Interaction of Polymorphonuclear Leukocytes and Viruses in Humans: Adherence of Polymorphonuclear Leukocytes to Respiratory Syncytial Virus-Infected Cells

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The nature of neutrophil-respiratory syncytial virus (RSV) interaction was investigated by assessing factors that influence neutrophil adherence to RSV-infected tissue culture monolayers. The adherence of neutrophils to infected cells was directly proportional to the degree of RSV replication as evidenced by infectious virus production, cytopathological changes, or viral antigen appearance. Sixty-one percent of the neutrophils adhered to the RSV-infected cells as compared with 52.7% on noninfected monolayers ($P < 0.05$). The addition of RSV-specific antibody markedly increased polymorphonuclear leukocyte adherence to 88.5% ($P < 0.001$). Complement in the absence of antibody augmented polymorphonuclear leukocyte adherence, but to a lesser degree, 69.0% ($P < 0.025$). Arachidonic acid metabolism appeared to play a critical role in the adherence process; thromboxane was the single most important arachidonic acid metabolite. Inhibition of thromboxane synthesis reduced antibody-dependent polymorphonuclear leukocyte adherence on RSV-infected cells to 52.3% ($P < 0.025$). These observations suggest a role for neutrophils in RSV infection. It is proposed that neutrophils may participate in RSV infection at the site of viral replication through the attachment to infected cells and the subsequent release of mediators of inflammation.

Lymphocytes and macrophages have traditionally been considered the major cellular defense system against viral infections. However, several pieces of available data suggest that the neutrophil may be important in nonspecific antiviral defense. For example, the study of peripheral blood leukocytes obtained from volunteers inoculated with a specific virus revealed increased neutrophil counts early in rhinovirus, influenza virus, and coxsackievirus infections and late in adenovirus infection (2). Predominantly neutrophilic inflammatory responses also have been observed at the site of virus replication in specific infections. For instance, neutrophils prevail in the cutaneous lesions of varicella zoster infections in the early and late phases of the illness (18). A brisk neutrophil response may be seen in the cerebrospinal fluid of children with viral meningitis (4). These observations suggest that viral infections provide a necessary stimulus for polymorphonuclear leukocyte (PMN) migration.

We have recently suggested a possible role for neutrophils in lower respiratory tract infections due to respiratory syncytial virus (RSV) by demonstrating in vitro activation of both oxidative and arachidonic acid metabolism of neutrophils with RSV immune complexes (3, 8). For neutrophils to interact with RSV immune complexes, they must migrate to the site of virus replication and adhere to the surface of the infected cell. The purpose of this study was to characterize the process underlying adherence of neutrophils to RSV-infected tissue culture monolayers. The results document increased neutrophil adherence to infected cells compared with noninfected cells. RSV-specific antibody markedly increased the adherence. Arachidonic acid (AA) metabolism appeared to be critical for the adherence process.

MATERIALS AND METHODS

Reagents. Zymosan (ICN Pharmaceuticals Inc., Cleveland, Ohio) was prepared as described previously (3), opso-

nized in human type AB serum, and suspended in a concentration 6.6 mg per ml in Hanks balanced salt solution (HBSS). Phorbol myristic acetate (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide and diluted in HBSS to a concentration 20 $\mu\text{g/ml}$; the final dimethyl sulfoxide concentration was 0.04%. Phenidone (1-phenyl-3-pyrazoline; Sigma) was dissolved in HBSS to a concentration 300 $\mu\text{g/ml}$. Nordihydroguaiaretic acid (Sigma) was initially dissolved in ethanol and then diluted to 150 $\mu\text{g/ml}$ in HBSS; the final alcohol concentration was 0.03%. FPL55712 (Fisons Corp., Bedford, Mass.) was dissolved in distilled water to a concentration of 300 $\mu\text{g/ml}$, filtered, and further diluted in HBSS to 90 $\mu\text{g/ml}$ immediately before use. Indomethacin (Sigma) was dissolved in Tris buffer (pH 8.4) to a concentration 10 mg/ml and further diluted in HBSS to 12 $\mu\text{g/ml}$. Imidazole (Sigma) was dissolved in HBSS to a concentration 15.2 mg/ml. Meclofenamate monohydrate (Warner Lambert Pharmaceuticals, Ann Arbor, Mich.) was dissolved in HBSS to a concentration 300 $\mu\text{g/ml}$.

