Inhibition of Viral Replication Reverses Respiratory Syncytial Virus-Induced NF- κ B Activation and Interleukin-8 Gene Expression in A549 Cells

MICHAEL A. FIEDLER,* KARA WERNKE-DOLLRIES, AND JAMES M. STARK

Division of Pulmonary Medicine, Department of Pediatrics, Children's Hospital Research Foundation, Cincinnati, Ohio 45229-3039

Received 29 May 1996/Accepted 4 September 1996

Previous studies have demonstrated that respiratory syncytial virus (RSV) infection of airway epithelial cells results in the expression of a number of cytokines, such as interleukin-8 (IL-8), that are transcriptionally regulated by nuclear factor κ B (NF- κ B). In the studies reported here, we demonstrate that treatment of RSV-infected A549 cells with 100 μ g of ribavirin (a viral replication inhibitor) per ml results in reversal of RSV-induced NF- κ B activation, IL-8 mRNA expression, and IL-8 protein production in A549 cells. These data confirm that viral replication is a key step in RSV-induced NF- κ B activation and IL-8 production.

Respiratory syncytial virus (RSV) lower respiratory tract infection is a major cause of morbidity and mortality in children less than 2 years of age. RSV causes an estimated 90,000 hospitalizations and 4,500 deaths annually in children in the United States (5). In addition, RSV infection is associated with increased morbidity and mortality in children with chronic lung disorders, such as cystic fibrosis and bronchopulmonary dysplasia (1, 9). Furthermore, a significant percentage of previously healthy children develop chronic pulmonary function abnormalities after RSV infection (4, 21).

In vitro and in vivo studies have indicated that airway inflammation plays a key role in RSV-induced airway injury and that proinflammatory cytokines are involved in the process (2, 13, 16). A number of cytokines produced by airway epithelial cells in response to RSV infection are regulated at the transcriptional level by NF- κ B. Studies in our laboratory and others have demonstrated that NF- κ B is critical in RSV-induced interleukin-8 (IL-8) gene expression (8, 12). Our working hypothesis was that inhibition of viral replication would result in a reversal of RSV-induced NF- κ B activation and subsequent cytokine production by infected airway epithelial cells. The results of the studies reported here indicate that inhibition of viral replication reversed the effects of RSV on NF- κ B activation, IL-8 gene expression, and IL-8 protein release without evidence of cell toxicity.

RSV infection increases IL-8 and RSV-F gene expression and IL-8 protein release. Previous studies in our laboratory and others demonstrated that RSV infection of airway epithelial cells resulted in IL-8 gene expression and protein release (7, 13). In attempt to correlate viral replication with IL-8 gene expression, Northern (RNA) blot analysis of control (cells treated with nonreplicative RSV [7]) and RSV-infected A549 cell RNA was performed. Results from these experiments indicated that as RSV-F expression (used as a measure of viral replication) increased, IL-8 gene expression increased. Furthermore, IL-8 protein production in RSV-infected A549 cells correlated with increasing viral replication.

RSV infection results in increased NF- κ B activation in A549 cells. In order to determine whether RSV infection induced NF- κ B activation, nuclear extracts were obtained from A549 cells exposed to UV-inactivated virus (control) or RSV at a multiplicity of infection of 1.0 for 2 h, washed in Dulbecco's modified Eagle medium, and subsequently maintained in Dulbecco's modified Eagle medium with 8% fetal calf serum for 1, 2, or 3 days. The nuclear extracts were subjected to electrophoretic mobility shift assays as previously described (20). For these studies the NF- κ B probe consisted of a double-stranded DNA which contained the NF- κ B consensus binding sequence GGGGACTTTCCC. The results are illustrated in Fig. 1. Equal amounts of nuclear protein were used for all reactions. A minimal, but consistent, increase in NF- κ B activation was observed in RSV-infected cells on day 1 compared with that in controls. NF- κ B activation increased on days 2 and 3 compared with that in control cells. Thus, increased RSV replication (as evidenced by increased RSV-F gene expression) was associated with increased NF- κ B activation.

In order to ensure that our probe was specific for NF- κ B, a 100-fold excess of "cold" (nonradiolabeled) competitor was added. Similarly, a probe containing a mutation in the NF- κ B binding site (mutation underlined), GGCGACTTTCCC, was employed. Additionally, an antibody to the p65 component (obtained from Santa Cruz Inc., Santa Cruz, Calif.) of NF- κ B was used to supershift the signal. As a control, antibody to the c-fos component of AP-1 was used to ensure that any supershifting observed was not secondary to nonspecific effects. As illustrated in Fig. 2, a 100-fold excess of nonlabeled probe competed with the labeled probe for the NF- κ B protein, but the mutant probe did not. Furthermore, the addition of the p65 antibody resulted in partial supershifting of the signal, but the antibody to c-fos did not. While these data do not identify all of the NF- κ B subunits involved, they confirm that the electrophoretic mobility shift assay employed is specific for NF- κ B detection.

