Acute Lung Injury (ALI) manifests itself as severe inflammatory lung disease. Clinical disorders commonly associated with ALI include sepsis, pneumonia, traumatic injury, and major surgery. No effective therapy for ALI has been developed to date, and mortality remains high. In ALI, the initial acute systemic inflammatory response leads to microvascular damage and, subsequently, to increased pulmonary vascular and epithelial permeability. This phase of ALI is characterized by the influx of protein-rich edema fluid into the air spaces and acute respiratory failure (30). Neutrophils have been implicated in the pathogenesis of ALI by mediating the microvascular damage and contributing to lung tissue damage. Accumulation of large numbers of neutrophils in the lungs occurs in ALI, and increased levels of neutrophils have been associated with poor survival (5). The main chemotactic factor for neutrophils in bronchoalveolar lavage (BAL) fluid of patients with ALI has been demonstrated to be IL-8 (18, 23).

IL-8, an important neutrophil activator (19), is responsible for ~70% of the neutrophil chemotactic activity in fluids from lungs of patients with ALI, and its concentration correlates with the neutrophil content of the fluids (18). However, we have reported that a significant portion of IL-8 in lung fluids from patients at risk and patients with ALI is associated with anti-IL-8 autoantibodies (anti-IL-8:IL-8 complexes) (14–16), and BAL fluid concentrations of these complexes correlate with development and outcome of ALI (15, 16). Therefore, the primary objective of this work was to explore the hypothesis that anti-IL-8:IL-8 complexes have the capacity to trigger an inflammatory response in the lung and, if so, to delineate cellular and intercellular mechanisms that regulate activity of these complexes in vitro.

**MATERIALS AND METHODS**

**Human subjects.** All studies involving human blood and pulmonary edema fluid were approved by the Human Subjects Investigation Committees of the University of California, San Francisco, and the University of Texas Health Center at Tyler. ALI was diagnosed in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Agnieszka Krupa, Hiroyuki Kato, Michael A. Matthay, and Anna K. Kurdowska. Proinflammatory activity of anti-IL-8 autoantibody:IL-8 complexes in alveolar edema fluid from patients with acute lung injury

us and other researchers (14, 24, 25). In addition, complexes were formed between a monoclonal anti-IL-8 antibody and recombinant human IL-8 (rhIL-8) (R&D Systems, Minneapolis, MN) and also a monoclonal anti-monoectheic peptide-1 (MCP-1) antibody (IgG1, R&D Systems) and rhMCP-1 (R&D Systems). These complexes were also purified with the A/G column. The monoclonal anti-IL-8 antibody was developed by Dr. Edward Leonard (National Cancer Institute, Frederick, MD) and is of IgG1 subclass as well (25). This antibody has similar properties to the anti-IL-8 autoantibody (12, 14, 25). Control antibodies were purified on the protein A/G column from normal human plasma or lung fluids that did not contain an anti-IL-8:IL-8 complexes as determined by the ELISA (see Measurement of concentration of anti-IL-8:IL-8 complexes) (14). Endotoxin was removed from the samples with Detoxi-Gel, and concentrations of endotoxin were measured in a Quantitative Chromatogic Lab assay (BioWhittaker, Walkersville, MD). Endotoxin content was <100 pg/ml. This concentration does not evoke appreciable biological effects in studied cells.

Western blot analysis was performed to evaluate the purified complexes (as routinely done in our laboratory) (13). Samples of purified complexes or control antibody were loaded into a 4–20% gradient SDS-PAGE gel, and separated proteins were transferred to a nitrocel- lulose membrane. The membrane was blocked, and then the anti-IL-8 antibody was incubated. Next, the membrane is incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody. After that, enhanced chemiluminescence (ECL) reagents (Perkin-Elmer Life Sciences, Boston, MA) were applied to the membrane. Then, the membrane was exposed to X-ray film (Fuji Super RX).

Measurement of concentration of anti-IL-8:IL-8 complexes. Anti-IL-8:IL-8 complexes were measured by an ELISA essay developed in our laboratory as previously described (14). Briefly, 96-well microtiter plates were coated with monoclonal anti-human IL-8 antibody. Then, the plates were incubated with samples followed by an HRP- conjugated antibody against human immunoglobulins.