Preparation of neutrophils. Heparinized blood (10 to 20 ml) was collected from healthy adult volunteers after obtaining signed informed consent. The donors were free of illness and medications for at least 1 week before donation. The heparinized blood was layered over a column of dextran-Hypaque. The resultant leukocyte-rich plasma was collected, layered over a column of Ficoll-Hypaque, and then centrifuged at 1,200 rpm for 10 min. Erythrocytes in the neutrophil pellet were lysed by sequential treatment with hypotonic and hypertonic saline. The neutrophils were suspended in HBSS in a concentration of 10^7 cells per ml. The neutrophil preparation was more than 98% pure. The viability of the cells exceeded 95% as determined by trypan blue dye exclusion method.

Preparation of sera. Serum was collected from the same group of donors. Anti-RSV antibody titers were determined for each donor by the fluorescent antibody technique to ensure the presence of RSV-specific antibody (9). The sera

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were pooled, and the titer of neutralizing anti-RSV antibody proved to be 1:64. Fresh pooled serum was used for experiments that were designed to assess the role of complement in adherence. Heat treatment of serum at 56°C for 30 min inactivated the complement. The sera from three subjects with a titer of anti-RSV neutralizing antibody less than 8 were used as negative controls in selected experiments. Fresh guinea pig serum (Cordis Laboratories, Miami, Fla.) was used as a source of complement after it had been tested by the fluorescent antibody method and found to contain no measurable RSV antibody.

Preparation of virus pool. The Long strain of RSV was initially grown in HEp-2 cell monolayers with minimal essential medium and 2% heat-inactivated fetal calf serum in 5% CO₂ at 37°C. The virus was harvested after cytopathological changes involved 90% of the monolayer. The cells were scraped from the surface and sonicated to release cell-associated virus. The sonicate was clarified by centrifugation at 2,000 rpm for 10 min at 4°C. Supernatant material was stored in 1-ml volumes at -70°C. The titer of virus was 1.5×10^6 50% tissue culture infective doses when assayed in HEp-2 cells.

Adherence assay. To determine the optimal number of neutrophils required for the adherence assay, three different numbers of cells, 10^5 , 10^6 , and 10^7 , were first tested. The 10^5 neutrophils adhered so well that too few cells were recovered in the supernatant fluid. In contrast, 10^7 PMN exceeded the capacity of the monolayer to bind in a dose-dependent fashion; thus, differences in adherence could not be readily discerned. The 10^6 neutrophils permitted convenient cell counting that demonstrated variations in adherence when the conditions of the experiments were altered. A sample of 0.1 ml of the PMN suspensions (10^6 PMN) was sufficient to cover the monolayer of HEp-2 cells in a single well of a 24-well plastic microtiter plate (Falcon Plastics, Oxnard, Calif.). The maintenance medium was removed, and the monolayer was washed once with HBSS before the addition of PMN. The PMN were incubated on the monolayer in a humid 5% CO₂ atmosphere at 37°C for 60 min. At the end of the incubation, 0.9 ml of warmed HBSS was added and then used to gently rinse the monolayer. The suspension was removed for a cell count in a standard hemacytometer chamber. Each assay was run in triplicate. The number of cells that adhered to the monolayer was calculated by subtracting the number of neutrophils in the aspirate from 10^6 PMN, the number of cells that had been placed originally in the well at the start of the experiment.

