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Immunology

Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2

(GROα/NAP-2/neutrophil activation/enzyme release/respiratory burst)

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ABSTRACT **Two monoclonal antibodies, anti-IL8R1 and anti-IL8R2, raised against both interleukin 8 receptors (IL-8R) of human neutrophils, IL-8R1 and IL-8R2, were used to study individual receptor functions after stimulation with IL-8, GROα, or NAP-2. Efficacy and selectivity of the antibodies were tested in Jurkat cells transfected with cDNA coding for one or the other receptor. The binding of ^{125}I-labeled IL-8 and IL-8-induced changes of the cytosolic free Ca^{2+} concentration were inhibited by anti-IL8R1 in cells expressing IL-8R1 and by anti-IL8R2 in cells expressing IL-8R2. In human neutrophils, release of elastase was observed after stimulation with IL-8 or GROα. The response to IL-8 was inhibited slightly by anti-IL8R1 and more substantially when both monoclonal antibodies were present, while the response to GROα was inhibited by anti-IL8R2 but was not affected by anti-IL8R1. These results indicate that both IL-8 receptors can signal independently for granule enzyme release. Superoxide production, a measure of the respiratory burst, was obtained with increasing concentrations of IL-8 with maximum effects at 25 to 50 nM, but no response was observed upon challenge with GROα or NAP-2 up to 1000 nM. The superoxide production induced by IL-8 was inhibited by anti-IL8R1, but was not affected by anti-IL8R2. Stimulation of neutrophils with IL-8, in contrast to GROα or NAP-2, also elicited phospholipase D activity. The effect of IL-8 was again inhibited by anti-IL8R1 but not by anti-IL8R2, indicating that this response, like the respiratory burst, was mediated by IL-8R1. Taken together, our results show that IL-8R1 and IL-8R2 are functionally different. Responses, such as cytosolic free Ca^{2+} changes and the release of granule enzymes, are mediated through both receptors, whereas the respiratory burst and the activation of phospholipase D depend exclusively on stimulation through IL-8R1.**

Chemokines constitute a large family of structurally related proteins which activate and attract leukocytes. Interleukin 8 (IL-8) and its analogs, GROα, GROβ, GROτ, NAP-2, and ENA-78, act predominantly on neutrophils and are called CXC chemokines because their first two conserved cysteines are separated by one amino acid (1). CXC chemokines share two distinct receptors, IL-8 receptors 1 (IL-8R1) and IL-8 receptor 2 (IL-8R2), which are both expressed in high numbers on neutrophils (1). IL-8 binds with high affinity (K_d values between 0.5 to 3 nM) to both receptors, whereas all the other CXC chemokines have high affinity for IL-8R2 only (2, 3). The two receptors have considerable amino acid sequence identity within the seven transmembrane domains and connecting loops, but differ almost totally in their NH_2- and COOH-terminal regions. This suggests that they not only possess distinct ligand-binding properties but also signal differently. Because the receptors are expressed together on neutrophils, their individual properties were studied in Jurkat cells after transfection of the respective cDNA. IL-8R1 and IL-8R2 were found to function independently and to be equivalent in mediating changes of cytosolic free Ca^{2+} (4), activation of mitogen-activating protein kinase (5) and chemotaxis (4) in response to IL-8. We have now generated monoclonal antibodies that selectively interact with IL-8R1 or IL-8R2 to assess the function of the single receptors in the neutrophils. In this paper we show that activation of phospholipase D (PLD) and superoxide production via the NADPH oxidase is triggered by IL-8R1 but not by IL-8R2, whereas the release of granule enzymes and the changes of cytosolic free Ca^{2+} are mediated by both receptors.

MATERIALS AND METHODS

Materials. RPMI 1640 medium and other cell culture supplements, including G-418 (Geneticin), were obtained from GIBCO/BRL. The chemokines IL-8, GROα, and NAP-2 were chemically synthesized (6) and generously provided by Ian Clark-Lewis (Adelaide, Australia). ^{125}I-Labeled NaI was obtained from Amersham and [9,10-^{3}H]oleic acid was from DuPont/New England Nuclear. All other reagents were purchased at the highest degree of purity from Merck, Fluka, or Sigma. Phosphatidylethanol was prepared as described (7).

