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Expression of Influenza Virus Hemagglutinin Activates Transcription Factor NF-κB

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Influenza virus infection initiates transcription of a variety of genes for cytokines such as tumor necrosis factor alpha (TNF-α), TNF-β, interleukin 1α, (IL-1α), IL-1β, IL-2, IL-4, IL-6, IL-10, granulocyte macrophage colony-stimulating factor, and gamma interferon. However, the mechanism by which virus infection elicits cytokine expression remains unknown. Six influenza virus-induced cytokine genes are targets for the inducible transcription factor NF-κB, a central regulator of the human immune response. Here, we show that expression of a single influenza virus protein, the virion surface hemagglutinin, strongly activates NF-κB DNA binding and transactivation. Activation is inhibited in the presence of the antioxidant dithiothreitol, suggesting that, similar to the findings for previously described inducers of NF-κB, hemagglutinin expression generates radical oxygen intermediates which activate the transcription factor. Hemagglutinin is the first secretory and structural viral protein reported to activate NF-κB and thus represents a new class of inducers for this transcription factor. We discuss these results in the context of clinical complications of influenza virus infection.

In the past 20 years, influenza virus infection has caused over 500,000 deaths in the United States alone. During the most severe pandemic of this century, in 1918, influenza killed 21 million people worldwide (24). In order to study acute influenza virus infection in vivo, murine influenza virus pneumonia is used as an animal model. With this system, several groups (8, 17) have shown that cells from infected mediastinal lymph nodes as well as from bronchoalveolar lavage synthesize large amounts of interleukin 1α (IL-1α), IL-1β, IL-2, IL-4, IL-6, IL-10, gamma interferon, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor beta (TNF-β) mRNAs. Using a different model, the infection of human monocytes with influenza virus, Sprenger et al. (22) observed significant increases in TNF-α, IL-1β, and IL-6 mRNAs in infected cells. However, the mechanism by which virus infection elicits cytokine gene transcription remains unknown.

The TNF-α, TNF-β, IL-1β, IL-2, IL-6, and GM-CSF genes are all target genes for the human transcription factor NF-κB (3). This protein plays an integral role in the immune response. It is present in an inactive, cytoplasmic form in almost all cell types. Upon cell stimulation by a large variety of pathogenic conditions, NF-κB is released from IκB, its inhibitory subunit (2). The activated factor can then translocate to the nucleus, where it binds its cognate DNA sequences, initiating transcription of numerous target genes encoding cytokines, immunoreceptors, cell adhesion molecules, acute-phase proteins, and hematopoetic growth factors (listed in detail by Baeuerle and Henkel [3]). NF-κB is activated by a variety of virus infections as well as by the expression of viral proteins (for a complete list, see reference 3). However, influenza virus infection has not previously been found to activate NF-κB. Here, we report that expression of a single structural viral protein, the influenza virus hemagglutinin (HA), activates NF-κB DNA binding and transcriptional activation. HA is the first structural viral protein shown to activate NF-κB and as such represents a novel class of NF-κB inducers.

All previously characterized NF-κB-activating stimuli seem to use a common signal transduction pathway to elicit their response: they increase the intercellular concentration of reactive oxygen intermediates, that is, they elicit oxidative stress (19). In some instances, this has been determined directly by measuring an increased production of H_2O_2 or superoxide in stimulated cells. Furthermore, exposure of some cell lines to micromolar concentrations of H_2O_2 activates NF-κB (21). Indirect evidence for reactive oxygen intermediates as signal transducers in this pathway comes from the finding that a large variety of chemically distinct antioxidants prevent the activation of NF-κB by all stimuli investigated so far (12, 20). HA is no exception to this rule. HA-mediated NF-κB activation is also inhibited by the antioxidant dithiothreitol (DTT). We discuss our results in the context of the severe clinical complications observed in patients suffering concurrent bacterial and influenza virus infections.

MATERIALS AND METHODS

Cell culture. HeLa cells and 293 cells (CCL 2 and CRL 1573, respectively; American Type Culture Collection) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 50 μg of penicillin-streptomycin per ml (all from Gibco-BRL, Eggenstein, Germany).

Transfections. HeLa cells were plated 12 to 16 h prior to transfection at a density of 10^6/60-mm-diameter dish. Transfections were performed by using calcium phosphate precipitation as previously described (7). The amounts of plasmids used are indicated in the figure legends.

