Effect of Respiratory Syncytial Virus and Virus-Antibody Complexes on the Oxidative Metabolism of Human Neutrophils

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The effect of respiratory syncytial virus (RSV) or mixtures of RSV and its specific antibody on the oxidative metabolic activity of human polymorphonuclear leukocytes was studied by the technique of luminol-dependent chemiluminescence. Peripheral blood neutrophils obtained from normal healthy donors were used. RSV alone failed to induce any chemiluminescent response by the neutrophils. However, mixtures of RSV and RSV antibody-positive serum regularly elicited significant neutrophil chemiluminescence. Ultracentrifugation, electron microscopy, and Raji cell immune complex assays of virus-antibody mixtures suggested that the neutrophil chemiluminescent response was related to the presence of specific immune complexes of RSV antigen-antibody. Heat inactivation of the serum significantly reduced the polymorphonuclear leukocyte chemiluminescence, and the response also appeared to be dependent on the dose of the virus and the antibody in the reaction mixture. It is proposed that interaction between the neutrophil and RSV-specific immune complexes may contribute to the pathogenesis of RSV infection via the possible release of metabolic products from the activated neutrophils.

Human infections with respiratory syncytial virus (RSV) are frequently associated with the development of bronchospasm and bronchiolitis, particularly in young infants. Previous studies have demonstrated significant alterations in virus-specific lymphocyte function with severe RSV-induced disease (34). However, the precise mechanism of RSV-induced bronchospasm remains to be determined. Although the bulk of available evidence suggests that neutrophils play a major role in bacterial diseases, recent studies have indicated that neutrophils may also participate in the early stages of viral infections. For example, neutrophils migrate to the site of viral replication in vivo (3, 11, 29). In vitro studies have demonstrated the release of leukocyte chemotactants during viral replication in tissue culture (32). Neutrophils from humans and a variety of animal species have also been shown to inhibit replication of several different viruses in tissue culture settings (9, 26). Recently, neutrophils have been shown to be associated with the synthesis of mediators that render cells resistant to viral infection (25). Several studies have also demonstrated that neutrophils in the presence of either antibody or complement are cytotoxic for virus-infected cells (17, 33). It has been shown that neutrophils can generate thromboxane-A_2 upon phagocytic stimulation. This substance has a potent bronchoconstrictive effect (13, 30). The present studies were undertaken to determine whether the concepts of virus-neutrophil interaction summarized above can be applied to the understanding of the pathogenesis of RSV infection in humans. Series of experiments were carried out to examine the effects of RSV on neutrophil function in vitro and to characterize the role of RSV-specific antibody on neutrophil-RSV interaction.

MATERIALS AND METHODS

Collection and preparation of neutrophils. Heparinized specimens of blood were collected from eight healthy donors. The donors ranged in age from 20 to 35 years and represented both sexes. None of the donors was suffering from any overt clinical illness or receiving any drugs at the time of and for 2 to 3 weeks before the specimen collection. The same donors were bled repeatedly, and all data presented are based on this uniform donor population. All donors were seronegative for antibody to RSV for several months before specimens were collected. Neutrophils were separated by the previously described procedures (4). Briefly, 10 ml of heparinized blood containing 10 U of heparin per ml of blood collected from the donors was layered on a dextran-Hypaque gradient. The leukocyte-rich plasma fraction was removed and centrifuged over a column of Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) for 10 min at 1,000 rpm in a siliconized glass tube. The neutrophil-erythrocyte pellet was then treated with hypotonic and hypertonic...
sodium to lyse the erythrocytes and subsequently centrifuged at 800 rpm for 10 min. The cells were suspended in Hanks balanced salt solution (HBSS; GIBCO, Grand Island, N.Y.) with luminol in a concentration of 10⁻⁶ polymorphonuclear leukocytes (PMN) per ml. The viability of PMN was greater than 98% as determined by trypan blue dye exclusion. The cell suspension contained about 98% PMN.

**RSV antibody testing.** The antibody activity against RSV in the sera of donors was determined by indirect immunofluorescence as described previously (35).

