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No apoptotic deaths and different levels of inductions of inflammatory cytokines in alveolar macrophages infected with influenza viruses

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Abstract

Influenza viruses are reported to infect mainly the respiratory tract epithelium of hosts. Our studies in a pig model show that influenza A viruses infect alveolar macrophages that constitutively reside in the respiratory tract, without causing apoptosis. Tumor necrosis factor alpha was the inflammatory cytokine most highly induced in these macrophages. In vivo, alveolar macrophages infected with human H3N2 influenza virus showed greater expression of tumor necrosis factor alpha than did alveolar macrophages infected with human H1N1 influenza virus. Induction of specific inflammatory cytokine such as TNF-α is a polygenic trait that involves the HA and NA genes. Markedly elevated expression of tumor necrosis factor alpha may be responsible for the high mortality rate caused by H3N2 influenza virus infection in elderly patients.

Keywords: Apoptotic death; Inflammatory cytokines; Influenza viruses

Introduction

Influenza is a leading cause of human morbidity and mortality worldwide (Monto, 1997). Each year in the United States alone, influenza is responsible for more than 30 000 excess deaths, 250 000 hospitalizations, and the loss of billions of dollars (Simonsen et al., 1997). The elderly account for about 90% of the excess mortality and 50% of the excess hospitalizations (Nordin et al., 2001). Studies have shown that influenza A viruses of the H3N2 subtype are the primary cause of the excess mortality, more than 90% of which occurs in persons 65 or more years of age (Thompson et al., 2003). Influenza virus infection triggers strong inflammatory responses that can result in fatal pneumonia (Stuart-Harris, 1961). Influenza viruses are thought to infect mainly the respiratory-tract epithelial cells of animals or humans. In vitro, influenza viruses are able to infect a variety of cells, including Madin–Darby canine kidney (MDCK) cells, human monocytes, and human monocyte-derived macrophages (Bussfeld et al., 1998; Hofmann et al., 1997). However, it has not been demonstrated whether influenza viruses can infect alveolar macrophages that constitutively reside in the respiratory tracts of natural influenza virus hosts.

Pigs have been proposed to serve as "mixing vessels" for the creation of reassortant human influenza strains, because the epithelial cells of their respiratory tracts have receptors for both avian and human influenza viruses (Ito et al., 1998; Scholtissek et al., 1985). Pigs are susceptible to the H1N1 and H3N2 subtypes of human influenza A viruses, and influenza in pigs was reported during the devastating 1918–1919 human influenza pandemic. The clinical signs in pigs include nasal discharge, coughing, fever, labored breathing, and conjunctivitis (Shope, 1931). When respiratory-tract epithelial cells and alveolar macrophages are infected with influenza viruses, they die by

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necrosis. Cell necrosis triggers inflammatory responses and a strong immune response by inducing the production of inflammatory cytokines whereas apoptosis does not induce inflammatory responses or strong immune responses. In apoptotic cells, chromatin aggregates at the nuclear membranes, forming apoptotic bodies that are engulfed by adjacent macrophages, thus preventing inflammatory responses (Duvall and Wyllie, 1986; Granville et al., 1998; Kerr and Harmon, 1991; Wyllie et al., 1980).

Previous studies have indicated that cells derived from nonrespiratory organs die of apoptosis, not of necrosis, when infected with influenza viruses (Fesq et al., 1994; Hinshaw et al., 1994; Lin et al., 2001; Nordin et al., 2001; Schultz-Cherry et al., 2001; Takizawa et al., 1993). These studies have examined MDCK cells infected with equine influenza virus (Lin et al., 2001) and MDCK, HeLa, and mink lung epithelial (Mv1Lu) cells infected with influenza A and B viruses (Hinshaw et al., 1994; Nordin et al., 2001). In contrast, human or porcine respiratory epithelial cells undergo necrosis rather than apoptosis when infected with influenza virus (Arndt et al., 2002; Seo et al., 2001), as shown in normal human bronchial epithelial cells (NHBE) infected with A/PR/8/34 (H1N1) influenza virus (Arndt et al., 2002) and St. Jude porcine lung epithelial cells (SJPL) infected with human, swine, or avian influenza viruses (Seo et al., 2001). However, the mechanism of cell death of alveolar macrophages that constitutively reside in the respiratory tract is not known.

We undertook studies in a pig model to determine whether influenza virus can infect alveolar macrophages in the respiratory epithelium, whether human H3N2 influenza A viruses induce stronger inflammatory responses in alveolar macrophages than do human influenza B or H1N1 viruses, and whether influenza infection causes apoptosis of alveolar macrophages.

Results

In vitro infection of cells

It is generally accepted that influenza viruses can infect respiratory epithelial cells in humans or animals. We wanted to determine in the pig model whether macrophages that constitutively reside in the respiratory tract can be infected with influenza virus. The cells isolated by BAL in post-mortem pigs were stained with macrophage-specific antibody to confirm their identity. More than 98% of cells were identified as macrophages (Fig. 1A). To confirm whether alveolar macrophages can be infected in vitro with influenza virus, cells were incubated with A/Sydney/5/97 (H3N2) virus and then immunologically stained. Infected cells were positively stained, but uninfected cells were not stained (Fig. 1B). Viral titers were measured in the supernatants of the infected alveolar macrophages as $\log_{10}EID_{50}$/ml. The infectivity titers of the three viruses were similar, and they peaked 48 h post-inoculation (p.i.) (Fig. 1C).

