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Interleukin-8 primes human neutrophils for enhanced superoxide anion production

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SUMMARY

Interleukin-8 (IL-8), a novel chemotactic cytokine, has been shown to play an important role in inflammation. In this study, we investigated the effect of recombinant human (rh) IL-8 on superoxide (O_2^-) production by neutrophils. We found that rhIL-8 (1–10 ng/ml) did not stimulate neutrophil O_2^- production on its own, but primed neutrophils for an enhanced response to other stimuli, such as *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), phorbol 12-myristate 13-acetate (PMA) and platelet-activating factor (PAF). The priming effect of rhIL-8 was dose dependent, rapid and long lasting. Recombinant human IL-8 increased both the maximal rate and the total O_2^- production, but did not prolong the response to FMLP. Stimulation of neutrophils with rhIL-8 increased intracellular-free calcium concentration ($[Ca^{2+}]_i$) by mobilizing calcium from internal stores and by increasing calcium influx. The increase in $[Ca^{2+}]_i$ was dose dependent and occurred in the same range of rhIL-8 concentrations that primed neutrophils for O_2^- production. In addition, rhIL-8 enhanced the FMLP-stimulated increase in $[Ca^{2+}]_i$. These observations suggest that calcium may play an important role in the priming phenomenon.

INTRODUCTION

An elevated number of neutrophils in synovium is a common feature of inflamed synovial joints of patients with rheumatoid arthritis (RA). Increased activity of neutrophils, in particular enhanced oxyradical generation and release of lysosomal enzymes, contributes to the joint destruction. A novel chemotactic cytokine, interleukin-8 (IL-8), capable of attracting neutrophils to the joints and activating their specific functions, may play a significant role in neutrophil-mediated tissue damage in RA.

There is increasing evidence for a role of IL-8 in the pathogenesis of RA. Elevated levels of IL-8 have been found in the synovial fluid of patients with RA,[1,2] and gout[3] but not in patients with systemic lupus erythematosus.[2] In the RA patient group there was a close correlation between the synovial fluid IL-8 concentration, the levels of C-reactive protein and synovial neutrophil numbers.[2] Furthermore, spontaneous production of IL-8 from cultured synovial cells,[1] peripheral blood mononuclear cells and synovial fluid mononuclear cells from patients with RA (but not normal controls) has been demonstrated.[4]

Abbreviations: CL, chemiluminescence; FMLP, formyl-methionyl-leucyl-phenylalanine; $[Ca^{2+}]_i$, intracellular-free calcium concentration; PMA, phorbol 12-myristate 13-acetate; PAF, platelet-activating factor; rhIL-8, recombinant human interleukin-8; O_2^-, superoxide anion.

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The action of IL-8 is pleiotropic in nature. For example, *in vitro* it has been shown to: (1) be chemotactic for neutrophils and lymphocytes;[5,6] (2) degranulate polymorphonuclear cells (PMN);[7] (3) augment *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated release of platelet-activating factor (PAF) by neutrophils; (4) enhance arachidonic acid release from neutrophil phospholipids; (5) enhance neutrophil superoxide (O_2^-) production;[8,9] (6) enhance neutrophil phagocytosis; (7) increase neutrophil CR1 and CR3 receptor expression.[10] It should be noted that many of these effects are not seen with IL-8 alone, but in conjunction with a second stimulus of cell activity.

Neutrophil oxidative metabolism plays an important role in the host defence system but exaggerated or prolonged production of oxyradicals may lead to substantial tissue damage, as is the case of the rheumatoid joint. Oxyradical production by activated neutrophils may be enhanced by various cytokines and mediators. For example, granulocyte–macrophage colony-stimulating factor (GM-CSF),[11,12] tumour necrosis factor (TNF)[13] and more recently IL-8[8,9] have all been shown to prime neutrophils for enhanced oxyradical production. The facilitating effect of IL-8 is of particular importance as, unlike GM-CSF and TNF, it causes rapid and prolonged neutrophil infiltration into tissue sites[14,15] and thus may further increase oxyradical production at inflammatory sites by increasing the numbers of cells.

However, the priming effect of IL-8 remains controversial, as some investigators have failed to demonstrate an effect of recombinant human (rh)IL-8.[16] In view of these conflicting

reports, we investigated the priming effect of rhIL-8 on neutrophil O_2^- production in response to FMLP, PMA and PAF, and have shown that rhIL-8 enhances O_2^- release induced by all of the above stimuli. Furthermore, we have demonstrated that rhIL-8 facilitates the FMLP-stimulated increase in intracellular free calcium, suggesting a further mechanism for the rhIL-8 priming.

