0026-895X/02/6201-173–180$7.00
MOLECULAR PHARMACOLOGY
Vol. 62, No. 1
1405/993071
Mol Pharmacol 62:173–180, 2002
Printed in U.S.A.

The Unique Property of the CC Chemokine Regakine-1 to Synergize with Other Plasma-Derived Inflammatory Mediators in Neutrophil Chemotaxis Does Not Reside in Its NH_2-Terminal Structure

MIEKE GOUWY, SOFIE STRUYF, FRANK MAHIEU, WILLY PUT, PAUL PROOST, and JO VAN DAMME

Laboratory of Molecular Immunology, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium

Received October 5, 2001; accepted April 8, 2002
This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The recently discovered CC chemokine, regakine-1, is constitutively present in bovine serum and synergizes with the CXC chemokine interleukin-8 (IL-8) to chemoattract neutrophils. Here we show that regakine-1 cooperates with the CXC chemokine receptor 2 ligand neutrophil activating protein-2 (NAP-2) and the anaphylatoxin C5a, two other mediators of inflammation present in the circulation. Neutrophil chemotaxis was 3-fold enhanced when regakine-1 (100 ng/ml) and C5a (30 ng/ml) were combined at concentrations present in bovine or human plasma, respectively. This synergy was also observed when neutrophils were preincubated with regakine-1. Plasma chemokines such as NAP-2, β-thromboglobulin, and hemofiltrate CC-chemokine-1 did not affect C5a chemotactic activity. The capability of regakine-1 to synergize with C5a, NAP-2, or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was not observed for monocyte chemotactic protein-3 (MCP-3), another CC chemokine that weakly chemoattracts neutrophils. Regakine-1 also failed to cooperate with MCP-3 and macrophage inflammatory protein-1α in neutrophil chemotaxis. The receptor of regakine-1 is not known yet. Competition with labeled fMLP or C5a for binding to neutrophils or receptor transfected cell lines demonstrated that regakine-1 did not alter receptor recognition. The protein kinase inhibitors 2′-amino-3′-methoxyflavone (PD98059), wortmannin and staurosporin had no effect on the synergy between C5a and regakine-1. Although NH_2-terminal truncation affects the chemotactic potency of most chemokines, it did not affect the synergistic capacity of regakine-1 with C5a on neutrophils. These findings indicate that the constitutive plasma chemokine regakine-1 is a stable enhancer of the inflammatory response and that its blockade might be beneficial in acute and systemic inflammatory disorders.

The chemokine family is composed of a number of structurally and functionally related chemotactic cytokines that regulate cell migration during healthy and pathological processes, including angiogenesis, hematopoiesis, infection, and cancer (Wuyts et al., 1999; Murphy et al., 2000; Rossi and Zlotnik, 2000). In particular, chemokines are key mediators of leukocyte migration during both the inflammatory response and normal homeostasis. The classification of chemokines in C, CC, CXC, and CX_3C subfamilies, according to the position of conserved cysteine residues, provides only a limited help to distinguish the functional activities of these mediators. Indeed, although many CXC chemokines [e.g., interleukin-8 (IL-8)], preferentially activate and chemoattract neutrophilic granulocytes, other members of this subfamily selectively stimulate migration of lymphocytes. Inversely, whereas most CC chemokines do not act on neutrophils, some [e.g., monocyte chemotactic protein-3 (MCP-3)] weakly chemoattract neutrophils in addition to monocytes and lymphocytes (Xu et al., 1995). This divergence in spectrum of target cells is dictated for each individual chemokine by the expression of specific G protein-coupled seven-transmembrane receptors that are recognized by the chemokine to exert its biological activities. In this respect, the CC chemokine receptor CCR1 is expressed on neutrophils, in addition to the CXC chemokine receptors CXCR1 and CXCR2 (Crisman et al., 1999; Zhang et al., 1999). Chemokine receptor recognition and hence

This work was supported by the Fund for Scientific Research of Flanders (FWO-Vlaanderen), the Concerted Research Actions of the Regional Government of Flanders, the InterUniversity Attraction Pole initiative of the Belgian Federal Government (IUAP), and the Biomed and Biotech Programs of the European Community. P.P. and S.S. hold fellowships from the FWO-Vlaanderen.

ABBREVIATIONS: IL, interleukin; MCP-3, monocyte chemotactic protein-3; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; NAP-2, neutrophil activating protein-2; C5a, C5 anaphylatoxin; RBL, rat basophilic leukemia; BSA, bovine serum albumin; PBS, phosphate-buffered saline; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FPR, formyl peptide receptor; RP-HPLC, reversed phase-high performance liquid chromatography; CTAP-III, connective tissue-activating peptide-III; MIP-1α, macrophage inflammatory protein-1α; HCC-1, hemofiltrate CC-chemokine-1; CI, chemotactic index; PD98059, 2′-amino-3′-methoxyflavone.

biological potency is strongly affected by NH_2-terminal processing of chemokines by proteases (Van Damme et al., 1999; Van den Steen et al., 2000; Struyf et al., 2001a).

