RAPID COMMUNICATION

The Role of Interleukin-1 in Neutrophil Leukocyte Emigration Induced by Endotoxin

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Chemotactic factors induce neutrophil emigration into tissues. Interleukin-1 (IL-1) was found to be several log times more potent in this respect than C5a des Arg, leukotriene B_4, and f-Met-Leu-Phe and of comparable potency to endotoxin. Kinetic studies revealed a rapid and transient neutrophil influx, with the peak rate at 30–90 minutes. Cross tachyphylaxis was observed between IL-1 and endotoxin; and this, together with its high potency and rapid onset of action, suggest that IL-1 mediates endotoxin-induced neutrophil emigration. (Am J Pathol 1986, 124:367–372)

THE EMIGRATION of neutrophil leukocytes (neutrophils) from blood into tissues is the hallmark of acute inflammation. This process occurs predominantly in postcapillary venules[1] and appears to be regulated by endothelial cells, which upon stimulation with factors chemotactic for neutrophils become "adhesive" for these cells.[2,3] *In vitro* studies suggest that the adhesive step involves the expression of a new membrane protein on endothelial cells and can be blocked by the inhibition of protein synthesis.[4–6] It is thought that after diapedesis through the endothelium into extravascular tissues neutrophils migrate along a chemotactic gradient.[1] A gradient may be formed by the local generation of host-derived factors, eg, complement component C5a des Arg and leukotriene B_4 (LTB_4), or microorganism-derived factors, eg, the chemotactic peptide f-Met-Leu-Phe released by *E coli*. Endotoxins, which are lipopolysaccharide-protein molecules released from Gram-negative bacteria, are not chemotactic for neutrophils,[7,8] yet are more potent on a molar basis in inducing neutrophil emigration than all chemotactic factors so far tested.[9] In addition, endotoxins can induce local or systemic thrombosis and provoke septic shock. The mechanisms of acute inflammation induced by endotoxins remain largely unknown, although it is known that endotoxins provide a potent stimulus for *in vitro* production of interleukin-1 (IL-1) by monocytes/macrophages.[10] The activities ascribed to IL-1 are common to a family of closely related proteins which are synthesized and secreted by many cells, but primarily by monocytes/macrophages, in response to various stimuli, including endotoxins.[10] *In vitro*, IL-1 activates endothelial cells to express "neutrophil adhesive" proteins[4,6,11] and may be chemotactic for neutrophils.[12] IL-1 may participate in eliciting local hemorrhage resembling the local Shwartzman reaction.[13] We have examined the potency and kinetics of IL-1-induced neutrophil emigration, compared its *in vivo* potency with that of other chemotactic factors, and investigated its role in acute inflammation elicited by endotoxin. Preliminary findings were reported in abstract form.[14]

Materials and Methods

Quantitation of Neutrophil Emigration

Neutrophil emigration into intradermal sites of 2.5–3.5 kg New Zealand albino rabbits was measured with ^{51}Cr-labeled allogeneic neutrophils. These were isolated from blood to greater than 90% purity by sedimentation with hydroxyethyl cellulose (Polysciences, Warrington, Pa) and density gravity centrifugation through Percoll (Pharmacia, Dorval, Quebec).[15] Cells were resuspended in a Ca^{2+}-, Mg^{2+}-free Tyrode's

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buffer containing 10% platelet poor rabbit plasma to a concentration of 10^8 leukocytes/ml and labeled for 20–30 minutes with 100–150 μCi/ml $Na_2{}^{51}Cr_4O_4$ (New England Nuclear, Lachine, Quebec, 200–500 mCi/mg). After labeling, the cells were pelleted by centrifugation, and the ^{51}Cr-labeled erythrocyte contaminent was removed by resedimentation of cells, which were resuspended in leukocyte-poor plasma containing unlabeled erythrocytes and 1% hydroxyethyl cellulose (saved from the initial sedimentation). This procedure yielded a greater than 80% recovery of ^{51}Cr-labeled neutrophils with less than 1% of the total radioactivity remaining free or associated with erythrocytes. Labeled neutrophils were aliquoted and transfused into rabbits at intradermal sites. Blood samples were obtained at the midpoint of the period during which neutrophil emigration was quantitated, and blood neutrophil specific activity was determined. The number of host neutrophils that emigrated per site was determined by dividing the ^{51}Cr radioactivity per site by the blood neutrophil specific actvity. Interanimal variability was standardized by expressing the number of neutrophils that emigrated into each site per 10^6 circulating neutrophils. Previous studies have validated this approach by demonstrating that ^{51}Cr-labeled allogeneic neutrophils marginate in a way identical to that of the host's unlabeled neutrophils in response to an intravenous injection of endotoxin[16] and emigrate into pleural exudates with the same efficiency as the host's cells.[17] With the use of this methodology, the minimal neutrophil emigration detectable was 10^5 neutrophils per site per 10^6 circulating neutrophils.