**Preparation of virus pool.** RSV (long strain) was grown in HEp-2 cell culture monolayers. After 3 days of incubation of 37°C, the virus was harvested by sonication of the infected cell cultures and stored at -70°C after quick freezing. The titer of the virus pool used for these studies was 10⁴ plaque-forming units per ml. Inactivated (killed) RSV was prepared by heat inactivation of the live virus stock at 56°C for 30 min. Uninfected cell culture monolayers prepared as described above were used as cell controls.

**Reagents for chemiluminescence.** Luminol, 5-amino-2,3-dihydro-1,4-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.), was dissolved in dimethyl sulfoxide in a 1.0 M concentration. This mixture was diluted to 2 × 10⁻⁴ M in HBSS. Zymosan (International Chemical and Nuclear Co., Plainview, N.Y.) was prepared as previously described (10). The zymosan particles were suspended in a concentration of 50 μg/ml in barbital buffer and were opsonized in human AB serum at 37°C for 30 min. Tetradecyl phorbol acetate (TPA) was obtained from Consolidated Midland Corp., Brewster, N.Y. It was prepared in dimethyl sulfoxide at 1 mg/ml and stored at -70°C. Before use, it was diluted in phosphate-buffered saline to 20 μg/ml, and subsequently 1 μg of TPA per 50 μl was used in each assay of chemiluminescence.

**Chemiluminescence assay.** Chemiluminescence was measured in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) according to the method previously described (10). Briefly, 3 ml of HBSS with luminol and 1 ml of PMN were mixed in a plastic scintillation vial (Fischer Scientific Co., Rochester, N.Y.) which had been dark adapted for 24 h. The specimens were counted for 0.2 min at 10-min intervals until the background counts stabilized. The PMN chemiluminescence was determined in consecutive sets of neutrophils from the same donor after treatment with 0.4 ml each of (i) RSV and HBSS alone, (ii) RSV pretreated with RSV antibody-positive serum, (iii) uninfected HEp-2 cell control with RSV antibody-positive serum, (iv) RSV antibody-positive serum alone, and (v) HBSS alone. The antibody-positive serum specimens used for these studies had an RSV antibody titer of 1:32. All preparations were preincubated at room temperature for 30 min before addition of neutrophils for scintillation counts.

**Detection of immune complexes.** All preparations listed above which were tested for chemiluminescence were also examined for the presence of immune complexes by the Raji cell radioimmunassay as described previously (31).

**Electron microscope examination.** Several preparations of RSV alone, virus-antibody, and cell control were treated with 1% ammonium acetate, negatively stained with 2% phosphotungstic acid, and examined under an electron microscope for the presence of RSV-specific immune aggregates.

**Statistical analysis.** Student's t-test was used to analyze the data.

**RESULTS**

Effect of RSV on zymosan- and TPA-induced chemiluminescence. Initially, experiments were performed to determine the effects of RSV on zymosan- and TPA-induced chemiluminescence of PMN. Significant zymosan-induced PMN chemiluminescence was observed with live RSV as well as with uninfected tissue culture controls (Fig. 1). However, a modest decline in chemiluminescence was observed with heat-inactivated RSV. Virus or tissue culture controls alone did not induce any PMN chemiluminescence in the absence of zymosan. Similar results were observed with TPA-induced chemiluminescence (data not shown). Therefore, all subsequent experiments were performed with live RSV without zymosan or TPA.

**Effect of RSV and antibody-virus mix-**

![Figure 1. Effect of RSV on zymosan-induced chemiluminescence. Symbols: (○) PMN plus zymosan plus RSV (live); (□) PMN plus zymosan plus RSV (killed); (△) PMN plus zymosan plus uninfected cell control. Zymosan-induced chemiluminescence was not significantly different with RSV or cell control in assay. Counts were significantly lower with heat-inactivated RSV](image-url)
turers on PMN chemiluminescence. RSV alone in the presence of HBSS did not induce PMN chemiluminescence (Fig. 2), nor did RSV antibody-positive serum preincubated with uninfected tissue culture cells. On the other hand, neutrophils treated with the preincubated mixture of RSV and RSV antibody-positive serum elicited a significant degree of chemiluminescence. The heat-inactivated antibody-positive serum and RSV mixture induced about $34 \times 10^3$ counts/0.2 min. However, the mixture of RSV and unheated antibody-positive serum generated $105 \times 10^3$ counts/0.2 min (Fig. 2). The degree of chemiluminescence observed with unheated serum was threefold higher than that with heated serum.