Noninfected cell monolayers were included as controls and were processed in a similar manner. In additional experiments with serum, complement, or inhibitors of arachidonic acid metabolism, these materials were added to the wells in 0.1-ml volumes. The total volume in control and experimental wells was kept equal to maintain the same density of neutrophils and to insure a similar degree of contact between the neutrophils and the monolayers.

Evaluation of the role of AA metabolism in PMN adherence. Inhibitors of cyclooxygenase, lipooxygenase, leukotriene metabolism, and thromboxane synthetase were added to the adherence assay in 0.1-ml volumes immediately before the addition of neutrophils. Controls included the appropriate corresponding solvent in concentrations that equaled those found in preparations of the inhibitor. The effect of inhibitors on PMN viability was assessed by trypan blue dye exclusion.

Antithromboxane antibody that had been prepared in rabbits was kindly supplied by P. S. Sato at the UpJohn Co.,

Kalamazoo, Mich. It was diluted 1:2,000 and added to the adherence assay in 0.1-ml volumes. Control medium included rabbit serum diluted 1:2,000 in the same gelatin buffer used for the preparation of the antibody.

Statistical analysis. Student's paired and unpaired *t* tests were used to analyze the data.

RESULTS

Temporal relationship between RSV replication, production of RSV antigen by fluorescent antibody, and appearance of cytopathological effect. Preliminary studies of RSV replication in HEp-2 tissue culture monolayers were conducted to determine the optimal time for the adherence assay. RSV antigen was first detected by immunofluorescence technique at 16 h (7) (Fig. 1). The earliest detectable positive fluorescence, however, was rather indistinct ($\pm$), and it was not consistently positive until 20 h. The initial fluorescence represented single cells; fluorescent syncytia were first noted at 30 h. Cytopathological changes were visualized by light microscopy as early as 18 to 20 h. These changes were limited to differences in the size and shape of cells. More characteristic morphological changes were detected at 24 h. By 48 h, changes in fluorescence and cytopathological effect were marked. Infected monolayers began to detach from the surface by 60 h, whereas noninfected control monolayers remained intact. The curve depicted in Fig. 1 represents the amount of infectious virus produced at various times during the incubation period. At 12 h after inoculating the tissue culture with 3×10^3 50% tissue culture infective doses of RSV, new virus production was demonstrated. The rate of virus replication began to plateau at 48 h with approximately 6×10^6 50% tissue culture infective doses per ml, a 2×10^3 -fold increase over the input dose of virus.

Neutrophil adherence assay. To determine the relationship

FIG. 1. Temporal relationship between RSV replication, detection of RSV antigen by fluorescence (FAT), and the appearance of cytopathological effect (CPE). RSV (3×10^3 50% tissue culture infective doses [TCID₅₀]) was inoculated onto monolayers of HEp-2 tissue culture and incubated at 37°C in a humid 5% CO₂ atmosphere for 60 h. At various intervals, the monolayers were examined for cytopathologic changes (by light microscopy), the presence of RSV antigen (by fluorescence), and infectious virus. The cytopathological effect and fluorescent antibody test changes were graded from no change (-) to marked changes (++++). The numbers within parentheses depict the numbers of experiments.

FIG. 2. Relationship between neutrophil adherence and virus replication. Neutrophils were applied to HEp-2 tissue culture monolayers in the presence of RSV antibody at various times after infection with RSV. The percentage of adherent neutrophils increased as the amount of infectious virus increased. TCID₅₀, 50% tissue culture infective dose.

between the number of adherent neutrophils and the amount of virus present, adherence was evaluated at various times during RSV replication. The proportion of adherent neutrophils on the infected tissue culture monolayer increased progressively as the amount of infectious virus increased (Fig. 2). Adherence on the noninfected tissue culture monolayer remained constant throughout the experiment (data not shown). These experiments were conducted in the presence of antibody-positive serum to better demonstrate changes in adherence.

