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Tumor Necrosis Factor Alpha Exerts Powerful Anti-Influenza Virus Effects in Lung Epithelial Cells

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Previous studies have associated influenza virus-induced expression of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), with influenza pathogenesis in the human respiratory tract and have suggested that alpha and beta interferons are the first cytokines recruited to counteract such infection. However, we report here that TNF-α has powerful anti-influenza virus activity. When infected with influenza virus, cultured porcine lung epithelial cells expressed TNF-α in a dose-dependent manner. Expression of TNF-α was induced only by replicating virus. TNF-α showed strong antiviral activity against avian, swine, and human influenza viruses, and the antiviral effect of TNF-α was greater than that of gamma or alpha interferon. These findings suggest that TNF-α serves as the first line of defense against influenza virus infection in the natural host.

Influenza is an acute respiratory viral infection that causes substantial morbidity and significant mortality. The clinical symptoms are sudden in onset and include malaise, fever, upper and lower respiratory symptoms, and headache. Influenza virus replicates throughout the upper and lower respiratory tracts of humans and pigs (3, 4, 15). Virus replication peaks 48 h after infection and subsequently declines; little virus is shed after 6 to 8 days. Influenza viruses efficiently induce interferons and are sensitive to the antiviral effects of these cytokines (19, 31, 39). However, the effect of tumor necrosis factor alpha (TNF-α) on influenza virus is not known.

Although TNF-α was first noted for its role in the killing of tumor cells (5), it has pleiotropic functions that include the inflammatory response and host resistance to pathogens (1). TNF-α is produced by activated macrophages, T and B lymphocytes, natural killer cells, astrocytes, endothelial cells, smooth muscle cells, some tumor cells, and epithelial cells (1, 2). Previous studies have shown that treatment of cells with TNF-α can inhibit the replication of vesicular stomatitis virus, encephalomyocarditis virus, and herpes simplex virus in a dose-dependent manner and can prevent the development of cytopathic effects (30). However, although TNF-α inhibited the replication of human immunodeficiency virus type 1 (HIV-1) in peripheral blood monocytes and alveolar macrophages (18, 27), it could also stimulate HIV-1 replication in chronically infected T cells and promonocytic cell lines (7, 8, 33).

Nasal lavage specimens from humans infected with influenza virus contain various proinflammatory cytokines, such as interleukin (IL)-6, TNF-α, gamma interferon (IFN-γ), IL-10, monocyte chemotactic protein 1, and macrophage inflammatory proteins 1α and 1β (9, 16, 23). IFN-α, TNF-α, and IL-1 were detected in bronchoalveolar lavage fluids from pigs infected with H1N1 influenza virus (38). Pigs are regarded as the mixing vessels of influenza viruses, because new pandemic strains are created when human and nonhuman influenza viruses undergo genetic reassortment in pigs (40). The clinical signs of influenza in pigs are similar to those in humans (15, 28).

We recently established a cell line from the lung epithelium of normal, healthy pigs; this cell line supports the replication of all subtypes of influenza viruses (41). Our experiments with this cell line showed that infection with influenza virus induces expression of TNF-α in lung epithelial cells and that TNF-α exerts powerful anti-influenza virus activity.

MATERIALS AND METHODS

Cells and viruses. St. Jude porcine lung (SJPL) epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, Md.) supplemented with 1.4% antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, Mo.). Cells were split in a 1:4 ratio every 3 days. Influenza viruses were obtained from the repository of St. Jude Children's Research Hospital. All viruses were grown in 11-day-old embryonated chicken eggs.

Reverse transcription-PCR detection of proinflammatory cytokines. Confluent SJPL cells in 75-cm^2 tissue culture flasks were infected with A/Sydney/5/97 (H3N2) at a multiplicity of infection (MOI) of 2, and total cellular RNA was isolated after 10 h by using Trizol reagent (Life Technologies). First-strand cDNA synthesis was achieved by using a preamplification system (Superscript Preamplification System for First Strand cDNA Synthesis; Life Technologies) and random hexamers as primers. The oligonucleotide primers used for amplification of the cDNAs of the porcine proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, TNF-α, and TNF-β were described elsewhere (6). PCR cycles comprised denaturing at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 45 s. The final extension period took place at 72°C for 10 min.