Regakine-1 is a recently discovered CC chemokine that is constitutively present at high concentrations in bovine plasma and to which neutrophil chemotactic activity has been ascribed (Struyf et al., 2001b). Although the natural 7.5-kDa protein of 70 residues contains the four conserved cysteines, it has less than 50% sequence identity with any known human or animal chemokine. No specific receptors for regakine-1 have been identified and no high affinity for the classical receptors on neutrophils (i.e., CXCR1 and CXCR2) was detected for regakine-1. However, a particular characteristic of the plasma-derived regakine-1 resides in its potential to synergize with IL-8, a ligand for both CXCR1 and CXCR2, to chemoattract neutrophils (Struyf et al., 2001b). CXCR2 is also functionally expressed on nonhematopoietic cells and accounts for the angiogenic activity of IL-8 and related CXCR2 agonists (Addison et al., 2000; Devalaraja et al., 2000). To better delineate the unexpected activity of this novel CC chemokine, parallel experiments were performed with either selective CXCR2 agonists [i.e., neutrophil activating protein-2 (NAP-2) or inflammatory mediators other than chemokines (e.g., complement factor C5a), both also present in human plasma]. It was found that NAP-2 and C5a synergize with regakine-1 in neutrophil chemotaxis, whereas other plasma- or tissue-derived CC chemokines failed to cooperate with C5a or regakine-1. This study demonstrates that regakine-1 possesses the unique capability to potentiate the inflammatory response in the blood circulation. It can be speculated that neutralization of this chemokine can reduce neutrophil-mediated injury.

Materials and Methods

Cells. Neutrophilic granulocytes were isolated from buffy coats, freshly derived from blood of healthy donors (Blood Transfusion Center of Antwerp and Laboratory of Experimental Immunology, University of Leuven). Mononuclear cells and granulocytes were separated by gradient centrifugation for 30 min at 400*g* on Ficoll-sodium metrizoate (Lymphoprep; Invitrogen, Groningen, The Netherlands). Erythrocytes in the granulocyte pellet were removed by sedimentation for 30 min at 37°C in hydroxyethyl-starch solution (Plasmasteril; Fresenius AG, Bad Homburg, Germany). Remaining erythrocytes were lysed by hypotonic shock (30 s) in bidistilled water. Neutrophilic granulocytes were used in the chemotaxis assay at a concentration of 10^6 cells/ml in Hanks' balanced salt solution (Invitrogen) supplemented with 1 mg/ml of human serum albumin (Belgian Red Cross).

Rat basophilic leukemia (RBL) cells stably transfected with epitope-tagged high-affinity *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor formyl peptide receptor (FPR) were developed (Ali et al., 1993) by Drs. H. Ali and R. Snyderman (Duke University, Durham, NC) and provided by Dr. J. M. Wang (National Cancer Institute, Frederick, MD). Cells were maintained in Dulbecco's minimum essential medium (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% fetal calf serum (Invitrogen) and 0.8 mg/ml Geneticin (G418; Invitrogen).

Chemoattractants. Regakine-1 was isolated from fetal calf serum (Invitrogen) derived from *Bos taurus* by subsequent adsorption to silicic acid, heparin-Sepharose affinity chromatography, mono-S cation-exchange chromatography (Amersham Biosciences, Uppsala, Sweden) and reversed phase-high performance liquid chromatography (RP-HPLC) (Perkin-Elmer, Norwalk, CT) as described previously (Struyf et al., 2001b). Natural human IL-8 (CXCL8) and human NAP-2 (CXCL7) were purified to homogeneity from monocyte-derived conditioned medium (Van Damme et al., 1989). A fraction containing natural connective tissue-activating peptide III (CTAP-III) (CXCL7) was purified from blood platelets (Van Damme et al., 1989). Natural chemokines contained less than 2.5 pg of lipopolysaccharide/μg of protein, as determined in the Limulus amoebocyte lysate assay (QCL chromogenic method; BioWhittaker). Recombinant human hemofiltrate CC chemo-