MATERIALS AND METHODS

Materials

Recombinant human IL-8, essentially endotoxin free (less than 0·1 ng/μg), was purchased from Promega (Sydney, Australia). The lyophilized rhIL-8 was reconstituted in phosphate-buffered saline (PBS) to a concentration of 10 μg/ml and stored in 50 μl aliquots at −70° and thawed only once before use. Cytochrome *c* (type IV, horse heart), FMLP, phorbol 12-myristate 13-acetate (PMA), PAF, luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) and RPMI-1640 (without phenol red) were purchased from Sigma (St Louis, MO). Dextran T-500 was purchased from Pharmacia (Uppsala, Sweden), Lymphoprep from Nycomed AS (Oslo, Norway), HEPES from Gibco (Grand Island, NY), Fura-2AM from Calbiochem (La Jolla, CA), superoxide dismutase (SOD) from Boehringer Mannheim (Mannheim, Germany) and Detoxi-Gel (endotoxin removing gels) from Pierce (Rockford, IL).

Isolation of human neutrophils

Peripheral blood was obtained from normal volunteers and was anticoagulated with EDTA. The leucocyte-rich fraction was obtained by sedimenting the erythrocytes with 1% dextran for 40 min at room temperature. Neutrophils were isolated by density-gradient centrifugation (400 ***g***, 20 min, 22°) using Lymphoprep followed by hypotonic lysis of residual erythrocytes. The cells were always >96% pure, as determined by Grünwald–Giemsa staining, and >98% viable by trypan blue exclusion. Neutrophils were resuspended in RPMI-1640 medium supplemented with 20 mM HEPES, 2 mM L-glutamine, 60 μg/ml penicillin and 8 μg/ml gentamycin.

Superoxide anion production

Neutrophil O_2^- production was measured as SOD-inhibitable reduction of ferricytochrome *c* (Cyt *c*). This was determined by the addition of 10 μl of 2 mg/ml SOD to duplicate samples in all experiments. Measurements were made in real time to enable determination of rates of production of O_2^-, and by end-point, to enable calculation of total O_2^- production by any given stimulus/manipulation as described below. All end-point experiments were performed in triplicate. The final concentration of cells in all experiments was adjusted to 10^6/ml.

In the initial dose–response experiments, neutrophils (5×10^6 cell/ml) were incubated with medium or the stated concentration of rhIL-8 (1–50 ng/ml) for 10 min at 37°. Two hundred microlitres of cells were transferred to a tube containing 800 μl of 100 μM Cyt *c* and medium or different concentrations (1, 5, 10, 100 nM) of FMLP. The mixture was incubated for a further 10 min. The reaction was stopped by addition of SOD and by immersion of the tubes in ice. The cells were pelleted by centrifugation at 4°. O_2^- production was quantified in cell supernatants by changes in absorption at 550 nm[17] and O_2^- production determined using an extinction coefficient of 21·1/mM/cm.[18]

In the time–course experiments, PMN were incubated with 10 ng/ml rhIL-8 or medium at 37°, and 200 μl (10^6 cells) was removed at stated times for stimulation with 100 nM FMLP, as in the dose–response experiments. The reaction was stopped and the measurements performed as outlined above.

The kinetics of O_2^- production was measured by following OD changes continuously in a thermostatic (37°) spectrophotometer. Neutrophils (5×10^6 cells) were preincubated with 10 ng/ml rhIL-8 or medium as control, for 10 min at 37°, then 200 μl of cells (10^6) transferred to cuvettes in the spectrophotometer containing 800 μl of prewarmed mixture containing 100 μM Cyt *c* and a stimulus (e.g. FMLP or PMA), and changes in OD at 550 nm were monitored.

Chemiluminescence

Luminol-enhanced chemiluminescence (CL) was employed as another method to assess respiratory burst using a Packard Pico Lite chemiluminometer. Before each experiment, luminol (20 μM) was mixed with prewarmed neutrophils (250 μl, $0{\cdot}5 \times 10^6$ cells) and the mixture was preincubated for 7 min at 37°. In the rhIL-8 dose–response experiments, background CL was measured for 100 seconds before rhIL-8 was added and subsequent changes in CL were measured over 5 min. In the priming experiments, the cells were preincubated for 10 min with medium or 10 ng/ml of rhIL-8 and were then stimulated with 100 nM FMLP for an additional 5 min. In these experiments, background CL was measured for 100 seconds before addition of FMLP.

