Impact of neutrophils on antiviral activity of human bronchoalveolar lavage fluid

Mitchell R. White, Tesfaldet Tecle, Erika C. Crouch, and Kevan L. Hartshorn

1Department of Medicine, Boston University School of Medicine, Boston, Massachusetts; and 2Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri

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White MR, Tecle T, Crouch EC, Hartshorn KL. Impact of neutrophils on antiviral activity of human bronchoalveolar lavage fluid. Am J Physiol Lung Cell Mol Physiol 293: L1293–L1299, 2007. First published August 24, 2007; doi:10.1152/ajplung.00266.2007.—Surfactant protein D (SP-D) and neutrophils participate in the early innate immune response to influenza A virus (IAV) infection. SP-D increases neutrophil uptake of IAV and modulates neutrophil respiratory burst responses to IAV; however, neutrophil proteases have been shown to degrade SP-D, and human neutrophil peptide defensins bind to SP-D and can cause precipitation of SP-D from bronchoalveolar lavage fluid (BALF). BALF has significant antiviral activity against IAV. We first added neutrophils to BALF during incubation with IAV. Addition of neutrophils to BALF caused significantly greater clearance of IAV from culture supernatants than from BALF alone, and this effect was significantly more pronounced when neutrophils were activated during incubation with the virus. In contrast, if activated neutrophils were incubated with BALF before addition of virus, they reduced antiviral activity of BALF. This effect correlated with depletion of SP-D from BALF. Activation of neutrophils with agonists that induce primary granule release (including release of human neutrophil peptide defensins) caused SP-D depletion, but activation with PMA, which causes only secondary granule release, did not. The ability of activated neutrophils to deplete SP-D from BALF was partially, but not fully, corrected with protease inhibitors but was unaffected by inhibition of neutrophil respiratory burst responses. These results suggest that chronic neutrophilic inflammation (e.g., in chronic smoking or cystic fibrosis) may reduce SP-D levels and predispose to IAV infection. In contrast, acute inflammation, as occurs in the early phase of IAV infection, may promote neutrophil-mediated viral clearance.

surfactant protein D; influenza A virus; human neutrophil peptide defensins; inflammation

Influenza A virus (IAV) infections are a major cause of morbidity and mortality, causing ∼40,000 deaths per year in the United States (20). Innate immune mechanisms provide important protection against IAV in the naïve host. In the present study, we evaluate the interactions of two important mediators involved in the early response to IAV infection, neutrophils and surfactant protein D (SP-D), with IAV. Neutrophils are recruited to the respiratory tract early in the course of IAV infection (18, 25, 28). Neutrophils bind to and take up IAV in the absence of antibodies, and IAV stimulates various activation signals and H2O2 generation by neutrophils (8, 10). Hence, these cells may play a direct role in viral clearance. Highly pathogenic IAV strains elicit more profound neutrophil influx (18, 31). Recent studies with the 1918 pandemic IAV strain demonstrate markedly increased neutrophil influx in the lungs of mice infected with this strain compared with infection with other recent human strains (31). In this model, depletion of neutrophils before infection reduced survival. Hence, in this case, neutrophils clearly play a protective role. Other studies have shown protective and adverse effects of neutrophil infiltration during IAV infection (2, 25).