Plasmids. The HA expression vector contains the entire HA coding sequence of the influenza virus strain A/FPV/Rostock/34 (H7N1) cloned into the vaccinia virus insertion vector pSC11 and was a generous gift of W. Garten and H. Klenk (University of Marburg, Marburg, Germany). The plasmid 6x-κB-tk-Luc contains three repeats of the human immunodeficiency virus type 1 (HIV-1) tandem NF-κB sites in front of a minimal thymidine kinase (TK) promoter and has been described previously (11). 6x-κB-tk-Luc, the parental tk-Luc, and the chloramphenicol acetyltransferase (CAT) expression vector were generous gifts of Markus Meyer, Heidelberg, Germany. The IκB expression vector Rc/CMV-IκB has been described previously (26); it contains the entire MAD-3 cDNA inserted as a *Hin*dIII fragment into Rc/CMV. The parental Rc/CMV vector was purchased from Invitrogen, Leek, The Netherlands.

Electrophoretic mobility shift assay (EMSA) and antibody supershifts. Total cell extracts were prepared by using a high-salt detergent buffer (Totex) described previously (2). Briefly, cells were harvested by centrifugation, washed once in ice-cold phosphate-buffered saline (Sigma, Deisenhofen, Germany), and

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resuspended in 4 cell volumes of Totex buffer, containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 350 mM NaCl, 20% (wt/vol) glycerol, 1% (wt/vol) Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 0.5 mM DTT, 0.1% phenylmethylsulfonyl fluoride (PMSF), and 1% aprotinin. The cell lysate was incubated on ice for 30 min and then centrifuged for 5 min at 13,000 × *g* at 4°C. The protein content of the supernatant was determined, and equal amounts of protein (10 to 20 μg) were added to a reaction mixture containing 20 μg of bovine serum albumin (Sigma), 2 μg of poly(dI-dC) (Boehringer, Mannheim, Germany), 2 μl of buffer D+ (20 mM HEPES [pH 7.9], 20% glycerin, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM DTT, 0.1% PMSF), 4 μl of buffer F (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1% PMSF), and 100,000 cpm (Cerenkov) of a ^{32}P-labeled oligonucleotide in a final volume of 20 μl. The reaction mixture was incubated at room temperature for 25 min. For the supershift assays, 2.5 μl of antibody was added to the reaction mixture simultaneously with the protein and the mixture was incubated as described above. Anti-p50, anti-p65, and anti-c-Rel antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, Calif. Anti-HA antibody was a generous gift of W. Garten and H. Klenk. NF-κB and AP-1 oligonucleotides (Promega, Heidelberg, Germany) were labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham, Braunschweig, Germany) and T4 polynucleotide kinase (Promega).

Luciferase assays. Cells were harvested 48 h posttransfection, and luciferase activity was determined precisely as described previously (23). The cell pellet obtained from one 60-mm-diameter dish was resuspended in 150 μl of lysis buffer (25 mM glycylglycine, 1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) and centrifuged at 13,000 × *g* at 4°C for 5 min. A 50-μl volume of the supernatant was assayed in 150 μl of assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KP_i [pH 7.5], 1 mM DTT, 1 mM ATP) with an LB 96 P luminometer (EG & G-Bertold, Bad Wildbad, Germany). Light emission was measured over a 30-s interval, and the results are given in relative light units.

RESULTS

Transfection of influenza virus HA activates NF-κB binding activity. 293 cells were transfected with 6 μg of an expression vector encoding the influenza virus HA or CAT or an empty vector, and total cell extracts were prepared 24 h after transfection. Equal amounts of protein were analyzed in an EMSA using a ^{32}P-labeled oligonucleotide with a high-affinity binding site for NF-κB. Figure 1 shows that expression of influenza virus HA protein (lane 2) strongly induced a novel DNA binding activity, which migrates more slowly than a constitutive complex. Transfection of the empty vector backbone (Fig. 1, lane 4) or of the CAT protein (lane 3) had no effect or only a marginal effect.