Inflammatory Agents

Working concentrations of inflammatory agents were prepared by diluting stocks in pyrogen-free saline (Travenol, Baxter Laboratories, Toronto, Ontario). The molarity of *E coli* 055:B5 lipopolysaccharide, endotoxin (Difco, Detroit, Mich; Lot 682197) was estimated with the use of a molecular weight of 12,000. Because endotoxin forms large-molecular-weight aggregates, our calculations may underestimate its potency. The molarity of C5a des Arg in zymosan-activated rabbit plasma was estimated on the assumption of a complete conversion of C5. f-Met-Leu-Phe (Sigma, St. Louis, Mo), 437.6 mol wt, was dissolved at 10^{-2}M in dimethyl sulfoxide. LTB_4 (100 μg per ml methanol), 336g mol wt, was a gift of Dr. J. Rokach, Merck Frosst Canada Inc. Human monocyte-derived IL-1 purified by affinity and gel filtration chromatography was purchased from Genzyme Corporation (Boston, Mass). Units of IL-1 activity were verified with the use of the mouse thymo-

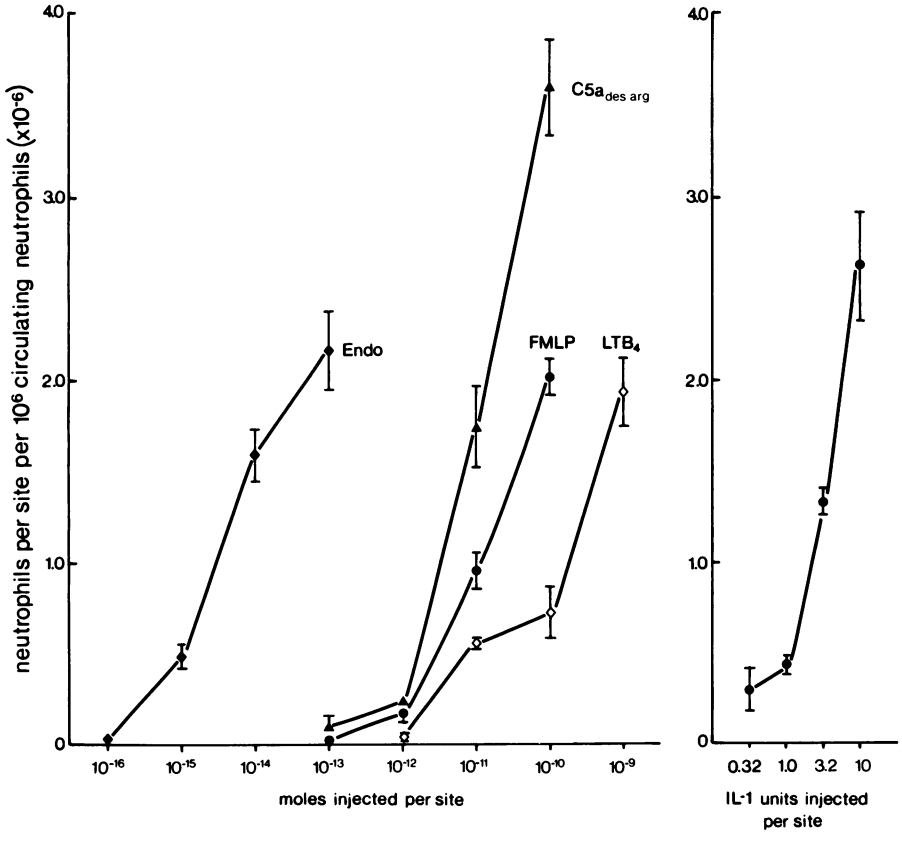

Figure 1—Neutrophil emigration into intradermal sites during a 2-hour period subsequent to injection of inflammatory agents. The means and standard errors (SEM) of triplicate injections are plotted for endotoxin, f-Met-Leu-Phe, C5a des Arg, and LTB_4 (*left panel*) and for IL-1 (*right panel*).