Receptor specificity

It is suggested that pigs can serve as "mixing vessels" for swine and human influenza viruses because their tracheal cells has both types of receptors (Ito et al., 1998). We therefore determined the receptor specificity of porcine alveolar macrophages (Fig. 1D). These cells expressed surface sialylglycoconjugates with Sia2–3Gal and Sia2–6Gal linkages, although expression of Sia2–6Gal (human virus) receptors was higher. The peak log fluorescence intensity of Sia2–3Gal-containing and Sia2–6Gal-containing receptors was 2.2 and 2.8, respectively.

In vivo infection

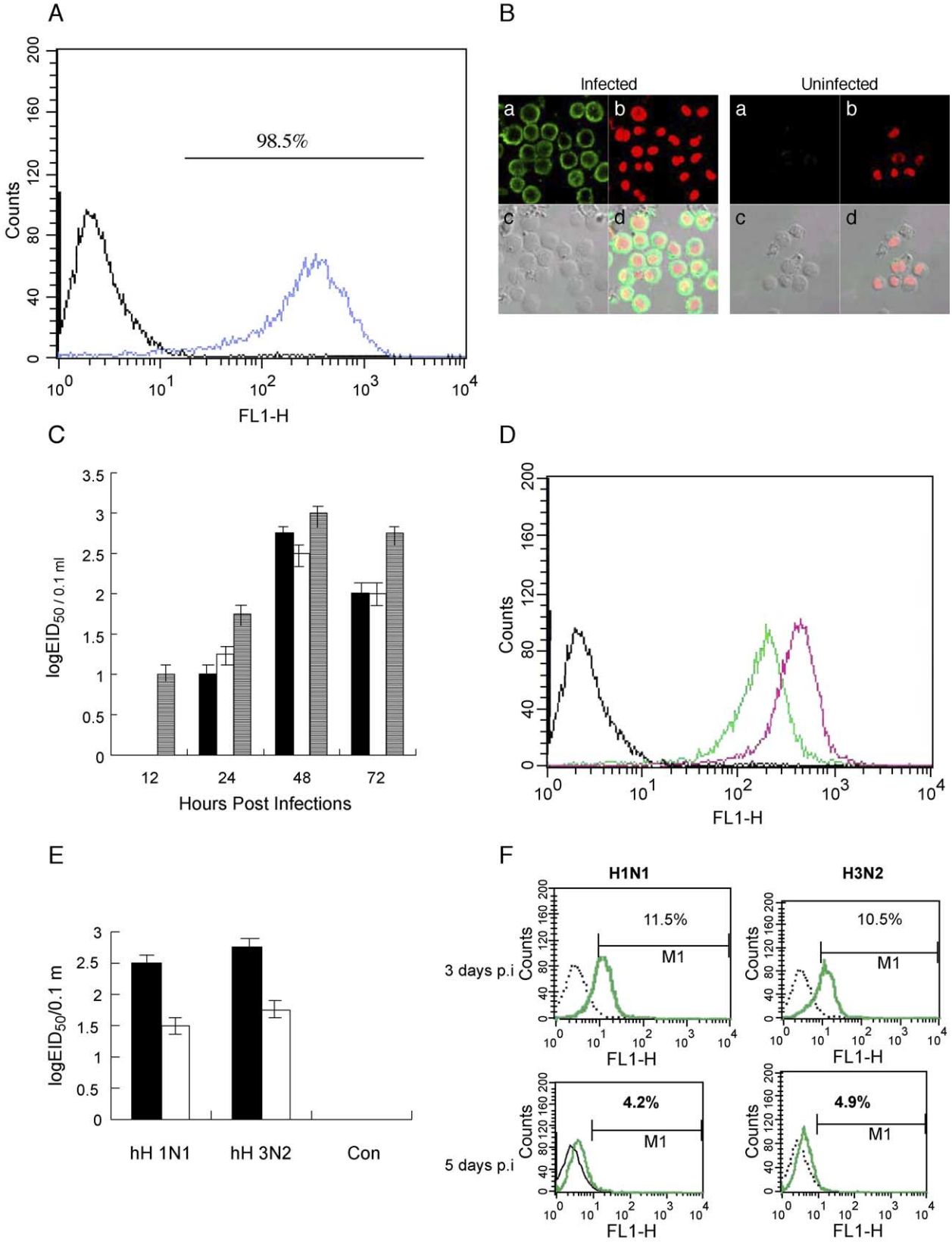
Previous studies showed that blood monocyte-derived human macrophages can be infected with influenza viruses in vitro (Fesq et al., 1994), but it was not known whether alveolar macrophages residing constitutively in the respiratory tracts of natural human or swine hosts can be infected with influenza viruses in vivo. We measured virus titers in alveolar macrophages obtained from pigs inoculated with human influenza A viruses (Fig. 1E). A/Sydney/5/97(H3N2) and A/New Caledonia/1/99 (H1N1) influenza viruses replicated well in alveolar macrophages at 3 and 5 days p.i., but viral titers were higher 3 days p.i. To determine what percentages of alveolar macrophages were infected, we stained alveolar macrophages with antibodies to H3N2 or H1N1 influenza viruses before flow cytometric analysis (Fig. 1F). The proportion of infected alveolar macrophages was <12% and <5% at 3 and 5 days p.i., respectively.

Death of infected alveolar macrophages by apoptosis vs. necrosis

In previous studies, human and porcine respiratory epithelial cells did not undergo apoptosis when infected with influenza viruses (Arndt et al., 2002; Seo et al., 2001). In porcine alveolar macrophages infected in vitro with A/New Caledonia/1/99 (H1N1), B/Shichuan/99, or A/Sydney/5/97(H3N2) viruses, we observed no characteristic apoptotic laddering of DNA, although alveolar macrophages treated with an apoptosis-inducing chemical, staurosporine, showed DNA laddering (Fig. 2A). During apoptosis, the specific calcium-dependent endonucleases nick DNA, causing double-stranded breaks (Duvall and Wyllie, 1986) that can be revealed by fluorescent staining when cells die of apoptosis. Flow cytometric analysis showed no apoptosis of alveolar macrophages infected with A/Sydney/5/97(H3N2) or of uninfected

control alveolar macrophages, whereas alveolar macrophages treated with staurosporine did undergo apoptosis (Fig. 2B).