SP-D has a particularly important role in restricting IAV replication and limiting the severity of inflammatory responses during the first several days of infection (3–6, 9, 17, 18). SP-D accounts for a substantial portion of the innate anti-influenza activity of human bronchoalveolar lavage fluid (BALF) (9, 12). SP-D not only directly inhibits infectivity of IAV, but it also modulates interaction of IAV with neutrophils. SP-D promotes uptake of IAV by neutrophils and can increase or reduce respiratory burst responses of neutrophils on exposure to IAV, depending on whether SP-D is first incubated with IAV or neutrophils (34). SP-D also protects neutrophils against IAV-induced neutrophil dysfunction (4, 9). In the absence of SP-D, influx of neutrophils into the lung of mice infected with IAV is greatly increased (18). There is concern, however, that activated neutrophils may also impair SP-D function and, hence, reduce antiviral activity of respiratory lining fluids. Depletion of SP-D from human BALF has been demonstrated in cystic fibrosis (23). The loss of SP-D in this setting correlates with chronic inflammation (21). This implies that chronic neutrophilic inflammation in the lung may cause depletion of SP-D. Neutrophil proteases have been shown to cleave SP-D in the carbohydrate recognition domain, leading to loss of bacteria-aggregating activity (15). In a murine model of bacterial pneumonia, SP-D accumulates in areas of neutrophil infiltration, and this coincides with development of cleaved SP-D in BALF (15). It is likely that human neutrophil defensins (HNPs) also participate in the early innate response to IAV infection, because defensins are abundant products of activated neutrophils and have strong antiviral activity. We have shown, however, that HNPs bind to the carbohydrate recognition domain of SP-D and that high concentrations of HNPs cause precipitation of SP-D from human BALF, leading to diminished antiviral activity of the fluid (13). Hence, although HNPs can neutralize IAV, they may also impair defense against IAV by inhibiting SP-D activity.

In the present study, we show that neutrophils promote clearance of the virus when neutrophils and IAV are simultaneously added to BALF. In contrast, preincubation of human BALF with activated neutrophils causes a marked decline in
SP-D levels, which correlated with loss of antiviral activity of the fluid. These results suggest that neutrophils and SP-D may collaborate in host defense during acute infection but that chronic neutrophilic inflammation preceding IAV infection may interfere with SP-D-mediated host defense.

MATERIALS AND METHODS

Buffers and reagents. Dulbecco’s PBS containing 0.9 mM calcium and 0.493 mM magnesium and PBS without calcium and magnesium were purchased from Invitrogen (Carlsbad, CA). PBS with added calcium and magnesium (pH 7.2) was used unless otherwise indicated. Formyl-methyl-leucinyl-phenylalanine (fMLP), cytochalasin B, and PMA were purchased from Sigma Chemical and IL-8 from Antigenix America (www.antigenix.com).

Virus preparations. IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as previously described (7). Sucrose was removed from the virus by dialysis against PBS without added calcium and magnesium, and the sample was aliquoted and stored at −80°C. The Philippines 82/H3N2 and 82/BS (Phi82 and Phi82/BS) strains were kindly provided by Dr. E. Margot Anders (University of Melbourne, Melbourne, Australia). The PR-8 strain was kindly provided by Dr. Jon Abramson (Wake Forest University, Winston-Salem, NC). The hemagglutinin (HA) titer of each virus preparation was determined by titration of virus samples in PBS with thoroughly washed human type O, Rh− red blood cells as described elsewhere (7). After they were thawed, the viral stocks contained ∼5 × 106 plaque-forming units/ml.

HA inhibition assay. HA inhibition was measured by serial dilution of collectin or other host defense protein preparations in round-bottom 96-well plates (Serocluster U-Vinyl plates, Costar, Cambridge, MA), with PBS used as a diluent. After addition of 25 μl of IAV, giving a final concentration of 40 HA U/ml or 4 HA U/well, the IAV–protein mixture was incubated for 15 min at room temperature, and 50 μl of a type O human erythrocyte suspension were added. The minimum concentration of protein required to fully inhibit the HA activity of the viral suspension was determined by noting the highest dilution of protein that still inhibited hemagglutination. Inhibition of HA activity in a given well is demonstrated by the absence of formation of an erythrocyte pellet.

Fluorescent focus assay of IAV infectivity. Madin-Darby canine kidney cell monolayers were prepared in 96-well plates and grown to confluence. The layers were then infected with diluted IAV preparations for 45 min at 37°C in PBS and tested for the presence of IAV-infected cells after 7 h with use of a monoclonal antibody directed against the IAV nucleoprotein (provided by Dr. Nancy Cox, Centers for Disease Control, Atlanta, GA) as previously described (24). After incubation of IAV for 30 min at 37°C with BALF, these viral samples were added to the Madin-Darby canine kidney cells.