Monoclonal Antibodies. Monoclonal antibodies against the IL-8 receptors, anti-IL8R1 (LS-33–5A12.5, IgG2b isotype) and anti-IL8R2 (LS-37–6C6.1, IgG1 isotype), were produced by immunizing mice with mouse pre-B lymphoma L1–2 cells (a kind gift of J. J. Campbell and E. C. Butcher, Palo Alto, CA) that were transfected with IL-8R1 or IL-8R2 cDNA. The antibodies were identified by flow cytometry, and hybridomas were subcloned by limiting dilution.

Cells. Stable Jurkat cell transfectants bearing functional IL-8R1 or IL-8R2 were generated as described (4) and maintained at 37°C in 5% CO_2 in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 0.8 mg of G-418 per ml. Human neutrophils were isolated from buffy coats of donor blood (8).

Binding Assays. IL-8 was iodinated by using 2 mCi (74 mBq) Na-^{125}I as described (2). Competition for IL-8 binding by the receptor antibodies was performed on Jurkat transfectants (2×10^6 cells/assay point) which were incubated at 4°C with 2 nM ^{125}I-labeled IL-8 and increasing concentrations of anti-IL8R1 or anti-IL8R2 (1–50 μg/ml), and the cell-associated radioactivity was determined.

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Abbreviations: IL-8, interleukin 8; IL-8R1 and IL-8R2, IL-8 receptors 1 and 2, respectively; PLD, phospholipase D; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration.

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Calcium Mobilization. Changes in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) were measured in cells loaded with Fura-2 by incubation for 30 min at 37°C with Fura-2-acetoxymethylester (0.1 nmol/10^6 cells) (9). Loaded cells were incubated for 30 min at 4°C in the presence or absence of anti-IL8R1 or anti-IL8R2 and stimulated at 37°C with a chemokine, and the fluorescence-related $[Ca^{2+}]_i$ changes were monitored.

Elastase Release. Neutrophils (2×10^7 cells/ml) were suspended in phosphate-buffered saline (PBS) (pH 7.4) containing 2.5 mg/ml bovine serum albumin (BSA) and incubated for 30 min at 4°C in the presence or absence of anti-IL8R1 or anti-IL8R2. The suspension was then diluted to 2.85×10^6 cells/ml, and incubated for 10 min at room temperature with 4 μg of cytochalasin B per ml. Stimulation was then performed with 10 nM IL-8 or 20 nM GROα and the release of elastase was monitored (8).

Superoxide Production. The rate of the superoxide dismutase-inhibitable cytochrome *c* reduction was monitored at 550–540 nm by using the extinction coefficient 19.1 $mM^{-1} \cdot cm^{-1}$ (10). Briefly, the cells were suspended in Krebs–Ringer buffer (100 mM NaCl/50 mM Hepes, pH 7.4/5 mM KCl/1 mM $MgCl_2$/1 mM NaH_2PO_4/1 mM $CaCl_2$/2 mM D-glucose), preincubated for 30 min at 4°C in the presence or absence of anti-IL8R1 or anti-IL8R2, and then stimulated at 37°C with a chemokine in the presence of cytochrome *c* (80 μM; horse heart type III) (10).

PLD. Neutrophils (10^7 cells/ml) were suspended in a buffered salt solution containing 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM D-glucose, and 10 mM Hepes (pH 7.5), and their phosphatidylcholine pool was labeled by incubation with 10 μCi of [^{3}H]oleic acid per ml for 20 min at 37°C. The cells were then washed, resuspended in a third of the original volume of buffered salt solution, treated with anti-IL8R1 or anti-IL8R2 for 20 min at 4°C, preincubated for 5 min at 37°C with 5 μg of cytochalasin B per ml, and finally stimulated (5×10^6 cells in 1 ml) with IL-8, GROα, or NAP-2. After 10 min, 3 ml of chloroform/methanol (1:2) was added, and the monophase split by addition of 0.75 ml chloroform and 0.75 ml water. The lower phase containing the phospholipids was recovered, supplemented with carrier phosphatidylethanol, and dried by speedvac centrifugation. The lipids were separated by thin-layer chromatography on silica gel 60 with chloroform/methanol/acetic acid (65:15:5) and stained with iodine vapor, and the phosphatidylethanol spots were quantified by liquid scintillation counting.