An intact NF- κ B site is required RSV-induced transcriptional activity of the 5' flanking region of the IL-8 gene. The next set of experiments were designed to confirm that binding of NF- κ B to the IL-8 gene was necessary for IL-8 mRNA expression. Mutational analysis of the 5' flanking region of the IL-8 gene was performed in order to determine the role of potential nuclear factor binding sites in RSV-induced IL-8

* Corresponding author. Mailing address: Division of Pulmonary Medicine, Department of Pediatrics, Children's Hospital Research Foundation, ASB 5, 3333 Burnet Ave., Cincinnati, OH 45229-3039. Phone: (513) 559-6771. Fax: (513) 559-4615.

FIG. 1. Effects of RSV infection on NF- κ B activation. Minus and plus signs indicate whether a 100-fold excess of cold (nonradiolabeled) probe was added as a competitor. NF- κ B activation increased over the 3 days of infection. Results are representative of four experiments.

gene expression. Preliminary studies demonstrated that a 200-bp fragment of the 5' flanking region of the IL-8 gene, which contains NF- κ B and NF-IL-6 binding sites and the AUG translational start site (14, 15), was transcriptionally active in response to RSV infection. This 20-bp fragment was cloned into reporter plasmid pGL2 (Promega, Madison, Wis.). Similarly, a 200-bp fragment with a mutated NF- κ B site (same

mutation described above: GGGGACTTTCCC to GGCGAC TTTCCC) was cloned into plasmid pGL2. The plasmid of interest was then transfected into A549 cells by calcium phosphate precipitation (22). To control for transfection efficiency, plasmid pGL2IL-8 was cotransfected with a plasmid containing the β -galactosidase gene under the transcriptional control of the cytomegalovirus promoter, which is constitutively active in A549 cells.

The cells were infected with RSV at 40% confluence. Two days after infection, cell lysates were obtained and luciferase (6, 18) and β -galactosidase activities were determined (7). The results, illustrated in Fig. 3 ($n = 12$), are reported as an increase over control (cells treated with UV-inactivated RSV). Cells containing the luciferase gene under the transcriptional control of the 200-bp fragment of the IL-8 gene demonstrate a sevenfold increase in luciferase activity in response to RSV infection ($P < 0.01$ compared with the results for the control, as determined by analysis of variance [ANOVA]). However, mutating the NF- κ B site resulted in a significant decrease in luciferase activity in response to RSV infection ($P < 0.01$

FIG. 2. Specificity of the electrophoretic mobility shift assay for NF- κ B activation in A549 cells. Ab, antibody.

FIG. 3. Transcriptional activity of the 5' flanking region of the IL-8 gene in response to RSV infection. 200 bp, 200-bp 5' flanking region of IL-8 gene; 200 bp NF- κ B mu, 200-bp fragment with a mutation of the NF- κ B site.

FIG. 4. Effects of ribavirin on IL-8 and RSV-F gene expression. Results are representative of three separate experiments.

compared with results for RSV treatment of cells containing the 200-bp fragment but not significantly different from results for the control). Finally, cells transfected with the promoterless plasmid pGL2 (control cells) did not demonstrate any detectable increase in luciferase activity in response to RSV infection. Data presented in the previous section demonstrated that NF- κ B activation occurred with viral infection, and these data demonstrate that an intact NF- κ B site is necessary for IL-8 gene transcription in response to viral replication.

Effects of the viral replication inhibitor ribavirin on RSV-induced IL-8 mRNA expression and IL-8 protein release. Since IL-8 mRNA expression and protein release were associated with viral replication, we sought to determine if inhibition of viral replication would reverse these phenomena. A549 cells were infected with RSV, and 18 h after exposure to virus, ribavirin was added to the cells in the 1- to 100- μ g/ml range. The cells were maintained in ribavirin-containing medium for 2 days. After the first 24-h period of ribavirin therapy, medium was removed and replaced with fresh medium and ribavirin. Twenty-four hours later, this medium was collected for assessment of viral particle release. Viral particle release was assessed by plaque assay as previously described (7). The addition of 1 μ g of ribavirin per ml resulted in a 75% decrease in viral particle release from the infected cells ($P < 0.05$ compared with results for RSV-infected cells not treated with ribavirin, as determined by ANOVA; $n = 4$). Similarly, the addition of 10 or 100 μ g of ribavirin per ml resulted in a 98% decrease in the number of viral particles released by RSV-infected cells ($P < 0.01$ compared with results for RSV-infected cells not treated with ribavirin, as determined by ANOVA; $n = 4$). Northern blot analysis of RNA obtained from the cells after 2 days of ribavirin treatment revealed decreased RSV-F gene expression with increasing doses of ribavirin (Fig. 4). These data indicated that ribavirin reversed RSV replication in A549 cells.