Assay of TNF-α in supernatant tissue culture medium. Confluent monolayers of SJPL cells in six-well plates were infected with different doses of the specified influenza viruses, and supernatant medium was collected at the specified intervals after infection. The concentration of TNF-α was measured by using a swine TNF-α immunoassay kit (Biosource International Inc., Camarillo, Calif.) as instructed by the manufacturer. Briefly, 50 μl of standard diluent buffer and 50 μl of tissue culture supernatant were added to each well of an eight-well strip plate coated with polyclonal antibody to TNF-α. Plates were incubated for 2 h at room temperature and washed with washing buffer; 100 μl of biotinylated anti-TNF-α solution was added, the mixture was incubated for 1 h at room temperature, and plates were washed again. Streptavidin-horseradish peroxidase working solution (100 μl) was added to each well, the plate was incubated for 30 min at room temperature, and stabilized chromogen was added. After stop solution was added, the optical density was measured at 450 nm on an enzyme-linked immunosorbent assay microplate reader (Bio-Rad, Hercules, Calif.), and the values were converted to concentration by using a standard curve provided with the assay kit. The reported values are the means ± standard deviation of three independent experiments.

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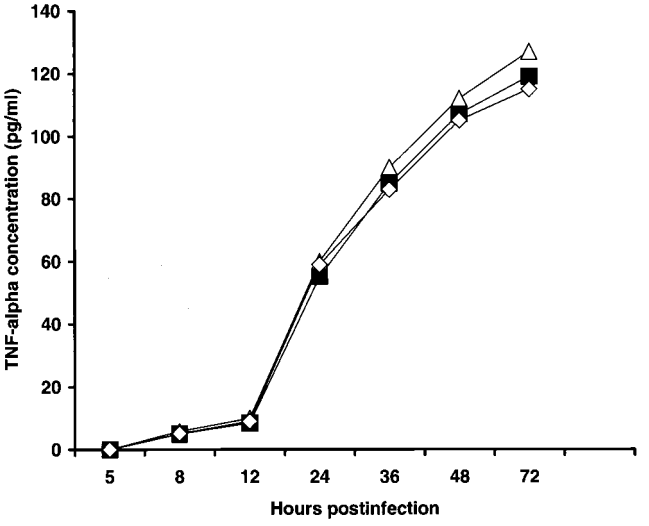

FIG. 1. TNF-α induction in SJPL cells infected with human influenza viruses. The TNF-α concentration was measured in supernatant medium of confluent SJPL cells infected with three human influenza viruses in the presence of TPCK-trypsin at the indicated times after infection. Each concentration shown is the mean of three independent experiments; the standard error was <4 pg/ml. Symbols: △, A/Moscow/10/99 (H3N2); ■, B/Sichuan/379/99; ◇, A/New Caledonia/20/99 (H1N1).

Inactivation of virus. The allantoic fluid of chicken eggs was harvested 2 days after infection with A/Sydney/5/97 (H3N2) and centrifuged at 10,000 rpm for 2 min to remove debris. The supernatant fluid was concentrated to 40,000 hemagglutination (HA) units per milliliter by using an Amicon concentrator (Amicon, Inc, Beverly, Mass.), and 37% formalin was added to yield a final concentration of 0.025%. The formalin-treated supernatant was refrigerated at 4°C for 3 days to inactivate virus; inactivated virus was then purified by centrifugation at 24,000 rpm on a 25 to 70% sucrose gradient at 4°C for 3 h. Viral inactivation was confirmed by inoculation of chicken eggs and SJPL cells.

