Fibrinogen Induces IL-8 Synthesis in Human Neutrophils Stimulated with Formyl-Methionyl-Leucyl-Phenylalanine or Leukotriene B<sub>4</sub>

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Fibrinogen Induces IL-8 Synthesis in Human Neutrophils Stimulated with Formyl-Methionyl-Leucyl-Phenylalanine or Leukotriene B₄

Douglas B. Kuhns,* Edward L. Nelson,* W. Gregory Alvord, † and John I. Gallin‡

Human exudative neutrophils have greatly increased stores of the neutrophil chemoattractant IL-8 compared with peripheral blood neutrophils, but the mechanism for the increase is not defined. In this report, we show that treatment of peripheral blood neutrophils with the chemotactic peptide fMLP or with leukotriene B₄ or fibrinogen results in little increase in the production of IL-8 by peripheral blood neutrophils. However, a chemotactically active dose of fMLP (5 × 10⁻⁹ M) or leukotriene B₄ (1 × 10⁻⁷ M) in the presence of a physiological concentration (2 mg/ml) of fibrinogen results in a receptor-mediated, pertussis toxin-sensitive, synergistic 30-fold increase in IL-8 synthesis. The levels of IL-8 attained are comparable to those observed in exudative cells. Higher concentrations of fMLP (1 × 10⁻⁷ M) are associated with reduced IL-8 protein synthesis without IL-8 degradation, indicating a sensitive regulatory mechanism for IL-8 production. Treatment of neutrophils with fibrinogen and fMLP resulted in minimal changes in the steady state levels of mRNA for macrophage inflammatory protein-1α and -1β and monocyte chemoattractant protein-1. In contrast, in the presence of fibrinogen, the steady-state level of neutrophil IL-8 mRNA increased 8-fold with 5 × 10⁻⁷ M fMLP, but was not decreased with 1 × 10⁻⁷ M fMLP, suggesting that neutrophils are specifically adapted to modulate neutrophil IL-8 synthesis through transcriptional and posttranscriptional mechanisms. The data indicate that fibrinogen can function not only as a substrate in the clotting cascade, but also as an important effector during the evolution of the innate immune response. The Journal of Immunology, 2001, 167: 2869–2878.

Interleukin-8 is an 8-kDa member of the α (CXC) subfamily of chemokines and a potent chemoattractant (ED₅₀ = 1–10 ng/ml) for neutrophils in vitro (1) as well as in vivo (2). In an experimental human model of inflammation in vivo, chemotactic levels of IL-8 accumulate locally (3). The exact source of IL-8 in this model is unknown but may include macrophages, lymphocytes, fibroblasts, and keratinocytes, which can be induced to make IL-8, as well as exudative neutrophils, which, compared with peripheral blood cells, have greatly increased cellular IL-8 that is spontaneously released in vitro (4). Local release of IL-8 by exudative neutrophils could amplify the inflammatory response by autocrine and/or paracrine signaling. However, regulation of IL-8 production in exudative neutrophils is poorly understood. It has been shown that treatment of peripheral blood neutrophils in vitro with an inhibitor of the endoplasmic reticulum Ca²⁺-ATPase, thapsigargin, results in a Ca²⁺-dependent induction of IL-8 mRNA and subsequent production of IL-8 to levels that are comparable to those found in exudative neutrophils (5).

fMLP and leukotriene B₄ (LTB₄) have long been recognized as potent chemoattractants for neutrophils in vitro and may play an important role during exudation in vivo. Low doses of fMLP (with an optimum concentration of ~1 × 10⁻⁸ M) cause chemotaxis of neutrophils through a specific receptor, whereas higher doses (1 × 10⁻⁹-1 × 10⁻⁶ M) are associated with activation of the NADPH oxidase and release of granule enzymes (6). LTB₄ is also chemotactically active through a specific receptor (7) at a concentration of 1–100 nM, although it is less capable of activating NADPH oxidase and degranulation than formylated peptides (8).

Fibrinogen (Fib), a 340-kDa protein made in the liver and found in plasma at a concentration of 1.90–3.65 mg/ml (9), is a complex molecule composed of three pairs of polypeptide chains, an α-chain (67 kDa), a β-chain (56 kDa), and a γ-chain (47 kDa). The proteolytic action of thrombin on Fib promotes the polymerization of the fibrin monomers, leading to fibrin clot formation. During the acute phase response, the plasma concentration of Fib is elevated 50% within 24 h and remains elevated 3-fold over a 3-wk period (10). Although the role of Fib in the clotting cascade has been well defined, its role during the acute phase response is less understood. It has been reported that mice subjected to systemic Fib depletion and then challenged with Bacteroides fragilis develop abdominal abscesses that are reduced in size and less purulent than those in control mice (11), demonstrating that Fib plays an important role in cell exudation. In this report, we show that Fib acts synergistically with fMLP and LTB₄ to induce neutrophil IL-8 production. The data suggest Fib has an important effector role in innate immunity.

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Materials and Methods

Materials

Reagents were obtained from the following sources: C-reactive protein, C5a, ceruloplasmin, collagen, fibrinectin, FMLP, human IgG, laminin, LTB4, thrombin, transferrin, vitronectin, and N-tetr-butylxycarbonyl-L-methionyl-L-tyrosyl-L-phenylalanine (N-Boc-MLF) were obtained from Sigma-Aldrich (St. Louis, MO); growth-related oncoprotein (GRO)-α was obtained from R&D Systems (Minneapolis, MN); Fib, fibrinopeptides A and B, and Fib D and E were obtained from Calbiochem (La Jolla, CA); serum amyloid A was obtained from BioSource International (Camarillo, CA); cyclosporin A and LTB4 dimethylamide were obtained from Biomol (Plymouth Meeting, PA); indole-1 AM and calcine AM were obtained from Molecular Probes (Eugene, OR); pertussis toxin was obtained from List Biological Laboratories (Campbell, CA); and Sephareryl S-300 was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Human recombinant Fib synthesized in Chinese hamster ovary cells (12) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Human recombinant Fib (20 mg) was loaded on a high resolution Sephacryl S-300 column (4.5 cm; Amersham Pharmacia Biotech) and eluted with PBS at a flow rate of 0.5 ml/min with 1.5-ml fractions. The column was calibrated with blue dextran 2000, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), BSA (67 kDa), OVA (43 kDa), and RNase (14 kDa). Fractions were analyzed on precast NuPAGE 4–12% gels, and stained with a SilverXpress silver staining kit (Invitrogen).