cyte comitogenesis assay as described by Mizel et al.[18] Its molarity was estimated on the assumption of a molecular weight of 15,000 and specific activity of 10^7 to 10^8 U/mg protein. Specific activities of 2.5×10^8, 4.37×10^7, and 7.6×10^6 U/mg were obtained with human blood monocyte-derived IL-1 purified to homogeneity.[19-21]

Injection Protocols

Three types of injection protocols were employed. In dose-response experiments, agents were injected into intradermal sites, and neutrophil emigration was quantitated over the subsequent 2 hours. The kinetics of neutrophil emigration were determined by serial injection of IL-1 (3.2 U/site) or endotoxin (10^{-14} moles per site) into intradermal sites and allowing ^{51}Cr-neutrophils to circulate for the final 30 minutes. In tachyphylaxis experiments intradermal sites were treated with IL-1 (3.2 U/site) or endotoxin (various doses) 8 hours before sacrifice. Two hours before sacrifice sites were treated, and neutrophil emigration was quantitated during the subsequent 2 hours. All injections were in triplicate.

Results

Dose-Response Experiments

On a molar basis endotoxin was three to four log times more potent than the chemotactic factors tested (Figure 1). This observation was consistently observed and has been reported by Colditz and Movat.[9] The minimal inflammatory doses injected per site were 10^{-15} moles of endotoxin, 10^{-12} moles of C5a des Arg and