Assay of TNF-α antiviral activity. SJPL cells that were approximately 90% confluent in wells of six-well tissue culture plates were treated with the indicated quantities of recombinant swine TNF-α (R & D Systems, Inc, Minneapolis, Minn.) for 24 h and then infected with influenza viruses (MOI, 0.0001). The infected cells were incubated for 72 h with DMEM containing 0.3% bovine serum albumin and 1 μg of trypsin treated with l-(1-tosylamido-2-phenyl)ethylchloromethylketone (TPCK) per ml, and the supernatant medium was then collected. The virus titer of the supernatant medium (log 50% tissue culture infectious doses [$TCID_{50}$] per milliliter) was measured in SJPL cells grown in 96-well plates.

RESULTS

Induction of TNF-α expression in SJPL cells. We first used reverse transcription-PCR to identify the proinflammatory cytokines whose expression was induced in SJPL cells infected with A/Sydney/5/97 (H3N2) influenza virus (MOI, 2). TNF-α was expressed, but IL-1α, IL-1β, IL-6, IL-8, and TNF-β were not detected (data not shown).

We then assayed TNF-α in the supernatant medium of SJPL cells infected with A/Moscow/10/99 (H3N2), B/Sichuan/379/99, or A/New Caledonia/20/99 (H1N1) (MOI, 0.0001) to demonstrate that these human influenza viruses induce expression of TNF-α in SJPL cells. The three viruses induced very similar levels of TNF-α expression (Fig. 1). The presence of TNF-α was first detected approximately 12 h after infection with these viruses (10 pg/ml), and the concentration of TNF-α continued to rise for 72 h after infection (127 pg/ml).

To investigate the effect of virus dose on induction of TNF-α expression in SJPL cells, we infected the cells with various doses of A/Sydney/5/97 (H3N2) influenza virus and measured TNF-α in the supernatant medium after 24 h. The concentration of TNF-α was dependent on the virus dose (Fig. 2). No TNF-α was produced by the uninfected SJPL cells.

To determine whether inactivated influenza virus could induce TNF-α expression, we assayed the supernatant medium of SJPL cells exposed to A/Sydney/5/97 (H3N2) virus that had been inactivated with formalin and purified by sucrose gradient centrifugation. Uninfected cells and cells exposed to inactivated virus (2048 HA units) expressed no TNF-α after 24 h, whereas those exposed to the noninactivated virus (MOI, 5) expressed a mean of 145 ± 4 pg of TNF-α per ml (data not shown).

Induction of TNF-α expression by different subtypes of influenza viruses. We next tested whether influenza viruses from other natural hosts affected induction of TNF-α expression in SJPL cells. SJPL cells were infected with 2 MOI of B/Lee/40, A/Port Chalmers/1/73 (H3N2), A/Swine/Ned/80 (H1N1), A/Swine/TX/4199-2/98 (H3N2), and A/Mallard/Alberta/52/97 (H12N5). The viruses induced comparable expression of TNF-α, as shown by assays of the supernatant medium (Fig. 3). The concentration ranged from 100 pg/ml (A/Swine/Ned/3/80) to 135 pg/ml (A/Swine/TX/4199-2/98). No TNF-α was produced by uninfected cells.

Inhibition of influenza virus replication by TNF-α. SJPL cells were pretreated with 100 ng of recombinant swine TNF-α per ml 24 h before infection with human influenza viruses (MOI, 0.0001). Replication of A/Moscow/4/99 (H3N2) influenza virus was reduced $10^{5.5}$-fold, replication of A/New Caledonia/20/99 (H1N1) was reduced $10^{7.9}$-fold, and replication of B/Sichuan/379/99 was reduced $10^{6.8}$-fold compared to virus replication in untreated SJPL cells (Fig. 4).