RESULTS

The monoclonal receptor antibodies anti-IL8R1 and anti-IL8R2 were first tested on Jurkat cells transfected with receptor cDNA and expressing either IL-8R1 or IL-8R2 and were subsequently used to investigate the responses of human neutrophils stimulated with IL-8, GROα, or NAP-2. The transfected receptors had similar affinities for IL-8 (K_d values of 8.0 ± 2.5 and 6.0 ± 3.0 nM for IL-8R1 and IL-8R2, respectively) and were expressed in similar numbers (13,000 ± 4000 IL-8R1 and 14,500 ± 9500 IL-8R2 per Jurkat cell). In the present study, the chemokines were mainly used at concentrations between 10 and 100 nM. Under these conditions, GROα and NAP-2 act exclusively through IL-8R2 (3). The antibodies were not stimulatory, and no responses of Jurkat cells or neutrophils were observed on addition of anti-IL8R1 or anti-IL8R2 alone or in combination.

Effects on Jurkat Transfectants. Cells expressing one or the other IL-8 receptors were exposed to increasing concentrations of the monoclonal antibodies and the binding of ^{125}I-labeled IL-8 was determined. As shown in Fig. 1*a*, anti-IL8R1 and anti-IL8R2 significantly decreased IL-8 binding to IL-8R1 and IL-8R2, respectively. Both antibodies also inhibited IL-8-induced functions in IL-8R1 and IL-8R2 expressing transfectants as shown for the transient rise in $[Ca^{2+}]_i$ (Fig. 1*b*). Despite the fact that inhibition of binding and the $[Ca^{2+}]_i$ changes were not complete, the amplitude of the effects observed and their selectivity clearly indicated that anti-IL8R1 and anti-IL8R2 were suitable to study the function of both receptors in neutrophils.

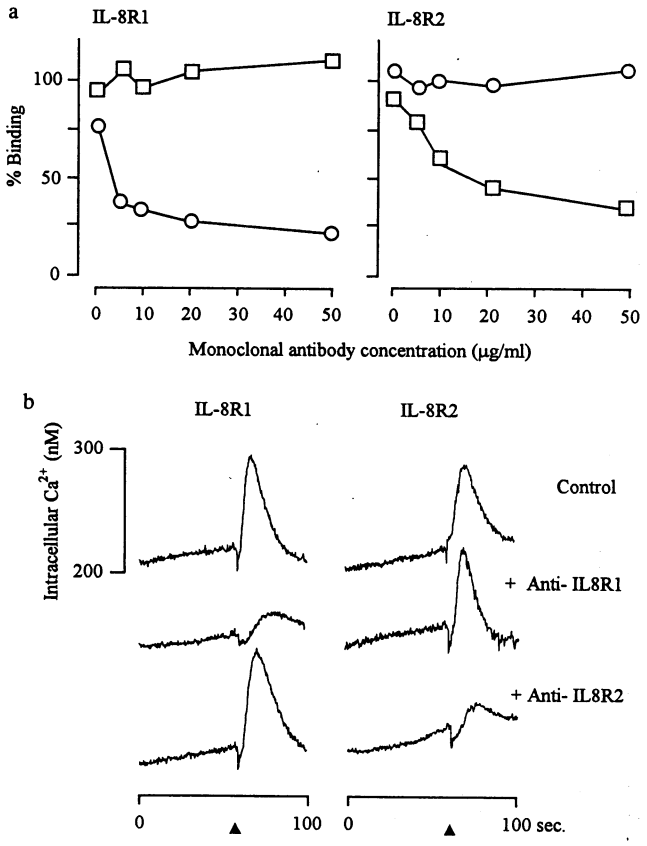

FIG. 1. Characterization of anti-IL8R1 and anti-IL8R2 monoclonal antibodies using Jurkat transfectants. (*a*) Competition for IL-8 binding. The curves show the relative binding of ^{125}I-labeled IL-8 to Jurkat cells expressing IL-8R1 or IL-8R2 in the presence of increasing concentrations of anti-IL8R1 (○) or anti-IL8R2 (□). (*b*) Inhibition of IL-8-mediated rise of the $[Ca^{2+}]_i$. Fura-2-loaded Jurkat cells expressing IL-8R1 or IL-8R2 (4×10^6 cells/ml) were preincubated with 25 μg of either anti-IL8R1 or anti-IL8R2 per ml and stimulated with 50 nM IL-8 (arrowhead), and the fluorescence changes were monitored.