Preparation of neutrophils. Human neutrophils from healthy vol- unteers were separated by dextran sedimentation and erythocyte lysis by the method of Boyum (3). Measurement of chemotactic activity of purified anti-IL-8:IL-8 complexes toward human blood neutrophils (neutrophil chemotaxis). Neutrophil chemotaxis was evaluated by the leading front method of Zigmond and Hirsch (32) as routinely done in our laboratory (14). Anti-IL-8:IL-8 complexes, control antibody, IL-8 (rhIL-8, R&D Sys- tems), or buffer (control) was placed in the lower well of a Boyden chamber. A 5-μm-pore-size, 100-μm-thick cellulose nitrate filter was placed on the surface, and the chamber was then assembled. A 200-μl aliquot of the neutrophil preparation (2 × 106 cells/ml) was added to the top of the filter and incubated at 37°C for 40 min. The filter was then fixed, stained, and mounted on a glass microscope slide. The leading front was determined by the distance that the leading two cells had moved through the filter. The measurements were made for five fields on three filters for each set of conditions. In some experiments the cells were preincubated with different concentrations of specific antibodies against IgG receptors [FcyRIIa [IV.3; F(ab)2] and FcyRIII [3G8; F(ab)2]; Medarex, West Lebanon, NJ], specific antibodies against IL-8 receptors (CXCR1 and CXCR2, 50 μg/ml; R&D Sys- tems), or anti-IL-8 monoclonal antibody (10 μg/ml, R&D). A mon- oclonal antibody against FcyRII [7.3, F(ab)2; Ancell, Bayport, MN], established to be equivalent to the IV.3 antibody, was used in the last series of experiments (as indicated) because the latter antibody was no longer available.

Competition between purified anti-IL-8:IL-8 complexes and 125I- labeled rhIL-8 for binding to IL-8 receptors on neutrophils (binding studies). rhIL-8 (R&D Systems) was labeled with 125I as previously described (8). Binding studies were performed on neutrophils sus- pended in PBS containing 1% BSA. The cells (1 × 10⁶) were incubated with 125I-labeled rhIL-8 in the presence or absence of different concentrations of unlabeled rhIL-8 or purified anti-IL-8:IL-8 complexes for 90 min at 4°C to reach equilibrium and then centrifuged. The pellet (‘bound’ counts) and supernatant (‘free’ counts) were counted in a gamma radiation spectrometer (11, 14). Measurement of the ability of purified anti-IL-8:IL-8 complexes to trigger superoxide release from human blood neutrophils. The generation of O2- was measured as the superoxide dismutase (SOD; Sigma Chemical, St. Louis, MO)-inhibitable reduction of ferricyto-chrome c (Cyt c; Boehringer Mannheim, Indianapolis, IN) as previ- ously described (20). Briefly, neutrophils (6 × 10⁶ cells/ml) are incubated with cytochalasin B (10 μg/ml, Sigma) for 15 min at 37°C. Then, the neutrophils were incubated with different concentrations of anti-IL-8:IL-8 complexes, control antibody, IL-8, or Hanks’ balanced salt solution (HBSS) in the presence of Cyt c for the additional 30 min (test samples). Parallel samples have SOD added before Cyt c (reference samples). The cells are pelleted by centrifugation, and O2- is quantified by changes of absorbance at 550 nm between test and reference samples. In some experiments, the cells were preincubated with specific antibodies against FcyRIIa (50 μg/ml), specific antibo- dies against IL-8 receptors (50 μg/ml), or anti-IL-8 monoclonal antibo- dy (10 μg/ml).

Measurement of the ability of purified anti-IL-8:IL-8 complexes to trigger neutrophil enzyme release (degranulation). Neutrophil en- zyme release was studied as routinely done in our laboratory (11). Briefly, neutrophils (6 × 10⁶ cells/ml) are incubated with cytochalasin B (10 μg/ml, Sigma) for 15 min at 37°C. After that the cells are incubated with different concentrations of anti-IL-8:IL-8 complexes, control antibody, IL-8, or HBSS for the additional 30 min. The cells are then centrifuged, and supernatants are removed. Myeloperoxidase (MPO) is measured by determining the change in absorbance of tetramethyl benzidine (Sigma) at 450 nm in the presence of hydrogen peroxide. Absorbances are read on a Titertek automated plate reader (Molecular Devices, Sunnyvale, CA), and results are expressed as nanomoles of oxidized substrate. In some experiments, the cells were preincubated with specific antibodies against FcyRIIa (50 μg/ml) or specific antibodies against IL-8 receptors (50 μg/ml) or anti-IL-8 monoclonal antibody (10 μg/ml).

Characterization of the crucial components of the FcγRIIa signal- ing pathway. Before the above-described assays for evaluating activity of anti-IL-8:IL-8 complexes were performed, neutrophils were preincubated with specific inhibitors of different components of the FcγRIIa signaling cascade [genistein (general inhibitor of tyrosine kinases, at 20 μM), PP2 (inhibitor of Src tyrosine kinase family, at 20 μM), piceatannol (Syk, at 20 μM), PD-98059 (ERK, at 20 μM), SB-203580 (p38, at 20 μM), wortmannin [phosphatidylinositol 3-ki- nase (PI 3-K), at 5 μM], U-73122 [phospholipases C (PLC), at 20 μM], and bisindolylimaleimide (BIM) [protein kinase C (PKC), at 20 μM; Calbiochem)].