In association with the inhibition of RSV replication, Northern blot analysis demonstrated a reversal of IL-8 gene expres-

FIG. 5. Effects of ribavirin on IL-8 protein release from the cells. RSV + 1, RSV + 10, and RSV + 100, RSV infection coupled with treatment with 1, 10, and 100 μ g of ribavirin per ml, respectively.

sion when increased doses of ribavirin were added. However, in contrast to the complete reversal of RSV-F gene expression noted, residual IL-8 gene expression was noted (Fig. 4). Evaluation of IL-8 released from the cells yielded similar results. Analysis of the supernatants from the second 24-h period of ribavirin treatment is illustrated in Fig. 5. As little as 1 μ g of ribavirin per ml resulted in a partial reversal of the effects of RSV on IL-8 protein release ($P < 0.01$, as determined by ANOVA; $n = 4$). Furthermore, increasing the dose of ribavirin to 100 μ g/ml resulted in a marked decrease in IL-8 release ($P < 0.01$, as determined by ANOVA). The differences in IL-8 release were significant for all conditions ($P < 0.01$, as determined by ANOVA; $n = 4$) except for RSV infection coupled with treatment with 10 μ g of ribavirin per ml and RSV infection coupled with treatment with 100 μ g of ribavirin per ml. Minimal IL-8 was detected in the cell lysates under all conditions, indicating that ribavirin did not inhibit the release of IL-8. Again, it is worth noting that a complete reversal of IL-8 release was not achieved despite complete inhibition of viral replication.

Effects of ribavirin on RSV-induced NF- κ B activation. In order to determine whether reversal of RSV replication resulted in an amelioration of NF- κ B activation, nuclear extracts were obtained from RSV-infected cells after treatment with ribavirin for 48 h. As illustrated in Fig. 6, electrophoretic mobility shift assay analysis revealed a marked reversal of RSV-induced NF- κ B activation by 100 μ g of ribavirin per ml. The effects of treatment with 10 μ g of ribavirin per ml varied but generally demonstrated a partial reversal of NF- κ B activation. The effects of treatment with 1 μ g of ribavirin per ml were minimal.

The effects of ribavirin were not secondary to toxicity. In order to ensure that the effects observed with ribavirin treatment were not secondary to cell toxicity, the release of lactate dehydrogenase (LDH) from the cells was determined (19). No increase in LDH release from the cells was noted with any concentration of ribavirin used. In fact, control cells released 1% of the total cellular LDH, in contrast to cells infected with RSV, which released 2% of the total cellular LDH. Treatment with 1, 10, or 100 μ g of ribavirin per ml resulted in a more complete reversal of the small increase in LDH release noted with RSV infection.

In summary, the studies reported here demonstrate that in airway epithelial cells, RSV infection resulted in increased NF- κ B activation, IL-8 gene expression, and IL-8 protein release, all in association with viral replication. Inhibition of viral

FIG. 6. Effects of ribavirin on NF- κ B activation. Results are representative of four separate experiments.

replication resulted in a reversal of these effects, implying that RSV replication is a key step in initiating and maintaining inflammation in the airway. While a number of cytokines are released in response to RSV, we chose to study IL-8 because it is the major chemotactic and activating agent for polymorphonuclear leukocytes in the airway. Also, as is the case with the transcriptional activation of other cytokines (3), NF- κ B is activation is absolutely required but is not sufficient for IL-8 gene expression (8, 10–12). Thus, understanding the role of NF- κ B activation in the response to RSV infection is important in understanding the inflammatory response.

We chose to study airway epithelial cells, as in vitro studies have demonstrated that RSV induces cytokine expression in respiratory epithelial cells, the precise cells targeted for infection by the virus. In vivo studies have not been performed to evaluate the response of airway epithelial cells to RSV with respect to cytokine production. Since such studies would likely involve surgical biopsy during a clinically critical stage of infection (17), they will be difficult to perform.