To study the effect of the dose of TNF-α on the replication of human influenza virus, we treated SJPL cells with various

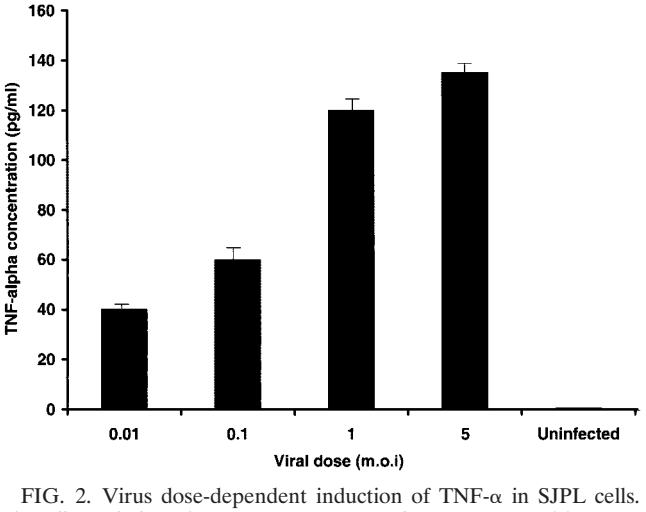

FIG. 2. Virus dose-dependent induction of TNF-α in SJPL cells. The effect of virus dose on TNF-α expression was assessed by measuring TNF-α concentration in the supernatant medium of SJPL cells 24 h after infection with the indicated doses of A/Sydney/5/97 (H3N2) influenza virus in the presence of 1 μg of TPCK-trypsin per ml. Each concentration shown is the mean of three independent experiments.

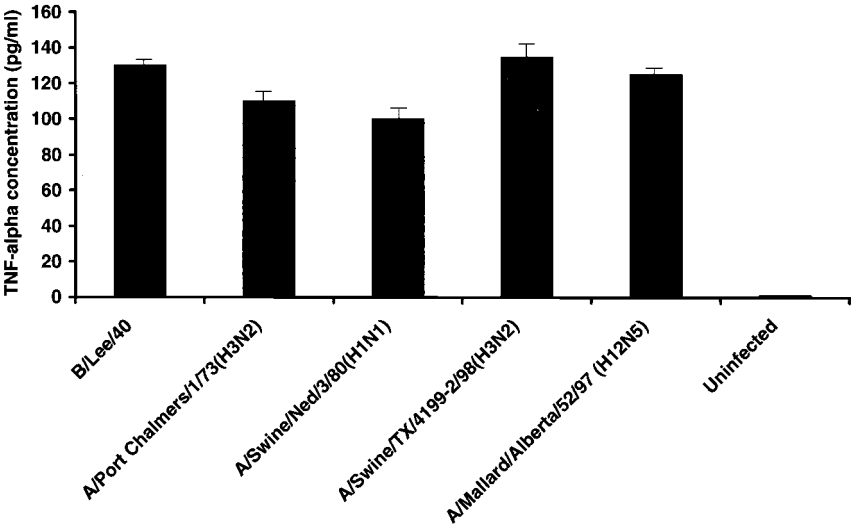

FIG. 3. Induction of TNF-α expression by different subtypes of influenza virus. The concentration of TNF-α was measured in the supernatant medium of SJPL cells 24 h after infection with the indicated subtypes of influenza virus (MOI, 2). Each concentration shown is the mean of three independent experiments.

doses of TNF-α 24 h before infection with A/New Caledonia/20/99 (H1N1) (MOI, 0.0001). Treatment with 200 ng/ml or more of TNF-α completely blocked virus replication (Fig. 5). The virus titer in the supernatant medium was less than 10^2 $TCID_{50}$/ml after treatment with 100 ng/ml and was 10^9 $TCID_{50}$/ml in the absence of treatment.

We also measured the effect of TNF-α on the replication of avian and swine influenza viruses in SJPL cells. Treatment with 100 ng of TNF-α per ml 24 h before infection (MOI, 0.0001) reduced replication of A/Swine/Ned/3/80 (H1N1) virus approximately $10^{6.8}$-fold, replication of A/Swine/TX/4199-2/98 (H3N2) virus approximately $10^{6.2}$-fold, replication of A/Mallard/Alberta/47/98 (H4N1) virus approximately $10^{5.8}$-fold, and replication of A/Shorebird/DE/11/95 (H11N9) virus approximately 10^6-fold compared to replication in untreated cells (Fig. 6).