Evaluation of activation of p38, ERK, and Akt triggered by anti- IL-8:IL-8 complexes (Western blot analysis). Western blot analysis was performed to detect specific elements of the signaling pathway (as routinely done in our laboratory) (13). After 2- or 5-min incubation with the samples (buffer, anti-IL-8:IL-8 complexes, or control anti- body), neutrophils were lysed in SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue]. Then, the samples were heated to 95°C and were centrifuged to remove the cell debris, and supernatants were used for further analysis. Samples of lysed neutrophils were loaded into a 4–20% gradient SDS-PAGE gel, and separated proteins were transferred to a nitrocellulose membrane. The membrane was blocked, and then the appropriate primary antibody (against phosphorylated or total p38, ERK, or Akt; Cell Signalign) was applied. Next, the membrane was incubated with an appropriate secondary antibody. After that ECL reagents (Perkin-Elmer Life Sciences) were applied to the membrane. Then the membrane was exposed to X-ray film (Fuji Super RX). Statistical analysis. Comparisons between groups were done using the Student’s t-test, or the nonparametric Mann-Whitney test when the data sets were not normally distributed, and the Fisher’s exact test.
Results are presented as means ± SD. All statistics was performed with SIGMASTAT (SPSS Science, Chicago, IL).

RESULTS

Anti-IL-8:IL-8 complexes and control antibody that were used in the following experiments were purified from plasma of healthy donors or alveolar edema fluids from patients with ALI (as indicated). Complexes purified from plasma are marked ICP, and complexes purified from edema fluids are marked ICEF. That was done because of limited availability of samples from patients with ALI and relatively low concentration of the complexes present in pulmonary edema fluids (14, 16). However, anti-IL-8:IL-8 complexes, regardless of source when evaluated at equivalent concentrations, display similar activity toward human neutrophils, as presented in this paper. Furthermore, plasma or edema fluid samples that were used to purify control antibody did not contain anti-IL-8:IL-8 complexes as determined by the specific ELISA (14).

Purification of anti-IL-8:IL-8 complexes and control antibody. Western blot was performed to detect anti-IL-8:IL-8 complexes (Fig. 1). Samples of the complexes (lane 1) or control antibody (lane 2, both purified from pulmonary edema fluids from patients with ALI) were loaded into 4–20% gradient SDS-PAGE gels. After the electrophoresis, one gel was stained with Coomassie blue (Fig. 1A), and the second was subjected to electrophoretic transfer (to a nitrocellulose membrane). The membrane was incubated with the anti-IL-8 antibody followed by secondary antibody and ECL reagents. Then, the membrane was exposed to X-ray film (Fig. 1B). As seen in Fig. 1A, both samples (lane 1, ICEF; lane 2, control antibody) contain equivalent amount of protein (IgG) with molecular weight of ~160,000 (under nonreducing conditions). However, only purified complexes (ICEF) reacted with the anti-IL-8 antibody (Fig. 1B, lane 1). In addition, no free IL-8 was detected.

Chemotactic activity of anti-IL-8:IL-8 complexes. Anti-IL-8:IL-8 complexes (ICP) displayed chemotactic activity for human neutrophils in vitro (Fig. 2). Equivalent concentrations of free IL-8 and IL-8 in complex (ICP) displayed similar activity (Fig. 2). The same concentrations of free and complexed IL-8 (ICP) were tested in the chemotactic assay. Concentration of IL-8 in the complex was calculated with a specific ELISA for measuring the concentration of IL-8 associated with the anti-IL-8 autoantibody that we developed (14). In addition, control antibody did not have appreciable chemotactic activity (Fig. 2), even though subsequent dilutions of the control antibody contained the same concentration of IgG as anti-IL-8:IL-8 complexes (ICP). Moreover, the complexes purified from alveolar edema fluids from patients with ALI (ICEF) were as active as the complexes obtained from plasma of healthy donors when identical concentrations were used (0.5 × 10^{-10} M) (compare Fig. 4, A with B).

Receptor binding studies (neutrophils). Our previous studies show that the anti-IL-8 autoantibody recognizes an epitope contained within residues 35–72 of IL-8 molecule (14). The amino-terminal residues (4–6) of the IL-8 molecule are critical for the binding of IL-8 to its receptors on neutrophils (4). Therefore, IL-8 in complex could still interact with the receptor. In fact, many anti-cytokine:cytokine immune complexes have the ability to bind to receptors specific for given cytokine (22). Thus anti-IL-8:IL-8 complexes could also behave in a similar way. Figure 3 shows ability of anti-IL-8:IL-8 complexes (ICP) to compete with ^125^I-rhIL-8 for binding to its receptors on human neutrophils. Different concentrations of the complexes (ICP) were incubated with ^125^I-rhIL-8 and neutrophils. Then, bound IL-8 was measured. The results are expressed as the percentage of total specific counts that are calculated by subtracting counts obtained for ^125^I-rhIL-8 mixed with 100-fold excess unlabeled IL-8 from counts for ^125^I-rhIL-8 alone. Anti-IL-8:IL-8 complexes (ICP) competed for ~40% of binding.