RSV-infected monolayers were incubated for 48 h after infection for use in the standard adherence assay; 48 h was selected because it represented the peak of virus replication (Fig. 1). The neutrophils and tissue culture monolayer were incubated together for 1 h at 37°C before the nonadherent cells were removed and counted. Approximately, 52.7% of the PMN adhered to noninfected monolayers (Fig. 3). Infec-

FIG. 3. Effect of RSV infection, antibody, and complement on the adherence of neutrophils to HEp-2 tissue culture monolayers. Adherence of neutrophils to RSV-infected cells (RSV control) was compared with adherence on noninfected cells (noninfected control). Adherence to RSV-infected cells in the presence of antibody and complement, antibody alone, or complement alone was compared to the RSV control. The bars represent means $\pm$ standard deviations. The numbers within parentheses represent the numbers of experiments.

tion with RSV increased adherence to 61.0% compared with adherence in the absence of infection. To determine whether the increased adherence was due to a soluble factor released from infected cells, neutrophils were preincubated in supernatants from infected monolayers and then applied to noninfected monolayers. The media from infected cells did not affect adherence of PMN to noninfected monolayers differently than preincubation in material from noninfected cells (data not shown). The possibility that adherent and nonadherent neutrophils represented two distinct cell populations was evaluated by adding the nonadherent cells from one assay to a fresh monolayer of infected cells. The proportion of adherent cells in the second assay remained the same as in the first one; thus, the nonadherent cells did not represent a different subpopulation of neutrophils (data not shown).

The role of RSV-specific antibody in PMN adherence to RSV-infected monolayers were next assessed. Fresh antibody-positive human serum was added to noninfected and infected monolayers in 0.1-ml volumes immediately before the addition of neutrophils. Adherence to infected monolayers increased to 91.2% in the presence of antibody compared with the RSV control, but antibody did not alter adherence to noninfected monolayers (Fig. 3). Adherence in the presence of RSV-specific antibody increased in a dose-dependent fashion with increasing concentrations of RSV antibody as performed with serial dilutions of serum (data not shown). Heat-inactivated, antibody-positive serum had a similar, although slightly lesser, effect. Antibody-negative serum did not affect PMN adherence on either infected or noninfected tissue culture monolayers (data not shown).

The addition of fresh guinea pig serum as a source of complement significantly increased adherence on infected tissue culture monolayers compared with the RSV control, but to a lesser degree than antibody (Fig. 3). Heat inactivation of the complement completely abrogated this effect (data not shown). Complement did not affect adherence on noninfected tissue culture monolayers.

The appearance of neutrophils attached to infected and noninfected tissue culture monolayers in the presence of antibody is depicted in Fig. 4 and 5. In the photographs with visual light microscopy, neutrophils are seen adhering avidly to the infected monolayers while only occasionally adhering to the uninfected monolayers. The electron micrographs in Fig. 5 more closely demonstrate the appearance of neutrophils that are adherent to the infected monolayers compared with the noninfected monolayers. At a lower magnification, the neutrophils can be seen attached to the tissue culture and not to the plastic surface between cells. At higher magnification, neutrophils attached to infected cells appear more active than those on noninfected cells, as evidenced by the accentuated ruffles, pseudopod formation, and greater contact between the neutrophil and the HEp-2 cell. The surface of the infected monolayers contains a stringy material that probably represents antibody-positive serum that had been retained and subsequently altered by the fixation process with glutaraldehyde.

To evaluate the importance of the AA metabolic pathway on the adherence of PMN to RSV-infected tissue culture monolayers, we conducted a series of experiments in the presence of inhibitors of AA metabolism. Imidazole, an inhibitor of thromboxane synthetase, was the most potent inhibitor of adherence, followed by phenidone, nordihydroguaiaretic acid, FPL 55712, and indomethacin (Table 1). The inhibitory effects of imidazole occurred in a dose-dependent manner (Fig. 6). The inhibitors did not adversely affect viability of the neutrophils in the concentrations tested.