Complexes binding the NF-κB target sequence may constitute a variety of hetero- or homodimers. These can be composed of five distinct subunits, all members of the NF-κB–Rel–dorsal family of transcription factors. We have analyzed the subunit composition of the HA-induced κB-binding complex, using an antibody supershift assay. The cell extract used in Fig. 1, lane 2, was used in an EMSA in which antibodies against the NF-κB subunit p50, p65, or c-Rel were added to the binding reaction mixture. The HA-induced complex binding to the κB probe is shown in Fig. 2, lane 1. The entire binding activity reacted with anti-p50 antibody, causing it to migrate more slowly in the gel (Fig. 2, lane 2). In contrast, anti-p65 (Fig. 2, lane 3) and anti-c-Rel (lane 4) each recognized only a portion of the NF-κB complex, leading to a partial reduction of DNA binding. Only when both anti-p65 and anti-c-Rel were added to the reaction mixture (Fig. 2, lane 5) was complex binding abolished completely. Anti-HA antibody was included as a control and did not alter the amount or the mobility of the NF-κB complex (Fig. 2, lane 6). Finally, competition analysis using an excess of unlabeled κB oligonucleotide (Fig. 2, lane 7) or the unrelated AP-1 binding site oligonucleotide (lane 8) confirmed that the complex binds specifically to the NF-κB site. These data identify the HA-induced NF-κB complex as a mixture of p50-p65 and p50–c-Rel heterodimers.

HA-induced NF-κB activates reporter gene expression. In order to test whether the HA-induced NF-κB is functional, we used a reporter gene assay. HeLa cells were transfected with a vector containing a minimal TK promoter preceded by three tandem repeats of the NF-κB sites found in the HIV long terminal repeat (6x-κB-tk-Luc). This reporter gene construct was cotransfected with increasing amounts of HA expression vector (Fig. 3). The total amount of DNA transfected was equalized by the addition of empty Rc/CMV vector, which was shown to have no effect on κB activity (Fig. 1, lane 4). Figure 3 shows that gene activation by influenza virus HA is dose dependent; 0.5 μg of expression plasmid achieved very little activation, while transfection of 3 and 6 μg of HA expression vector increased reporter gene activity 8- and 16-fold, respectively. In order to determine the relative levels of HA protein expressed, we stained HeLa cells transfected with 6 μg of HA expression vector with an anti-HA antibody. By fluorescence microscopy, low levels of HA were detected in approximately 10% of the cells. Thus, small quantities of the protein suffice to activate NF-κB-dependent gene expression.

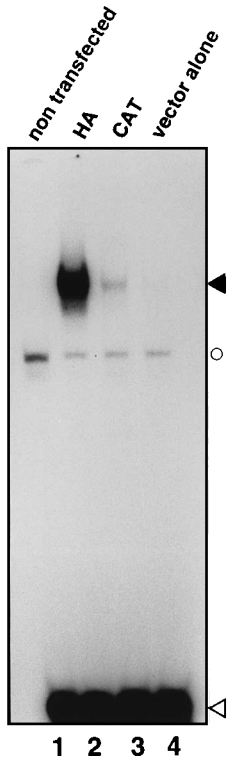

FIG. 1. Expression of influenza virus HA protein activates NF-κB. Results of an EMSA using total 293 cell extracts of untransfected cells (lane 1) or cells transiently transfected with 6 μg of HA expression vector (lane 2), 6 μg of CAT expression vector (lane 3), or 6 μg of empty Rc/CMV vector (lane 4) are shown. Specific NF-κB complexes (filled arrowhead), nonspecific binding to the probe (open circle), and unbound oligonucleotide (open arrowhead) are indicated.

HA-induced reporter gene activity depends specifically on the κB sites and is repressed by IκB. We investigated whether the HA-induced luciferase activity (Fig. 3) was mediated by the activation of NF-κB, as the EMSA data suggested (Fig. 1). HeLa cells were transfected with either the 6x-κB-tk-Luc plasmid or the parental tk-Luc vector, which contains only the minimal TK promoter driving luciferase expression. Four additional plasmids were cotransfected: the HA or the CAT expression vector together with either an expression vector encoding the inhibitory subunit IκB or the empty Rc/CMV vector. The results of these experiments are shown in Fig. 4. Expression of neither HA nor CAT protein affected the basal