Human neutrophil and BALF preparation. Neutrophils from healthy volunteers were isolated to >95% purity by dextran precipitation followed by Ficoll-Paque gradient separation for removal of mononuclear cells and then hypotonic lysis to eliminate any contaminating erythrocytes as previously described (7). Cell viability was determined to be >98% by trypan blue staining. The isolated neutrophils were resuspended at the appropriate concentrations in control buffer (PBS) and used within 2 h. Normal volunteer donor BALF was obtained from healthy volunteer donors. Neutrophils and BALF were collected with informed consent as approved by the Institutional Review Board of Boston University School of Medicine.

Measurement of SP-D levels in BALF. SP-D levels in BALF were measured by ELISA as previously described with use of MAb 245-04 followed by donkey anti-mouse IgG coupled to horseradish peroxidase (9). Two different BALF samples were used. With use of a standard curve obtained with recombinant human SP-D, the levels of SP-D in these BALF samples were 229 and 270 ng/ml. Results are expressed as optical density at 450 nm (OD450). SP-D was also detected by Western blot using the antibodies and chemiluminescence described elsewhere (6).

Measurement of neutrophil release of HNPs. HNP release was measured using a commercially available sandwich ELISA (Cell Sciences, Canton, MA). The assay includes a standard and detects concentrations between 41 and 10,000 pg/ml. Neutrophil supernatants were diluted before assay, and HNP concentrations for at least two 10-fold dilutions were obtained for each sample. Neutrophils were maintained in buffer or activated by treatment with cytochalasin B (5 μg/ml) for 5 min followed by addition of fMLP (10−7 M), IL-8 at various concentrations, or IAV (1,320 HA U/ml). Other samples were treated with PMA (250 ng/ml). The effects of diphenyleylenoniodium (DPI; Sigma; 5.6 μM final concentration) on HNP release or H2O2 generation by neutrophils were tested where indicated. In addition, in some experiments, the effect of the protease inhibitor cocktail (catalog no. P8340, Sigma Chemical) on HNP release or other neutrophil activities was tested. The protease inhibitor cocktail stock solution, which contained 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E64, 2 mM leupeptin, and 1.5 mM pepstatin A, was added at a final dilution of 1:100 during neutrophil activation.

Measurement of IAV uptake by neutrophils. FITC-labeled IAV (Phi82 strain) was prepared as described elsewhere (11). Uptake of virus by neutrophils was measured as previously described (5). Briefly, IAV was incubated with neutrophils for 30 min at 37°C in the presence of control buffer or BALF and/or cytochalasin B and fMLP (or IL-8). Extracellular fluorescence was quenched by addition of trypan blue (0.2 mg/ml) to the samples. After they were washed, the neutrophils were fixed with paraformaldehyde, and neutrophil-associated fluorescence was measured using flow cytometry. The mean neutrophil fluorescence (>1,000 cells/sample) was measured.

Measurement of neutrophil H2O2 production. H2O2 production was measured by assessment of scopoletin fluorescence reduction as previously described (8) with a POLARstar OPTIMA fluorescence plate reader (BMG Labtech, Durham, NC).

Statistics. Statistical comparisons were made using Student’s paired, two-tailed t-test or ANOVA with Tukey’s post hoc test. ANOVA was used for multiple comparisons with a single control.

RESULTS

Activated neutrophils increase viral uptake in the presence of BALF. Neutrophils were incubated with FITC-labeled IAV, and viral uptake was measured by flow cytometry. As previously reported, BALF alone increased viral uptake (Fig. 1, top). A much more marked increase in uptake was observed when neutrophils were activated with fMLP in control buffer or buffer containing BALF. Incubation of neutrophils with IL-8 also caused a dose-related increase in viral uptake (Fig. 1, bottom).

Simultaneous incubation of neutrophils with IAV in BALF leads to increased clearance of the virus. In an effort to mimic acute infection with IAV, we added IAV and neutrophils simultaneously to BALF and measured viral HA activity remaining in the culture supernatant after a 30-min incubation. Addition of unstimulated neutrophils to BALF caused a modest, but statistically significant, reduction in virus (Fig. 2). Activation of neutrophils with fMLP caused a significantly greater decrease in virus in the supernatant than in BALF alone or BALF + unstimulated neutrophils.

