We have previously shown that the residues Glu-Leu-Arg (ELR) preceding the first cysteine at the N terminus of the 72-residue form of interleukin-8 (IL-8) are essential for receptor binding and neutrophil activation (Clark-Lewis, I., Schumacher, C., Baggiolini, M., and Moser, B. (1991) J. Biol. Chem. 266, 23128-23134). We have now synthesized a series of analogs of IL-8(4-72), the truncated form of IL-8 with the N-terminal sequence ELRC, as potential IL-8 antagonists. Among 26 analogs with deletions or amino acid replacements in the ELR region several inhibited IL-8 function. The most potent were IL-8(6-72), with Arg at the N terminus, and IL-8, AAR(7-72) with N-terminal Ala-Ala instead of Glu-Leu. They inhibited IL-8 receptor binding, exocytosis (IC50 0.3 μM), as well as chemotaxis and the respiratory burst. Inhibition was restricted to responses elicited by IL-8, GROα, or NAP-2, and no effect was observed when the unligated agonists fMet-Leu-Phe or C5a were used as stimuli. These results demonstrate that selective antagonists that prevent or attenuate the action of IL-8 and its related chemotactic cytokines are obtained by modification of the ELR sequence at the N terminus.

Interleukin-8 (IL-8) belongs to a family of small cytokines which are structurally related to platelet factor 4. It is produced by activated phagocytes and mesenchymal cells and activates neutrophils inducing chemotaxis, exocytosis, and the respiratory burst (1, 2). IL-8 is generated as a precursor of 99 amino acids and is secreted after cleavage of a leader sequence. Proteolytic processing at the N terminus yields several variants, the most prominent of which consists of 72 residues (3). The 4 conserved cysteines, which are characteristic for all members of the IL-8 family, form two disulfide bonds that are essential for biological activity (2, 4). The disulfide bond links a short conformationally flexible N-terminal region to the core structure consisting of three antiparallel β-strands followed by a C-terminal α-helix.

As part of a study of the relationships between the structure and function of IL-8, we chemically synthesized analogs of the 72-residue form of IL-8 that were shortened at the N- or C-terminal ends. The results demonstrated that the integrity of each of the 3 residues, Glu-Leu-Arg (ELR), that precede the first cysteine (Table I) is critical for receptor binding and neutrophil-stimulating activity (5). IL-8(4-72), the derivative with Glu at the N terminus, had maximal neutrophil-stimulating activity and binding affinity. Elimination of Glu (derivative IL-8(5-72)) led to a marked reduction in activity, and further truncation yielded two completely inactive derivatives, IL-8(6-72) and IL-8(7-72) (5). Evidence for the importance of the N terminus for IL-8 activity was also obtained in a mutagenesis study showing that replacement of Glu, Leu, or Arg with alanine leads to inactivation (6).

IL-8 is a potent neutrophil attractant in vivo (7-9), and its expression is enhanced in a variety of inflammatory conditions (2). The potential role of IL-8 as a mediator of inflammation has stimulated the search for inhibitors that could be used therapeutically. In view of the critical role of the N-terminal domain, we have attempted to produce such inhibitors by modification of the ELR sequence. In this paper, we describe several analogs that selectively bind to IL-8 receptors and inhibit IL-8-mediated neutrophil responses, and thus qualify as IL-8 receptor antagonists.

EXPERIMENTAL PROCEDURES

Chemical Synthesis—IL-8, GROα, NAP-2, and the IL-8 analogs listed in Table I were synthesized by solid-phase methods using the tertiary butyloxycarbonyl and benzyl protection strategy (10). After deprotection with hydrogen fluoride, the material was folded by air oxidation and purified by reverse-phase HPLC. Purity was assessed by reverse-phase HPLC and isoelectric focusing. Amino acid incorporation was monitored during synthesis, and the final composition was determined by amino acid analysis. The exact procedure has been reported previously (10).

Biological Assays—Human neutrophils were isolated from buffy coats of donor blood (4). The final suspension containing 10^6 cells/ml in 0.15 M NaCl supplemented with 0.05 mM CaCl2 was kept at 10 °C until use. In vitro chemotaxis was measured in multiwell chemotaxis chambers (NeuroProbe, Cabin John, MD) equipped with polycarbonate membranes (Nucleopore) with pores of 5 μm (11). The chemotactic index is defined as the ratio of the numbers of cells migrated in the presence and absence of chemotactant. Elastase release from cytochalasin B-pretreated neutrophils (4) and the respiratory burst (12) were determined as described previously.

Receptor Binding Assays—IL-8 iodination, binding assays, and the calculation of binding parameters were performed as described (5).