Treatment with pertussis toxin

Neutrophils (2 x 10^6/ml of HBSS-HEPES) were treated in the absence or presence of pertussis toxin (0.1 µg/ml) for 3 h at 37°C, washed twice with HBSS without divalent cations, and resuspended in HBSS-HEPES before use. Pertussis toxin treatment reduced FMLP-induced O_2^- production by 85% without altering the PMA-induced response.

Determination of changes in intracellular Ca^{2+} concentration ([Ca^{2+}]_i)

Neutrophils (1 x 10^6/ml HBSS-HEPES) were incubated with the cell-permeant dye, indo-1 AM, in the dark at 37°C for 45 min. The neutrophils were then pelleted by centrifugation at 2000 x g, resuspended in HBSS-HEPES, and the procedure was repeated to remove remaining extracellular indo-1 AM. The cells were resuspended at 2.5 x 10^6/ml HBSS-HEPES. Changes in [Ca^{2+}]), were monitored on a DeltaScan spectrophotometer (Photon Technology International, Lawrenceville, NJ) using a thermostatically controlled cuvette holder. Data were collected as the ratio (R) of the λ emissions (λem=502 nm,λex=405 nm) where excitation was at 358 nm. [Ca^{2+}]), was calculated as previously described (13). The parameters Rmax and Rmin were determined empirically by addition of ionomycin (1 µM) and EGTA (12.5 mM), respectively.

O_2^- production by cytochrome c reduction

Neutrophils (1 x 10^6/ml HBSS) were preincubated at 37°C for 15 min in the absence or presence of Fib (2 µg/ml). Cytochrome c (150 nmol) was added to each tube before the addition of the indicated concentration of FMLP. The incubation was continued for 30 min. Superoxide dismutase (100 µg/ml) was added to an identical tube as a blank. Samples were analyzed using an analytical wavelength at 549.5 nm and background wavelengths at the isobestic points, 541.0 and 556.0 nm. A micromolar extinction coefficient of 0.0211 was used to convert absorbance values to nanomoles of O_2^- released.

Release of granular enzymes

Neutrophils (2 x 10^6/ml of HBSS with 10 mM HEPES) were incubated in the presence and absence of Fib for 15 min before the addition of FMLP. After 8 h, the cells were isolated from the cell suspension by centrifugation at 37°C at 4°C. Both the neutrophil pellet and the supernatant fraction were analyzed for granular enzyme content. Data are expressed as the percentage of the total found in the supernatant fraction.

Lactoferrin was determined by an ELISA as previously described (14). Levels of β-glucuronidase were determined by measuring the release of 4-methylumbelliferone from 4-methylumbelliferyl-β-D-glucuronide. Briefly, an aliquot of the sample (0.01 ml) was incubated with 5 mM substrate in 100 mM sodium acetate buffer (pH 4.8) for 30 min at 37°C (final volume of 0.05 ml). The reaction was terminated by the addition of 0.2 ml of ammonium glycine buffer (0.2 M NH_4 OH and 0.05 M glycine (pH 10.5)), and the fluorescence of the samples was determined using a CytoFluor II 96-well fluorescence plate reader (PerSeptive Biosystems, Framingham, MA). β-Glucuronidase activity was determined using a 4-methylumbelliferone standard curve.

Chemotaxis

Chemotaxis was determined using calcium AM-loaded neutrophils and a disposable 96-well chemotaxis chamber (NeuroProbe, Gaithersburg, MD) as previously described (15). A polycarbonate filter with a pore size of 8 µm and a pore density of 1000 pores/mm^2 was used for the analysis. Migrating neutrophils were suspended in the presence or absence of Fib (2 mg/ml) before migration through the filter. After incubating the filter chamber at 37°C for 60 min, the top of the filter was washed three times with HBSS without divalent cations. The fluorescence of the filter and plate was determined on a CytoFluor II fluorescence plate reader. The number of migrating cells was determined using a standard curve of calcium-labeled Fib ENHANCEMENT OF PMN IL-8 PRODUCTION

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neutrophils. In control studies, there was <10% spontaneous release of calcine.

**Analysis of neutrophil mRNA**

Neutrophils (2.5 × 10⁶/ml HBSS with 10 mM HEPES) were incubated for 1 h and then harvested by centrifugation at 200 × g at room temperature. Total cellular RNA was isolated using TRIzol (Life Technologies, Gaithersburg, MD). The OD₂₆₀ nm/OD₂₈₀ nm of the extracted RNA was usually 1.6; total RNA yields were 20–30 μg/5 × 10⁶ neutrophils. RNA concentrations were determined using the RiboGreen RNA quantitation system (Molecular Probes). The RiboQuant RNase protection assay kit and the hCKS-5 RiboQuant human cytokine multi-probe template set (BD Pharmingen, San Diego, CA) were used according to the manufacturer’s instructions with the exception that the probes labeled with [³²P]UTP (Amersham, Arlington Heights, IL) were purified using TE Micro Select-D, G-25 microcentrifuge spin columns (5 Prime→3 Prime, Boulder, CO). Protected fragments were resolved on 6% polyacrylamide sequencing gels and quantitated on a BAS 1000 phosphor imager (Fungi Medical Systems, Fairfield, NJ). To measure steady-state mRNA levels for IL-8, MIP-1α, and MIP-1β, the amount of total RNA added was reduced to 5 ng (the manufacturer’s recommendation is 1–20 μg) to avoid saturation of these components of the multi-probe template set. Higher concentrations of total RNA that were run initially demonstrated the integrity of the RNA from all samples and yielded approximately equal signals for the GAPDH housekeeping gene and 18S ribosomal RNA probe. The RNA concentrations of the diluted samples were confirmed by the RiboGreen RNA quantitation system.