Intracellular calcium

Changes in intracellular-free calcium ($[Ca^{2+}]_i$) were measured using Fura-2 loaded neutrophils. After separation 10^7 neutrophils were incubated with 2 μM Fura-2AM for 30 min at 37° in Hanks' buffer. During this time the Fura-2AM entered the cells where it was hydrolysed to the acid form Fura-2 and trapped inside the cells. The cells were washed twice with Hanks' buffer to remove any unesterified Fura-2AM. For our studies the cells (10^6/ml) were then placed in a spectrophotofluorimeter (Perkin Elmer LS-50, Emeryville, CA). The various test components were added and changes in fluorescence (excitation 340 nm, emission 510 nm, slit widths 10 nm) monitored continuously. Levels of intracellular calcium were then calculated using the formula developed by Tsein and colleagues:[19,20]

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

where F is the fluorescence of the cell sample, F_{max} the maximum fluorescence signal (obtained at the end of each measurement by releasing all intracellular calcium by treatment of the cell suspension with excess Triton, 0·1%), and F_{min} the minimum fluorescence value (the value obtained when there is no calcium bound to Fura-2), obtained by the addition of excess EGTA (2 mM) (buffered with 25 mM Tris) to the above. K_d is the dissociation constant for Fura-2 (220 nM).

Statistics

Data were analysed by Student's *t*-test for paired data, using a Macintosh computer and the Statview II™ statistics package. Each experiment was performed in triplicate and the results are expressed as mean ±SEM (where n = number of experiments).

RESULTS

The effect of rhIL-8 on FMLP-stimulated superoxide anion production

Incubation of neutrophils with concentration of up to 1 μg/ml rhIL-8 alone had no effect on O_2^- production (data not shown). To determine whether rhIL-8 was able to prime neutrophils for enhanced O_2^- production in response to FMLP, the cells were preincubated for 10 min at 37° in the presence of 1–50 ng/ml rhIL-8 (or medium as a control), and subsequently, stimulated with various concentrations of FMLP (Fig. 1a). Because of the donor-dependent variability of FMLP-stimulated neutrophil O_2^- production, the data were normalized and expressed as a percentage of the control FMLP response. Recombinant human IL-8 enhanced the response to FMLP in a dose-dependent manner. The maximum effect was seen at 10 ng/ml rhIL-8 for each FMLP concentration tested. Preincubation of cells with 10 ng/ml rhIL-8 increased O_2^- production by 104 ± 13, 110 ± 28, 115 ± 17 and 104 ± 18% of FMLP response at 1, 5, 10 and 100 nM FMLP respectively (Fig. 1a) but did not shift the dose response to FMLP to the left (Fig. 1b).

The priming effect of rhIL-8 was not caused by endotoxin contaminating the rhIL-8 preparation (endotoxin level at rhIL-8 concentration of 10 ng/ml was less then 1 pg/ml). Recombinant human IL-8 free of endotoxin (from which endotoxin was removed on Detoxi-Gel) primed neutrophils in the same manner as untreated rhIL-8 (data not shown).

The priming effect of rhIL-8 was dependent on the incubation time (Fig. 2). When cells were incubated with medium alone, and the data expressed as a percentage of FMLP response at time 0, the responsiveness of neutrophils to FMLP declined gradually and at 2 hr was 51%. The rhIL-8 induced enhancement of the FMLP-stimulated O_2^- production was already evident at 1 min, reached maximum at 6 min and was still present at 2 hr.

The effect of rhIL-8 on PAF-stimulated superoxide production

Recombinant human IL-8 also enhanced PAF-stimulated O_2^- production. Neutrophils were incubated for 10 min with medium or 10 ng/ml of rhIL-8 and then stimulated with 1 μM PAF (for 6 min). In these experiments, O_2^- production was enhanced from 6·1 ± 1·0 to 8·2 ± 1·3 nmol/10^6 cells ($P = 0{\cdot}02$, $n = 3$). Thus rhIL-8 priming of neutrophils increased PAF-stimulated O_2^- production by 37·1 ± 2·2%.