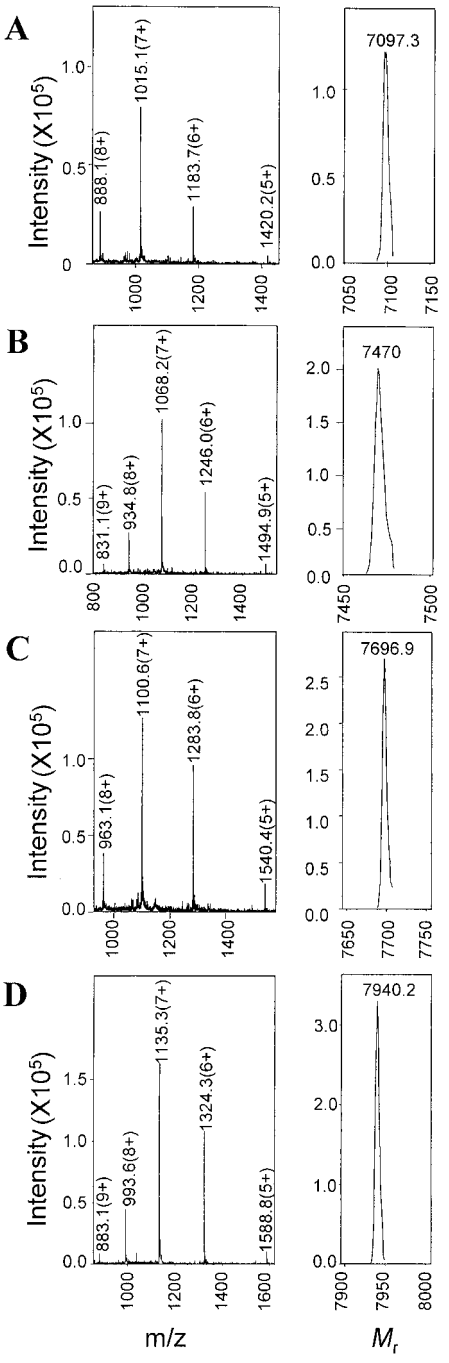

Fig. 1. Mass spectrometry of folded synthetic Regakine-1 forms. RP-HPLC purified and folded synthetic regakine-1(9–70) (A), regakine-1(5–70) (B), regakine-1(3–70) (C), and regakine-1(1–70) (D) were subjected to electrospray ion trap mass spectrometry. Left, average of 800 to 1000 spectra of the multiple charged ions resulting in an expected error for the molecular mass of the uncharged molecules of less than 1 Da. The *m/z* values are indicated above the peaks that are derived from the regakine-1 proteins, whereas the number of charges for the multiple charged ions are indicated between brackets. The right part of the figure shows the uncharged deconvoluted spectra for the different regakine-1 forms with the average molecular mass indicated on top of the peaks.

kine-1 (HCC-1, CCL14) was purchased from Peprotech (Rocky Hill, NJ) and the bacterial-derived chemotactic peptide fMLP was obtained from Sigma (St. Louis, MO). The CC chemokines MCP-3 (CCL7) and MIP-1α/LD78β (CCL3-L1) were synthesized by solid-phase peptide synthesis using fluorenylmethoxycarbonyl chemistry (see next paragraph) as described previously (Struyf et al., 2001a). The anaphylatoxin C5a was either obtained from Sigma (recombinant anaphylatoxin C5a) or purified from human plasma by heparin-Sepharose affinity chromatography (Amersham Biosciences), Resource S cation-exchange chromatography (Amersham Biosciences) and RP-HPLC on a Resource RPC column (Amersham Biosciences). For identification, the NH_2-terminal residues of C5a (TLQKKIEEIA) were determined by Edman degradation on a capillary protein sequencer (Procise 491cLC; Applied Biosystems, Foster City, CA) and corresponded to that of intact C5a. The average relative molecular mass of natural C5a was determined by ion trap mass spectrometry (Esquire LC, Bruker Daltonik, Bremen, Germany) to be 10437 Da. Because the average molecular mass of unglycosylated C5a is 8268 Da, considerable posttranslational modification by glycosylation is evident.

Chemical Synthesis of Different Forms of Regakine-1. Intact regakine-1 and NH_2-terminally truncated forms missing the first two, four, or eight amino acids, designated regakine-1(1–70), regakine-1(3–70), regakine-1(5–70), and regakine-1(9–70), respectively, were chemically synthesized in a single run using amino acids with 9-fluorenylmethoxy-carbonyl–protected α-amino groups on a model 433A solid-phase peptide synthesizer using the standard FastMoc programs with conditional double coupling and acetic anhydride capping (Applied Biosystems). The final deprotection and cleavage of the peptide from the resin was performed by incubating the synthesis product for 2 h at room temperature in the following cleavage mixture: 10 ml of trifluoroacetic acid, 0.5 ml of water, 0.5 ml of thioanisole, 0.25 ml of ethanedithiol, and 0.75 g of crystalline phenol. The synthetic chemokine was separated from the resin on a medium-porosity glass filter, precipitated into cold methyl *t*-butyl ether, washed, dissolved in water, and subsequently lyophilized. Crude full-length and shorter isoforms of synthetic regakine-1 were separated from peptide fragments by RP-HPLC on a Resource RPC column (Amersham Biosciences). After purification, the disulfide bridges were formed by incubation at room temperature for 1.5 h in 150 mM Tris, pH 8.6; 2 M ureum, 3 mM EDTA, 0.3 mM oxidized glutathione, and 3 mM reduced glutathione. The folded peptides were repurified by RP-HPLC on an Aquapore RP-300 column (Applied Biosystems). The purity and the molecular mass of folded intact or truncated regakine-1 proteins were confirmed by ion trap electrospray mass spectrometry (Fig. 1). For all regakine-1 forms, the experimentally determined average molecular mass differed by less than 1 Da from the theoretical average molecular mass, indicative of a correct synthesis and folding (Table 1). Purified synthetic regakine-1 isoforms were free of detectable lipopolysaccharide ($<$ 2.5 pg of lipopolysaccharide/μg of regakine-1).