**Statistics**

Data were evaluated with a variety of statistical techniques including linear and nonlinear regression, ANOVA, analysis of covariance, repeated-measures analysis, profile analysis, descriptive techniques, and post hoc tests. In many cases, data were transformed to their common logarithms to satisfy homogeneity of variance requirements. The data presented represent the mean ± SEM.

A conservative strategy was used to define synergy. Following global tests of significance, if the treatment of neutrophils with both Fib and chemoattractant yielded a significantly higher mean response (i.e., p < 0.01) than Fib alone and a significantly higher mean response than chemoattractant alone, then the effect of Fib and chemoattractant were defined to be synergistic. As a further validation, the aggregate response of Fib alone and of chemoattractant alone was compared with the response of both ligands, and similar significance was observed.

**Results**

**Effect of Fib and fMLP/LTB₄ on neutrophil IL-8 production**

Freshly isolated neutrophils have low, but detectable, levels of IL-8 (0.04 ± 0.01 ng/10⁶ cells) (Fig. 1A; t = 0). As shown previously (4), incubation of neutrophils in buffer alone for 8 h resulted in a small but significant increase in the level of total IL-8 (sum of the cellular and extracellular) compared with freshly isolated neutrophils. Treatment of neutrophils for 8 h with a chemotactic concentration of the peptide fMLP (5 × 10⁻⁹ M) alone (Fig. 1A), or a chemotactic concentration of LTB₄ (1 × 10⁻⁷ M) alone (Fig. 1B), resulted in small increases in the levels of total IL-8 compared with freshly isolated neutrophils (buffer treated = 0.36 ± 0.04 ng IL-8/10⁶ cells, p < 0.001; fMLP treated = 1.14 ± 0.13 ng IL-8/10⁶ cells, p < 0.01; and LTB₄ treated = 3.42 ± 0.77 ng IL-8/10⁶ cells, p < 0.001). Thus, fMLP and LTB₄ are able to induce neutrophil IL-8 production, but not at levels that could account for the observed production of IL-8 in exudative neutrophils.

Treatment of neutrophils for 8 h with a physiologic concentration of Fib (2 mg/ml) resulted in a small, yet significant, increase in the level of total IL-8 compared with control neutrophils (buffer treated = 0.36 ± 0.04 ng IL-8/10⁶ cells; and Fib treated = 2.43 ± 0.59 ng IL-8/10⁶ cells, p < 0.001). Incubation of neutrophils with both Fib and fMLP (5 × 10⁻⁹ M) resulted in a 30-fold increase in total neutrophil IL-8 that was detectable within 1 h and reached a plateau by 4 h. Treatment of neutrophils with Fib plus fMLP yielded a significant synergistic response vs neutrophils treated with Fib alone or fMLP alone at t = 2, 4, and 8 h (p < 0.01 at all time points) (Fig. 1A).

**FIGURE 1.** Effect of Fib on IL-8 production. A and B, Neutrophils, suspended at 1–2 × 10⁹/ml HBSS-HEPES in polypropylene tubes, were preincubated for 10 min at 37°C. Agonists (no addition, ■; Fib (2 mg/ml), □; fMLP (5 × 10⁻⁹ M), ○; Fib plus fMLP; ●, and B, LTB₄ (1 × 10⁻⁷ M), ○; Fib plus LTB₄, ●) were added to the neutrophil suspension at t = 0. Cells were removed from the incubation at the designated times after the addition of fMLP or LTB₄. The cell suspension was centrifuged at 4°C for 10 min at 200 × g. The cell pellets and supernatants were harvested. Detergent-solubilized cell pellets and supernatants were harvested for IL-8 content. The data are presented as the sum of the cellular and extracellular fractions and represent the mean ± SEM of four experiments. Paired comparisons were performed on the samples treated with Fib alone, chemoattractant alone, and the combination of Fib and chemoattractant. Synergy is indicated when the response to the combination of Fib and chemoattractant differs significantly (*, p < 0.01; **, p < 0.001) from the response due to Fib alone or chemoattractant alone. C and D, Neutrophils were preincubated with different concentrations of Fib for 15 min. The samples were further incubated for 8 h in the absence (○) or presence (●) of fMLP (5 × 10⁻⁹ M) (C; n = 5) or LTB₄ (1 × 10⁻⁷ M) (D; n = 4). The samples were analyzed as described above. E and F, Neutrophils were incubated for 8 h at 37°C with fMLP (E; n = 12) or LTB₄ (F; n = 4) in the absence (○) or the presence (●) of Fib (2 mg/ml). Samples were analyzed as described above.

Similarly, treatment of neutrophils with both Fib and LTB₄ (1 × 10⁻⁷ M) resulted in an increase in total IL-8 that was detected within 1 h and continued to increase through 8 h. Fib plus LTB₄ yielded significant synergistic responses vs neutrophils treated with Fib alone or LTB₄ alone at t = 1, 2, 4, and 8 h (p < 0.01 at all time points) (Fig. 1B).
Dose response of Fib

Exposure of neutrophils to increasing concentrations of Fib alone (1–5 mg/ml) resulted in small (p < 0.05) increases in total IL-8 (Fig. 1C). Treatment of neutrophils with increasing concentrations of Fib plus a constant concentration of fMLP (5 × 10⁻⁹ M) greatly increased neutrophil IL-8 at all doses of Fib tested (p < 0.01). Similarly, treatment of neutrophils with increasing concentrations of Fib plus LTB₄ (1 × 10⁻⁷ M) increased neutrophil IL-8 at 0.5–5 mg/ml of Fib (p < 0.01) (Fig. 1D). Removal of Fib by washing the cells abrogated its synergistic effect on IL-8 production, yielding a response comparable to that of fMLP alone (data not shown).