The effect of rhIL-8 on the rate of superoxide production

In order to study the kinetics of the priming effect of rhIL-8, neutrophils were preincubated with 10 ng/ml rhIL-8 or medium for 10 min at 37° and then 10^6 cells were transferred to prewarmed cuvettes containing Cyt *c* and 10 ng/ml PMA or 100 nM FMLP. The O_2^- production was measured continuously and the results from a representative experiment are shown in Fig. 3. Recombinant human IL-8 increased the maximal rate of O_2^- production induced by both PMA and FMLP. The PMA-stimulated rate of O_2^- production increased from 83 ± 16 pmol/10^6 cell/second to 107 ± 16 pmol/10^6 cell/second ($P < 0{\cdot}001$, $n = 5$) and the FMLP-stimulated rate of O_2^- production increased from 145 ± 10 pmol/10^6 cell/second to 220 ± 13 pmol/10^6 cell/second ($P < 0{\cdot}005$, $n = 9$). The termination of the FMLP response in rhIL-8-treated or untreated cells occurred at the

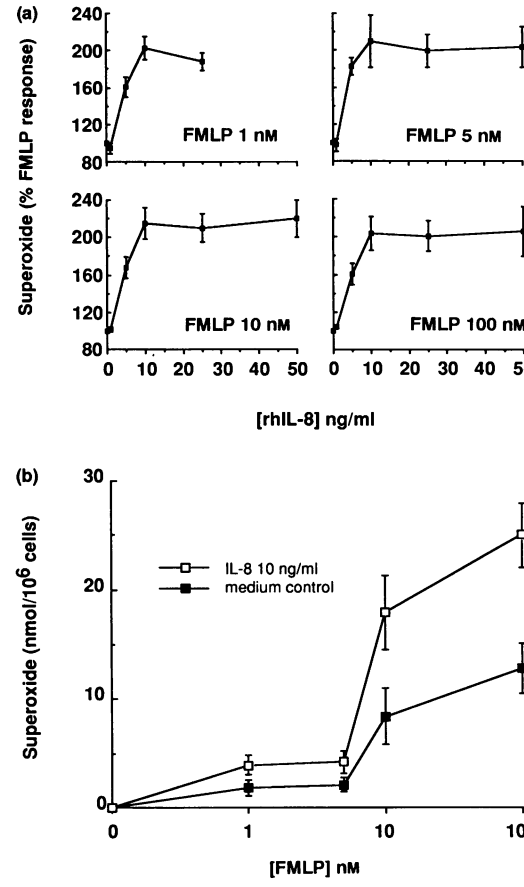

Figure 1. The effect rhIL-8 on FMLP-stimulated O_2^- production. Neutrophils (10^6) were preincubated for 10 min at 37° in the presence of 1–50 ng/ml rhIL-8 (or medium as a control) and were then stimulated with 1, 5, 10 or 100 nM FMLP for an additional 6 min (a). Results are normalized and expressed as a percentage of the control FMLP response. (b) The effect of rhIL-8 (10 ng/ml) on the FMLP dose-response curve. Values represent means ± SE of four experiments performed in triplicate.

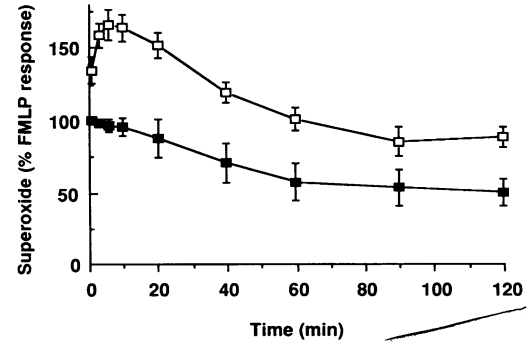

Figure 2. The effect of rhIL-8 on FMLP stimulated O_2^- production as a function of time. Neutrophils (5×10^6/ml) were incubated with medium (—■—) or 10 ng/ml rhIL-8 (—□—) at 37° for indicated periods of time. At each time-point 10^6 cells were transferred to tubes containing cyt *c* and 100 nM FMLP for an additional 6 min stimulation. Results are normalized and expressed as a percentage of the control FMLP response at time zero. Values represent means ± SE of seven experiments performed in triplicate.