Mechanism of chemotactic activity of anti-IL-8:IL-8 complexes. Because anti-IL-8:IL-8 complexes can interact with IL-8 receptors, we examined the ability of specific antibodies directed against IL-8 receptors (CXCR1 and CXCR2) to inhibit chemotactic activity of the complexes that were purified from alveolar edema fluids from patients with ALI (ICEF). We
Fig. 3. Competition for binding of 125I-labeled recombinant human (rh) IL-8 to human neutrophils by anti-IL-8:IL-8 complexes (purified from normal human plasma, ICP). The results represent the average of 4 experiments (means ± SD).

We observed an ~29% reduction in the chemotactic activity of anti-IL-8:IL-8 complexes in the presence of these antibodies (Fig. 4A). On the other hand, it is well established that activity of immune complexes is primarily mediated by IgG receptors (27). In humans there are two types of IgG receptors (FcRs) that bind immune complexes, FcγRII and FcγRIII, and neutrophils express FcγRIIa and FcγRIIb (27). We used antibodies directed against FcγRIIa [IV.3; F(ab)2] and FcγRIIIa and FcγRIIIb (27). The latter antibody reacts with both FcγRIIa and FcγRIIb.

Neutrophils were preincubated with the antibodies against FcγRIIa [IV.3; F(ab)] and FcγRIII [3G8; F(ab)2]. Only anti-FcγRIIa antibody significantly (P < 0.05) suppressed chemotactic activity of human neutrophils (Fig. 4, A and B). About a 70% decline in the chemotactic activity was observed when neutrophils were preincubated with the antibody (Fig. 4, A and B). Data shown in Fig. 4B were generated from anti-IL-8:IL-8 complexes that were purified from normal human plasma (ICP). Moreover, anti-IL-8 antibody did not affect chemotactic activity of the complexes (Fig. 4A). This antibody, however, completely abrogated neutrophil chemotaxis triggered by 10M anti-IL-8:IL-8 complexes.

Fig. 4. A: effect of antibodies against CXCR1 and CXCR2 (CXCR), anti-FcγRIIa, anti-FcγRIII, or anti-IL-8 on chemotactic activity of human neutrophils triggered by anti-IL-8:IL-8 complexes (purified from pulmonary edema fluids from patients with ALI, ICP, 0.5 × 10^{-10} M). Effect of anti-CXCR and anti-IL-8 on chemotaxis induced by IL-8 is also shown. Chemotaxis of human neutrophils induced by anti-IL-8:IL-8 complexes (ICEF) (solid bars) in the presence or absence of anti-CXCR, anti-FcγRIIa or anti-FcγRIII, anti-IL-8, or buffer (HBSS, open bar). Also shown is chemotaxis of human neutrophils induced by IL-8 (solid bars) in the presence or absence of anti-CXCR or anti-IL-8. *P < 0.05. The results represent the average of 5 experiments (means ± SD). B: effect of anti-FcγRIII or anti-FcγRIIa on chemotactic activity of human neutrophils triggered by anti-IL-8:IL-8 complexes (purified from normal human plasma, ICP). Chemotaxis of human neutrophils induced by buffer only (HBSS, negative control, ■), anti-IL-8:IL-8 complexes alone (0.5 × 10^{-10} M; positive control) (ICP, ▼), or anti-IL-8:IL-8 complexes in the presence of increasing concentrations of anti-FcγRIIa (○, dashed line) or anti-IL-8:IL-8 complexes in the presence of increasing concentrations of anti-FcγRIIa (■, solid line). Each point represents the mean of 15 measurements. The results represent the average of 5 experiments (means ± SD). C: effect of antibodies against CXCR1 and CXCR2 (CXCR), anti-FcγRII (clone 7.3), anti-FcγRIII, or anti-IL-8 on chemotactic activity of human neutrophils triggered by anti-IL-8:IL-8 complexes (purified from normal human plasma, ICP). Effect of anti-FcγRII (clone 7.3) or anti-FcγRIII on chemotaxis induced by anti-MCP-1:MCP-1 complexes (IC2*, 5.0 × 10^{-10} M) is also shown. Chemotaxis of human neutrophils induced by anti-IL-8:IL-8 complexes (IC1) (solid bars) in the presence or absence of anti-CXCR, anti-FcγRII (clone 7.3), anti-FcγRIII, anti-IL-8, or buffer (HBSS, open bar). Also shown is chemotaxis of human neutrophils induced by anti-MCP-1:MCP-1 complexes at a concentration of 0.5 × 10^{-10} M (IC2) or 5.0 × 10^{-10} M (IC2*, solid bars) in the presence or absence of anti-FcγRII (clone 7.3) or anti-FcγRIII. *P < 0.05. The results represent the average of 4 experiments (means ± SD).
Anti-IL-8 autoantibody: IL-8 complexes in lung injury