Figure 3 presents the cumulative data obtained in 12 additional experiments. The individual and mean values of PMN chemiluminescence generated by unheated or heat-inactivated antibody-positive serum and virus mixtures were significantly higher ($P < 0.001$) than the control results. Little or no chemiluminescence was observed in control experiments.

The degree of PMN chemiluminescence generated by virus-antibody mixtures appeared to be determined by the concentration of the antibody and the virus. Dilution of either antibody or virus markedly reduced the chemiluminescence activity (Table 1). The effect, however, was more pronounced with the dilution of virus than with the dilution of antibody.

In the next series of experiments, RSV and antibody-positive serum mixtures or controls were centrifuged at $60,000 \times g$ for 1 h to concentrate chemiluminescence-generating components. The centrifuged pellets were resuspended

![Fig. 3. Effect of RSV and virus-antibody mixtures on neutrophil chemiluminescence responses. Geometric mean (± standard deviation) values of 12 experiments are presented. From left to right: PMN plus virus plus unheated antibody-positive serum; PMN plus virus plus heat-inactivated antibody-positive serum; PMN plus uninfected cell control plus heat-inactivated antibody-positive serum; PMN plus virus plus HBSS. Counts generated by virus and heat-inactivated or unheated antibody-positive serum were significantly greater ($P < 0.001$) than the counts generated by the controls.]

| Table 1. Effect of various dilutions of antigen and serum on chemiluminescence |
|-----------------|-----------------|-----------------|-----------------|
| Serum dilution  | $10^6$          | $10^4$          | $10^2$          |
| Undiluteda      | 67,178          | 12,727          | 7,787           |
| $10^{-1}$       | 40,320          | 13,007          | 3,865           |
| $10^{-1.5}$     | 15,936          | 9,860           | 3,264           |

a Input virus (plaque-forming units/milliliter).

b Initial antibody titer, 1:32.
in HBSS to the original volume. The pellets of RSV and antibody-positive serum produced peak counts of $218 \times 10^3/0.2$ min (Fig. 4), and the supernatant fluid generated low counts in the range of $8 \times 10^3/0.2$ min. The addition of fresh virus to the ultracentrifuged supernatant from RSV and heat-inactivated serum mixtures generated low background counts, as did the pellet material from control preparations.

**Immune complexes.** As mentioned earlier, all preparations were assayed for the presence of immune complexes by the Raji cell radioimmunoassay. Mixtures of RSV and unheated or heated antibody-positive serum induced appreciable immune complex activity, which was found to be two to three times higher than the activity observed in the control (Table 2).

**Electron microscope study.** Several RSV and antibody-positive serum mixtures were studied by electron microscopy. Viral aggregates were observed in samples which generated high chemiluminescence (data not shown). No such aggregates were observed in the preparations of virus or antibody alone or in the controls which induced little or no chemiluminescence.

**DISCUSSION**

The observations summarized in this report suggest that although RSV or its specific antibody independently have no discernible effect on induction of PMN chemiluminescence, mixtures of virus and antibody-rich serum provide a potent stimulus for PMN chemiluminescence. The effects appeared to be specific for virus-antibody interaction. The degree of chemiluminescence was directly proportional to the concentration of the virus and antibody in the mixture. The induction of chemiluminescence was significantly impaired by heat inactivation of the antibody-containing serum. This may reflect a possible role of heat-labile components of complement in the virus-antibody-induced chemiluminescence phenomenon. Ultracentrifugation experiments (Fig. 4) indicate that the chemiluminescence-inducing activity is largely associated with virus-antibody complexes. Furthermore, appreciable immune complex activity was

![Figure 4: Effect of ultracentrifugation of virus-antibody mixtures on neutrophil chemiluminescence.](http://iai.asm.org/)

**Table 2. Relationship of immune complex activity and the PMN chemiluminescence responses observed in various preparations of RSV and RSV antibody**

<table>
<thead>
<tr>
<th>Prepn*</th>
<th>Immune complex (µg/ml)</th>
<th>CL response ($\times 10^3$ mean counts ± SD/0.2 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V + Au</td>
<td>30</td>
<td>116.9 ± 24.6</td>
</tr>
<tr>
<td>V + Ah</td>
<td>25.6</td>
<td>41.7 ± 12.8</td>
</tr>
<tr>
<td>Uc + Ah (control)</td>
<td>15.0</td>
<td>12.4 ± 5.9</td>
</tr>
<tr>
<td>Au + HBSS (control)</td>
<td>12.8</td>
<td>12.4 ± 5.9</td>
</tr>
</tbody>
</table>

*V, RSV; Au, RSV antibody-positive serum (unheated); Ah, RSV antibody-positive serum (heated); Uc, uninfected tissue culture cells.