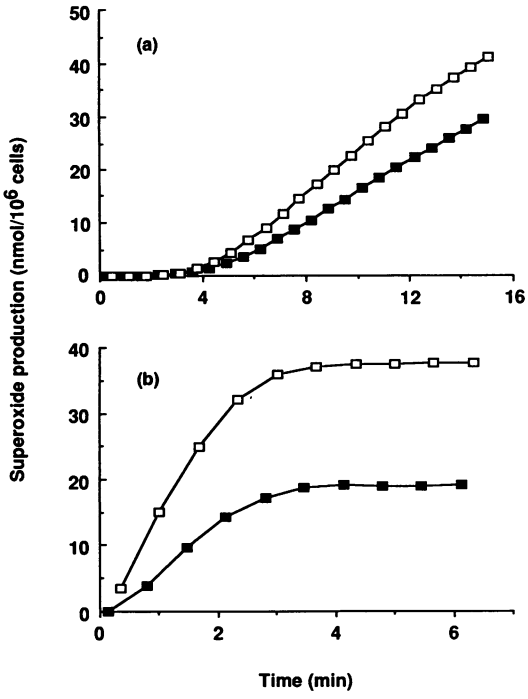

Figure 3. The effect of rhIL-8 on the kinetics of PMA- and FMLP-stimulated O_2^- production. Neutrophils (5×10^6/ml) were preincubated with medium (—■—) or 10 ng/ml of rhIL-8 (—□—) for 10 min at 37° and then 10^6 cells were transferred to prewarmed cuvettes containing Cyt *c* and (a) 10 ng/ml PMA or (b) 100 nM FMLP in which the O_2^- production was measured continuously. The results are from one experiment representative of five (a) and nine (b).

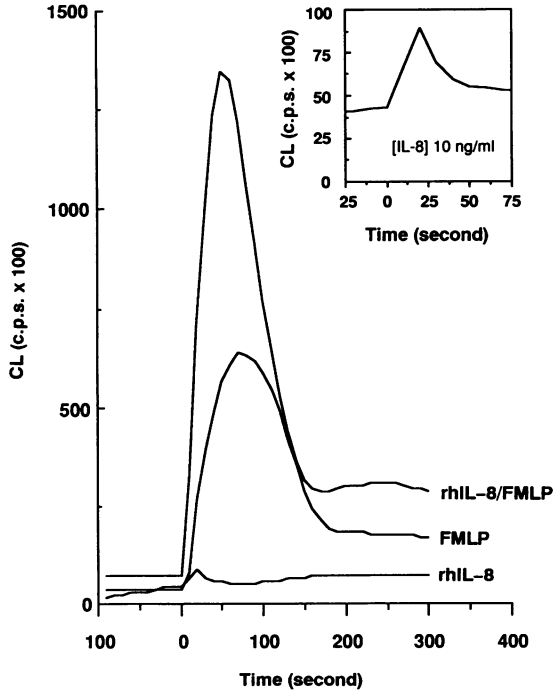

Figure 4. Luminol-enhanced CL response induced by 10 ng/ml rhIL-8 alone (insert) and in neutrophils that were preincubated with medium or 10 ng/ml of rhIL-8 for 10 min at 37° and were then stimulated with 100 nM FMLP. The peak of CL corresponds to the maximum rate of oxyradical production. The results are from one experiment representative of four.

same time. There was a high correlation ($r^2 = 0{\cdot}91$, $n = 9$) between the increase in the maximal rate and the total O_2^- production induced by rhIL-8 in FMLP-stimulated cells.

The effect of rhIL-8 on chemiluminescence

The priming effect of rhIL-8 and the kinetics of the FMLP response were further investigated using luminol-enhanced CL. The peak of CL represents the maximum rate of oxyradical production. Preincubation of neutrophils with 10 ng/ml rhIL-8 increased the rate of FMLP-stimulated oxyradical production twofold. Maximum CL was reached at 60–70 seconds after addition of FMLP in the control cells but occurred 20 seconds earlier in the rhIL-8-primed cells (Fig. 4). In addition, rhIL-8 alone at 10 ng/ml produced a small but significant CL that reached a maximum at 10–20 seconds and reverted to the baseline by 60 seconds (Fig. 4 insert). This direct CL response of rhIL-8 was dose dependent. At the highest concentration tested (500 ng/ml) the rhIL-8-induced CL response was 15% of that induced by FMLP alone (data not shown).

The effect of rhIL-8 on intracellular free calcium concentration

To determine whether enhancement of O_2^- production was associated with changes in $[Ca^{2+}]_i$, Fura-2-loaded neutrophils were stimulated directly with rhIL-8 concentrations from 1 to 25 ng/ml (Fig. 5). Recombinant human IL-8 stimulated an increase in $[Ca^{2+}]_i$, which paralleled closely the results obtained from O_2^- experiments. For example, the concentration of rhIL-8 (10 ng/ml), shown to be optimal for the facilitation of O_2^- response, induced maximal increase in $[Ca^{2+}]_i$. Neutrophils stimulated with 10 ng/ml rhIL-8 increased $[Ca^{2+}]_i$ levels by 573 ± 100 nM. However, when neutrophils were stimulated in calcium-free buffer, the increase in $[Ca^{2+}]_i$ was reduced to 221 ± 43 nM and even more (129 ± 32 nM) when 2 mM EGTA was added to the buffer. The rhIL-8 response was restored to 71% of the original response when calcium was added to the cells suspended in the calcium-free buffer (Fig. 6). These results suggest that the increase in $[Ca^{2+}]_i$ induced by rhIL-8 is caused by both an influx