Preincubation of BALF with activated neutrophils decreases antiviral activity of the fluid. BALF was preincubated with unstimulated or fMLP-activated neutrophils for 30 min at 37°C. Control BALF samples were incubated with fMLP and cytochalasin B alone. After these incubations, the neutrophils
were removed by centrifugation, and BALF-containing supernatants were tested for their ability to inhibit HA activity or infectivity of IAV. As previously reported, untreated BALF supernatant has substantial HA inhibitory and neutralizing activity against IAV. After treatment with resting neutrophils, antiviral activities of the fluid were not diminished (data not shown). The HA inhibitory activity of BALF containing resting neutrophils was taken as the control condition (expressed as 100% in Fig. 3, top). When BALF was preincubated with neutrophils activated with fMLP, HA inhibitory activity significantly declined (Fig. 3, top). Comparison of neutralizing activity of BALF that had been preincubated with unstimulated vs. fMLP-stimulated neutrophils also showed that the stimulated neutrophils impaired the neutralizing activity of BALF: more infectious virus remained after incubation with BALF that had been pretreated with activated neutrophils (Fig. 3, bottom).

We next compared HA inhibitory activity of BALF against a panel of viral strains with varying susceptibility to inhibition by SP-D (Fig. 4). In Figs. 1–3, we used the Phil82 strain, which is representative of recent human strains and sensitive to inhibition by SP-D. The Phil82/BS strain was developed through repeated passage in the presence of bovine serum and is partially resistant to SP-D by virtue of loss of a high-mannose oligosaccharide on the globular tip of its HA. The PR-8 strain is a mouse-adapted strain that is fully resistant to human SP-D because of the absence of high-mannose oligosaccharides on its HA or neuraminidase. Untreated BALF inhibited the PR-8 strain significantly less than the Phil82 strain (Fig. 4), and treatment of BALF with activated neutrophils did not reduce activity of the fluid against the Phil82/BS or PR-8 strain.

Activated neutrophils cause depletion of SP-D from BALF. As shown in Fig. 4, SP-D levels in BALF treated with activated neutrophils were substantially reduced compared with control BALF or BALF treated with resting neutrophils. The neutrophils used in Fig. 4 were activated with cytochalasin B and fMLP. Effects of other neutrophil stimulants were compared with effects of fMLP and cytochalasin B in Fig. 5A. In these experiments, fMLP-stimulated neutrophils again caused marked depletion of SP-D in BALF. fMLP and cytochalasin B in

**Fig. 1.** Activation of neutrophils increases uptake of influenza A virus (IAV). Neutrophil uptake of FITC-labeled IAV was assessed by flow cytometry. Top: addition of bronchoalveolar lavage (BAL) fluid (BALF) significantly increased IAV uptake by neutrophils. Activation of neutrophils with formyl-methyleucinyl-phenylalanine (fMLP) during incubation with IAV caused a much more marked increase in viral uptake in the presence of control buffer alone and BALF (n = 4). Results are significantly different (by ANOVA). Bottom: IL-8 increased uptake of IAV by neutrophils.

were removed by centrifugation, and BALF-containing supernatants were tested for their ability to inhibit HA activity or infectivity of IAV. As previously reported, untreated BALF supernatant has substantial HA inhibitory and neutralizing activity against IAV. After treatment with resting neutrophils, antiviral activities of the fluid were not diminished (data not shown). The HA inhibitory activity of BALF containing resting neutrophils was taken as the control condition (expressed as 100% in Fig. 3, top). When BALF was preincubated with neutrophils activated with fMLP, HA inhibitory activity significantly declined (Fig. 3, top). Comparison of neutralizing activity of BALF that had been preincubated with unstimulated vs. fMLP-stimulated neutrophils also showed that the stimulated neutrophils impaired the neutralizing activity of BALF: more infectious virus remained after incubation with BALF that had been pretreated with activated neutrophils (Fig. 3, bottom).