Dose response of fMLP and LTB₄

Incubation of neutrophils with Fib and increasing concentrations of fMLP yielded an approximately bell-shaped curve with maximum IL-8 production at 2–5 × 10⁻⁹ M fMLP (ED₅₀ = 1.15 ± 0.05 nM), doses associated with chemotaxis (16) and binding to the high affinity fMLP receptor (Fig. 1E). A striking reduction in neutrophil IL-8 was observed with higher doses of fMLP (up to 1 × 10⁻⁷ M), indicating sensitive regulation of neutrophil IL-8 synthesis. A second peak of IL-8-inducing activity, exhibiting marginal synergy (p < 0.05), was observed in the range of 5 × 10⁻⁶–1 × 10⁻⁵ M fMLP, doses associated with binding of fMLP to the low affinity fMLP receptor (17). The dose-response characteristics of IL-8 synthesis in response to Fib plus fMLP are similar to the neutrophil chemotactic response to fMLP.

The dramatic decrease in IL-8 production observed at doses of 5 × 10⁻⁹–1 × 10⁻⁷ M fMLP was not due to increased proteolytic activity in the extracellular fluid. In experiments in which exogenous [¹²⁵]I-labeled IL-8 was added to neutrophils exposed to Fib plus fMLP (5 × 10⁻⁹ M) for 8 h and then the cell-free supernatant fluid analyzed by SDS-PAGE and autoradiography, there was no degradation of IL-8 (data not shown). In addition, treatment of neutrophils with a higher dose of fMLP (1 × 10⁻⁷ M) 4 h after treatment with optimal conditions of Fib plus fMLP (5 × 10⁻⁹ M) resulted in no decrease in IL-8 production, indicating that no mediators were released at a higher dose of fMLP that degraded newly synthesized IL-8.

Treatment of neutrophils with Fib plus LTB₄ also resulted in significant synergistic responses at doses of 2 × 10⁻⁸–1 × 10⁻⁷ M Fib (Fig. 1F; p < 0.01 at all doses). However, in contrast to the response with fMLP, higher doses of LTB₄ (up to 10⁻⁶ M) were not associated with reduced levels of IL-8. No increase in neutrophil IL-8 was observed when neutrophils, in the presence or absence of Fib, were incubated with the chemoattractants platelet-activating factor (PAF), complement fragment, C5a, or GRO-α (Table I). Because GRO-α and IL-8 share a common CXCR (CXCR-2), the failure of GRO-α to synergize with Fib indicates that IL-8 does not affect its own synthesis through CXCR-2.

fMLP- and LTB₄-induced increases in neutrophil IL-8 are receptor mediated

To determine whether the increases in neutrophil IL-8 induced by Fib plus fMLP and Fib plus LTB₄ were mediated by their respective chemoattractant receptors, neutrophils were treated with either N-BOC-MLF, a competitive antagonist of fMLP (18), or LTB₄ dimethylamide, a competitive antagonist of LTB₄ (19). Treatment with these receptor antagonists abrogated the fMLP- and LTB₄-induced responses by 75 and 70%, respectively (Fig. 2), but had only minor effects on the thapsigargin-induced increase in neutrophil IL-8.

![Image](http://www.jimmunol.org/)

**FIGURE 2.** Specific chemoattractant receptors mediate increases in neutrophil IL-8. Neutrophils were preincubated in the presence of buffer or Fib (2 mg/ml) and N-BOC-MLF (10 μM) or LTB₄ dimethylamide (1.0 μM) for 20 min at 37°C before the addition of fMLP (5 × 10⁻⁹ M), LTB₄ (1 × 10⁻⁷ M), or thapsigargin (1 × 10⁻⁷ M). The samples were then treated as described in Table I. The data represent the mean ± SEM of four experiments. Paired comparisons were performed on the control vs antagonist-treated samples, and significant differences are indicated (*, p < 0.05).

<table>
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<th>Buffer</th>
<th>+ Fib</th>
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<tr>
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<tr>
<td>fMLP (5 × 10⁻⁹ M)</td>
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<td>10.0</td>
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<td>100.0</td>
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* Neutrophils (1–2 × 10⁶/ml HBSS with HEPES) were incubated for 15 min at 37°C in the absence or presence of Fib prior to the addition of the indicated chemoattractants. After the addition of chemoattractant, the incubations were continued for an additional 8 h. The cell suspension was centrifuged at 4°C for 10 min at 200 × g. The cell pellets and supernatant fluids were harvested. Detergent-solubilized cell pellets and supernatant fluids were analyzed for IL-8 content. The data are presented as the sum of the two fractions and represent the mean ± SEM. The number in parentheses refers to the number of experiments performed. Paired comparisons were performed on those samples treated with Fib alone, chemoattractant alone, and the combination of the both. Synergy is indicated when the response to the combination of the indicated chemoattractant and Fib differs significantly (*, p < 0.01) from the response due to chemoattractant alone or Fib alone.

Pertussis toxin sensitivity of fMLP- and LTB₄-induced increase in neutrophil IL-8

To determine whether the increases in neutrophil IL-8 induced through the fMLP receptor and LTB₄ receptor were mediated by...
heterotrimeric G proteins, neutrophils were pretreated with pertussis toxin to inactivate the G proteins before the addition of agonists. As shown in Fig. 3, treatment of neutrophils with pertussis toxin reduced the responses to Fib plus fMLP and Fib plus LTB₄ to the level observed with Fib alone. However, the increases in neutrophil IL-8 in response to buffer vs Fib alone (p < 0.01) or to thapsigargin alone (p < 0.001) were not significantly altered by treatment with pertussis toxin, indicating that signaling through these agonists was independent of G proteins.