Neutrophil activation triggered by anti-IL-8:IL-8 complexes. Because anti-IL-8:IL-8 complexes displayed chemotactic activity toward neutrophils, we next evaluated the effect of these complexes on neutrophil superoxide release and degranulation (MPO activity). As shown in Fig. 5, anti-IL-8:IL-8 complexes (ICP) triggered a respiratory burst of human blood neutrophils. The effect of the complexes (ICP) was significant (P < 0.05) and concentration dependent (Fig. 5). On the other hand, control antibody was not active (Fig. 5). Complexes purified from alveolar edema fluids from patients with ALI (ICEF) were equally effective in stimulating neutrophils when identical concentrations were used (8 nM) (compare Figs. 5 and 6). Furthermore, superoxide release induced by these complexes (ICEF) was significantly (P < 0.05) suppressed by the anti-FcγRIIa antibody but not by the combination of the antibodies against IL-8 receptors (anti-CXCR1 and anti-CXCR2) (Fig. 6). These antibodies, on the other hand, significantly inhibited IL-8-triggered superoxide release (P < 0.05, Fig. 6). MPO release was also significantly (P < 0.05) augmented by anti-IL-8:IL-8 complexes (ICP) but not by control antibody (Fig. 7). Complexes from pulmonary edema fluids (ICEF) induced similar MPO release from human neutrophils when identical concentrations were used (8 nM) (compare Figs. 7 and 8). Anti-FcγRIIa antibody substantially (P < 0.05) inhibited the MPO release induced by these complexes (ICEF), whereas the combination of the antibodies against IL-8 receptors (anti-CXCR1 and anti-CXCR2) had no effect (Fig. 8). The latter antibodies completely abrogated MPO release induced by IL-8 (P < 0.05, Fig. 8).

Fig. 5. Superoxide release from neutrophils stimulated by anti-IL-8:IL-8 complexes (ICP), control antibody (purified from normal human plasma), or buffer (HBSS, negative control). Superoxide release induced by different concentrations of anti-IL-8:IL-8 complexes (ICP, solid bars), buffer (HBSS, open bar at left), or control antibody (open bar at right). *P < 0.05. The results represent the average of 5 experiments (means ± SD).

Fig. 6. Effect of antibodies against CXCR1 and CXCR2 (CXCR) or anti-FcγRIIa on respiratory burst of human neutrophils triggered by anti-IL-8:IL-8 complexes (purified from pulmonary edema fluids from patients with ALI, ICEF). Superoxide release induced by anti-IL-8:IL-8 complexes (ICEF, 8 nM; solid bars) in the presence or absence of anti-CXCR or anti-FcγRIIa, or buffer (HBSS, open bar). *P < 0.05. The results represent the average of 5 experiments (means ± SD).

Fig. 7. Myeloperoxidase (MPO) released from neutrophils stimulated by anti-IL-8:IL-8 complexes (ICP), control antibody (purified from normal human plasma), or buffer (HBSS, negative control). MPO release induced by different concentrations of anti-IL-8:IL-8 complexes (ICP, solid bars), buffer (HBSS, open bar at left), or control antibody (open bar at right). The results represent the average of 5 experiments (means ± SD). *P < 0.05. TMB, tetramethyl benzidine.
Characterization of the crucial components of the FcγRIIa signaling pathway. Because we determined that anti-IL-8:IL-8 complexes promote chemotaxis and activation of human neutrophils through FcγRIIa, we wished to define which proteins of the FcγRIIa signaling cascade are activated by the complexes. Although signaling through FcγRIIa has been investigated to some extent, there are still many gaps in understanding of the precise sequence of the signaling events controlling neutrophil function, and most certainly nothing is known about the signaling mechanisms by which anti-IL-8:IL-8 complexes stimulate neutrophils. We used the following inhibitors [of the proteins indicated in the signaling cascade initiated by the engagement of FcγRIIa (1, 2, 21)]: genistein (general inhibitor of tyrosine kinases), PP2 (inhibitor of Src tyrosine kinase family), piceatannol (Syk), PD-98059 (ERK), SB-203580 (p38), wortmannin (PI 3-K), U-73122 (PLCγ), and BIM (PKC). Then, neutrophil activation and chemotaxis induced by anti-IL-8:IL-8 complexes purified from alveolar edema fluids from patients with ALI (ICEF) were evaluated in the presence and absence of the specific inhibitors. Neutrophil respiratory burst was inhibited by genistein, PP2, PD-98059, wortmannin, and U-73122, whereas SB-203580 and BIM had no effect (Fig. 9), suggesting that tyrosine phosphorylation as well as activation of a protein from Src tyrosine kinase family, ERK, PI3-K, and PLCγ are evoked by FcγRIIa engagement that leads to the respiratory burst triggered by anti-IL-8:IL-8 complexes. In case of neutrophil degranulation, PD-98059 and U-73122 were not effective, but the inhibition was observed in the presence of genistein, PP2, piceatannol, SB-203580, U-0126, wortmannin, and BIM (Fig. 10). These results indicate that tyrosine phosphorylation along with activation of a protein from the Src tyrosine kinase family, Syk, p38, PI3-K, and PKC mediate neutrophil degranulation induced by anti-IL-8:IL-8 complexes. Furthermore, the chemotactic activity of the complexes was measured in the presence or absence of the selected inhibitors. PP2 and SB-203580, as well as U-73122, significantly \( P < 0.05 \) reduced the activity of anti-IL-8:IL-8 complexes, indicating that activation of a protein from the Src tyrosine kinase family, p38 as well as PLCγ, is important for the chemotactic activity of the complexes (Fig. 11).