FIG. 4. Light micrograph ($\times 400$) of the adherence of neutrophils to (a) noninfected (arrows on neutrophils) and (b) RSV-infected tissue culture monolayers in the presence of antibody after 48 h of incubation (small arrows on neutrophils, large arrows on infected HEp-2 cells).

FIG. 5. Scanning electron micrographs of the adherence of neutrophils to noninfected and RSV-infected tissue culture monolayers in the presence of antibody after 48 h of incubation. (a) Noninfected, $\times 500$; (b) infected, $\times 500$; (c) noninfected, $\times 5,000$; (d) infected, $\times 5,000$.

TABLE 1. Effect of AA metabolic inhibitors on PMN adherence to RSV-infected cells

Treatment group ($\mu\text{g/ml}$)	No. of expts	% Adherence $\pm$ SD	P
None	7	91.1 $\pm$ 2.5	
Imidazole (520)	3	52.3 $\pm$ 6.0	<0.025
Nordihydroguaiaretic acid (50)	2	77.5 $\pm$ 5.0	NS ^a
Phenidone (100)	2	77.5 $\pm$ 12.0	NS
FPL55712 (30)	2	82.5 $\pm$ 3.5	NS
Indomethacin (4)	3	92.0 $\pm$ 3.0	NS

^a NS, Not significant.

Indomethacin, an inhibitor of cyclooxygenase, did not affect PMN adherence in concentrations up to 40 $\mu\text{g/ml}$. Indomethacin from a different lot was found to yield the same results. Meclofenamate, another inhibitor of cyclooxygenase, also failed to impair adherence in concentrations up to 100 $\mu\text{g/ml}$. On the other hand, indomethacin inhibited PMN adherence to RSV-infected cells to a slight degree when the assay was conducted in the absence of serum; however, the inhibitory effect was minimal in comparison to imidazole.

The results of the experiments with inhibitors of AA metabolism suggested that thromboxane may have played a major role in PMN adherence, whereas products of the lipoygenase pathway had a lesser role. Because inhibitors of thromboxane synthesis may have affected more than one pathway, we next added antithromboxane antibody, prepared in rabbits, to the adherence assay in an attempt to block specifically the action of thromboxane. Normal rabbit serum served as a control. Anti-thromboxane antibody reduced adherence on RSV-infected monolayers from 85 to 65% in two separate experiments, thus supporting the importance of thromboxane in neutrophil adherence.

DISCUSSION

Active participation of neutrophils in viral infections requires the attachment of the phagocytes to the infected tissue. As seen in the present report, neutrophils adhered readily to tissue culture monolayers *in vitro*. Approximately 50% of the neutrophils that were applied to the noninfected surface adhered. Infection of the monolayer with RSV increased PMN adherence. This was directly related to the degree of virus replication or dysmorphic changes in the monolayer due to viral replication (or both). Additional viruses, including poliovirus, measles virus, herpes simplex virus, and adenovirus, similarly affected PMN adherence to endothelial tissue culture monolayers in an earlier report (12). The basis for increased PMN adherence in the presence of active viral replication is not known, although material released from infected monolayers has been proposed as a stimulus for PMN attachment (12). However, supernatant fluid removed from RSV-infected monolayers did not increase the adherence of neutrophils onto noninfected HEp-2 cells in the present study.

RSV-specific antibody increased PMN adherence to infected cells in a dose-dependent fashion. In contrast, serum without RSV antibody failed to augment adherence. Complement increased the adherence of neutrophils to infected cells; however, complement-dependent adherence was significantly less than that mediated by antibody, 69 versus 88.5%. The increased attachment of neutrophils in the presence of complement suggests that the complement stuck to the infected cells. Kaul et al. (10) have previously demonstrated the presence of complement on RSV-infected epithelial cells during natural infection. Smith et al. (16) have corroborated complement attachment to RSV-infected cells

in vitro. The mechanism of attachment of complement to RSV-infected cells in the absence of specific antibody is not known. Cines et al. (1) have suggested that certain viruses may induce complement receptors during infection. The presence of complement receptors on the infected HEp-2 cells was not evaluated in the present report, but another recent study from our laboratory demonstrated fixation of C3 to RSV-infected HEp-2 cells exclusively (T. N. Kaul, H. Faden, R. Baker, and P. L. Ogra, *J. Clin. Exp. Immunol.*, in press). These data illustrate the importance of antibody and complement on the adherence of neutrophils to RSV-infected cells. Antibody was clearly the more important component in an *in vitro* setting; however, in the presence of lower levels of antibody, complement may have contributed more to the adherence process.