**Specificity of Fib**

To determine the specificity of the response to Fib, other extracellular matrix proteins including human fibronectin, vitronectin, collagen, and laminin were tested and failed to mimic the effect of Fib on IL-8 production (Fig. 4). Of the acute-phase proteins tested, ceruloplasmin and C-reactive protein both mimicked the effect of Fib, but to a lesser extent.

**Properties of Fib and Fib-related molecules**

Heat-treated Fib (30 min at 56°C) and a fibrin clot were unable to synergize with fMLP to induce IL-8 production (Table II). The fibrinoepitopes A and B, released from Fib by thrombin during the clotting process, and the proteolytic Fib fragments D (85 kDa) and E (50 kDa), derived from the complete hydrolysis of Fib, were also unable to support the production of IL-8 from neutrophils stimulated with fMLP.

Gel filtration of commercially obtained Fib on a Sephacryl S-300 column resulted in a single peak of IL-8-inducing activity that coeluted with both the protein peak and the Fib peak (Fig. 5A). PAGE analysis of the peak fractions under reducing conditions yielded three bands, consistent with the three subunits of Fib (Fig. 5, shown as insert). The addition of thrombin to Fib to promote clot formation and the subsequent removal of the fibrin clot by centrifugation resulted in loss of the three Fib subunits and abrogation of the IL-8 response, indicating that Fib was responsible for synergistic induction of neutrophil IL-8 production (Fig. 5B). To

**FIGURE 3.** G protein coupling of chemoattractant-induced increases in neutrophil IL-8. Neutrophils were treated with pertussis toxin as described in Materials and Methods. Neutrophils were then treated in the presence of buffer or Fib (2 mg/ml) for 20 min at 37°C before the addition of fMLP (5 x 10⁻⁹ M), LTB₄ (1 x 10⁻⁷ M), or thapsigargin (1 x 10⁻⁷ M). The samples were then treated as described in Table I. The data represent the mean ± SEM of four experiments. Paired comparisons were performed on the control vs pertussis toxin-treated samples, and significant differences are indicated (*, p < 0.05).

**FIGURE 4.** Specificity of Fib for IL-8 production. Neutrophils were preincubated with the indicated proteins for 15 min at 37°C before the addition of fMLP (5 x 10⁻⁹ M). The samples were then treated as described in Table I. The data represent the mean ± SEM of four experiments. Paired comparisons were performed on the samples treated with protein alone, fMLP alone, and protein and fMLP. Synergy is indicated when the response to the combination of the added protein and fMLP differs significantly (*, p < 0.01) from the response due to protein alone or fMLP alone.
Table II. Fib-related molecules fail to support neutrophil IL-8 production with fMLP

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Buffer</th>
<th>fMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.46±0.04 (31)</td>
<td>1.08±0.11 (31)</td>
</tr>
<tr>
<td>Fib (2.0 mg/ml)</td>
<td>1.76±0.21 (31)</td>
<td>11.11±1.32 (31)**</td>
</tr>
<tr>
<td>Δ-Treated Fib</td>
<td>0.64±0.20 (3)</td>
<td>3.86±1.80 (3)</td>
</tr>
<tr>
<td>Clotted Fib</td>
<td>0.40±0.15 (3)</td>
<td>2.58±1.27 (3)</td>
</tr>
<tr>
<td>Fib fragment D (20 μg/ml)</td>
<td>0.53 (1)</td>
<td>1.50 (1)</td>
</tr>
<tr>
<td>Fib fragment E (10 μg/ml)</td>
<td>0.48 (1)</td>
<td>1.29 (1)</td>
</tr>
<tr>
<td>Fibrinopeptide A (50 μg/ml)</td>
<td>0.42±0.09 (2)</td>
<td>1.25±0.76 (2)</td>
</tr>
<tr>
<td>Fibrinopeptide B (50 μg/ml)</td>
<td>0.44±0.11 (2)</td>
<td>1.52±1.01 (2)</td>
</tr>
</tbody>
</table>

*Neutrophils were preincubated with Fib or Fib-related molecules for 15 min at 37°C prior to the addition of fMLP (5 × 10⁻⁹ M). The incubation was continued for an additional 8 h. Δ-Treated Fib was prepared by incubation of Fib (20 mg/ml PBS) at 56°C for 30 min, followed by centrifugation to remove the precipitate. Clotted Fib was prepared by incubating Fib (2 mg/ml HBSS) with human AB sera (5%) for 2 h at 37°C. The clot was centrifuged and the supernatant fluid removed prior to the addition of neutrophil suspension. The samples were treated as described in Table I. The data represent the mean ± SEM. The number in parentheses refers to the number of experiments performed. Paired comparisons were performed on those samples treated with Fib or Fib-related molecule alone, fMLP alone, and the combination of both. Synergy is indicated when the response to the combination of fMLP and Fib or Fib-related molecule differs significantly (+, p < 0.01 or **, p < 0.001) from the response due to fMLP or Fib molecule alone.

exclude the possible contribution of a contaminant(s) in the native Fib, recombinant Fib was also tested. In a single experiment, treatment of neutrophils with human recombinant Fib (2 mg/ml) and fMLP resulted in a 4-fold increase in neutrophil IL-8 compared with neutrophils treated with recombinant Fib alone. Although the response to recombinant Fib was somewhat reduced compared with native Fib (7-fold increase), its effectiveness excluded a contaminant of the commercial Fib as the active agent.

Responsiveness of neutrophil populations

To address whether a specific subpopulation of neutrophils or some other contaminating cell type contributed to increased levels of IL-8, neutrophils were permeabilized and stained for intracellular IL-8. Neutrophils treated with Fib (2 mg/ml) and fMLP (5 × 10⁻⁹ M) responded as a single population of cells with an apparent shift in the peak of intracellular IL-8 staining to the right as compared with untreated (control) cells (data not shown).