Comparison of antiviral activity of cytokines. Interferons are thought to participate in the initial host defense against viral infection (31). There are two kinds of interferons, IFN-α/β and IFN-γ. We treated SJPL cells with IFN-α, IFN-γ, or TNF-α, infected them with A/New Caledonia/20/99 (H1N1) human influenza virus (MOI, 0.0001), and measured the virus titer of the supernatant medium after 72 h (Fig. 7). TNF-α had a markedly greater effect on influenza virus replication than IFN-α or IFN-γ at all doses tested (10, 100, and 1,000 pg/ml) (Fig. 7).

DISCUSSION

Although influenza virus infection is known to induce expression of TNF-α in animals and humans (16, 17, 24, 38), whether lung epithelial cells can produce TNF-α has not previously been established, nor has the activity of TNF-α against influenza virus been demonstrated. This report is the first to describe the expression of TNF-α by infected lung epithelial cells and the powerful activity exerted by the cytokine against influenza virus infection.

TNF-α expression in lung epithelial cells, which are the key targets of influenza virus infection, appears to be crucial to control of influenza virus infection in the host respiratory tract. This powerful inflammatory cytokine's role in recruiting various host cells, including monocytes and T and B lymphocytes, to sites of infection (1) suggests that TNF-α plays an important role in clearing influenza virus infection in the respiratory tract before the secondary immune response is activated. Previous studies showed that influenza virus infection of human macrophages triggers production of TNF-α (20, 21, 29, 35), IL-1β, IL-18, and IFN-α/β (35). It is possible that $CD4^+$ and $CD8^+$ T cells can be very important sources of TNF-α, since these cells

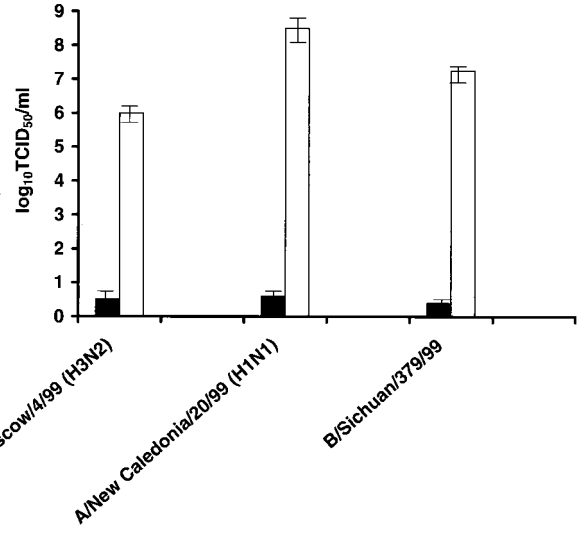

FIG. 4. TNF-α exerts antiviral activity against human influenza viruses. SJPL cells were treated with recombinant swine TNF-α (100 ng/ml) for 24 h before infection in the presence of TPCK-trypsin, and the virus titer was measured in the supernatant medium 72 h after infection. Values are the means of three independent experiments. Symbols: ■, TNF-α treated; □, untreated.