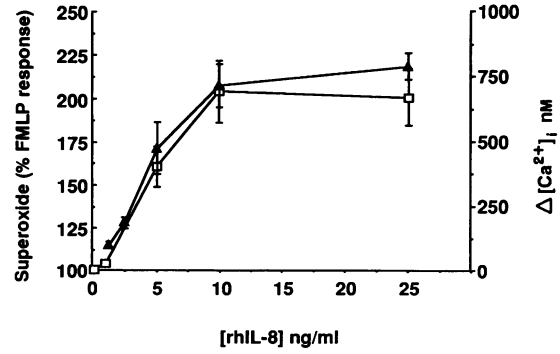

Figure 5. The effect of rhIL-8 on $\Delta[Ca^{2+}]_i$ (—▲—) and FMLP-stimulated O_2^- production (—□—). For the study of changes in $[Ca^{2+}]_i$, Fura-2 loaded neutrophils were stimulated with various concentrations of rhIL-8. Values represent means ± SE of four experiments. For O_2^- production the cells were treated as in Fig. 1 and stimulated with 100 nM FMLP.

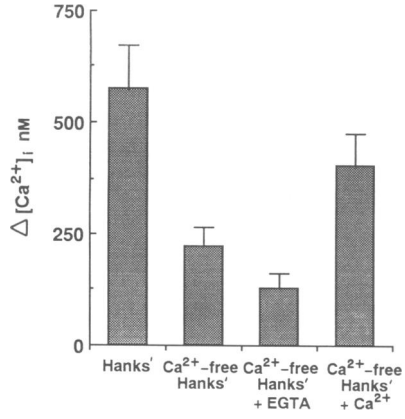

Figure 6. The effect of extracellular calcium on the rhIL-8-induced $\Delta[Ca^{2+}]_i$. Neutrophils prepared as outlined in Materials and Methods were loaded with Fura-2 in complete Hanks' buffer and divided into two preparations. Each was then washed and resuspended in either Hanks' buffer or calcium-free Hanks' buffer. Approximately 10^6 cells from each preparation were then stimulated with 10 ng/ml rhIL-8. In addition, neutrophils that were resuspended in calcium-free Hanks', were either repleted with 1·25 mM Ca^{2+} or treated with 2 mM EGTA for 5 min at 37° before stimulation with rhIL-8. Values represent means ± SE of four experiments.

of extracellular calcium and release of calcium from intracellular stores. Furthermore, preincubation of neutrophils with rhIL-8 facilitated the FMLP-stimulated increase in $[Ca^{2+}]_i$ and this effect of rhIL-8 was dependent on preincubation time. In these experiments neutrophils were preincubated with 10 ng/ml rhIL-8 or medium for the indicated times and were then stimulated with 100 nM FMLP. The maximal facilitating effect of rhIL-8 was seen at 5 min when the FMLP-stimulated elevation in $[Ca^{2+}]_i$ was increased by 1027 ± 80 nM (220 ± 43% FMLP response) and gradually declined to 308 ± 136 nM (140 ± 20% FMLP response) at 15 min (Fig. 7). This time–response pattern complements the time–course of the priming response for FMLP-stimulated O_2^- production (Fig. 2).

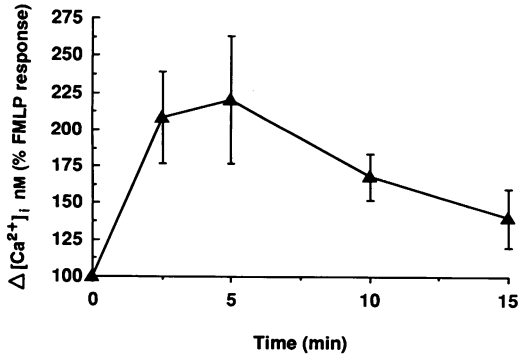

Figure 7. The effect of rhIL-8 on FMLP-stimulated $[Ca^{2+}]_i$ as a function of time. Neutrophils (10^6) were preincubated with medium or 10 ng/ml of rhIL-8 at 37° for indicated times and were then stimulated with 100 nM FMLP. Results are expressed as a percentage of the FMLP responses. Values represent means of five experiments.