Effect of Fib on other neutrophil functions

The synergy observed with Fib plus fMLP was not observed for other neutrophil functions. Fib had no effect on the dose-response curve of fMLP for alterations in [Ca²⁺], chemotaxis, O₂ production, or degranulation of either specific (lactoferrin) or azurophilic (β-d-glucuronidase) granules (data not shown).

Studies of neutrophils from patients with leukocyte adhesion deficiency (LAD)

Fib responses in neutrophils have been attributed to interactions with the β₂ integrins (20, 21). To investigate the potential role of the β₂ integrins in mediating the effects of Fib on neutrophil IL-8 production, neutrophils were isolated from patients who lack the β₂ integrins (LAD). LAD neutrophils supported Fib plus fMLP-induced IL-8 production normally, indicating that the β₂ integrins were not the receptors mediating the Fib effects (Table III). Interestingly, treatment of LAD neutrophils with fMLP in the absence of Fib resulted in an IL-8 response that was significantly elevated vs normal subjects.

Effect of inhibitors of Ca²⁺ metabolism

The levels of IL-8 attained after treatment of neutrophils with Fib plus fMLP (12.48 ± 1.11 ng IL-8/10⁶ cells) and Fib plus LTB₄ (23.01 ± 2.31 ng IL-8/10⁶ cells) were comparable to concentrations in exudative neutrophils (4) and thapsigargin-treated neutrophils (5). The Ca²⁺ chelator, EGTA (2.5 mM), inhibited the Fib production with fMLP a

FIGURE 5. Fib cocultures with IL-8-inducing activity. A, Fib (20 mg) was loaded on a Sephacryl S-300 gel filtration column and eluted with PBS at a flow rate of 0.5 ml/min. Fractions (1.5 ml) were analyzed for protein and Fib content and for the ability to induce neutrophil IL-8 production in the presence of fMLP (5 × 10⁻⁹ M). The arrows above the chromatogram represent the void volume, V₀, and the peak fraction of m.w. standards. The inset represents PAGE analysis under reducing condition of fractions 25–33, showing the three subunits of Fib. Lane C contains control Fib. B, Fib (4 mg/ml in HBSS) was treated at 37°C for 3 h with thrombin (0.05 U/ml) to promote clotting or heat-inactivated thrombin (15 min at 100°C) as a control. The fibrin clot was removed by centrifugation, and the remaining supernatant fluid was collected and tested for IL-8-inducing activity. The results obtained with untreated Fib (19.41 ± 3.47 ng IL-8/10⁶ cells) did not differ significantly from the results obtained with Fib treated with heat-inactivated thrombin. The data represent the mean ± the SEM of four experiments. The inset represents PAGE analysis under reducing conditions of Fib treated with thrombin, Fib treated with heat-inactivated thrombin, and untreated Fib.
plus fMLP response by <50%, whereas the calcineurin inhibitor, cyclosporin A (200 ng/ml), had no effect, indicating that the neutrophil response to Fib plus fMLP, unlike the response to thapsigargin (5), was not dependent on Ca\textsuperscript{2+} (Table IV).

**Effect of protein synthesis inhibitors on Fib plus fMLP-induced IL-8 production**

The production of IL-8 by Fib plus fMLP was dependent on protein synthesis de novo. Treatment of neutrophils with cycloheximide (10 μg/ml) inhibited the production of IL-8 with Fib plus fMLP by 89% (8.69 ± 2.15 ng/1 × 10\textsuperscript{6} cells vs 0.95 ± 0.33 ng/1 × 10\textsuperscript{6} cells; p = 0.0004). IL-8 production induced by Fib plus fMLP was inhibited (89%) by the RNA synthesis inhibitor, actinomycin D (10 μg/ml) (22.23 ± 8.19 ng/1 × 10\textsuperscript{6} cells vs 2.61 ± 0.15 ng/1 × 10\textsuperscript{6}; p = 0.0368), a dose which has been shown to block completely the increased expression of IL-8 mRNA (22) and formyl peptide receptor mRNA (23) in neutrophils.

The induction of IL-8 synthesis was accompanied by changes in the steady-state level of IL-8 mRNA. Using RNase protection assays of total RNA isolated from neutrophils, the level of IL-8 mRNA could be detected using as little as 5 ng of total neutrophil RNA, much less than usually needed for RNase protection assays. Analysis of neutrophil RNA revealed that treatment of neutrophils with chemotactically active (16) doses of fMLP (5 × 10\textsuperscript{-9} M) alone, LTB\textsubscript{4} (1 × 10\textsuperscript{-7} M) alone, or Fib alone had little effect on the steady-state level of IL-8 mRNA compared with untreated neutrophils incubated for 1 h (Fig. 6), although small, but significant, increases in neutrophil IL-8 were observed (Fig. 1, A and B). At a high dose of fMLP alone (1 × 10\textsuperscript{-7} M), associated with reduced chemotactic responsiveness (16), there was a 3-fold increase in the steady-state level of IL-8 mRNA but no further increase in neutrophil IL-8 synthesis. Addition of Fib to fMLP (5 × 10\textsuperscript{-9} M) or LTB\textsubscript{4} (1 × 10\textsuperscript{-7} M) resulted in an 8- or 4-fold increase in the steady-state level of IL-8 mRNA, respectively, and a 30-fold increase in neutrophil IL-8 (Fig. 6). The complete inhibition of IL-8 synthesis observed with Fib plus fMLP at 1 × 10\textsuperscript{-7} M vs Fib plus fMLP at 5 × 10\textsuperscript{-9} M (Fig. 1E) could not be explained by a reversal in the steady-state level IL-8 mRNA (Fig. 6), indicating that additional regulatory mechanisms were involved in down-regulating IL-8 synthesis with 1 × 10\textsuperscript{-7} M fMLP.