We next investigated whether alveolar macrophages infected with influenza virus undergo apoptosis in vivo (Fig. 3). At 5 days p.i., less than 2% of alveolar



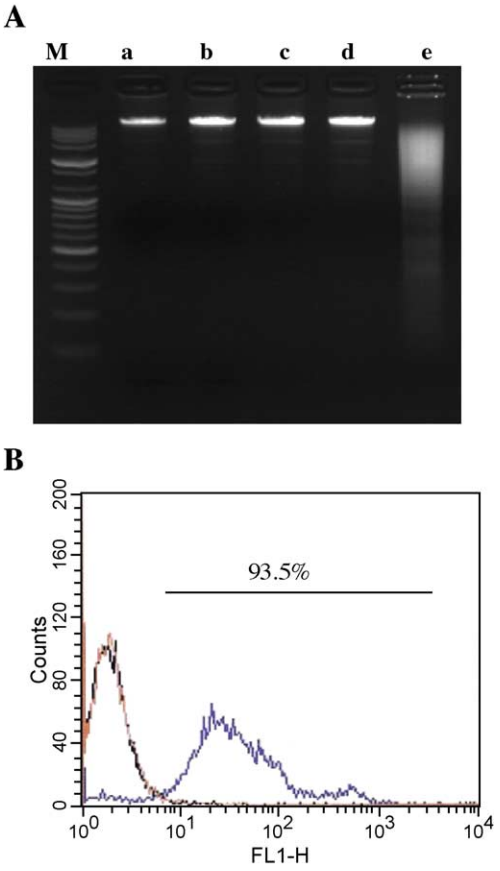


Fig. 2. Detection of apoptosis in alveolar macrophages. (A) Alveolar macrophages were incubated with human influenza viruses before DNA fragmentation assay. Molecular markers (M); cells incubated for 48 h with A/New Caledonia/1/99 (H1N1) (a), B/Shichuan/99 (b), A/Sydney/5/97 (H3N2) (c), PBS (d), or staurosporine (0.01 μg/ml) (e). (B) Alveolar macrophages were incubated for 48 h with A/Sydney/5/97 (H3N2) virus before flow cytometric analysis. Cells were incubated with PBS (black line), A/Sydney/5/97 (H3N2) (yellow line), or staurosporine (0.01 μg/ml) (blue line).

macrophages from pigs inoculated with A/New Caledonia/1/99 (H1N1) virus (Fig. 3A) or A/Sydney/5/97(H3N2)-infected (Fig. 3B) or from uninfected pigs (Fig. 3C) were apoptotic, compared to 58.24% of alveolar macrophages treated in vitro with staurosporine (Fig. 3D). These results suggest that influenza virus-infected alveolar macrophages in the respiratory tract do not die of apoptosis in vivo.

Induction of inflammatory cytokines

If alveolar macrophages infected with influenza viruses do not die of apoptosis, then they may induce strong inflammatory responses (Arndt et al., 2002). Inflammatory cytokines were previously shown to be induced in blood monocyte-derived murine and human macrophages infected with influenza viruses (Fesq et al., 1994), but there was no information about cytokine induction in the alveolar macrophages of natural hosts of influenza viruses. We quantified cytokines in alveolar macrophages infected in vitro with influenza viruses (Fig. 4A). The inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α were induced. Among these, TNF-α was most highly induced, and H3N2 influenza viruses induced significantly greater expression of TNF-α than did H1N1 or influenza B viruses ($P < 0.05$). The copies of TNF-α per 1000 copies of β-actin induced by A/Sydney/5/97(H3N2), A/New Caledonia/1/99 (H1N1), or B/Shichuan/99 were 9500, 3500, and 3000, respectively. To compare TNF-α protein expression induced by the three human influenza viruses, we assayed TNF-α by enzyme-linked immunosorbent assay (ELISA) in infected macrophages. TNF-α protein increased as the duration of infection increased (Fig. 4B). Human H3N2 virus induced significantly higher production of TNF-α protein than did human H1N1 or B viruses ($P < 0.05$). The amount of TNF-α by A/Sydney/5/97(H3N2), A/New Caledonia/1/99 (H1N1), or B/Shichuan/99 at 48 h p.i were 550, 150, and 50 pg/ml, respectively.

We next determined whether the inflammatory cytokines could be induced in alveolar macrophages in vivo (Figs. 4C,D). Six pigs in each group were infected with influenza A viruses, and alveolar macrophages were collected and pooled 3 or 5 days p.i. The infected pigs showed clinical symptoms similar to those of humans, such as fever (>39 °C), difficulty in breathing, runny nose, and loss of appetite. IL-6 and TNF-α were detected. Unlike the in vitro findings, IL-1β and IL-8 were not detected. H3N2 virus induced greater expression of TNF-α than did H1N1 virus. The TNF-α copy numbers per 1000 β-actin copies