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**Fig. 2.** Simultaneous incubation of neutrophils with IAV increases ability of BALF to clear IAV. Neutrophils (PMNs) were incubated with IAV in the presence of BALF, and hemagglutinin (HA) titers were measured in neutrophil-free BALF supernatants. These experiments differ from those in Figs. 3–5, where neutrophils were preincubated with BALF and IAV was added later to cell-free BALF supernatants. Addition of resting neutrophils to BALF during IAV incubation significantly reduced HA titers. fMLP-activated neutrophils caused a significantly greater decline in HA titer in IAV containing BALF than in resting neutrophils (n = 7). Results were significantly different (by ANOVA).
In the absence of neutrophils, SP-D levels of BALF were not altered (data not shown). PMA activation of neutrophils caused a modest reduction in SP-D levels, significant by t-test but not by ANOVA. Neutrophils stimulated with IL-8 (2.5 μg/ml, preceded by fMLP) caused significant depletion of SP-D (Fig. 5B). The ability of fMLP-stimulated neutrophils to reduce SP-D levels in BALF could relate to oxidant production, release of proteases, release of HNPs, or other effects. Because PMA stimulates robust oxidant production from neutrophils, the release of oxidants may not be a major factor in SP-D reduction. Indeed, pretreatment of neutrophils with DPI did not significantly alter the effects of fMLP-stimulated neutrophils on SP-D levels (Fig. 5A), despite ablation of the respiratory burst response (Fig. 5D). On the other hand, release of proteases appeared to contribute to reduced SP-D levels caused by fMLP or PMA, because addition of protease inhibitors partially restored SP-D levels in BALF in both cases (although PMA-induced changes in SP-D level were not significant by ANOVA). The protease inhibitor cocktail has been previously shown to inhibit neutrophil-mediated proteolysis of SP-D in a murine model of bacterial infection (15).

Activated neutrophils release HNPs in BALF. Neutrophils were incubated in BALF and stimulated with fMLP or PMA. Those treated with fMLP released significantly greater amounts of HNPs than those treated with PMA or control resting neutrophils (Fig. 6). DPI or protease inhibitor treatment did not significantly alter the release of HNPs from neutrophils. IL-8 also caused a dose-related release of HNPs (Fig. 7). We compared the amount of HNPs remaining in cell-free supernatants when neutrophils were activated in the presence of BALF with the amount of HNPs remaining when the cells were incubated with PBS (Fig. 7) and found significantly lower HNP levels when neutrophils were incubated in BALF.

**DISCUSSION**

There is evidence that neutrophils contribute to clearance of IAV during acute infection (2, 31). Neutrophils are the predominant cell present in the early inflammatory infiltrate after IAV infection and are able to ingest IAV. Uptake of IAV is...
increased in the presence of SP-D, SP-A, or BALF (34). We now demonstrate that activation of neutrophils with fMLP or IL-8 substantially increases uptake of IAV. We also show that simultaneous incubation of IAV with BALF and activated neutrophils causes significantly greater clearance of IAV in vitro than incubation with BALF alone or BALF + resting neutrophils. These findings support a role for activated neutrophils in early host defense against IAV. Although fMLP could be considered an artificial activating stimulus in this context, IL-8 levels are increased during IAV infection (14, 19, 27). Results differed considerably, however, when incubation of BALF with activated neutrophils was followed by addition of