Moreover, Western blot analysis was performed to evaluate activation of p38, ERK, and Akt (a protein downstream from PI3-K) (Fig. 12). A significantly increased phosphorylation was observed after stimulation with anti-IL-8:IL-8 complexes purified from alveolar edema fluids from patients with ALI.

**Fig. 8.** Effect of antibodies against CXCR1 and CXCR2 (CXCR) or anti-FcγRIIa on MPO release from human neutrophils triggered by anti-IL-8:IL-8 complexes (purified from pulmonary edema fluids from patients with ALI, ICEF). MPO release induced by anti-IL-8:IL-8 complexes (ICEF, 8 nM; solid bars) in the presence or absence of anti-CXCR, anti-FcγRIIa, or buffer (HBSS, open bar). The results represent the average of 5 experiments (means ± SD). \( *P < 0.05 \).

**Fig. 9.** Effect of the inhibitors of the main proteins of the FcγRIIa signaling pathway on respiratory burst of human neutrophils triggered by anti-IL-8:IL-8 complexes (purified from pulmonary edema fluids from patients with ALI; ICEF). Superoxide release induced by anti-IL-8:IL-8 complexes (ICEF, solid bars) in the presence or absence of PP2 (inhibitor of Src tyrosine kinase family), piceatannol (P; Syk), PD-98059 (PD; ERK), SB-203580 (SB; p38), wortmannin [W; phosphatidylinositol 3-kinase (PI 3-K)], U-73122 (U; PLCγ), or bisindolylmaleimide (BIM; PKC), or buffer (HBSS, open bar). \( *P < 0.05 \). The results represent the average of 5 experiments (means ± SD).

**Fig. 10.** Effect of the inhibitors of the main proteins of the FcγRIIa signaling pathway on MPO release from human neutrophils stimulated by anti-IL-8:IL-8 complexes (purified from pulmonary edema fluids from patients with ALI, ICEF). MPO release induced by anti-IL-8:IL-8 complexes (ICEF, solid bars) in the presence or absence of PP2 (inhibitor of Src tyrosine kinase family), piceatannol (Syk), PD-98059 (ERK), SB-203580 (p38), wortmannin (PI 3-K), U-73122 (PLCγ), or BIM (PKC), or buffer (HBSS, open bar). \( *P < 0.05 \). The results represent the average of 5 experiments (means ± SD).
death in patients with ALI (16). These data suggest that the presence of anti-IL-8:IL-8 complexes in lung fluids of patients with ALI is an important prognostic indicator for the development and outcome of ALI (15, 16).

In the current study, we explored the hypothesis that anti-IL-8:IL-8 complexes may exhibit proinflammatory activity in vitro. There are only a few reports in the literature showing the ability of defined soluble immune complexes containing IgG to induce neutrophil chemotaxis (26) and trigger neutrophil respiratory burst and degranulation (28, 31). Thus the ability of anti-IL-8:IL-8 complexes to chemoattract and activate neutrophils was examined. Anti-IL-8:IL-8 complexes displayed chemotactic activity for human neutrophils similar to that of free IL-8, whereas control complexes had negligible activity. It should be stressed that these complexes are extremely stable (25); therefore, the chemotactic activity of anti-IL-8:IL-8 complexes cannot be due to the free IL-8 that was released from the complexes. Furthermore, anti-IL-8 autoantibodies bind IL-8 with high affinity ($\sim 10^{-12}$ M) (14). Neutrophils express specific receptors for IL-8 (19), and our data show that anti-IL-8:IL-8 complexes competed with $^{125}$I-rhIL-8 for binding to its receptors on human neutrophils. Previous studies by Sylvester et al. (25) indicate that anti-IL-8:IL-8 complexes cannot bind to IL-8 receptors on neutrophils. However, close analysis of the graph presented in that study reveals that there is concentration-dependent inhibition of binding of $^{125}$I-rhIL-8 to its receptors on neutrophils and that, at the highest complex concentration, 20% of the binding was suppressed (25). Furthermore, it is not possible to compare both studies directly, because the complexes were quantified by different methods (14, 25). Nevertheless, the previous study presents results (not conclusions) similar to ours. Perhaps the authors concluded that 20% of inhibition is too low to be significant, although statistical analysis of these data is not presented. Moreover, we may have used higher concentrations of the complexes.