Effects on Neutrophil Elastase Release. The release of granule enzymes is a characteristic response of neutrophils to CXC chemokines such as IL-8 (8), NAP-2 (11), and the GRO proteins (12). The effect of the receptor antibodies on the release of elastase is illustrated in Fig. 2. The response to IL-8 was slightly inhibited by anti-IL8R1 but was not affected by anti-IL8R2. The release induced by GROα, on the other hand, was markedly inhibited by anti-IL8R2 and was not affected by anti-IL8R1. As expected, the IL-8 response was markedly inhibited when the neutrophils were pretreated with both antibodies together. These experiments indicate that both CXC chemokine receptors signal for enzyme release. The slight inhibition of the effect of IL-8 by anti-IL8R1, as opposed to anti-IL8R2, suggests that IL-8 acts preferentially through IL-8R1. Because elastase release is induced through both receptors and can be precisely quantified, we used this assay to test for agonistic activities of the antibodies. As shown in Table 1, the fluorescence readings reflecting elastase release in the presence of anti-IL8R1, anti-IL8R2 or both monoclonal antibodies together did not differ from the values obtained with

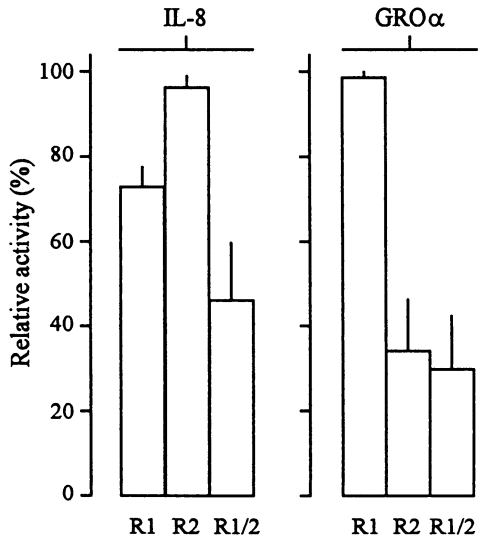

FIG. 2. Effect of anti-IL8R1 and anti-IL8R2 monoclonal antibodies on chemokine-induced elastase release by human neutrophils. Elastase release after stimulation with 10 nM IL-8 or 20 nM GROα in the presence of anti-IL8R1 (R1), anti-IL8R2 (R2) alone, or in combination (R1/2). The antibody concentration was 10 μg/ml. The activity is expressed in percent of the release obtained on stimulation with IL-8 or GROα in the absence of antibody. Values (mean ± SD) from four independent experiments performed in duplicate with neutrophils from different donors are shown.

buffer alone. This indicates that neither monoclonal antibody possesses agonistic properties.

Effects on the Respiratory Burst. The production of superoxide ensues from the stimulus-dependent assembly and activation of the NADPH oxidase. By monitoring the superoxide dismutase-inhibitable reduction of cytochrome *c* we found a concentration-dependent activation by IL-8 with a maximum at 25–50 nM, but no response to stimulation with either GROα (Fig. 3*a*) or NAP-2 (data not shown) at concentrations up to 1000 nM. The effect of IL-8 was rapid and transient, and superoxide production leveled off after 20–30 sec (Fig. 3*c*). The maximum rate observed was consistent with previous results (13). The difference in efficacy between IL-8 and the other CXC chemokines was not modified by pretreatment of the cells with cytochalasin B which enhances receptor-dependent responsiveness (14). These results suggested that the respiratory burst could only be elicited through IL-8R1. As shown in Fig. 3, this hypothesis was fully confirmed using the receptor antibodies: The IL-8-dependent superoxide production was inhibited in a concentration dependent manner by anti-IL8R1, but was not affected by anti-IL8R2.

Effects on PLD. Activation of PLD is observed when neutrophils are stimulated with fMet-Leu-Phe and some other chemoattractants (15). Phosphatidic acid, the product obtained, has been shown to elicit the respiratory burst (16), and therefore PLD activation is considered a potential intermediate step in the activation of the NADPH oxidase. Using

Table 1. Lack of agonist effects by anti-IL8R1 and anti-IL8R2 antibodies as assessed by elastase release

	Fluorescence units			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Buffer	2	8	3	12
Anti-IL8R1	1	6	4	10
Anti-IL8R2	3	7	3	9
Anti-IL8R1/IL8R2	2	7	4	10
IL-8, 10 nM	450	403	267	121
GROα, 20 nM	276	245	151	87