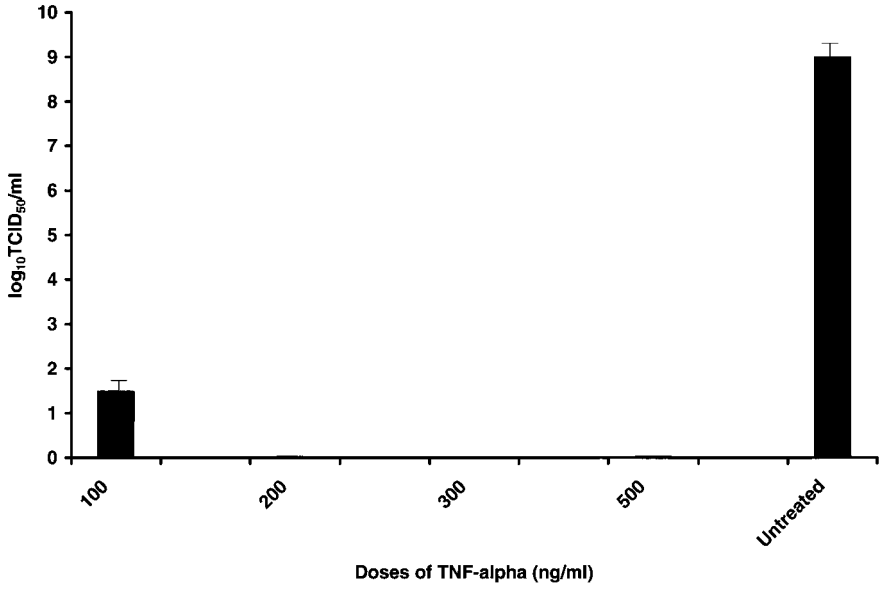

FIG. 5. Dose-dependent inhibition of viral replication. SJPL cells were treated with the indicated doses of recombinant swine TNF-α before infection with A/New Caledonia/20/99 (H1N1) human influenza virus. Virus titer was measured in the supernatant medium 72 h after infection. Values are the means of three independent experiments.

produce a considerable amount of TNF-α in an infected host (1).

In our study, expression of TNF-α in porcine lung epithelial cells was induced only by replicating virus. However, in the mouse model of influenza, purified influenza virus neuraminidase (NA) induced expression of TNF-α in peritoneal macrophages (21). The disparity between the findings of these two studies may reflect the different properties of the two cell types. Macrophages, unlike epithelial cells, can engulf and degrade foreign antigens, leading to cytokine induction (1). In other studies, macrophages have expressed various cytokines in response to bacterial lipopolysaccharide or bacterial cell wall proteins in the absence of bacterial infection (10, 32, 47).

We found TNF-α expression in lung epithelial cells to be proportional to the titer of replicating virus. Thus, specific subtypes of influenza virus may induce expression of TNF-α in host lung epithelial cells at levels that reflect the subtype's rate of replication. Furthermore, a subtype of influenza virus that has a novel mechanism to counteract TNF-α may replicate to high titers, thus inducing a high level of TNF-α expression. An in vivo study of influenza virus infection in humans found that the concentration of the inflammatory cytokines IL-6, TNF-α, IFN-γ, and IL-10 in nasal lavage fluids increased in proportion to influenza virus titers (9, 16).

Our study is the first to show that TNF-α exerts strong antiviral activity against influenza viruses. Other investigators, in contrast, have hypothesized that the inflammatory cytokine response is related to influenza pathogenesis (9, 16, 38, 42). Fritz and coworkers (9) found that in humans, virus titers peaked 2 or 3 days after intranasal inoculation with A/Texas/36/91 (H1N1) influenza virus and declined to basal levels after 5 days, whereas TNF-α peaked 3 days after inoculation and declined to basal levels after 5 to 6 days. The clinical symptoms were associated with increasing virus titer and cytokine concentration (9). These findings suggested that TNF-α might have antiviral activity, but the anti-influenza virus activity of the cytokine was not tested. Other studies found no association between influenza virus-induced fever and the concentrations of inflammatory cytokines (23, 36). In a ferret model of influenza, the level of endogenous pyrogen expression induced by influenza virus was not correlated with the concentrations of cytokines such as IL-1, IL-6, and TNF-α (23).