Fig. 1. Infection of porcine alveolar macrophages with human influenza viruses. (A) Purified alveolar macrophages were stained with macrophage-specific antibody MCA874F (blue line) or isotype-matched control antibody (black line) to confirm their identity. (B) Alveolar macrophages in a chambered slide were incubated for 48 h with A/Sydney/5/97 (H3N2) or PBS and labeled with antinucleoprotein monoclonal antibody and FITC-labeled secondary antibody (X151). (a) FITC-labeled antibody staining; (b) propidium iodide staining; (c) negative image; (d) merged panels. (C) Alveolar macrophages were infected with human influenza viruses and the $\log_{10}EID_{50}$/0.1 ml of virus was determined in aliquots of supernatant collected at various times. Data are the means and standard errors of three independent experiments. A/New Caledonia/1/99 (H1N1) (■), B/Shichuan/99 (□) A/Sydney/5/97 (H3N2) (▤). (D) Purified alveolar macrophages were stained to reveal receptors for human or avian influenza viruses. Control (black line), Sia2–3Gal (green line), Sia2–6Gal (pink line). (E and F). Six pigs per group were intranasally inoculated with 3×10^6 $\log_{10}EID_{50}$ of A/New Caledonia/1/99 (H1N1) or A/Sydney/5/97 (H3N2) virus. Alveolar macrophages were collected at 3 or 5 days p.i. and purified before viral titers ($\log_{10}EID_{50}$/0.1 ml) and percentage of infected cells were determined. (E) Viral titers at 3 (■) and 5 (□) days p.i. (F) Percentage of virus-positive cells was determined by flow cytometric analysis. Pigs were infected with A/New Caledonia/1/99 (H1N1) or A/Sydney/5/97 (H3N2) virus, and 3 and 5 days later, viral infections in alveolar macrophages were determined by anti-NP antibody and FITC-labeled secondary antibody. Alveolar macrophages from uninfected pigs (dotted black line); alveolar macrophages from infected pigs (green line). Data are one of five experiments.

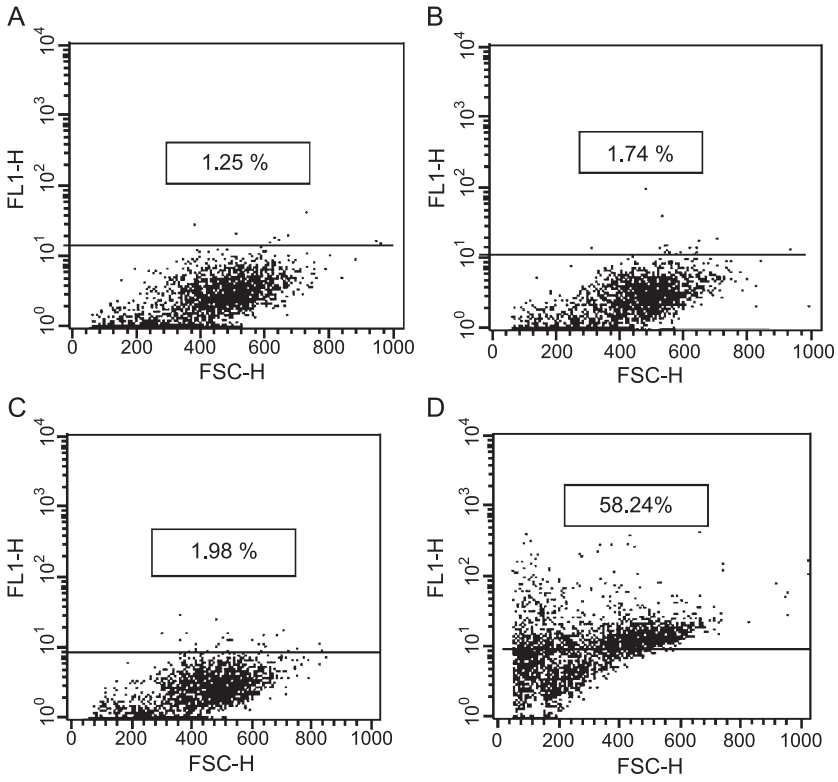


Fig. 3. Determination of apoptosis in alveolar macrophages in vivo. Six pigs in each group were inoculated with A/Sydney/5/97(H3N2) or A/New Caledonia/1/99 (H1N1) influenza virus. Alveolar macrophages were isolated and pooled 5 days p.i. Apoptosis was determined by flow cytometry as described in Materials and methods. (A) Alveolar macrophages from pigs infected with A/New Caledonia/1/99 (H1N1). (B) Alveolar macrophages from pigs infected with A/Sydney/5/97(H3N2). (C) Alveolar macrophages from uninfected pigs. (D) Alveolar macrophages treated in vitro with staurosporine to induce apoptosis.

were 155 for H1N1 and 450 for H3N2 viruses at 3 days p.i. and 750 for H1N1 and 3000 for H3N2 viruses at 5 days p.i.

The role of viral genes in TNF-α induction by H3N2 influenza viruses

We used reverse genetics to determine what genes of H3N2 viruses are involved in their greater induction of TNF-α in alveolar macrophages (Hoffmann et al., 2000). Recombinant viruses were constructed using the backbone of A/PR/8/34 (H1N1) (Table 1) and were allowed to replicate to similar titers in alveolar macrophages (data not shown). The TNF-α copy numbers in alveolar macrophages infected with the recombinant virus A/PR/8/34/NS containing the NS gene of A/Sydney/5/97 (H3N2) virus were similar to those in macrophages infected with A/PR/8/34 (H1N1). The PB2, PB1, PA, NP, and M genes of H3N2 viruses also failed to affect the cytokine elevation (Table 1). When alveolar macrophages were infected with recombinant viruses containing the HA (A/PR/8/34/HA) or NA (A/PR/8/34/NA) gene of A/Sydney/5/97 (H3N2), TNF-α copy numbers were higher than those of A/PR/8/34 (H1N1), but lower than those of A/Sydney/5/97 (H3N2). In a further study, when alveolar macrophages were infected with recombinant viruses containing both HA and NA genes of A/Sydney/5/97 (H3N2), TNF-α copy numbers were similar to those of A/Sydney/5/97 (H3N2).