DISCUSSION

Activated neutrophils produce oxyradicals that are essential for their microbicidal and cytotoxic activities and play an important role in the host defence system. This is clearly demonstrated in chronic granulomatous disease, which is characterized by decreased oxidative burst. Patients with this disease suffer from recurrent bacterial infections. However, in chronic inflammatory diseases, such as RA, release of oxyradicals from activated neutrophils may lead to severe local tissue damage and exacerbation of disease. There have been conflicting reports regarding the role of IL-8 in the enhancement of oxyradical production by neutrophils. Recent studies by Yuo *et al.*[8] and Daniels *et al.*[9] have shown that IL-8 primes neutrophils for enhanced O_2^- production in response to FMLP, but other investigators have failed to demonstrate an effect of IL-8 in the same assay system.[16] In the present study, we confirmed the finding that IL-8 facilitates FMLP-stimulated neutrophil O_2^- production using the Cyt *c* reduction assay and also demonstrated this effect in a luminol-enhanced CL assay. We also demonstrated that IL-8 enhances neutrophil O_2^- production to two other stimuli, PMA and PAF. The priming response of rhIL-8 was rapid, long lasting and reached maximum at 10 ng/ml of IL-8. Since IL-8 concentrations of up to 15 ng/ml have been found in synovial fluids of patients with RA,[2] it is likely that IL-8 may enhance synovial neutrophil oxyradical production and contribute to the joint destruction. Alternatively, neutrophils may be primed when exposed to low chemotactic levels of IL-8 during migration before entering the synovium. Similar concentrations of IL-8 to those required for maximal O_2^- priming effect have been reported to be optimal for IL-8-induced neutrophil chemotaxis and transendothelial migration.[21,22] In contrast, circulating neutrophils are less likely to be primed, as IL-8 released to the blood could be absorbed by red blood cells[23] or its activity neutralized by circulating anti-IL-8 antibody.[24]

The mechanisms by which IL-8 and other cytokines exert their priming effect on neutrophil oxyradical production are unknown. Since the demonstration that GM-CSF and TNF-α enhance neutrophil oxyradical production, there has been considerable interest in mechanisms underlying the priming effect. Subsequent investigations have shown that preincubation of neutrophils with GM-CSF or TNF-α modulates the number and affinity of FMLP receptors[11,25] and suggested that the receptor changes may explain the priming effect. However, studies by McColl *et al.*[26] challenged this hypothesis by demonstrating that GM-CSF or TNF-α prime neutrophils by inducing changes in signal transduction pathways that are independent of and distal to the FMLP receptor. This was further supported by O'Flaherty *et al.*[27] who demonstrated that receptor numbers alone cannot explain the priming phenomenon by showing that, although TNF-α enhanced neutrophil degranulation responses to FMLP, leukotriene B_4 (LTB_4) and PAF, it increased the number of FMLP receptors but down-regulated LTB_4 receptors and only transiently up-regulated PAF receptors. An alternative mechanism of priming, whereby the cells could be primed by their own metabolites, was proposed by Bauldry *et al.*,[28] who showed that TNF-α enhances neutrophil phospholipase A_2 activity, thus increasing arachidonic acid release, LTB_4 production and PAF synthesis. Since these neutrophil metabolites have been shown previously to

prime neutrophil oxidative metabolism,[29] they could prime the cells in an autocrine fashion.

Although there is no single satisfactory answer to explain what causes cell priming, the above studies suggest that changes in activity of components of intracellular signal transduction pathways leading to oxyradical production must take place following cell exposure to the priming agent. These changes most probably occur in a part of the pathway that is common to the different stimuli, as we have shown that rhIL-8 affects FMLP, PMA and PAF responses. Furthermore, these changes are associated with the rate of O_2^- production rather than the duration of the response. For example, the rhIL-8-increased rate of O_2^- production in both PMA- and FMLP-stimulated neutrophils correlated closely with the total O_2^- production. In FMLP-stimulated cells, where the O_2^- response is normally terminated after 2–4 min, rhIL-8 did not prolong but slightly shortened the duration of the response. Moreover, rhIL-8 increased O_2^- production by the same value (% FMLP response) at any FMLP concentration tested and did not shift the dose–response curve to the left. Together, these findings suggest that the catalytic activity rather than the number of activated NADPH-oxidase molecules in the cell is increased by rhIL-8, although the other possibility cannot be excluded.