*CL, PMN chemiluminescence response; SD, standard deviation.
evidenced by the Raji cell radioimmunoassay, and demonstrable viral aggregates were observed by electron microscopy in chemiluminescence-inducing preparations of RSV and antibody-positive mixtures. Thus, based on these data, it is proposed that immune complexes of RSV and its specific antibody may act as potent stimulants for the generation of neutrophil chemiluminescence.

A number of studies have clearly demonstrated that various other stimuli, including zymosan, latex particles, opsonized bacteria, and aggregated immunoglobulins, can excite the oxidative metabolic pathway of neutrophils (1, 14, 18). One recent report has suggested increased chemiluminescence response and heightened oxidative metabolism in human phagocytic cells after their interaction with killed mumps virus antigen and specific antibody (5).

Bronchiolitis due to RSV is a disease primarily of children less than 6 months old, with a peak incidence at 2 months (24). Severe bronchiolitis has also been observed in children who received an experimental killed vaccine (6, 12). It has been postulated that natural infection with RSV in these infants resulted in more severe disease, perhaps due to immune complex injury (6). In other studies, an exaggerated cell-mediated immune response was shown to correlate with more severe disease (34). Gardner et al. (16) have proposed that bronchiolitis in RSV is, in part, due to a widespread type 1 allergic reaction after a second encounter with RSV antigen. The prolonged presence of cell-bound immunoglobulin E in the respiratory epithelial cells of patients with RSV bronchiolitis tends to support this possibility (34a).

Viral antigen-antibody complexes occur in a variety of viral infections of humans and animals. Deposition of immune complexes has been demonstrated in the renal glomeruli, liver, and spleen of patients with cytomegalovirus and hepatitis B virus infections (20, 23, 28). In mice, immune complex-mediated disease has been observed in infections with lymphocytic choriomeningitis and cytomegalovirus (21, 22).

Although immune complexes have been identified in various target organs, the mechanisms of tissue damage remain unclear. Neutrophils have recently been shown to play an essential role in immune complex-induced tissue damage (8, 15, 19). In laboratory studies with animals, neutrophils played an important role in glomerular damage of nephrotic nephritis induced by mammalian antisera and depletion of PMN in these animals prevented the development of proteinuria. It is also interesting to note that PMN failed to accumulate in the glomerular basement membrane if the animals were depleted of complement (7). The interaction between neutrophils and immune complexes has been shown to trigger the release of enzyme granules and a burst of oxidative metabolism (5, 18). Thus, it is conceivable that the release of these and other potentially toxic substances contributes, in part, to the pathogenesis of viral immune complex-induced tissue damage.

Many young patients with RSV infections possess high levels of maternal antibody directed against RSV. It has been suggested that bronchiolitis in these patients may be due to the development of immune complexes in the presence of maternally derived specific immunoglobulin G antibody after natural exposure of RSV (2, 6). However, the few histological studies currently available have failed to reveal the presence of immune complexes in the bronchopulmonary tissues of patients with fatal RSV infections (6, 16). It is possible that the target cells for immune complex interactions in RSV infection may be PMN rather than bronchopulmonary epithelial cells.

Earlier studies have shown that the metabolic activity of PMN as evidenced by Nitro Blue Tetrazolium dye reduction is significantly increased in children with RSV-associated acute bronchiolitis (27). In the present study, RSV in the absence of antibody did not induce increased metabolic activity in the neutrophils, although significant enhancement of chemiluminescence was observed with virus-antibody complexes. Based on these findings and the observations summarized above, it is suggested that the interaction of neutrophils with RSV-specific immune complexes during the acute phase of RSV infection may contribute to the pathogenesis and severity of clinical disease. These effects may be mediated through the release of thromboxane-A2 and other pharmacological products from metabolically activated neutrophils.

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LITERATURE CITED