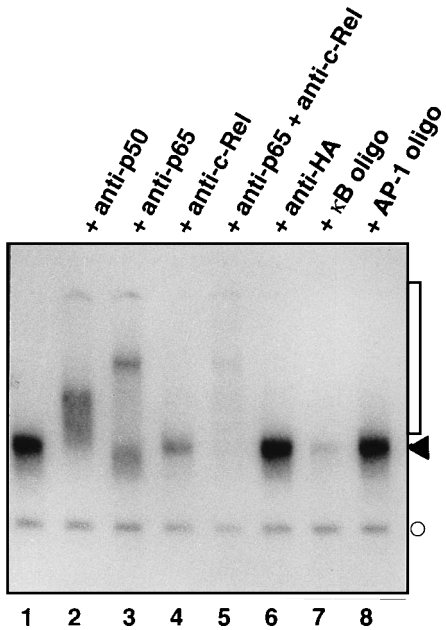

FIG. 2. Identification of the NF-κB subunit composition. Results of an EMSA of total cell extracts from 293 cells transiently transfected with 6 μg of HA expression vector are shown. Lane 1, control; lanes 2 to 6, extracts incubated with the indicated antibodies; lane 7, extracts incubated with a 50-fold excess of unlabeled NF-κB oligonucleotide; lane 8, extracts incubated with a 50-fold excess of unlabeled AP-1 oligonucleotide. The specific NF-κB complex (filled arrowhead), the nonspecifically binding complex (circle), and the supershifted bands (bracket) are indicated.

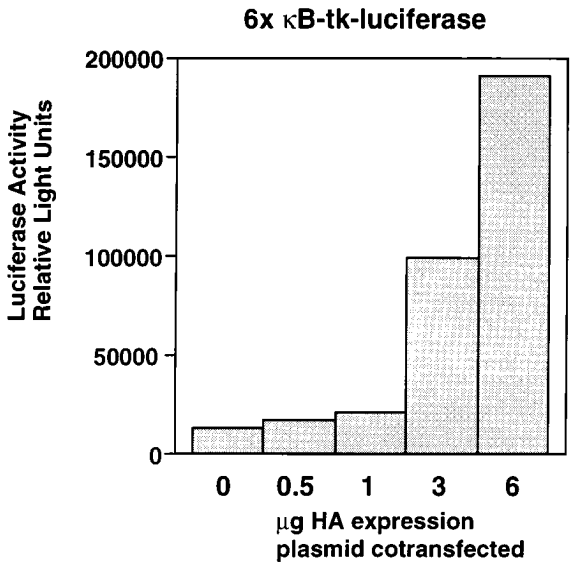

FIG. 3. HA protein activates gene expression. HeLa cells were transiently transfected with 2.5 μg of 6x-κB-tk-Luc reporter plasmid together with the indicated amounts of HA expression vector. The total amount of DNA transfected was equalized by the addition of empty Rc/CMV vector. Cells were harvested 48 h after transfection, and the luciferase activity was determined. Results are given in arbitrary relative light units. The experiment was performed three times with similar results, and results of a representative experiment are shown.

TK promoter activity. However, expression of HA protein strongly induced luciferase activity in cells transfected with the κB-dependent reporter construct (6x-κB-tk-Luc). Transfection of the CAT expression plasmid increased reporter gene activity very slightly. This parallels the slight induction of NF-κB binding activity observed in Fig. 1 (lane 3) following CAT expression. The NF-κB-dependent luciferase activity was abrogated entirely by cotransfection of IκB but not by the empty vector (Fig. 4). These data show that expression of influenza virus HA activates gene expression specifically through NF-κB binding sites.

HA-mediated NF-κB activation is inhibited under antioxidant conditions. Since all NF-κB-activating stimuli described to date are inhibited by antioxidants, we tested whether this is also true of HA-mediated NF-κB activation. 293 cells were preincubated in the presence of the antioxidant DTT (1 to 5 mM) for 1 h prior to either stimulation with TNF (Fig. 5, lanes 2 to 5) or transfection with 6 μg of HA expression vector (Fig. 5, lanes 6 to 9). Cell extracts were prepared 4 h after stimulation or transfection and assayed for NF-κB activity in an EMSA. Since DTT loses its antioxidative effect over time, cells were transfected for only 4 h, during which much less HA protein is produced than after 24 h of transfection. Thus, the level of HA-mediated NF-κB activation in this experiment (Fig. 5, lane 6) is lower than that in Fig. 1 (lane 2) or than the level of TNF-mediated activation (Fig. 5, lane 6). While incubation with 1 mM DTT was ineffective in preventing NF-κB activation (Fig. 5, lanes 3 and 7), addition of either 2.5 or 5 mM DTT prevented induction by both TNF and HA (lanes 4, 5, 8, and 9). Thus, the influenza virus virion protein is unable to activate NF-κB under antioxidant conditions. This suggests that, like all other known NF-κB inducers, HA increases the production of reactive oxygen intermediates within the cell, which may act as second messengers to activate the transcription factor. The mechanism by which HA expression elevates intercellular reactive oxygen intermediate levels remains to be investigated.