[^{3}H]oleic acid labeled neutrophils we studied PLD activation after stimulation with IL-8, GROα, and NAP-2 by measuring phosphatidylethanol, a product formed in the presence of ethanol (17). As shown in Fig. 4*a*, PLD was activated in a concentration-dependent manner in neutrophils stimulated with IL-8 or fMet-Leu-Phe (which was taken as positive control) but not with NAP-2. NAP-2 and GROα, which act through IL-8R2, were compared with IL-8 in six separate experiments with neutrophils from different donors and were inactive in each case. The monoclonal antibodies were then used to identify the receptor mediating the effect of IL-8. As shown in Fig. 4*b*, anti-IL8R1 markedly decreased IL-8-dependent activation of PLD while anti-IL8R2 was without effect, indicating that the response was totally dependent on IL-8R1.

DISCUSSION

This paper shows that the two IL-8 receptors expressed on human neutrophils, IL-8R1 and IL-8R2, are functionally different. Several responses such as $[Ca^{2+}]_i$ changes, the release of granule enzymes, as well as chemotaxis (4) were found to be induced by both receptors, whereas the respiratory burst and the activation of PLD were exclusively dependent on IL-8R1. As the only ligand with high affinity for IL-8R1 (3), IL-8 stands out among the neutrophil-activating chemokines. The present demonstration that some major neutrophil responses are induced through IL-8R1 but not IL-8R2, suggests that the role of IL-8 as a neutrophil attractant may differ from that of the other CXC chemokines.

Early studies of the action of C5a and fMet-Leu-Phe on neutrophils have shown that chemoattractants induce, in addition to migration, a complex pattern of responses including actin polymerization, activation of phospholipase C and D, $[Ca^{2+}]_i$ changes, expression of complement receptor 1 and 3, the release of granule enzymes, and the respiratory burst (14, 18). On the basis of such activities, we originally characterized IL-8 as a potential chemotactic agonist (8, 13) and subsequently attributed similar properties to NAP-2 (11), GROα (19), GROβ, and GROτ (12), and ENA-78 (20). The respiratory burst response to the IL-8-related chemokines, however, was very weak and detectable only thanks to a highly sensitive chemiluminescence assay for H_2O_2 (21). The present demonstration that IL-8R2 does not signal for the respiratory burst suggests that the activities reported previously were due to interaction with IL-8R1.

Receptor-specific monoclonal antibodies have been used in other laboratories to assess possible differences in the ability of IL-8R1 and IL-8R2 to elicit chemotaxis. Chuntharapai and Kim, (22) suggested that IL-8R2 is primarily involved in neutrophil recruitment, while IL-8R1 may have a more active role at the site of inflammation. Hammond *et al.* (23), however, came to the opposite conclusion, suggesting that chemotaxis to IL-8 depends predominantly on IL-8R1. We have found it difficult to consistently inhibit *in vitro* chemotaxis with either one of the antibodies selected for this study (S.Q. and S.A.J., unpublished observation). Since migration depends on multiple responses, such as adherence, cytoskeletal changes, enzyme release, etc., and is not assessed in real time, we consider chemotaxis unsuitable for the study of receptor usage by means of competing antibodies. Earlier reports demonstrating that IL-8 and related CXC chemokines attract neutrophils *in vitro* and *in vivo* (1), and the observation that Jurkat cells expressing either IL-8R1 or IL-8R2 show identical *in vitro* chemotaxis in response to IL-8, provide unquestionable evidence that both IL-8 receptors signal for migration (4).

Our finding that activation of PLD depends exclusively on IL-8R1 are in full agreement with the results of L'Heureux *et al.* (24) who showed that in human neutrophils PLD is activated by stimulation with IL-8 but not with GROα or