Discussion

We have shown in a pig model of influenza infection that human H3N2 influenza viruses trigger greater induction of TNF-α in alveolar macrophages than do human H1N1 or human influenza B viruses and that alveolar macrophages do not undergo apoptosis when infected with influenza viruses. Taken together, our findings suggest that alveolar macrophages are involved in the pathogenesis of influenza virus infection and in the associated immune response.

Alveolar macrophages infected with influenza viruses in the pig model did not undergo apoptosis. In previous studies, MDCK cells underwent apoptosis when infected with influenza viruses; however, dogs are not natural hosts of influenza virus (Hinshaw et al., 1994). The observed disparity may reflect the different origins of the cells. The apoptotic death of numerous alveolar macrophages would seriously impair the immune response in the respiratory tract, which involves the release of inflammatory and anti-viral cytokines, such as interferons, IL-1, IL-6, and IL-8, by alveolar macrophages. Therefore, the absence of an apoptotic response in these cells is consistent with their role in protecting the host respiratory tract from infection. Previous findings in human

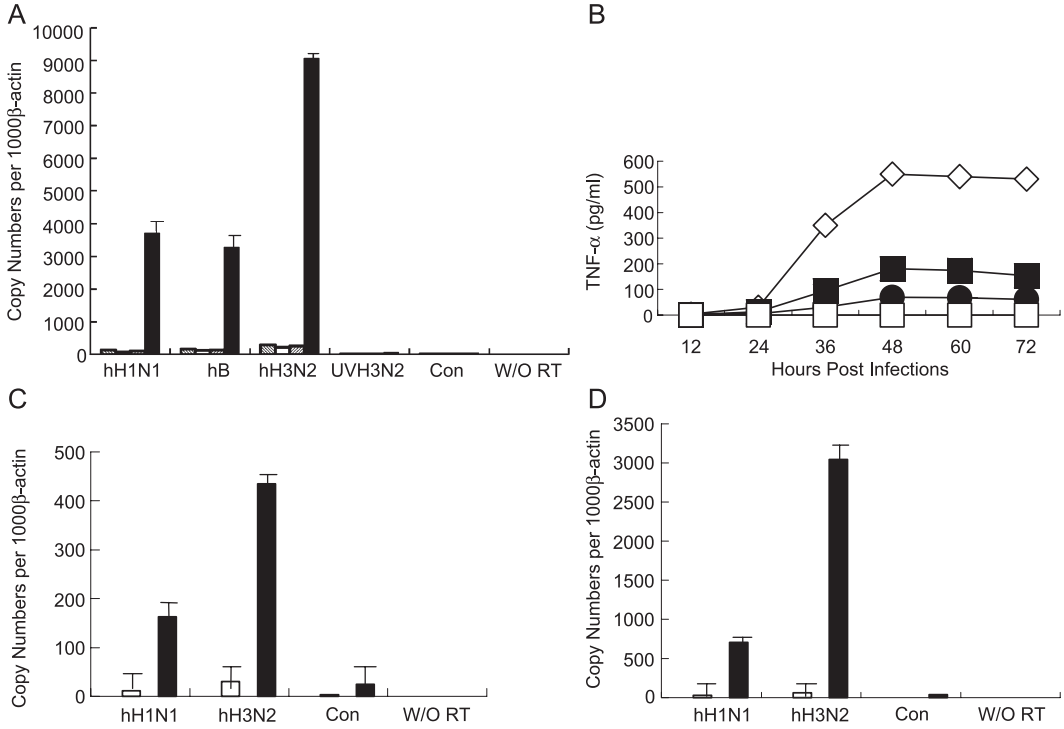


Fig. 4. Induction of inflammatory cytokines in alveolar macrophages infected with human influenza viruses. (A) Purified alveolar macrophages were incubated in vitro with human influenza viruses for 7 h before inflammatory cytokines were quantified by real-time PCR. IL-1β (▨), IL-6 (□), IL-8 (▨), TNF-α (■). Data show the mean and standard error of three independent experiments. hH1N1, A/New Caledonia/1/99 (H1N1), hB, B/Shichuan/99; hH3N2, A/Sydney/5/97 (H3N2); UVH3N2, UV-inactivated A/Sydney/5/97 (H3N2); Con, cells mock-infected with PBS; W/O RT, cDNA synthesis without reverse transcriptase. (B) ELISA comparison of TNF-α induction in alveolar macrophages incubated with influenza viruses. Aliquots of supernatant were collected at the times indicated after incubation with A/New Caledonia/1/99 (H1N1) (■), B/Shichuan/99 (●) A/Sydney/5/97 (H3N2) (□), or PBS (□). Data are the mean of three independent experiments. (C, D) Inflammatory cytokines induced in vivo were quantified by real-time PCR in alveolar macrophages collected from pigs 3 days p.i. (C) or 5 days p.i. (D). IL-6 (□), TNF-α (■). hH1N1, A/New Caledonia/1/99 (H1N1); hH3N2, A/Sydney/5/97 (H3N2); Con, PBS mock-infected; W/O RT, without reverse transcriptase. Data are the mean and standard error of results obtained from three pigs. IL-1β and IL-8 were not detected.

and porcine respiratory epithelial cells, which are also vital for immune defense, support our results (Arndt et al., 2002; Seo and Webster, 2002). When human bronchial epithelial cells were infected with an A/PR/8/34 (H1N1) influenza virus, they died of necrosis, not of apoptosis (Arndt et al., 2002). In addition, our previous studies showed that porcine lung epithelial cells (SJPL) infected in vitro with A/Sydney/5/97 (H3N2), A/Chicken/NY/13307-3/95 (H7N2), or A/Swine/IA/17672/88 (H1N1) influenza viruses did not undergo apoptosis (Seo et al., 2001). The absence of apoptosis is consistent with these cells' ability to produce a greater quantity of inflammatory cytokines. In a recent study (Brydon et al., 2003), the human nasal septum squamous cell carcinoma cell line RPMI-2650 and the human muco-epidermoid bronchiolar carcinoma cell line NCIH292 died of apoptosis after infection with influenza A viruses. Interestingly, when apoptosis of the infected bronchiolar cells was blocked with the caspase-8 inhibitor Z-IETD-fmk and the pan-caspase inhibitor (Z-VAD-fmk), expression of the chemokine, RANTES and the cytokines, IL-6 and IL-8 was increased.