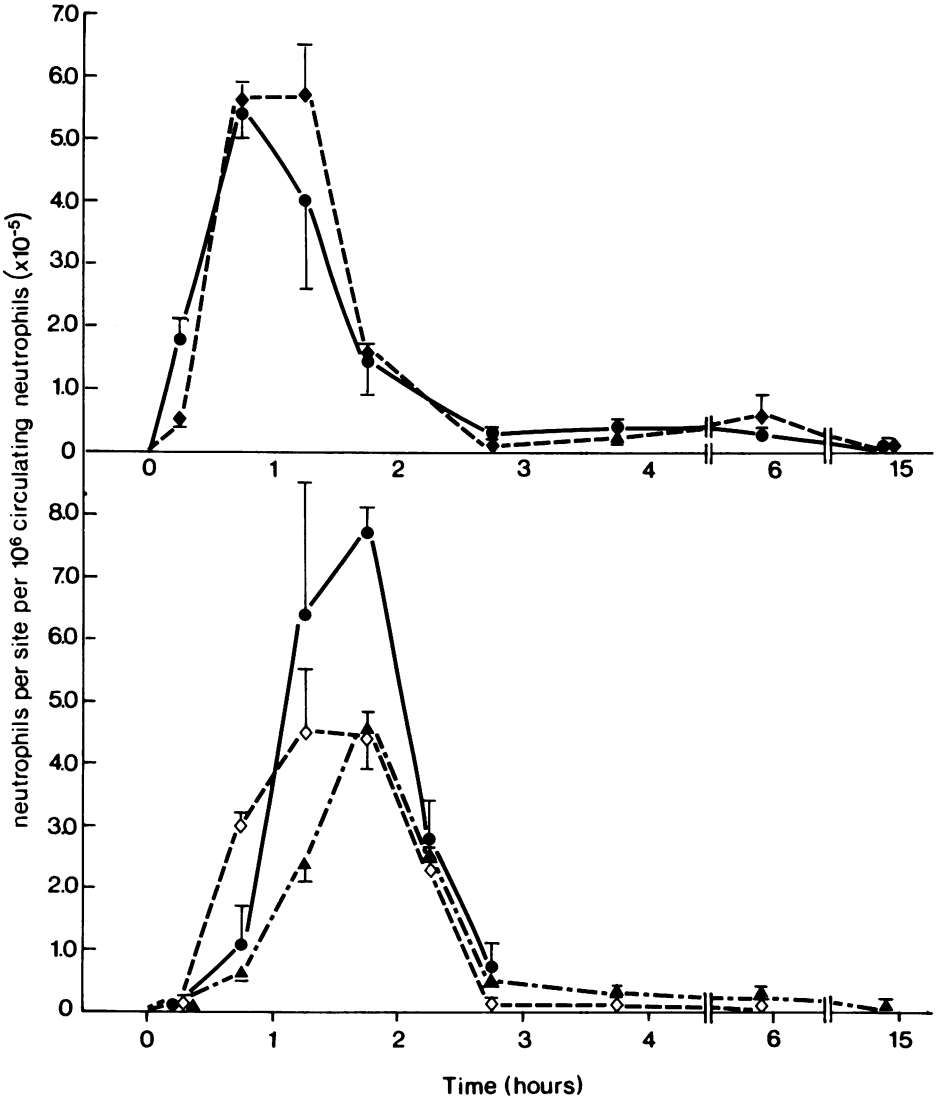

Figure 2—Kinetics of neutrophil emigration into intradermal sites sequentially injected with 3.2 units of IL-1 per site (*upper panel*) or 10^{-14} moles of endotoxin per site (*lower panel*). Neutrophils labeled with ^{51}Cr were allowed to circulate for 30 minutes before sacrifice. Means ± SEM of triplicate injections in 2 animals (IL-1) and 3 animals (endotoxin).