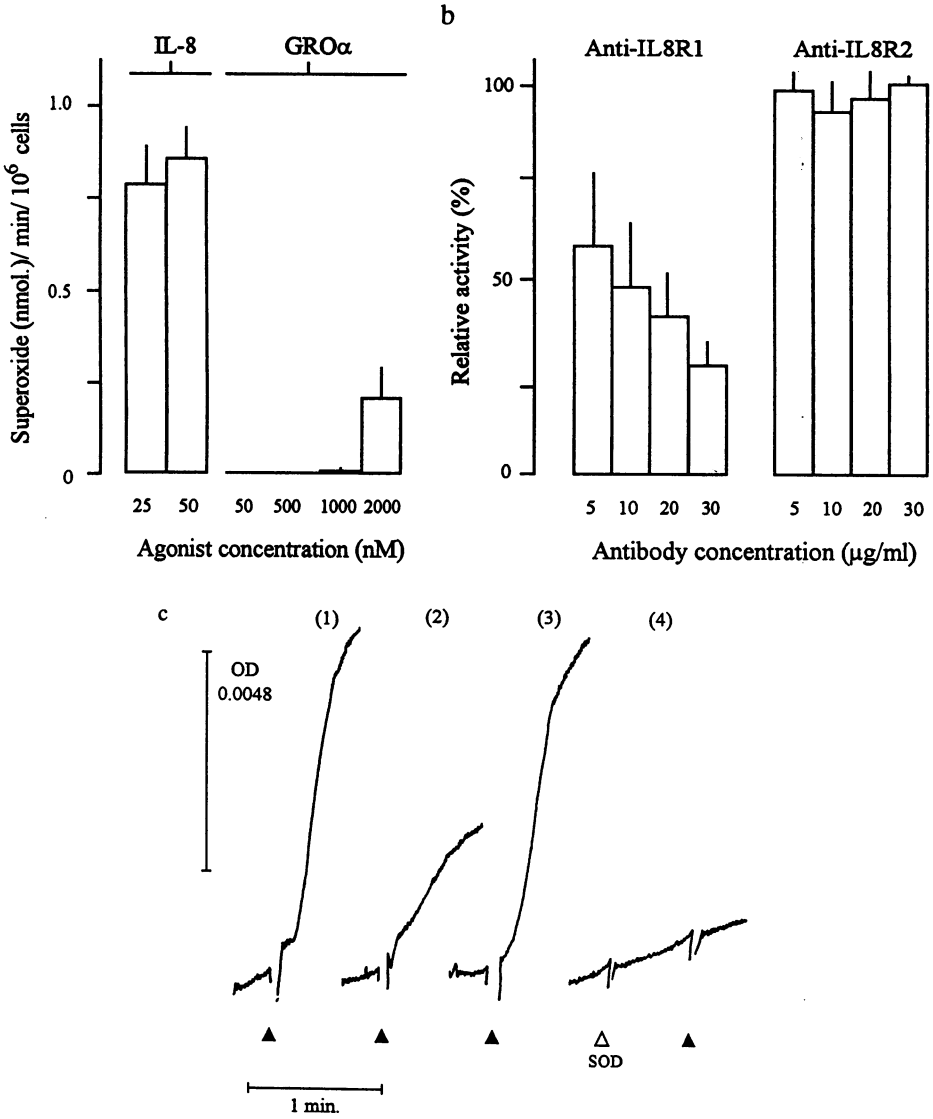

FIG. 3. Chemokine-induced superoxide production by human neutrophils. (*a*) Superoxide production induced by optimal concentrations of IL-8 and increasing concentrations of GROα. Values (mean ± SD) from three independent experiments performed with neutrophils from different donors are shown. (*b*) Effect of anti-IL8R1 and anti-IL8R2. Superoxide production after stimulation with 25 nM IL-8 in the presence of increasing concentrations of anti-IL8R1 or anti-IL8R2. The activity is expressed in percent of the values obtained in the absence of antibody. Values (mean ± SD) from three independent experiments performed with neutrophils from different donors are shown are shown. In the controls (100% value) the maximum rate of superoxide production was 0.72 ± 0.1, 0.94 ± 0.12, and 0.91 ± 0.08 nmol min^{-1} per 10^6 cells (mean ± SD). (*c*) Original cytochrome *c* reduction recordings illustrating the transient effect of IL-8. The cells were incubated with buffer alone (*1*), 20 μg of anti-IL8R1 per ml (*2*), or anti-IL8R2 (*3*) and stimulated with 25 nM IL-8 (arrowhead). Addition of IL-8 in the presence of 50 μg of superoxide dismutase per ml (SOD) represents the baseline (*4*).

NAP-2. All CXC chemokines, on the other hand, appear to elicit a number of common signal transduction events. L'Heureux *et al.* (24) found no differences in the $[Ca^{2+}]_i$ changes and the patterns of phosphorylation after stimulation with IL-8, GROα, or NAP-2, and we obtained the same degree of activation of the p42/p44 mitogen-activating protein kinase in IL-8-stimulated Jurkat transfectants expressing either IL-8R1 or IL-8R2 (5).