DISCUSSION

The mechanism by which influenza virus infection mediates cytokine production is unknown. Here, we have shown that

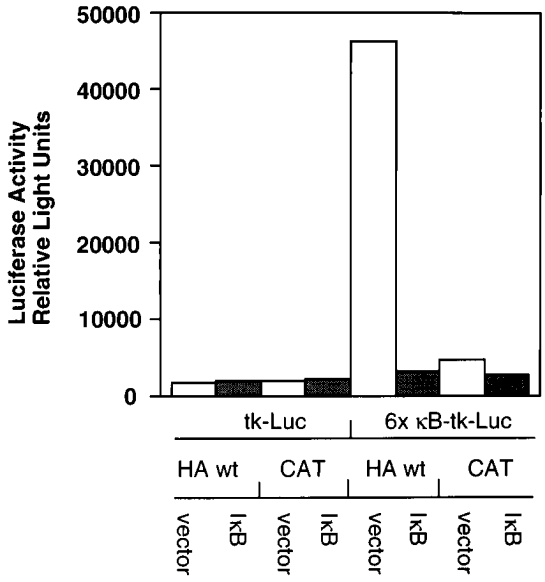

FIG. 4. HA-activated gene expression is NF-κB dependent. HeLa cells were transfected with 2.5 μg of either tk-Luc or 6x-κB-tk-Luc plasmid as indicated. A 6-μg sample of either the HA or the CAT expression vector was cotransfected together with 5 μg of either IκB expression vector or empty Rc/CMV vector as indicated. Cells were harvested 48 h posttransfection, and luciferase activity was determined. Results are averages of duplicate experiments. wt, wild type.

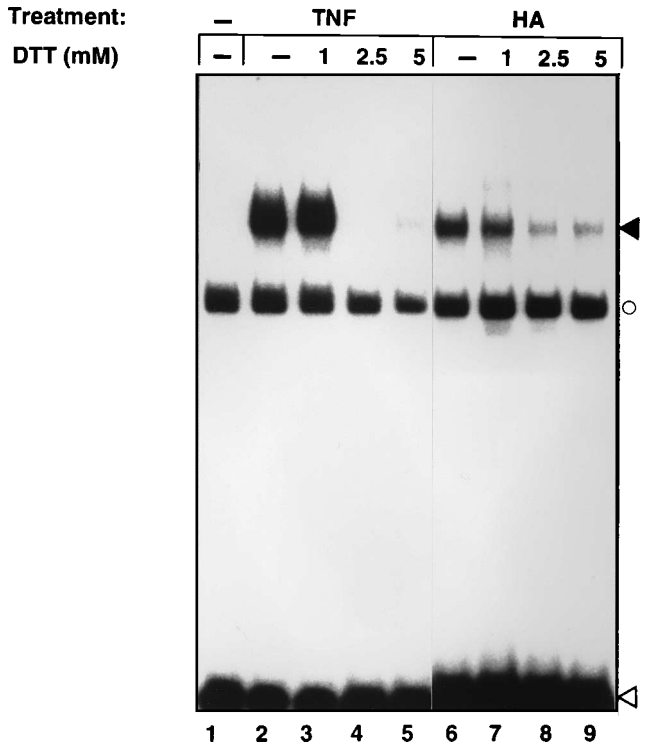

FIG. 5. HA-mediated NF-κB activation is inhibited by DTT. 293 cells were pretreated for 1 h with the indicated concentrations of DTT. Subsequently, cells were either stimulated with 200 μg of TNF per ml or transfected with 6 μg of HA expression plasmid as indicated above the lanes. Lanes 1 and 6 show untransfected controls. Total cell extracts were prepared 4 h after stimulation or transfection, and equal amounts of protein were used in an EMSA. Specific NF-κB complexes (filled arrowhead), nonspecific binding to the probe (open circle), and unbound oligonucleotide (open arrowhead) are indicated.