We then explored the mechanism of activity of anti-IL-8: IL-8 complexes. We found that blocking of IL-8 receptors inhibits $\sim 29\%$ of chemotactic activity of the complexes but has no effect on the oxidative burst or degranulation of neutrophils that was induced by the complexes. IgG receptors that bind immune complexes, FcγRIIa and FcγRIIIb, are also present on the surface of neutrophils (27). When human neutrophils were preincubated with the antibody against FcγRIIa or the antibody against FcγRIIIb, only the former antibody significantly ($P < 0.05$) suppressed chemotactic activity of

DISCUSSION

Previous studies from our laboratory revealed the presence of anti-IL-8:IL-8 complexes in alveolar edema fluids and lavage fluids from the lungs of patients with ALI (14–16). We found that the amount of complexes was significantly decreased ($P < 0.05$) in ALI survivors over time. In contrast, in nonsurvivors the presence of the increased amount of complexes (not quantified) was statistically significant, although the amount of complexes was only $\sim 20\%$ suppressed ($P < 0.05$) in ALI survivors over time. In addition, the presence of the increased amount of complexes in alveolar edema and lavage fluids from the lungs of patients with ALI (14) and was associated with

![Fig. 11. Effect of the inhibitors of the main proteins of the FcγRIIa signaling pathway on chemotaxis of human neutrophils triggered by anti-IL-8:IL-8 complexes (purified from pulmonary edema fluids from patients with ALI, ICEF). Neutrophils were incubated with buffer (HBSS, B), anti-IL-8:IL-8 complexes (ICEF), and control antibody (C) for 2 or 5 min. The results are representative of 3 experiments. p, Phosphorylated; t, total.](image)

![Fig. 12. Phosphorylation of p38, ERK, and Akt in response to stimulation with anti-IL-8:IL-8 complexes (purified from pulmonary edema fluids from patients with ALI, ICEF). Neutrophils were incubated with buffer (HBSS, B), anti-IL-8:IL-8 complexes (ICEF), and control antibody (C) for 2 or 5 min. The results are representative of 3 experiments. p, Phosphorylated; t, total.](image)

![Fig. 13. FcγRIIa signaling cascade. Interaction between FcγRIIa and Src tyrosine kinase family triggers activation of Syk-tyrosine kinase, PI3-K, Akt, MAP kinases, PLCγ, and PKC in human neutrophils.](image)
neutrophils. Moreover, the antibody against FcγRIIa completely inhibited respiratory burst and enzyme release of neutrophils stimulated with anti-IL-8:IL-8 complexes. These results indicate that activity of anti-IL-8:IL-8 complexes toward neutrophils is primarily mediated by FcγRIIa. It is known that soluble immune complexes of different size and composition are also capable of interacting with complement receptors (17). However, these in vitro experiments (chemotaxis, receptor binding, and neutrophil activation) were done in the absence of serum or other sources of complement, and in such conditions complement activation cannot occur (28). (We do not use serum because it contains immune complexes.)

In summary, our studies demonstrate that the IgG receptor FcγRIIa is a predominant receptor involved in cellular activation mediated by anti-IL-8:IL-8 complexes. However, the chemotactic activity of the complexes depends also on stimulation through IL-8 receptors. Furthermore, the facts that anti-IL-8: IL-8 complexes competed only for ~40% of IL-8 binding sites on neutrophils (Fig. 3) and antibodies directed against IL-8 receptors suppressed as little as 29% of chemotactic activity of the complexes (Fig. 4) suggest that anti-IL-8:IL-8 complexes bind more readily to FcγRIIa. That would explain why the activity of the complexes is primarily mediated by FcγRIIa.