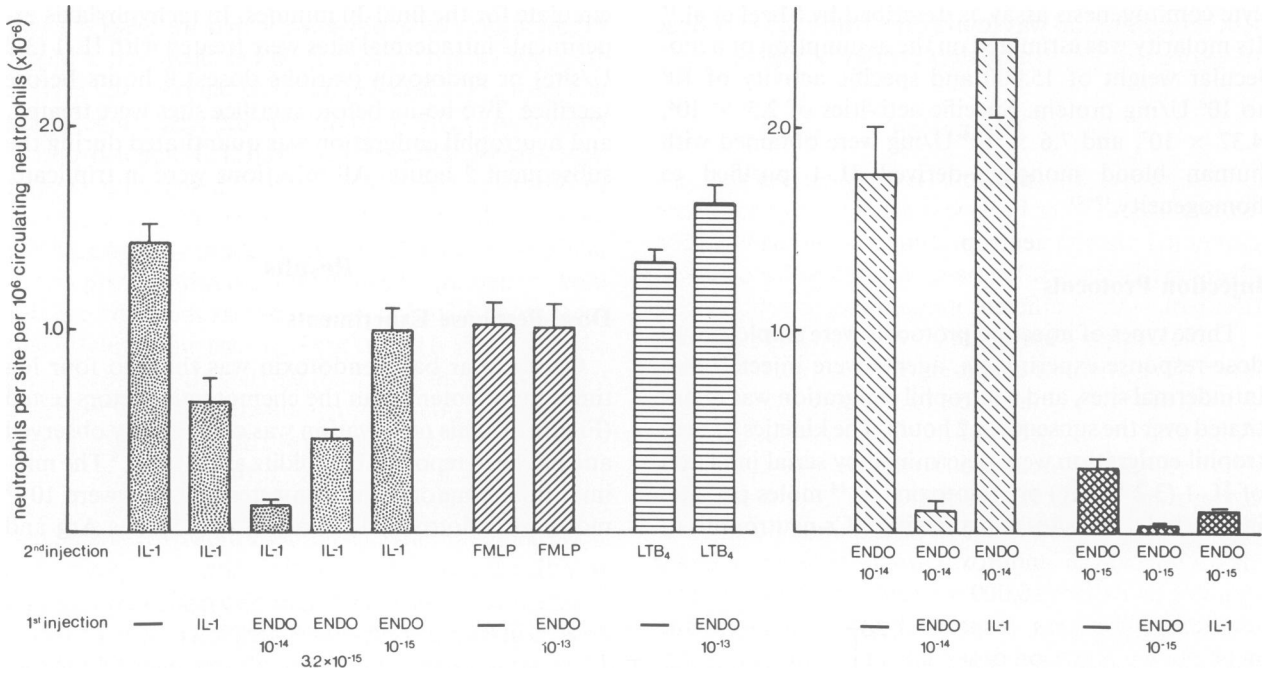

Figure 3—Tachyphylaxis experiments. Eight hours before sacrifice (1st injection) intradermal sites were either treated with IL-1 (3.2 U/site) or endotoxin (moles indicated) or did not receive any injections. Two hours before sacrifice (second injection) sites were retreated, and neutrophil emigration was quantitated during the subsequent 2 hours. The *left panel* shows tachyphylaxis to IL-1 (3.2 U/site) in sites previously treated with IL-1 ($P < 0.01$, analysis of variance), endotoxin, 10^{-14} and 3.2×10^{-15} moles ($P < 0.001$), and endotoxin, 10^{-15} moles ($P < 0.05$). Tachyphylaxis to f-Met-Leu-Phe (10^{-12} moles) or LTB_4 sites (10^{-10} moles) was not observed in sites prestimulated with endotoxin (10^{-13} moles). The *right panel* shows homologous tachyphylaxis to endotoxin at both higher and lower doses ($P < 0.01$). Tachyphylaxis to endotoxin in sites prestimulated with 3.2 units of IL-1 was observed only with 10^{-15} moles of endotoxin ($P < 0.025$).

f-Met-Leu-Phe, and 10^{-11} moles of LTB_4. Earlier studies with synthetic platelet-activating factor indicated that its minimal inflammatory dose was 10^{-11} moles/site.[9]

IL-1 induced detectable neutrophil emigration at doses as low as 0.32 U/site, corresponding to approximately 2×10^{-15} to 2×10^{-16} mol/site (Figure 1). Polymyxin B sulfate (40 μg/site), when injected together with IL-1, did not diminish neutrophil emigration. When injected with endotoxin (10^{-13} moles) the extent of neutrophil emigration was reduced by 90%. Treatment of IL-1 with heat (90 C for 30 minutes) significantly diminished neutrophil accumulation, whereas endotoxin was heat-stable.

Kinetics of Neutrophil Emigration

Neutrophil emigration into sites given injections of IL-1 was rapid (Figure 2, top). Emigration was detected within the first 30 minutes, and the maximal rate was between 30 and 90 minutes. In contrast, the kinetics of neutrophil emigration into intradermal sites injected with endotoxin were shifted to the right (Figure 2, bottom). In the first hour only minimal emigration occurred, and the maximal rate was observed at 60–120 minutes. After 3 hours significant neutrophil emigration did not occur.

Tachyphylaxis Experiments

Tachyphylaxis or local desensitization of tissues to a specific agent was described by Colditz and Movat,[22–24] who found that when intradermal sites were reinjected with the same agent, the subsequent neutrophil emigration was diminished in comparison with sites that received no prior injections or sites that received previous injections of different agents. Homologous tachyphylaxis to IL-1 was observed in the present study (Figure 3, left), with neutrophil emigration decreased by approximately 50%. Prestimulation with endotoxin induced heterologous tachyphylaxis to IL-1 (Figure 3, left). The most marked inhibition of neutrophil accumulation was observed in sites prestimulated with 10^{-14} moles of endotoxin, and slight inhibition with 10^{-15} moles. In contrast, inhibition was not observed when intradermal sites that were prestimulated with a large dose of endotoxin were treated with f-Met-Leu-Phe or LTB_4. When intradermal sites previously injected with IL-1 were reinjected with endotoxin, diminished neu-

trophil emigration was found only with the lower dose of endotoxin (10^{-15} mol/site) (Figure 3, right).