RESULTS

The effects of the truncated IL-8 analogs IL-8(5-72), IL-8(6-72), and IL-8(7-72) on the binding of IL-8 to its receptor and on IL-8-dependent neutrophil activation were compared. As shown in Fig. 1A, IL-8(6-72) inhibited the release of elastase induced by IL-8. The effect was concentration-de-
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**TABLE I**

*N-terminal modifications of IL-8*

Analog with substitutions of Glu, Leu, or Arg were designated with one letter code for the N-terminal amino acids preceding the first cysteine.

| Residues | Kd \(\text{nM}\) | IC\(_50\) \(\mu\text{M}\) |
|----------|----------------|-----------------|
| IL-8(1-72) | Ser Ala Lys Glu Leu Arg Cys Gin Cys | 0.25 0.22 |
| IL-8(5-72) | Glu Leu Arg Cys Gin Cys | 0.8 0.5 |
| IL-8(6-72) | Leu Arg Cys Gin Cys | 50 0.3 |
| IL-8(7-72) | Cys Gin Cys | >10,000 |
| IL-8, ELQ(7-72) | Glu Leu Gin Cys Gin Cys | 700 1.1 |
| IL-8, ELL(7-72) | Glu Leu Leu Cys Gin Cys | 1,300 0.7 |
| IL-8, ELK(7-72) | Glu Leu Orn Cys Gin Cys | 5.4 |
| IL-8, ELOn(7-72) | Ala Ala Arg Cys Gin Cys | 8 0.3 |
| IL-8, ELOm(7-72) | Ile Arg Cys Gin Cys | 10 0.3 |
| IL-8, ELL(7-72) | Gin Arg Cys Gin Cys | 50 2.1 |

*a Kd values were determined in 1\(^{25}\)I-IL-8(1-72) competition binding experiments with increasing concentrations of unlabeled IL-8 analogs, and the binding data were evaluated by computer modeling involving iterative least square fitting to either one- or two-binding site models according to Refs. 13 and 19.

*b The IC\(_50\) values were determined from the inhibition curves obtained for the IL-8(1-72)-induced release of elastase.

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**FIG. 1. Antagonist effects of IL-8(6-72).**

A: elastase release from cytochalasin B-pretreated neutrophils stimulated with 10 nM IL-8(1-72) in the presence of increasing concentrations of IL-8(6-72) (△), IL-8(6-72) (●), or IL-8(7-72) (■). B: competition for 1\(^{25}\)I-IL-8 binding to human neutrophils by unlabeled IL-8(1-72) (△), IL-8(5-72) (●), IL-8(6-72) (■), and IL-8(7-72) (■). 100% of 1\(^{25}\)I-IL-8(1-72) bound corresponds to the fraction of iodinated IL-8 bound in the absence of unlabeled IL-8 or IL-8 analogs (40-50% receptor occupancy).

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*IL-8(6-72) inhibited the release of elastase elicited by IL-8, but not by fMet-Leu-Phe or by C5a, two chemotactic agonists that act through different receptors, indicating that its antagonistic effect is selective for the IL-8 receptor (Fig. 2A). In addition to exocytosis, IL-8(6-72) inhibited in vitro chemotaxis (Fig. 2B) and the respiratory burst (Fig. 2C). Inhibition was again restricted to the responses induced by IL-8, and no effect was observed when neutrophils were stimulated with fMet-Leu-Phe (data not shown). IL-8(6-72) also prevented neutrophil activation by the IL-8 homologs GRO\(_\alpha\) and NAP-2 as shown in Fig. 2D for the release of elastase. These results are in agreement with our previous observations that IL-8, GRO\(_\alpha\), and NAP-2 share the same receptors on neutrophils (13) and further support the evidence that IL-8(6-72) acts as a selective antagonist. Since IL-8(6-72) binds to IL-8 receptors, its activity as an agonist was thoroughly tested. IL-8(6-72) did not elicit exocytosis (Fig. 2A) or the respiratory burst up to a concentration of 10 \(\mu\text{M}\), but showed weak chemotactic activity at 1 and 3 \(\mu\text{M}\) (Fig. 2B).

In addition to the three truncated forms of IL-8(4-72), 23 analogs with modifications to the Glu-Leu-Arg (ELR) sequence were synthesized and tested for neutrophil activation and IL-8 antagonism. Seven of them markedly inhibited IL-8-induced elastase release (Table I). IL-8,AAR(7-72) was particularly effective. It was roughly equipotent with IL-8(6-72) (Fig. 3A), and agonized to a similar extent the responses to IL-8, GRO\(_\alpha\), and NAP-2. In addition, IL-8,AAR(7-72) also reduced IL-8-elicited chemotaxis. Analog with substitution of Arg, namely, IL-8,ELQ(7-72) and IL-8,ELL(7-72) (Fig. 3A) as well as IL-8,ELK(7-72) and IL-8,ELOm(7-
IL-8 Receptor Antagonists