Finally, we determined which of the main proteins of the FcγRIIa signaling cascade are activated by anti-IL-8:IL-8 complexes. It has been established that the engagement of FcγRIIa initiates a tyrosine kinase cascade dependent on the cytoplasmic tail of this receptor, which contains one copy of an immunoreceptor tyrosine-based activation motif, a substrate for phosphorylation by members of the Src tyrosine kinase family (Fig. 13). The phosphorylated immunoreceptor tyrosine-based activation motif can bind to and activate Syk. Tyrosine kinase, which subsequently activates a number of effector pathways (1, 7, 9, 10) (Fig. 13). Ultimately, neutrophil activation is initiated (10).

The outline of the signaling cascade described above represents a compilation of data presented in several studies focused on defining cellular signals triggered by ligation of FcγRIIa. However, the effect of soluble immune complexes was examined in only a few of these papers. Other triggers of FcγRIIa activation differ in their ability to engage particular elements of this cascade. Zymosan, for example, behaves differently from immune complexes (9). Furthermore, even more importantly, activity of anti-IL-8:IL-8 complexes has never been studied before. Our goal was then to delineate the signaling pathways that are set in motion by anti-IL-8:IL-8 complexes.

Our results suggest that tyrosine phosphorylation, as well as activation of tyrosine kinase from the Src tyrosine kinase family, Syk, ERK, PI3-K, and PLCγ, is evoked by FcγRIIa engagement, which leads to the respiratory burst triggered by anti-IL-8:IL-8 complexes. On the other hand, tyrosine phosphorylation, along with activation of a protein from the Src tyrosine kinase family, Syk, p38, PI3-K, and PKC, mediates neutrophil degranulation induced by anti-IL-8:IL-8 complexes. Furthermore, chemotactic activity of the complexes was measured in the presence or absence of the selected inhibitors. PP2 and SB-203580, as well as U-73122, significantly reduced the activity of anti-IL-8:IL-8 complexes, indicating that activation of a protein from the Src tyrosine kinase family, p38, as well as PLCγ, is important for the chemotactic activity of the complexes. Because Syk is not a part of the IL-8 signaling cascade (1), the facts that the inhibitor of Syk (piceatannol) had no effect on the chemotactic activity of the complexes but that SB-203580 (inhibitor of p38) significantly reduced the activity confirm the role of IL-8 receptors in mediating the chemotactic activity of anti-IL-8:IL-8 complexes. Figure 13 summarizes our findings.

The idea that immune complexes have the ability to activate neutrophils in pathological conditions is not new and, in fact, was explored already in the early 1980s (6). However, only recently it became clear that, depending upon its characteristics, such as size, composition, and reactivity with its complement system and Fc receptors, an immune complex may trigger diverse cellular responses (28). Soluble complexes differ substantially from insoluble counterparts and, at least in the case of neutrophils, soluble immune complexes are able to activate only primed cells, whereas insoluble complexes do not require priming to display full activity toward neutrophils (20, 28). In contrast, anti-IL-8: IL-8 complexes are not active in insoluble form (unpublished observations) and attract and stimulate unprimed neutrophils. Furthermore, different classes of Fc receptors are involved, i.e., either FcγRIIa or FcγRIIb, or frequently both, according to complex type (28).

Our studies demonstrate that even though the IgG receptor FcγRIIa is a predominant receptor involved in cellular activation mediated by anti-IL-8:IL-8 complexes, the chemotactic activity of the complexes depends also on stimulation through IL-8 receptors. That is because anti-IL-8:IL-8 complexes can interact with IL-8 receptors as well (Fig. 4, A and C).

Furthermore, we show that anti-IL-8:IL-8 complexes are also unique in another respect. Anti-IL-8:IL-8 complexes (prepared with the anti-IL-8 monoclonal antibody) but not anti-MCP-1:1-MCP-1 complexes exhibited chemotactic activity for human neutrophils when identical concentrations were used (0.5 × 10^{-10} M) (Fig. 4C). The activity of anti-IL-8:IL-8 complexes (prepared with the anti-IL-8 monoclonal antibody) was similar to that of purified complexes and was mediated by both IL-8 receptors and FcγRIIa, as it was the case with purified complexes (compare Fig. 4, A with C). Anti-MCP-1:1-MCP-1 complexes, on the other hand, were chemotactic for neutrophils only at the concentration that was 10 times higher than the effective concentration of anti-IL-8:IL-8 complexes (Fig. 4C). This activity was inhibited by the antibody against FcγRIII but not by anti-FcγRIIa (Fig. 4C). Anti-IL-8:IL-8 complexes (prepared with the anti-IL-8 monoclonal antibody) also induced neutrophil activation (as it was the case with purified complexes), whereas anti-MCP-1:1-MCP-1 complexes did not (data not shown).

Finally, little has been done to define effects of soluble immune complexes mediated through FcγRIIa in human neutrophils, and the signaling cascade (due to FcγRIIa engagement) activated by anti-IL-8:IL-8 complexes has never been studied before (1, 2, 7).

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REFERENCES