Discussion

The present and previous reports[9] found that endotoxin on a molar basis was three to four log more potent in inducing neutrophil emigration than various chemotactic factors. We have confirmed this observation with several different preparations of endotoxin, including polysaccharide-deficient mutants. Endotoxin is not chemotactic or chemokinetic *in vitro*,[7,8] but when deposited *in vivo* may elicit local neutrophil emigration either by directly stimulating endothelial cells or by generating local production of an endogenous chemotactic factor. If C5a des Arg or LTB_4 were mediating the endotoxin-induced inflammatory response, each molecule of endotoxin would have to generate as much as 1000 or 10,000 molecules of these two chemotactic substances, respectively, to account for the neutrophil emigration observed. This is not likely, because endotoxin is not a potent stimulator of LTB_4 production,[25] and relatively high concentrations are required for complement activation.[8] In rabbits depleted of circulating complement by cobra venom factor, the potency of endotoxin in eliciting neutrophil emigration was not diminished (results not shown). On the basis of the units of activity and on the specific activity, we have estimated that IL-1 is of comparable potency to endotoxin, with neutrophil emigration detectable with 0.32 units or 2×10^{-15} to 2×10^{-16} moles injected per site. A similar potency was observed by Habicht and Beck[13] with murine IL-1. In addition, we have tested recombinant human IL-1α (gift of Drs. Peter T. Lomedico and Alvin S. Stern, Hoffmann-La Roche, Nutley, NJ; see Gubler et al[26]) and were able to detect intense neutrophil emigration with 10^{-15} moles per site. Thus, a single molecule of IL-1 generated by each molecule of endotoxin would account for the degree of neutrophil emigration observed. Cells which can synthesize IL-1 when stimulated include monocyte/macrophages, Langerhans cells, keratinocytes, smooth muscle cells, and endothelial cells.[10]

Neutrophil emigration into sites treated with IL-1 is rapid, and its onset and maximal rate precede those of endotoxin sites by 30–60 minutes. The 30–60-minute delay in neutrophil emigration following the injection of endotoxin may be time required for the synthesis and secretion of IL-1. IL-1 is not stored preformed, but has to be newly synthesized upon stimulation.[27] On injection into tissues it appears to elicit neutrophil emigration rapidly, and its duration of action is brief.

We attribute the brevity of IL-1 inflammation to tachyphylaxis or local desensitization of tissues to IL-1. Homologous tachyphylaxis, which we observed with IL-1, has been detected with other chemotactic factors at 2 hours.[22,24] Cross-tachphylaxis was observed between endotoxin and IL-1. Endotoxin induced a dose-dependent desensitization to IL-1, and IL-1 desensitized sites to the lower dose of endotoxin. These results are consistent with the hypothesis that endotoxin can stimulate local IL-1 synthesis and secretion, which would then mediate neutrophil emigration and subsequently induce local desensitization to IL-1, as was shown by a diminished neutrophil influx into IL-1 sites prestimulated with endotoxin. This is not to say that IL-1 synthesis and secretion, or other biologic functions attributed to IL-1, are inhibited concomitantly with the cessation of neutrophil emigration. Desensitization to IL-1 and other chemotactic agents is likely to occur at the level of endothelial cells,[23] which have been shown to regulate neutrophil adherence *in vitro*.[2-6] In fact, tachyphylaxis or desensitization as described *in vivo* has also been noted *in vitro* in conjunction with generation of a tissue factor-like procoagulant activity by cultured endothelial cells stimulated by IL-1 and was referred to as "hyporesponsiveness" of the endothelial cells.[28] A hyporesponsiveness of the endothelial monolayer, with respect to neutrophil adherence, following IL-1 stimulation, was also observed.[4] Tachyphylaxis may be responsible for regulating the sensitivity of tissues to IL-1, thus preventing inflammation by physiologic levels of IL-1[29] or by chronically elevated levels of IL-1.[30] Studies with f-Met-Leu-Phe indicated that local desensitization can be prolonged up to several days after a single exposure, and repeated exposures induced more pronounced tachyphylaxis.[24]

The measurement of neutrophil emigration into intradermal sites treated with IL-1 is a sensitive *in vivo* assay of IL-1 activity and detects neutrophil emigration despite the presence of inhibitors which mask IL-1 activity in the mouse thymocyte comitogenesis assay. This *in vivo* assay is rapid and quantitative and enables measurement of multiple samples in the same animal, which provides an inbuilt control and allows direct comparisons of potency with other agents. Our results with this assay suggest that IL-1 plays an important role in mediating endotoxin-induced neutrophil emigration; and thus it may be instrumental in mounting the inflammatory response to infection of tissues by Gram-negative bacteria.

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