PMA stimulates O_2^- production in neutrophils by binding to and directly activating protein kinase C (PKC).[30] In FMLP-stimulated cells, two sequential pathways leading to oxyradical production have been recognized: a PKC-independent pathway that initiates and a PKC-dependent pathway that sustains the response.[31] As O_2^- production induced by both stimuli depends on PKC activity, IL-8 could, for example, increase PKC activity by translocating it to the cell membrane.[32] Alternatively, IL-8 could accelerate the catalytic activity of NADPH-oxidase in a PKC-independent manner, by acting on component(s) of the signal transduction pathway that are more proximal to this enzyme than PKC. Since NADPH-oxidase consists of many cytosolic and membrane-associated components that are assembled together upon activation,[33-36] it is possible that IL-8 speeds up the assembly process by inducing a redistribution of the cytosolic components to the cell membrane.[37] Further mechanisms may include an effect of IL-8 on (1) the number of NADPH-oxidase molecules that are being assembled and activated by a second stimulus,[38] (2) the affinity of NADPH-oxidase for its substrate, or (3) substrate availability by an as yet unknown mechanism.

It is of interest to note that, although GM-CSF and TNF-α prime neutrophils for enhanced oxyradical production by FMLP, the preincubation time required for their optimal effect is much longer than that required by IL-8. Furthermore, neither of these cytokines can prime neutrophils for the direct PKC activator PMA, or increase $[Ca^{2+}]_i$ in these cells.[12,39,40] We have shown that rhIL-8 stimulates increases in $[Ca^{2+}]_i$ over the same concentration range as it facilitates O_2^- production of FMLP-stimulated neutrophils. In addition, FMLP-stimulated $[Ca^{2+}]_i$ increase was enhanced further when cells were preincubated with rhIL-8 prior to FMLP stimulation. These observations suggest that elevations in $[Ca^{2+}]_i$ may play an important role in the IL-8 priming effect. It has been shown previously that elevations in $[Ca^{2+}]_i$ can facilitate oxyradical production in neutrophils stimulated with PMA or FMLP.[41,42] This effect was thought to be mediated by increased PKC activity, resulting from PKC translocation to the cell membrane as well as increased affinity for phorbol esters. More recently, activated PKC has been implicated in phosphorylation-dependent translocation to the cell membrane of cytosolic oxidase factors p47-phox and p67-phox, which are essential for NADPH-oxidase assembly and activation.[34,43] Although our present studies did not aim to address the role of IL-8-induced $[Ca^{2+}]_i$ rises in NADPH-oxidase assembly, they alerted us to this possibility.

In agreement with a previous report by Daniels *et al.*,[9] who were also unable to demonstrate a direct effect of rhIL-8 on O_2^- production using the Cyt *c* assay, we did not detect any O_2^- production in neutrophils that were stimulated with up to 1 μM rhIL-8 alone. However, we demonstrated a direct effect of rhIL-8 in the luminol-enhanced CL assay, which was very weak. The rhIL-8 concentration of 10 ng/ml, found to have maximal priming effect on neutrophils only slightly increased CL, and even concentrations as high as 500 ng/ml induced a response that was less than 15% of the FMLP response. The direct effect of rhIL-8 on luminol-enhanced CL has been previously demonstrated,[44] and it was suggested that the apparent discrepancy between the results from the two different assays was due to a greater sensitivity of the CL assay.[45] However, a further explanation may be the different specificity of these assays. Cyt *c* is specific for O_2^- and therefore reflects the activity of NADPH-oxidase alone, whereas luminol may be oxidized by many different oxyradicals and oxidants, and thus may reflect the activity of other radical-generating enzymes. This is relevant as Wang *et al.*[46,47] have recently demonstrated that nitric oxide, produced by nitric oxide synthase, increases luminol-enhanced CL. Whatever the explanation, the high IL-8 concentrations required to stimulate neutrophil oxyradical release directly and the low magnitude of this response question its physiological significance, in contrast to the priming effect, where very low concentrations of IL-8 can dramatically enhance neutrophil responses.

Thus, we believe that our studies clearly demonstrate that IL-8 facilitates neutrophil oxyradical production and provide important new information for the assessment of IL-8 in inflammation. On the molecular level, this study alerted us to the possible role of cytosolic calcium in neutrophil priming with particular reference to NADPH-oxidase assembly and activation. However, further studies are required to determine the exact mechanism by which IL-8 exerts its priming effect on neutrophils as we anticipate that many intracellular pathways may be involved.

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