It is conceivable that IL-8R1 and IL-8R2 activate different signaling pathways, possibly through the coupling to G proteins with distinct α subunits as shown by Gierschik *et al.* (25) for the fMet-Leu-Phe receptor. Several studies have shown that single neutrophil functions such as the shape change (which is a correlate of motion), enzyme release, and activation of the NADPH oxidase are regulated at different levels of the signal transduction sequence (26). Inhibitors of protein kinase C, for instance, block superoxide production but not granule exocytosis (27). Protein kinase C is activated by diacylglycerol which is produced by phosphatidylinositol- or phosphatidylcholine-specific phospholipases C or, via phosphatidic acid, by PLD (15, 28). It has been reported that the phosphatidylinositol-specific phospholipase C is activated by IL-8 (29), but little is known regarding the activation of the other phospholipase C isoforms (30). In addition to diacylglycerol, the immediate product of PLD, phosphatidic acid, is considered an activator of NADPH oxidase (16, 31). From these considerations it is plausible that PLD and NADPH oxidase activation are linked and, therefore, affected to a similar extent by the antibodies that block IL-8R1.

The present study documents that the two IL-8 receptors elicit distinct patterns of responses in neutrophils, and suggests that IL-8, which is the only CXC chemokine with high affinity to IL-8R1, may have functions that cannot be fulfilled by its analogs, the GRO proteins, NAP-2, and ENA-78. Since all CXC chemokines, however, are fully capable of eliciting chemotaxis and neutrophil accumulation in the tissues (1), the

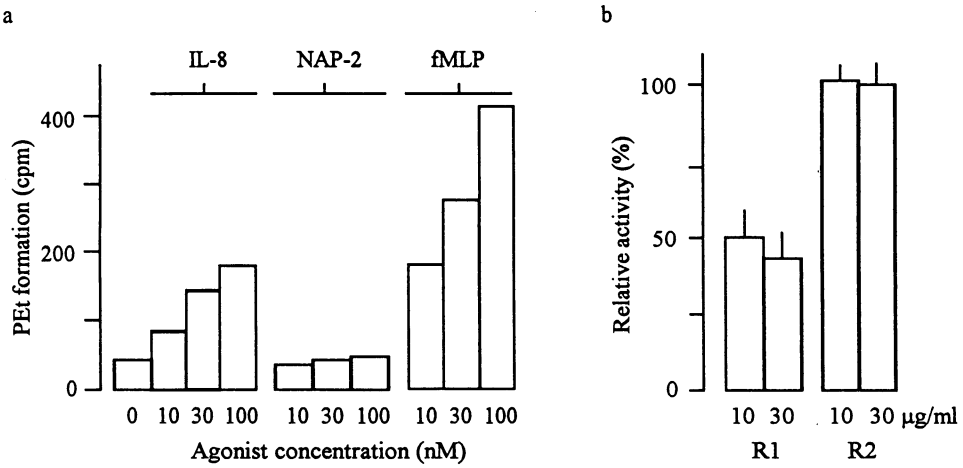

FIG. 4. Chemoattractant-induced PLD activation in human neutrophils. (*a*) Response to IL-8, NAP-2, and fMet-Leu-Phe (fMLP). (*b*) Effect of anti-IL8R1 and anti-IL8R2 on PLD activity after stimulation with 25 nM IL-8 in the presence or absence of 10 or 30 μg of anti-IL8R1 (R1) or anti-IL8R2 (R2) per ml. The activity is expressed in percent of the values obtained in the absence of antibody. Values (mean ± SD from three independent experiments performed with neutrophils from different donors are shown. The control values (100%) expressing the amount of radioactivity incorporated into phophatidylethanol corresponded to 384, 234, and 180 cpm.

present results indicate that these functions do not depend on PLD and the activation of the respiratory burst. It is conceivable that chemokines acting through IL-8R2 may function under physiological conditions and may primarily regulate the diapedesis that is required for eliminating senescent neutrophils and sustaining neutrophil turnover. Such physiological functions should not be compromised by potential side effects arising from the respiratory burst. IL-8, on the other hand, may be chiefly involved in neutrophil recruitment and activation under pathological conditions such as infection and inflammation where optimum recruitment and release responses are required for effective host defense.

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