REFERENCES

- Abman, S. H., J. W. Ogle, N. Butler-Simon, C. M. Rumack, and F. J. Accurso. 1988. Role of respiratory syncytial virus in early hospitalizations for respiratory distress of young infants with cystic fibrosis. *J. Pediatrics* **113**:826–830.
- Arnold, R., B. Humbert, H. Werchau, H. Gallati, and W. Konig. 1994. Interleukin-8, interleukin-6, and soluble tumor necrosis factor receptor type II release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. *Immunology* **82**:126–133.
- Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF- κ B in the immune system. *Annu. Rev. Immunol.* **12**:141–179.
- Breese Hall, C., W. J. Hall, C. L. Gala, F. B. McGill, and J. P. Leddy. 1984. Long term prospective study in children after respiratory syncytial virus infection. *J. Pediatrics* **105**:358–364.
- Centers for Disease Control and Prevention. 1994. Update: respiratory syncytial virus activity—United States, 1994–1995 season. *Morbidity and Mortality Weekly Rep.* **43**:920–922.
- de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**:725–737.
- Fiedler, M. A., K. Wernke-Dollries, and J. M. Stark. 1995. Respiratory syncytial virus increases IL-8 gene expression and protein release in A549 cells. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* **269**:L865–L872.
- Fiedler, M. A., K. Wernke-Dollries, and J. M. Stark. 1996. Respiratory syncytial virus increases IL-8 gene transcription via NF- κ B activation in A549 cells. *Pediatr. Pulmonol.* **12**(Suppl.):275 (Abstract.)
- Groothuis, J. R., K. M. Gutierrez, and B. A. Lauer. 1988. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. *Pediatrics* **82**:199–203.
- Jamaluddin, M., R. Garofolo, P. L. Ogra, and A. R. Brasier. 1996. Inducible translational regulation of the NF-IL6 transcription factor by respiratory syncytial virus infection in pulmonary epithelial cells. *J. Virol.* **70**:1554–1563.
- Marini, M., E. Vittori, J. Hollemberg, and S. Mattoli. 1992. Expression of the potent inflammatory cytokines, granulocyte-colony-stimulatory factor and interleukin-6 and interleukin-8, in bronchial cells of patients with asthma. *J. Allergy Clin. Immunol.* **89**:1001–1009.
- Mastronarde, J. G., B. He, M. M. Monick, N. Mukaida, K. Matsushima, and G. W. Hunninghake. 1996. Induction of interleukin-8 (IL-8) gene expression by respiratory syncytial virus involves activation of nuclear factor (NF)- κ B and NF-IL-6. *J. Infect. Dis.* **174**:262–267.
- Mastronarde, J. G., M. M. Monick, and G. W. Hunninghake. 1995. Oxidant tone regulates IL-8 production in epithelium infected with respiratory syncytial virus. *Am. J. Respir. Crit. Care Med.* **13**:237–244.
- Mukaida, N., S. Okamoto, Y. Ishikawa, and K. Matsushima. 1994. Molecular mechanism of interleukin-8 gene expression. *J. Leukocyte Biol.* **56**:554–558.
- Mukaida, N., M. Shiroo, and K. Matsushima. 1989. The genomic structure of the human monocyte derived neutrophil chemotactic factor IL-8. *J. Immunol.* **143**:1366–1371.
- Noah, T. L., and S. Becker. 1993. Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* **265**:L472–L478.
- Noah, T. L., F. W. Henderson, I. A. Wortman, R. B. Devlin, J. Handy, H. S. Koren, and S. Becker. 1995. Nasal cytokine production in viral acute upper respiratory infections of childhood. *J. Infect. Dis.* **171**:584–592.
- Ow, D. W., K. V. Wood, M. DeLuca, J. R. DeWet, D. R. Helinski, and S. H. Howell. 1986. Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234**:856–859.
- Rice, W. R., K. C. Osterhout, and J. A. Whitsett. 1984. Effect of cytochalasins on surfactant release from alveolar type II cells. *Biochim. Biophys. Acta* **805**:12–18.
- Warner, B. B., L. Stuart, S. Gebb, and J. R. Wispe. 1996. Redox regulation of manganese superoxide dismutase. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* **265**:L150–L158.
- Webb, M. S. C., R. L. Henry, A. D. Milner, G. M. Stokes, and A. S. Swarbrick. 1985. Continuing respiratory problems three and a half years after acute viral bronchiolitis. *Arch. Dis. Child.* **60**:1064–1067.
- Wigler, M., S. Silverstein, L. Lee, A. Pellicer, Y. Cheng, and R. Axel. 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11**:223–232.