Several investigators have suggested that influenza virus infection is initially controlled by interferon-mediated antiviral activity (11, 12, 13, 14, 26, 31, 34, 39, 43, 44). Our findings

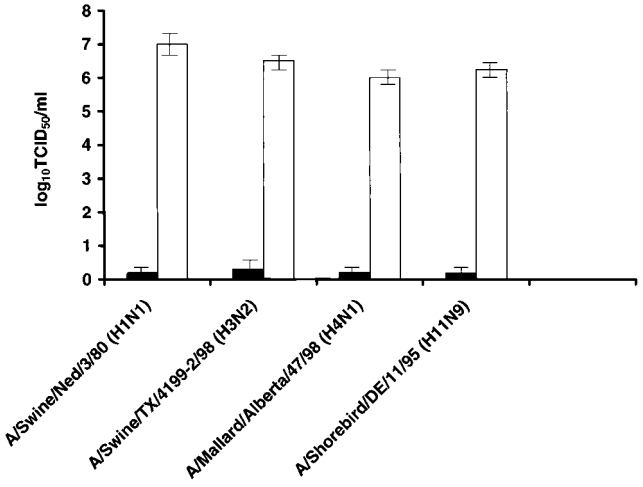

FIG. 6. TNF-α antiviral activity against avian and swine influenza viruses. Treated (100 ng/ml) SJPL cells were infected with avian or swine influenza viruses. Virus titer was measured in the supernatant medium 72 h after infection. Values are the means of three independent experiments. Symbols: ■, TNF-α treated; □, untreated.