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A

B

C

D

EL-8(6-72) (−logM)

IL-8(6-72) (−logM)

IL-8(6-72) (−logM)

FIG. 2. IL-8(6-72) inhibits IL-8 induced neutrophil responses. A, inhibition by IL-8(6-72) of elastase release from cytochalasin B-pretreated neutrophils stimulated with 10 nM IL-8(1-72) (○), 10 nM fMet-Leu-Phe (○), or 1 nM C5a (△). The effect of IL-8(6-72) alone (□) is also shown. B, effect on neutrophil chemotaxis. IL-8(6-72) was present in the lower wells of a multiwell chemotaxis chamber either alone (○) or together with 10 nM IL-8(1-72) (○). C, inhibition of the respiratory burst. Rate of \( \text{H}_{2}\text{O}_{2} \) production induced by 10 nM IL-8(1-72) alone (a), and in the presence of 10 nM (b), 100 nM (c), and 1 µM (d) IL-8(6-72). D, inhibition of GROα and NAP-2 induced elastase release. Relative activity expressed as the percent of the release obtained with 10 nM IL-8(1-72) (○), 10 nM GROα (△), or 30 nM NAP-2 (○) in the absence of IL-8(6-72) which corresponded to 258, 177, and 123 units, respectively.

72) (Table I) were somewhat less effective as inhibitors and markedly less potent as competitors for IL-8 receptor binding (Fig. 3B). Two analogs of IL-8(5-72) with substitution of Leu\(^6\), IL-8,IR(7-72) and IL-8,QR(7-72), also acted as antagonists, but showed in addition some agonistic activity at high concentrations (Fig. 3C). Inhibition of elastase release was accompanied by competition for IL-8 receptor binding, although all analogs with substitution of Arg\(^6\) had lower affinity for the IL-8 receptor than those with conserved Arg\(^6\) (Fig. 3, B and D, and Table I).

The ELR tripeptide itself and other oligopeptides containing the ELR sequence were not active as agonists or antagonists and did not bind to receptors, suggesting that additional structural features are required for IL-8 function.

DISCUSSION

It had been previously shown that elimination of the N-terminal ELR sequence abolishes IL-8 receptor binding and biological activity, indicating that this region is essential for IL-8 function (5, 6). The present results show that modification of ELR can yield IL-8 derivatives that have lost neutrophil-stimulating activity but still bind to the receptors, thereby acting as selective IL-8 antagonists.

Two types of N-terminal modification were found to generate antagonists: the substitution or elimination of Glu\(^4\) and Leu\(^6\) with conservation of Arg\(^6\), or the substitution of Arg\(^6\) with conservation of Glu\(^4\) and Leu\(^6\). The present results indicate that Arg\(^6\) is the most critical residue of the ELR sequence for high-affinity binding to the IL-8 receptor. Anologs with substitution of Arg\(^6\) showed comparable potency as those with conserved Arg\(^6\) for inhibiting neutrophil activation, but, for reasons that could not be established, had lower affinity for the IL-8 receptor as assessed by competition assays.

Among the IL-8 derivatives with antagonistic properties IL-8(6-72) and IL-8, AAR(7-72) appear to be especially promising. IL-8(6-72) affected IL-8-induced chemotaxis, exocytosis (elastase release), and the respiratory burst, and thus behaved as an overall inhibitor of receptor-dependent neutrophil activation. The effect on chemotaxis, however, differed somewhat from that on exocytosis and the respiratory burst. At concentrations between 1 and 10 µM IL-8(6-72) displayed weak chemotactic activity without inducing enzyme release or the respiratory burst. Since it has been shown that the various neutrophil responses depend on different levels of receptor occupancy (14) and signal transduction control (15), it is conceivable that a receptor-bound antagonist may transmit a signal for chemotaxis, but not for exocytosis or the respiratory burst.

IL-8(6-72) and IL-8, AAR(7-72) inhibited to similar extents
the elastase release induced in neutrophils by IL-8 and its homologs, GROs and NAP-2. This observation is of major practical significance, because it is known that in different types of stimulated cells IL-8 is often generated together with related chemotactic cytokines (16–18). These factors are believed to act in concert in pathological conditions by recruiting and activating neutrophils. It is, therefore, desirable to obtain inhibitors, like those described in this study, that diminish or prevent the action of all chemotactic cytokines of the IL-8 family.

The present results further emphasize the importance of the ELR motif in IL-8 receptor binding and triggering. However, the ELR motif alone, as a peptide or in peptide constructs, is not sufficient for effective receptor interaction. The challenge will be to identify the additional structural requirements for the design of future generations of inhibitors with potential therapeutic application.

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