Table 1
Role of H3N2 genes in induction of TNF-α

TNF-α	Viruses (copies per 1000 β-actin)
A/PR/8/34 (H1N1)	3500 ± 35
A/Sydney/5/97 (H3N2)	8950 ± 53
A/PR/8/34/NS (H3N2)	3650 ± 32
A/PR/8/34/HA(H3N2)	7550 ± 41
A/PR/8/34/NA(H3N2)	7370 ± 39
A/PR/8/34/NP(H3N2)	3470 ± 50
A/PR/8/34/M (H3N2)	3370 ± 41
A/PR/8/34/PA (H3N2)	3452 ± 35
A/PR/8/34/PB1 (H3N2)	3550 ± 34
A/PR/8/34/PB2 (H3N2)	3520 ± 36
A/PR/8/34/HA.NA (H3N2)	8690 ± 48
Uninfected control	30 ± 4

Induction of cytokines appears to depend on polygenic influenza virus traits. Our results showed that the higher induction of TNF-α by human H3N2 viruses compared to human H1N1 and human B influenza viruses is due partially to the HA and NA genes and is not due to the NS gene. However, the quantity of TNF-α induced by a recombinant H1N1 virus containing H3N2 HA or NA was not equal to that induced by whole H3N2 viruses. A previous similar study found that H5N1 influenza viruses induced higher production of inflammatory cytokines because of the NS gene (Cheung et al., 2002). However, this study did not compare the effects of the HA or NA genes of different

viruses on cytokine induction. Further, the NS gene of H3N2 influenza viruses does not contain glutamic acid at position 92 that was found in the H5N1/97 influenza viruses to inhibit the effects of interferon (Seo et al., 2002).

To date, most studies of influenza virus-induced inflammatory cytokines have been based on macrophages and monocytes infected in vitro (Bussfeld et al., 1998). However, in vivo cytokine induction profiles differ from those observed in vitro. IL-1β and IL-8 were not induced in alveolar macrophages infected in vivo but were induced in alveolar macrophages infected in vitro. The ratio of infected cells may cause this difference. Flow cytometric analysis showed that most alveolar macrophages were infected with influenza viruses in vitro, but fewer than 12% were infected in vivo. The previous study also showed that alveolar macrophages could be infected with influenza viruses in vivo, but this study did not enumerate the infected cells by flow cytometric analysis (Jung et al., 2002).

Our experiments in vivo showed TNF-α copy numbers to be higher at 5 days p.i than at 3 days p.i., although viral titers were higher on day 3. TNF-α induction may not have been closely related to viral titers due to the feedback effect of cytokine induction on these cells. Cytokine concentration has been known to influence the synthesis of the cytokine in question and that of other cytokines (Beutler, 1995; Rossi and Zlotnik, 2000). Therefore, TNF-α produced at 3 days p.i. may have induced greater production of TNF-α in alveolar macrophages at 5 days p.i.

It is possible that inflammatory cytokines produced in alveolar epithelial cells may be involved in lung pathogenesis. In our study in influenza-infected alveolar epithelial cells, TNF-α was produced among inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α (manuscript in preparation).

The role of T lymphocytes in lung immunopathology cannot be ruled out. The previous studies showed that T cells in influenza-infected mice were involved in cell injury in lungs (Humphreys et al., 2003; Small et al., 2001). In HA-transgenic mice expressing the A/Japan/57 influenza HA, the adaptive transfer of HA-specific $CD8^+$ T cells caused the type II alveolar cells to produce the chemokine monocyte chemoatractant protein 1. This study suggests that $CD8^+$ T cells may cause tissue injury in lungs by producing proinflammatory mediators (Small et al., 2001). In a study of OX40 role in T-cell-mediated immunopathology in influenza-infected mice, the blocking of OX40/OX40L ameliorated influenza-driven T cell immunopathology in lungs by reduced inflammatory responses and enhanced apoptosis (Humphreys et al., 2003).

On the basis of our findings in the pig model system, we hypothesize that the higher mortality rate caused by human H3N2 influenza viruses in elderly patients is due to the high induction of TNF-α in the respiratory tract. Although TNF-α in low quantities causes local inflammation, in high quantities it causes systemic clinical and pathologic abnormalities (Brydon et al., 2003) that include hypothalamus-mediated induction of fever and the inhibition of myocardial contractility. TNF-α also induces an acute inflammatory response in leukocytes and respiratory tract endothelium. The results of previous studies suggested that the high mortality caused by H5N1 influenza virus infection is based on greater induction of TNF-α (Cheung et al., 2002; Seo et al., 2002). Similarly, high induction of TNF-α by H3N2 viruses in elderly patients may cause death by impairing normal pulmonary function. Further studies using more strains of influenza viruses are warranted to demonstrate the role of H3N2 viruses in the elderly mortality.

