Control (C) uninfected cultures were treated similarly. Apoptosis was quantified at 24 h p.i. for bronchiolar (a) and nasal (b) epithelial cells. Graphs show results from a single representative experiment of three.

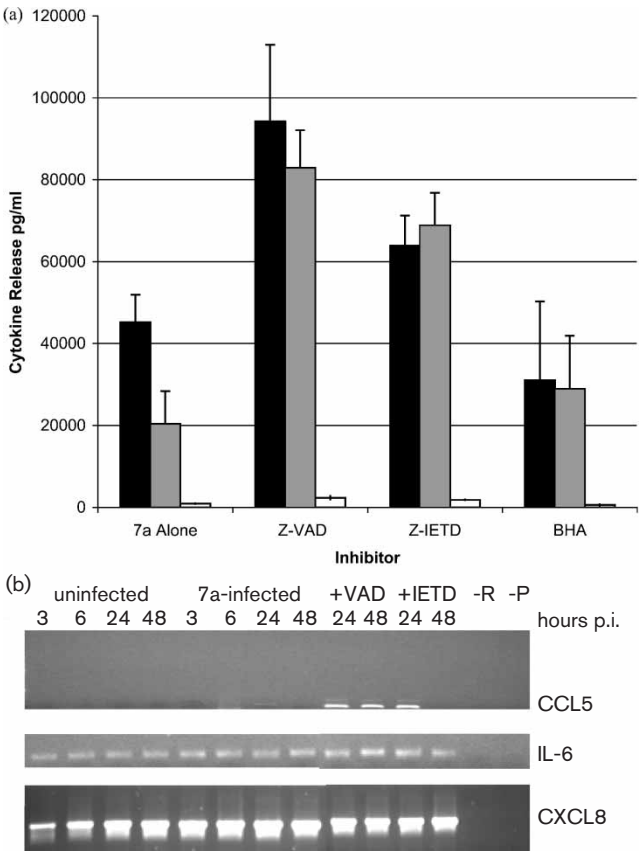


**Fig. 5.** Inhibition of apoptosis increases pro-inflammatory cytokine release and expression. Uninfected and clone 7a-infected human bronchiolar epithelial cell monolayers were incubated in the absence or presence of 100 μM of apoptosis inhibitors Z-VAD-fmk (pan-caspase inhibitor), Z-IETD-fmk (caspase-8 inhibitor) and BHA (ROS inhibitor). (a) Supernatants taken 24 h p.i. were tested by ELISA for IL-6 (black), CXCL8 (grey) and CCL5 (white). Results are the means (±SD) of a single representative of three independent experiments. (b) Total RNA was extracted at 3, 6, 24 and 48 h p.i. from uninfected cells, 7a-infected cells and 7a-infected cells treated with either Z-VAD-fmk (+VAD) or Z-IETD-fmk (+IETD) at 100 μM. CCL5, IL-6 and CXCL8 mRNA was amplified by RT-PCR using specific primers. Results are representative of three experiments. −R = negative RT control, −P = negative PCR control.

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 ($P<0{\cdot}01$ in each case) (Fig. 5a). Z-IETD-fmk also increased cytokine/chemokine release ($P<0{\cdot}02$ 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