The results from the present study also support the concept that AA metabolism is essential in the process of adherence. The observed reduction of adherence in the presence of imidazole and anti-thromboxane antibody further suggests that thromboxane may be the key AA metabolite that is responsible for adherence. The results of these experiments, however, must be viewed critically and as suggestive rather than as conclusive evidence, because actual determinations of the quantity and activity of AA metabolites in the preparations were not performed. Furthermore, inhibitors of AA metabolism may not function as specifically as previously thought and may alter the metabolic pathway at more than one site even though the inhibitors were used in concentrations and in situations that were thought previously to be appropriate. Similarly, the results of the experiments with anti-thromboxane antibody need to be confirmed in future experiments by actually demonstrating the reduction in functional thromboxane activity after treatment with specific anti-thromboxane antibody.

The reason for the failure of cyclooxygenase inhibitors to affect adherence adversely in the present study is unclear. Spagnuolo and associates (17) demonstrated significant impairment of PMN adherence to plastic in the presence of indomethacin, albeit less than with imidazole. One possible explanation for the apparent discrepancy might be the presence of serum and its binding capacity for selected inhibitors. Spagnuolo and associates did not use serum in the assay system, whereas serum comprised 33 to 50% of the assay system in the present report. Thus, it is conceivable that the amount of free drug available for inhibition in the present study was relatively low due to the binding capacity to the serum proteins. A similar explanation was put forward by Kelly et al. (11), who demonstrated that serum significantly reduced the inhibitory effects of indomethacin on lymphocyte function. Another confounding element in the present study was the potential metabolic activity of the tissue culture monolayer itself. Although Fitzpatrick and Stringfellow (5) reported that prostaglandin synthesis by virus-infected tissue culture monolayers was uncommon, Ingerman-Wojenski et al. (6) demonstrated prostaglandin and thromboxane production by endothelial cells in culture. The possibility that the monolayer released products that induced PMN adherence was tested, in fact, when neutrophils were incubated in supernatant fluids from infected monolayers. Neutrophils treated in such a manner adhered similarly to noninfected monolayers, as did neutrophils incubated in control medium. Thus, the evidence suggests that AA metabolic inhibitors affected the neutrophils rather than the tissue culture cells.

The data from this study further demonstrates the similarity in the adherence process of neutrophils to HEp-2 tissue culture monolayers compared with other tissue culture

FIG. 6. Effect of imidazole on neutrophil adherence to RSV-infected tissue culture monolayers in the presence of antibody. Increasing concentration of imidazole progressively reduced the degree of neutrophil adherence.

monolayers, plastic, or nylon wool (12–14, 17). As in previous reports, serum was not essential for the adherence process; however, in the case of virus-infected tissue culture monolayers, specific antibody or complement in the serum increased the binding of neutrophils.

The present observation that neutrophils adhere to RSV-infected cells *in vitro* suggests that the same process may occur *in vivo*. Although PMN adherence during natural RSV infection has not been previously studied, peripheral blood neutrophils collected from children with documented infection with RSV exhibit elevated resting metabolic activity (15). Further studies from our laboratory have shown that RSV immune complexes may activate the oxidative and AA metabolism of neutrophils (3, 8). We propose that neutrophils migrate from the peripheral blood to the site of RSV replication, adhere to the infected cells, especially in the presence of virus specific antibody, and undergo metabolic activation with the release of mediators of inflammation that may contribute to disease production.

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