Materials and methods

Viruses

The influenza viruses A/Sydney/5/97 (H3N2), A/New Caledonia/1/99 (H1N1), and B/Shichuan/99 were grown in 11-day-old embryonated chicken eggs for use in this study.

Animals and cells

Four-week-old pigs were obtained from local farms and were serologically negative for exposure to porcine respiratory and reproductive syndrome virus, *Mycoplasma hyopneumoniae*, and H1 and H3 influenza viruses. Alveolar macrophages were collected from the pigs by bronchoalveolar lavage (BAL) with phosphate-buffered saline (PBS) (pH 7.2). Alveolar macrophages were purified from the BAL specimen by centrifugation for 30 min at 900 × *g* on a gradient (Histopaque-1083, Sigma Diagnostics, Inc., St. Louis, MO). The macrophages were further purified in tissue culture flasks with RPMI-1640 (Sigma) supplemented with 5% bovine serum albumin, 1% sodium pyruvate, 1% L-glutamine, 1.4% MEM nonessential amino acids, and 1% antibiotic–antimycotic solution (Sigma). Two hours after cells were plated, unbound cells were removed by washing three times with RPMI-1640 medium, and bound alveolar macrophages were collected by treatment with 3× trypsin–EDTA (0.15% trypsin and 1.50 mM EDTA (Sigma) in PBS (pH 7.2).

Verification of alveolar macrophages by flow cytometry

The purified alveolar macrophages (3×10^6) were fixed by treatment with 3.7% formaldehyde on ice for 30 min and permeabilized with 0.1% Triton-X 100 in PBS on ice for 45 min. The permeabilized cells were stained with the fluorescein isothiocyanate (FITC)-labeled anti-macrophage antibody MCA874F (Serotech Inc., Raleigh, NC, USA), and with FITC-labeled isotype-matched control antibody (Serotech). The stained cells were analyzed with a FACSCalibur fluorospectrometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Indirect immunofluorescence assay for virus infection

Porcine alveolar macrophages (2×10^6) were cultured in RPMI-1640 in a Lab-Tek chamber slide (Nalge Nunc International, Naperville, IL, USA) for 4 h before exposure to influenza virus. Cells were washed with PBS (pH 7.2) twice and incubated with A/Sydney/5/97 (H3N2) virus (multiplicity of infection [m.o.i.], 2) for 1 h in a humidified incubator containing 5% CO_2 at 37 °C. Cells were then washed twice with warm PBS (pH 7.2), incubated for 24 h with RPMI-1640 containing 5% BSA in a humidified incubator (37 °C, 5% CO_2), and fixed with cold 80% acetone in water. The fixed cells were incubated at room temperature with a mouse anti-nucleoprotein monoclonal antibody for 45 min, and then washed three times with cold PBS (pH 7.2) containing 0.05% Tween 20. Cells were incubated on ice with FITC-labeled anti-mouse IgG antibody for 45 min and washed four times with cold PBS (pH 7.2) containing 0.05% Tween 20. To stain nuclei, cells were incubated with propidium iodide (50 μg/ml) (Sigma). The stained cells were evaluated for infectivity by using an LSM 510 confocal laser scanning microscope (Carl Zeiss International) at the Korea basic science institute (Daejeon, Korea).

Inoculation of swine with human influenza A viruses

Three groups of six 4-week-old pigs kept in biosafety level 2 conditions were sedated by intramuscular injection of 20 mg/kg of ketamine hydrochloride (Yuan Corporation, Seoul, Korea). Six pigs were intranasally (i.n.) inoculated with 3×10^6 $\log_{10}$ 50% egg infectious dose ($\log_{10}EID_{50}$) of A/Sydney/5/97 (H3N2) virus, six were inoculated i.n. with 3×10^6 $\log EID_{50}$ of A/New Caldedonia/1/99 (H1N1) virus, and six were mock-inoculated i.n. with PBS. Pigs were killed 3 days or 5 days after inoculation by intravenous injection of high doses of ketamine hydrochloride, and alveolar macrophages were collected for determination of viral titer and apoptosis.

DNA fragmentation assay

Purified porcine alveolar macrophages (3×10^6) were incubated for 48 h with A/Sydney/5/97 (H3N2), A/New Caldedonia/1/99 (H1N1), or B/Shichuan/99 influenza viruses (m.o.i., 2). Cells were then washed in PBS (pH 7.2), resuspended in 500 μl ice-cold lysis buffer (10 mM Tris, pH 7.5; 0.5% Triton X-100 [Sigma]), and incubated on ice for 40 min. The lysates were centrifuged for 10 min at 12 000 × *g* at room temperature to remove cellular debris, and the supernatants was extracted from by centrifuging once with buffered phenol and once with buffered phenol–chloroform. DNA was precipitated from the supernatants by adding ethanol plus 3 M sodium acetate (pH 5.2). DNA was dissolved in 20 μl sterile water and treated with RNase A (Sigma), and approximately 1 μg of DNA sample was separated by electrophoresis in 2% agarose (GTG SeaKem agarose; FMC BioProducts, Rockland, ME) in TAE buffer (0.04 M Tris–acetate, pH 7.2; 0.001 M EDTA).

Quantification of cytokine mRNA by real-time RT-PCR

Total RNA was isolated from alveolar macrophages (2×10^6) incubated for 7 h with A/Sydney/5/97 (H3N2), A/New Caldedonia/1/99 (H1N1), or B/Shichuan/99 virus (m.o.i. 2) and from the alveolar macrophages (2×10^6) of influenza virus-inoculated pigs by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The first cDNA was synthesized by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) for RT-PCR with 2 μg of total RNA and 0.5 μg of oligo (dT) 12–18 as a primer. mRNAs were quantified by real-time PCR in a Rotor-Gene 3000 instrument (Corbett Research, Mortlake, Australia) at conditions of 50 °C for 2 min, 95 °C for 10 min (1 cycle) and 95 °C for 15 s, 58 °C for 60 s (45 cycles). The oligonucleotide primers used for real-time quantification of the porcine inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α and the housekeeping gene product β-actin were previously described (Dozois et al., 1997; Seo et al., 2002).