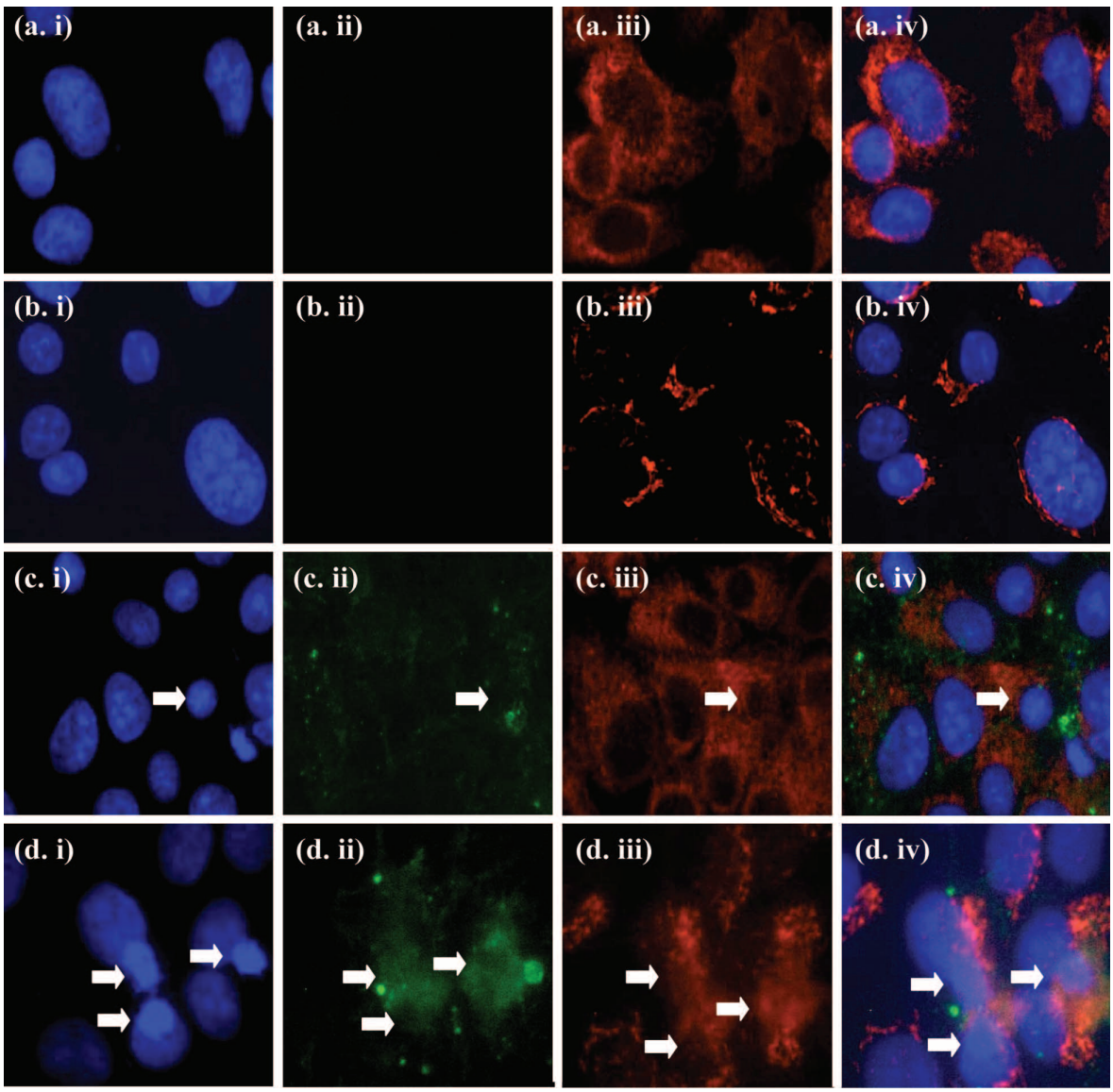


**Fig. 6.** Secretory organelle morphology in human bronchiolar epithelial cells during influenza A virus-induced apoptosis. Fluorescence micrographs depicting the morphological state of the ER (a) and Golgi ribbon (b) in uninfected healthy control cells and of the ER (c) and Golgi ribbon (d) in cells 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-$NP_{147}$), (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