expression of the viral HA protein activates the cellular transcription factor NF-κB. NF-κB induces transcription of a variety of cytokines released during influenza virus infection (3). NF-κB activation thus represents one mechanism by which influenza virus infection increases cytokine transcription. However, several influenza virus-induced cytokines, such as IL-10 and gamma interferon, are not known to be NF-κB target genes. Therefore, the virus must employ other mechanisms of gene activation as well.

NF-κB is activated during various viral infections. The following viruses have been shown to induce the factor: HIV-1, human T-cell leukemia virus type I (HTLV-I), hepatitis B virus, herpes simplex virus type 1, human herpesvirus 6, Newcastle disease virus, Sendai virus, Epstein-Barr virus, and adenovirus (for a review, see reference 3). HIV-1 is the most studied virus in this context, and it is interesting that HIV-1 itself harbors two NF-κB binding sites in its long terminal repeat (13). The virus thus exploits the cellular transcription factor, which it activates by infection, by using it to enhance transcription of the viral genome. Infection with an intact virus is not always required for NF-κB activation. In several cases, the expression of a single viral protein is sufficient to activate the factor. This has been reported for $p40^{tax}$ protein from HTLV-I, HBx and $MHBs^t$ from hepatitis B virus, and EBNA-2 and latent membrane protein from Epstein-Barr virus (3). However, the mechanisms by which these proteins activate gene expression may be quite distinct. All viral proteins which activate NF-κB have transactivating or signal-transducing functions on their own. For example, $p40^{tax}$ is a pleiotropic transcriptional activator without DNA binding activity of its own (25). Influenza virus HA is the first structural viral protein shown to activate NF-κB. This raises interesting questions about the biology of the HA protein. Is it indeed only a structural protein, or does it function as a signal transducer? So far, there is no evidence suggesting that the HA protein possesses intrinsic signal-transducing activity. Rather, we suggest that the synthesis of large amounts of HA during viral infection leads to an accumulation of protein in the endoplasmic reticulum. We have recently shown that intracellular accumulation of proteins leads to NF-κB activation (14). This represents a likely mechanism by which a structural viral protein can activate this transcription factor.

The data implicating NF-κB in influenza virus signal transduction have important clinical significance. It is known that patients presenting with combined influenza and bacterial infections frequently suffer serious complications (1, 10). Gong and colleagues (5) have shown that bacterial lipopolysaccharide (LPS) stimulates TNF release from influenza virus-infected cells more than sevenfold. Since LPS is also a potent inducer of NF-κB (3), we propose that the dual and continuous NF-κB stimulation by influenza virus and LPS mediates the increased TNF release. Large quantities of circulating TNF can cause high fever, cell damage, cachexia, and shock symptoms (4). Thus, inhibiting NF-κB activation may prove therapeutic in patients with combined influenza virus and bacterial infections. Several antioxidative agents have been shown to prevent NF-κB activation (18). These agents are also being used in clinical trials for the treatment of AIDS (16). As mentioned above, NF-κB plays a critical role in HIV gene activation. It is possible that influenza virus infection, by activating NF-κB, may trigger HIV provirus transcription, thus exacerbating the disease.

Another pathological characteristic of influenza virus infection may also involve NF-κB. Influenza virus was shown to induce apoptosis in tissue culture cells (9, 23). Cells stably transfected with Bcl-2, an inhibitor of apoptosis, were much less sensitive to virus-mediated cytotoxicity and showed no signs of apoptosis following infection (9). It has recently been demonstrated that NF-κB is activated in cells undergoing apoptosis (6). In addition, the expression of Bcl-2 inhibited NF-κB-mediated gene activation. Perhaps NF-κB activates genes involved in programmed cell death, and influenza virus infection invokes apoptosis by causing prolonged NF-κB activation. We therefore suggest that the transcription factor NF-κB plays a pivotal role in influenza virus-mediated morbidity and mortality. NF-κB antagonists may be used therapeutically in patients suffering from viral pneumonia, which is refractory to treatment and incurs a high mortality.

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