Assay of TNF-α in tissue culture supernatants

Alveolar macrophages (2×10^6 per well in six-well plates) were incubated for various times with A/Sydney/5/97(H3N2), A/New Caldedonia/1/99 (H1N1), and B/Shichuan/99 viruses (m.o.i., 0.1). Cytokines in the supernatants were assayed by using a swine TNF-α kit (Biosource International Inc., Camarillo, CA) as recommended by the manufacturer. Briefly, 50 μl of standard diluent buffer was added to each well of an eight-well strip plate precoated with polyclonal antibody to specific cytokines, and then 50 μl of tissue culture supernatant was added. Plates were incubated for 2 h at room temperature before washing with wash buffer; 100 μl of biotinylated antisera was added, and plates were incubated for 1 h at room temperature before washing with wash buffer. Streptavidin-HRP working solution (100 μl) was added, plates were incubated for 30 min at room temperature, and stabilized chromogen was added to develop color. After stop solution was added, the optical density was measured at 450 nm on an ELISA microplate reader (Bio-Rad, Hercules, CA). The readings were converted to pg/ml by using a standard curve provided with the assay kit.

Detection of apoptosis by flow cytometry

Apoptosis was detected by using the kit as instructed by the manufacturer (R&D Systems, Minneapolis, MN). Briefly, 3×10^6 alveolar macrophages incubated with A/Sydney/5/97 (H3N2) (m.o.i., 1) for 48 h or 3×10^6 alveolar macrophages obtained from pigs infected with A/Sydney/5/97(H3N2) or A/New Caldedonia/1/99 (H1N1) virus were

fixed in 3.7% formaldehyde for 10 min at room temperature before washing with PBS (pH 7.2). The fixed cells were sequentially washed with 2 ml of 100%, 95%, and 70% ethanol before permeabilization with 100 μl of cytonin for 30 min at room temperature. TdT labeling buffer (1×) was added, and cells were labeled with 50 μl of labeling reaction mix (1 μl each of TdT-dNTP, 50× cation stock, and TdT enzyme; 50 μl of 1× TdT labeling buffer) by incubation for 1 h in a humidity chamber (37 °C). The cells were incubated at room temperature for 20 min with 50 μl of streptavidin–FITC detection solution, washed twice with 1× PBST (0.1% Tween 20 in 1× PBS), and analyzed with a FACSCalibur fluorospectrometer (Becton Dickinson).

Flow cytometric analysis of virus receptor expression on alveolar macrophages

This analysis used the Digoxigenin (DIG) Glycan Differentiation Kit (Roche Molecular Biochemicals, Indianapolis, IN). Alveolar macrophages were resuspended at a concentration of 2×10^6 cells per milliliter in the binding medium (Tris-buffered saline, pH 7.6; 0.5% BSA, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 1 mM Mn^{2+}) and incubated for 30 min at room temperature with the DIG-labeled lectins *Maackia amurensis* agglutinin (MAA), which specifically binds to Sia2–3Gal, or *Sambucus nigra* agglutinin (SNA), which specifically binds to Sia2–6Gal. Cells were washed three times with cold PBS (pH 7.2) containing 0.05% Tween 20, and FITC-labeled anti-DIG antibody diluted in PBS (pH 7.2) containing 0.5% BSA was added. After a 30-min incubation on ice, the cells were washed three times with cold PBS (pH 7.2) containing 0.05% Tween 20, and the fluorescence intensity of the cells was analyzed with a FACSCalibur Fluorospectrometer. Because FITC-labeled anti-DIG antibodies were obtained from a commercial source, we could not double-label cells with two different colors.

Determination of virus titers

Alveolar macrophages (5×10^6) collected from pigs inoculated with A/Sydney/5/97 (H3N2) or A/New Caledonia/1/99 (H1N1) virus were resuspended in 1 ml of PBS (pH 7.2), and were then disrupted by freezing and thawing three times. Serial 10-fold dilutions were made from the supernatants, and each dilution was inoculated into four eggs. The presence of viruses was confirmed by hemagglutination assay using 0.5% turkey red blood cells in PBS (pH 7.2). Viral titers in tissue culture supernatants were determined as described above. Virus titers were calculated as $\log_{10}EID_{50}$/ml by the method of Reed and Muench (1938).

Generation of recombinant viruses

Recombinant viruses were generated by reverse genetics as previously described (Hoffmann et al., 2000). We cloned cDNAs of the HA, NA, NP, PB1, PB2, PA, M and NS genes of A/Sydney/5/97 (H3N2) to generate A/PR/8/34 (H1N1) recombinant viruses containing the HA, NA, NP, PB1, PB2, PA, M, and NS genes of A/Sydney/5/97 (H3N2). The recombinant viruses were designated A/PR/8/34/HA (H3N2), A/PR/8/34/NA (H3N2), A/PR/8/34/NS (H3N2), A/PR/8/34/NP(H3N2), A/PR/8/34/M (H3N2), A/PR/8/34/PA (H3N2), A/PR/8/34/PB1 A/PR/8/34/PB2 (H3N2), and A/PR/8/34/HA.NA (H3N2).

Statistical analysis

Cytokine data were analyzed by Student's *t* test or one-way analysis of variance. A P value <0.05 was considered to indicate a significant difference between compared groups.

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