The increase in the steady-state level of IL-8 mRNA was detected within 15 min, reached a maximum by 60 min, and remained elevated at 2 h (Fig. 7). Treatment of neutrophils with Fib plus LTB\textsubscript{4} (1 × 10\textsuperscript{-7} M) yielded a lower steady-state level of IL-8 mRNA than Fib plus fMLP (5 × 10\textsuperscript{-9} M), but the net production of IL-8 was higher with LTB\textsubscript{4} than fMLP, indicating additional ligand-specific differences in posttranscriptional regulation of IL-8 synthesis.

The increase in the steady-state level of IL-8 mRNA was cytochrome specific. Unlike IL-8 mRNA, which was easily detected in freshly isolated neutrophils and did not increase with incubation in

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**Table III. Fib plus fMLP-induced IL-8 production in neutrophils from patients with LAD**

<table>
<thead>
<tr>
<th>Clinical Condition</th>
<th>Buffer</th>
<th>1 × 10\textsuperscript{-10} M</th>
<th>5 × 10\textsuperscript{-9} M</th>
<th>1 × 10\textsuperscript{-7} M</th>
<th>Buffer</th>
<th>1 × 10\textsuperscript{-10} M</th>
<th>5 × 10\textsuperscript{-9} M</th>
<th>1 × 10\textsuperscript{-7} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 7)</td>
<td>0.50 ± 0.11</td>
<td>0.59 ± 0.14</td>
<td>1.14 ± 0.18</td>
<td>0.99 ± 0.14</td>
<td>2.12 ± 0.52</td>
<td>3.05 ± 0.56</td>
<td>11.10 ± 3.03</td>
<td>3.42 ± 0.60</td>
</tr>
<tr>
<td>LAD (n = 3)</td>
<td>0.45 ± 0.04</td>
<td>0.48 ± 0.05</td>
<td>3.11 ± 0.74*</td>
<td>0.92 ± 0.14</td>
<td>2.28 ± 0.40</td>
<td>2.97 ± 0.14</td>
<td>15.46 ± 4.34</td>
<td>3.77 ± 0.59</td>
</tr>
</tbody>
</table>

* Neutrophils were treated as described in Table I and analyzed for IL-8 production. Significant differences between patients with LAD and normal subjects using a Student’s t test are indicated (*, p < 0.01).

**Table IV. Effect of Ca\textsuperscript{2+} inhibitors on IL-8 production**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-8 (ng/10\textsuperscript{6} cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.42 ± 0.10</td>
</tr>
<tr>
<td>+ EGTA</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>+ Cyclosporin A</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>8.94 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>4.63 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>9.01 ± 1.77</td>
</tr>
<tr>
<td></td>
<td>19.38 ± 3.07</td>
</tr>
<tr>
<td></td>
<td>0.56 ± 0.14*</td>
</tr>
<tr>
<td></td>
<td>5.27 ± 1.19*</td>
</tr>
</tbody>
</table>

* Neutrophils (1–2 × 10\textsuperscript{6} ml HBSS with HEPES) were treated with EGTA (2.5 mM) or cyclosporin A (200 ng/ml) for 15 min prior to the addition of either thapsigargin (100 nM) or Fib (2 mg/ml) and fMLP (5 × 10\textsuperscript{-9} M). The data represent the mean ± SEM of three experiments. Significant differences using a Student’s paired t test are indicated (*, p < 0.05).
the absence of a stimulus, the steady-state level of mRNA for both MIP-1α and MIP-1β was not detected in freshly isolated cells, yet it was detected in low amounts upon incubation for 1 h without a specific stimulus (Fig. 7). No further increases in the steady-state levels of MIP-1α and MIP-1β mRNA were observed under the various treatment regimens. The failure to induce changes in the steady-state levels of MIP-1α and MIP-1β mRNA and the minimal changes in the synthesis of other neutrophil cytokines, IL-1β, TNF-α, and IL-1ra (Table V), attest to the specificity of the IL-8 response to Fib plus fMLP.

**Discussion**

The increased levels of IL-8 observed in exudative neutrophils compared with peripheral blood neutrophils (4) suggest that during neutrophil diapedesis, neutrophils accumulate IL-8. The mechanism for the increase in neutrophil IL-8 is not yet understood. In this report, we demonstrate that Fib synergizes with chemotactic doses of fMLP or LTB₄ to induce neutrophil IL-8 synthesis. This is a previously unrecognized function for Fib that may relate to its role as an acute-phase protein in infection and inflammatory disease. This suggestion is supported by the findings of other investigators who have shown that a 75% reduction in the level of plasma Fib in mice results in smaller and less purulent abscess formation (11), indicating that Fib plays a role in the pathogenesis of purulent abscess formation.

The synergistic induction of neutrophil IL-8 synthesis is specific for Fib plus fMLP or LTB₄; no synergy is observed with Fib plus the chemoattractants C5a, PAF, and GRO-α. Because GRO-α and IL-8 share a common CXCR (CXCR-2), the failure of GRO-α to synergize with Fib suggests that IL-8 does not affect its own synthesis through CXCR-2. However, it does not preclude IL-8 mediation of its own synthesis through CXCR-1. Browning et al. (24) recently reported that immobilized IL-8 induced IL-8 production in monocytes; however, these authors failed to demonstrate any significant IL-8 production in neutrophils. Furthermore, these authors showed that the stimulation of neutrophils through CXCR-2 (with immobilized GRO-α) did not induce IL-8 production, confirming the results presented in Table I. The synergistic increases in neutrophil IL-8 observed with both Fib plus fMLP and Fib plus LTB₄ are sensitive to specific receptor antagonists and pertussis toxin and are therefore mediated by their respective G protein-coupled chemoattractant receptors. In contrast, the modest increase in neutrophil IL-8 observed with Fib is insensitive to pertussis toxin and is therefore independent of a G protein-coupled receptor-signaling event.

The synergy of Fib plus fMLP is limited to IL-8 synthesis because other fMLP-mediated neutrophil functions (chemotaxis, O₂⁻ production, and degranulation) are not altered in the presence of Fib.