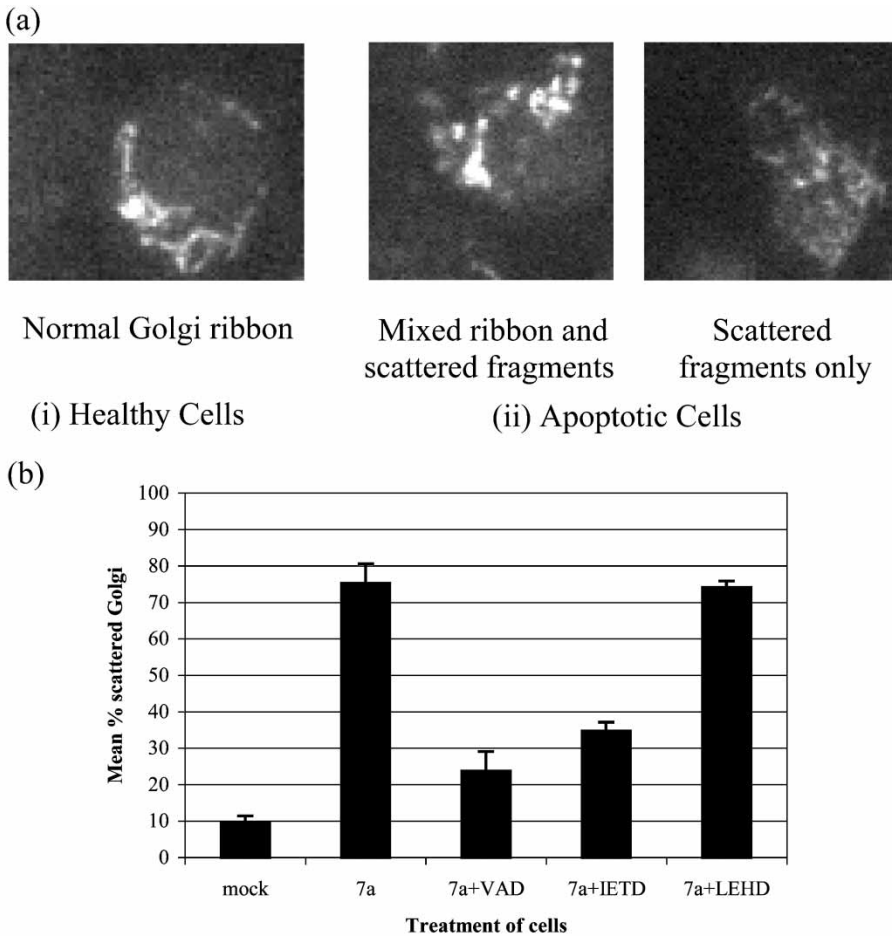


**Fig. 7.** Quantification of Golgi ribbon fragmentation in infected human bronchiolar cells with or without treatment with caspase inhibitors. (a) Fluorescence micrographs depicting the morphological state of the Golgi ribbon in uninfected healthy control cells (i) and cells undergoing apoptosis due 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 ($\pm$SD) of one representative of three independent experiments.

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 ($P<0{\cdot}02$ and $P<0{\cdot}01$ 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 ($P<0{\cdot}02$). 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.

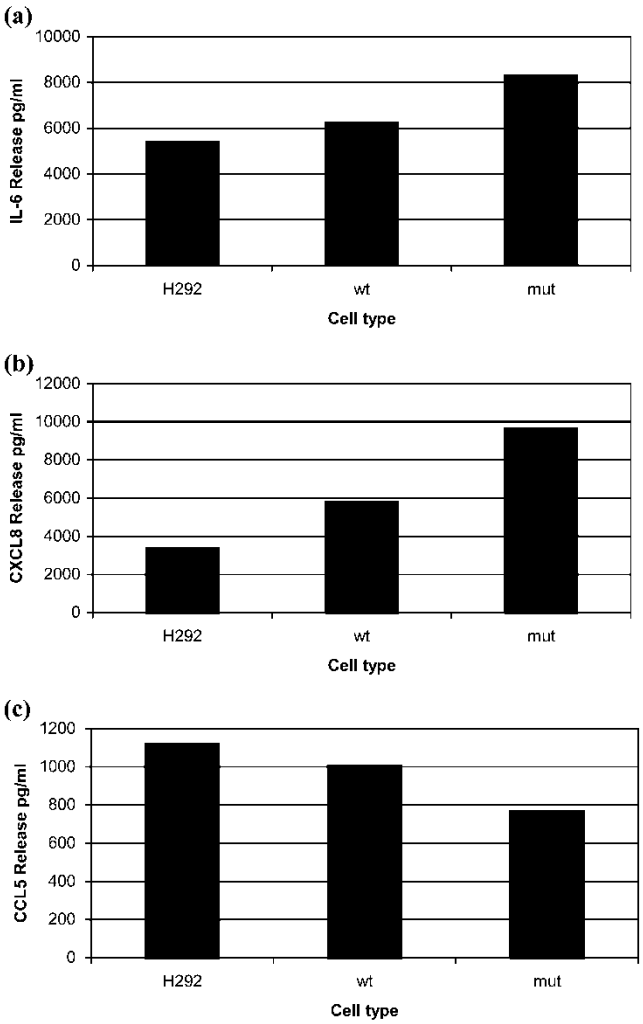


**Fig. 8.** Transfection of human bronchiolar cells with a caspase-resistant form of GRASP65 partially rescues cytokine secretion. Human bronchiolar cells (H292) were transfected with wild-type (wt) or a caspase-resistant mutated (mut) rat GRASP65-GFP using lipofectAMINE PLUS. Cells grown to confluence in 48-well plates were infected with 5 $EID_{50}$/cell of clone 7a and the supernatants analysed for IL-6 (a), CXCL8 (b) or CCL5 (c) content by ELISA at 48 h p.i. The results show the means of three pooled experiments; analysis was done using 2-way ANOVA with replicates.

## 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-$\alpha/\beta$) 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 caspases 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 caspases 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-2$\alpha$. 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-$\beta$ (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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