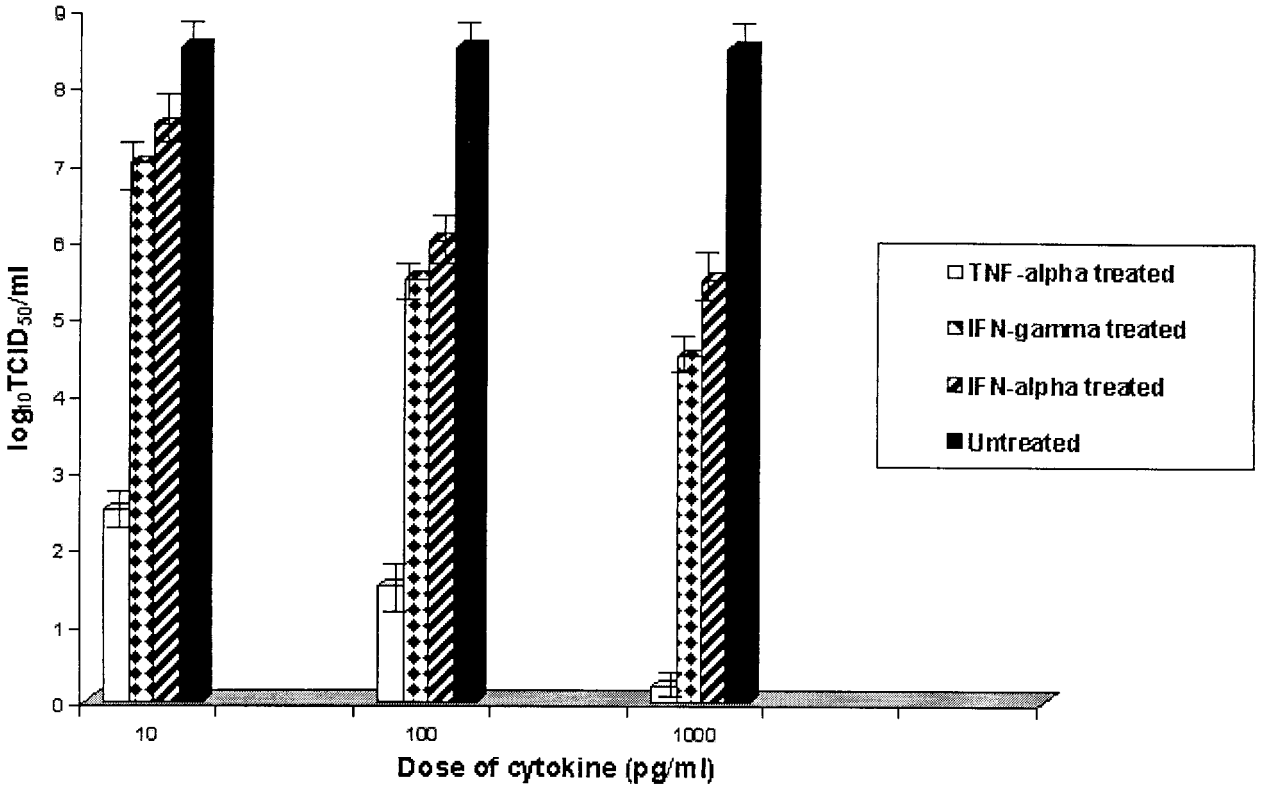

FIG. 7. Comparison of anti-influenza virus activity of cytokines. SJPL cells were treated with the indicated doses of recombinant swine TNF-α, IFN-γ, or IFN-α 24 h before infection with A/New Caledonia/20/99 (H1N1) influenza virus (MOI, 0.0001). Virus titers were measured 72 h after infection. Values are the means of three independent experiments.

showed that although the interferons do exert anti-influenza virus activity, the effect of TNF-α against influenza virus in vitro is greater. The previous study showed that the study with the mouse model of influenza on antiviral activity by TNF-α and IFN-α/β or IFN-γ is different from our study. Blocking of TNF-α by monoclonal antibody did not affect virus clearance (22), and mice unable to respond to IFN-α/β, IFN-γ, or both interferons did not differ from wild-type mice in the outcome of influenza virus clearance (37).

In humans, there is strong correlation between the level of virus multiplication and the amount of interferon induced in the respiratory tract (31). The reason for the difference may be the difference in the viral replication cycle among hosts. In mice infected with mouse-adapted A/PR/8/34 (H1N1) influenza virus, virus titers remained high even at 8 days postinfection (37), but influenza viruses were undetectable at 5 days postinfection in humans and pigs (15, 28, 31). To address antiviral activity by these cytokines, we need to use a better animal model, such as pigs.

TNF-α is known to inhibit the replication of other viruses (25, 27, 30, 46) to various extents. Treatment of Vero E6 cells with 50 ng of recombinant TNF-α per ml reduced the quantity of sin nombre virus nucleocapsid protein 1, 3, 4, 5, and 7 days after inoculation but did not affect the percentage of infected cells (25). TNF-α also inhibited the replication of HIV-1 in peripheral blood monocytes and alveolar macrophages. Treatment of peripheral blood monocytes with 50 ng of TNF-α per ml reduced the reverse transcriptase activity of HIV-1 by 70%, whereas treatment with 5 ng/ml reduced it by 30% (27). In A549 human lung carcinoma cells treated with 10,000 ng of TNF-α or IFN-γ per ml, the replication of vesicular stomatitis virus, encephalomyocarditis virus, adenovirus type 2, and herpes simplex virus type II was inhibited only 10- to 100-fold. However, simultaneous treatment of the cells with both cytokines inhibited virus replication 1,000- to 10,000-fold (45). The differences observed in response to TNF-α may reflect either viral sensitivity or the cellular systems used.

TNF-α antiviral activity seems to be species specific. In a study of TNF-α inhibition of influenza virus replication in MDCK cells infected with A/PR/8/34 (H1N1) (45), TNF-α inhibition effect was transient. At 48 h postinfection, the difference between virus yield from control and recombinant murine TNF-α-treated MDCK cells was no longer found. This study did not determine TNF-α induction in MDCK cells. Our study is in accordance with this study. Treatment of MDCK cells with recombinant swine or human TNF-α did not inhibit the replication of influenza virus (data not shown).

Whether TNF-α can inhibit human cells remains to be studied. At present, we do not have a good human cell line to productively support influenza virus and to test TNF-α inhibition. Furthermore, whether TNF-α can be induced in cells from other human sources, such as endothelial cells and dendritic cells, needs to be addressed.

In conclusion, our findings suggest that TNF-α plays an important role in resolving influenza virus infection in the host

respiratory tract. The molecular mechanisms by which TNF-α inhibits influenza virus replication warrant further study.

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