The effect of Fib on IL-8 production is both dose dependent and specific. The effective doses of Fib range from the levels of Fib found in normal plasma to the levels found during the acute-phase response. Heat-treated Fib, fibrinopeptides A and B (proteolytic fragments of Fib released during clot formation), as well as a fibrin clot fail to exhibit synergy with fMLP for enhanced neutrophil IL-8 production. Analysis of Fib by gel filtration chromatography results in a single peak of IL-8-inducing activity that coelutes with

![Image](http://www.jimmunol.org/)

**FIGURE 7.** Induction of IL-8 mRNA expression. Neutrophils were treated with Fib (2 mg/ml), fMLP (5 x 10⁻¹⁰ M), and LTB₄ (1 x 10⁻¹⁰ M) as indicated at the top of lanes 3–17. RNA was obtained and RNase protection assays were performed as described in Fig. 6. Lane 1. Free Probe, represents the undigested probes MIP-1β, MIP-1α, MCP-1, IL-8, and IFN-γ-inducible protein-10 (IP-10) from top to bottom. Yeast RNA (lane 2) represents the negative control, whereas RNA from thapsigargin-treated (100 nM) neutrophils (lane 18) represents the positive control. Lane 3. Fresh, represents freshly isolated neutrophils. The data are from one of two experiments.

<table>
<thead>
<tr>
<th>Cytokine Produced</th>
<th>Picograms of Analyte per 10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>IL-8</td>
<td>454.3 ± 39.8</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>717.2 ± 36.3</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>23.4 ± 2.7</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>42.9 ± 7.4</td>
</tr>
</tbody>
</table>

* Neutrophils were treated as described in Table I and analyzed for the indicated analytes. The data represent the mean ± SEM of six experiments. Synergy is indicated when the response to the combination of Fib plus fMLP differs significantly (*, p < 0.01 or **, p < 0.001) from the response due to fMLP alone or Fib alone.
both the peak of protein activity and the peak of Fib activity. Removal of the Fib by treatment with thrombin abrogates the IL-8-inducing activity, supporting the role of Fib in inducing neutrophil IL-8 production. Finally, the effectiveness of recombinant Fib in inducing neutrophil IL-8 production excludes a contaminant of Fib in our commercial preparation as the active agent and further supports the role of Fib. The reduced response of recombinant Fib compared with native Fib probably reflects glycosylation differences of the recombinant protein.

Extracellular matrix proteins such as vitronectin, fibronectin, laminin, collagen, and thrombospondin and acute-phase proteins such as serum amyloid A and transferrin are unable to mimic the effects of Fib. The acute-phase proteins, ceruloplasmin and C-reactive protein, are able to mimic the synergy observed with Fib, although the extent of IL-8 production is diminished. Interestingly, elevated levels of Fib and C-reactive protein are predictors of long-term risk of death from cardiac causes (25), and it is intriguing to speculate that the observation presented in this report may relate to the pathogenesis of atherosclerosis.

Using RNase protection assays of total RNA isolated from resting neutrophils, the mRNA for IL-8, MIP-1α, and MIP-1β was detected. Stimulation of neutrophils with Fib plus fMLP or Fib plus LTB₄ increased the steady-state levels of IL-8 mRNA, but not MIP-1α and MIP-1β. The increase in protein translation was relatively specific, with a 30-fold increase in the levels of IL-8 compared with control, a 4- to 6-fold increase in the level of IL-1β and TNF-α, and little or no increase in the levels of IL-1α, MIP-1α, and MIP-1β. The preferential induction of neutrophil IL-8 vs other cytokines/chemokines indicates that neutrophils are specifically adapted to amplify IL-8 synthesis and suggests that there are complex regulatory mechanisms involved.

Fib plus fMLP induction of neutrophil IL-8 was tightly regulated, with a biphasic dose-response curve. Low doses of fMLP (5 × 10⁻⁹ M) caused a 30-fold increase in IL-8 synthesis, whereas at a higher dose (1 × 10⁻⁷ M), the response was ablated; increasing fMLP to 1 × 10⁻⁵ M resulted in a second smaller response. The biphasic dose-response curve is the same as the chemotactic response and may relate to high and low affinity fMLP receptors (16). The depressed IL-8 synthesis at 1 × 10⁻⁷ M compared with 5 × 10⁻⁹ M could not be attributed to the release of proteolytic enzymes degrading fMLP or IL-8 or to inhibition of IL-8 synthesis. Further investigation of the mechanism of the posttranslational regulation of IL-8 synthesis may offer important clues as to normal regulation of inflammation.

Cellular interactions with Fib are thought to be mediated through lower affinity binding to promiscuous surface integrins rather than through higher affinity binding via a specific Fib receptor. Fib has been shown to bind to endothelial cells through the integrin αᵥβ₃ receptor (26) and thereby has been implicated in angiogenesis and wound healing. Fib has also been shown to activate platelets through the α₅β₃ receptor (27) and is thought to play an important role in activating platelet aggregate and clot retraction. Neutrophil interactions with surface-bound Fib via β₂ integrins results in a substantial increase in the production of IL-8 and IL-1β, a response that is abrogated in CD18-deficient mice (28). However, the synergistic effect of Fib plus fMLP on neutrophils described here is independent of the β₂ integrins because neutrophils from three patients with leukocyte adhesion deficiency, whose cells lack these receptors, exhibit responses to Fib plus fMLP that are normal (Table III).

The production of IL-8 in vitro by neutrophils mimics that observed in exudative neutrophils and indicates that migrating neutrophils are a potential source of IL-8 found at an inflammatory focus. Moreover, this observation provides a plausible rationale for the logarithmic amplification of the inflammatory response through neutrophil IL-8-mediated recruitment of additional neutrophils. Finally, insight into the regulatory mechanisms that precipitate neutrophil IL-8 production may provide therapeutic targets for modulation of the innate immune response.

Acknowledgments
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References