Fig. 5. Activated neutrophils deplete SP-D from BALF. Human neutrophils were incubated in BALF as described in Fig. 2 legend, and SP-D levels in BALF supernatants were measured by ELISA and expressed as optical density at 450 nm (OD450). A: SP-D levels were significantly reduced by fMLP-activated neutrophils. Values are means ± SE; n = 6 with separate neutrophil donors. *P < 0.001 (by ANOVA or t-test). Pretreatment of neutrophils with protease inhibitors (PI), but not with diphenylene iodonium (DPI), altered the effect of fMLP activation on SP-D levels. **Significantly greater than fMLP alone but significantly less than unactivated neutrophils (by ANOVA). PMA-activated neutrophils caused modest but significant reductions of SP-D levels (P < 0.02, by t-test), and this effect was again reduced by PI (P < 0.03 vs. PMA alone); however, effects of PMA were not significant when analyzed by ANOVA. B: IL-8-activated neutrophils also reduced SP-D levels in BALF. *P < 0.01 vs. control. C: representative Western blot for detection of SP-D in BALF treated with resting or fMLP-activated neutrophils. D: representative traces of H2O2 generation in fMLP-treated neutrophils. Addition of DPI blocked H2O2 production, but PI did not. Results are representative of 4 experiments with different blood donors. fMLP- and IL-8-treated samples were preincubated with cytochalasin B as well.
IAV. In this setting, the neutrophils significantly reduced antiviral activity of BALF. These results suggest that neutrophilic inflammation before or during IAV infection could also impair antiviral activity of BALF. SP-D contributes strongly to the antiviral activity of BALF against common human strains of IAV (9). Prior studies showed that activated neutrophils release serine proteases, which can degrade SP-D (15), and that HNPs bind to SP-D and can cause it to precipitate from BALF (13). Indeed, we found that activated neutrophils markedly reduced SP-D levels in BALF, which could account for the reduced activity against IAV. This is further supported by the finding that treatment of BALF with activated neutrophils did not reduce HA inhibitory activity against SP-D-resistant strains of IAV. Other components of BALF that inhibit SP-D-resistant strains include scavenger receptor-rich glycoprotein 340, SP-A, and mucins (12, 30, 33).

Proteolytic activity released by the activated neutrophils did account, to a significant extent, for reduction of SP-D levels, because a protease inhibitor cocktail resulted in partial restoration of SP-D levels in the presence of activated neutrophils. In contrast, oxidant activity of the neutrophils did not appear to contribute to the loss of immunoreactive SP-D. Preincubation of neutrophils with DPI, which blunted the respiratory burst response, did not prevent the reduction in SP-D levels. Furthermore, activation of neutrophils with PMA, a potent respiratory burst stimulus, resulted in a minor reduction of SP-D levels in BALF that was not significant by ANOVA. Reduction of SP-D levels likely relates to release of primary granules of neutrophils, because fMLP and IL-8 at the concentrations used in our study (and when preceded by cytochalasin B) cause degranulation of primary granules, whereas PMA predominantly results in degranulation of secondary granules only. Proteases released by the granules include elastase, proteinase 3, and cathepsin G, among others (22). These proteases have previously been shown to degrade SP-A and SP-D in vitro (15, 24) and probably contributed to loss of SP-D in our study. Our findings related to loss of SP-D in the presence of activated neutrophils extend on those previously reported using a murine model by confirming similar effects with use of human cells and BALF. Since our experiments were done in vitro, it remains possible that neutrophils recruited to the lung in vivo might behave differently (26). We believe, however, that this is unlikely on the basis of confirmation of SP-D degradation resulting from neutrophilic inflammation in a murine model (15).

HNPs also are present in primary granules, and we provide some evidence that HNPs contribute to reduction of SP-D levels in BALF. HNPs were released in response to fMLP or IL-8 stimulation, but not in response to PMA, which, as indicated above, primarily stimulates the release of secondary granules. The levels of HNP measured in the supernatant were substantially lower in activated neutrophils incubated with BALF than in activated neutrophils incubated in PBS. It is possible, therefore, that HNPs released in the presence of BALF bind to SP-D and precipitate out, as we showed previously using purified HNPs (13). HNPs could also contribute to the ability of activated neutrophils to take up more IAV than resting neutrophils, since we recently showed that incubation of IAV with HNPs substantially increased viral uptake by neutrophils, probably by inducing viral aggregation (29).

Overall, our findings are consistent with in vivo evidence that neutrophils contribute to clearance of IAV during acute
infection but that neutrophilic inflammation may also impair innate defense against IAV, in part by depleting SP-D from BALF. This could account for increased susceptibility to viral infection in some conditions characterized by chronic neutrophilic inflammation, such as heavy smoking or cystic fibrosis. Both of these conditions are known to be associated with reduced SP-D levels and increased risk of IAV infection (1, 16, 23, 32).

REFERENCES