Chemotaxis Assay. Cell migration was measured with the Boyden microchamber technique (Neuro Probe, Gaithersburg, MD). Cell fractions and samples were diluted in Hanks' balanced salt solution supplemented with 1 mg/ml human serum albumin (dilution buffer) and tested in triplicate. The lower compartment, containing the test sample or control dilution buffer, was separated from the upper compartment, containing neutrophils (1×10^6 cells/ml), by a polyvinylpyrrolidone-free polycarbonate membrane with a 5-μm pore size (Nuclepore; Corning Costar, Acton, MA). After migration (45 min at 37°C) the filters were incubated, fixed and stained using Hemacolor solutions (Merck, Darmstadt, Germany). The migrated cells were counted microscopically in 10 oil immersion fields at a 500× magnification. The chemotactic activity was expressed as a chemotactic index (CI), the number of cells migrated to the test sample divided by the number of cells migrated to the dilution buffer (negative control). In experiments in which the synergistic effect of two chemoattractants was investigated, both molecules were added to the lower wells of the microchamber. In desensitization experiments, neutrophils were preincubated with regakine-1 for 10 min at 37°C and subsequently washed twice with dilution buffer before transfer of the cells to the chemotaxis chamber. To study the effect of protein kinase inhibitors on the synergistic effect, neutrophils were treated with the kinase inhibitors PD98059 (Calbiochem Merck Eurolab, Lutterworth, UK), wortmannin (Sigma-Aldrich, St. Louis, MO) or staurosporin (Sigma) and loaded into the upper wells of the Boyden chamber. Statistical differences between chemotactic indexes were determined by the Mann-Whitney *U* test.

TABLE 1
NH_2-terminal forms of regakine-1

	NH_2-Terminal Sequence	Theoretical Relative Molecular Mass
Regakine-1 (1–70)	NEEPAGNMRVCC	7940.07 Da
Regakine-1 (3–70)	EPAGNMRVCC	7696.86 Da
Regakine-1 (5–70)	AGNMRVCC	7470.61 Da
Regakine-1 (9–70)	RVCC	7097.18 Da

Binding Assays. Competition for fMLP binding was measured using purified neutrophils or FPR transfected rat basophilic leukemia cells (RBL cells) as described previously (Le et al., 1999). Briefly, a cell suspension [FPR/RBL cells, 2×10^6 cells/200 μl in RPMI 1640 medium containing 2 mg/ml bovine serum albumin (BSA; Sigma) and 5 mg/ml NaN_3] was incubated for 30 min at 37°C under constant rotation with 30 nM [^{3}H]fMLP (PerkinElmer Life Sciences, Boston, MA) and varying concentrations of unlabeled fMLP or regakine-1. Each experiment was done in duplicate. After incubation, the samples were filtered onto Whatman GF/C discs (Whatman International, Kent, UK) on a 12-well manifold, followed by washing three times with 5 ml of ice-cold phosphate-buffered saline (PBS). The discs were air-dried, submerged in liquid scintillation fluid, and counted for β-emission.

Competition for C5a binding was measured on freshly isolated blood neutrophils. Cells (5×10^6) were incubated for 2 h at 4°C in PBS containing 2 mg/ml BSA with 0.04 nM ^{125}I-C5a (PerkinElmer Life Sciences) and several different concentrations of unlabeled recombinant C5a (Sigma) and/or natural regakine-1. Duplicate samples were incubated. After incubation the cells were washed three times with PBS containing 2 mg/ml BSA and then counted for γ-emission.

Results

Synergy between Plasma-Derived Regakine-1 and NAP-2 in Neutrophil Chemotaxis. It has previously been observed that the chemotactic effect of IL-8 on neutrophils was significantly enhanced in the presence of the CC chemokine regakine-1 (Struyf et al., 2001b). To verify whether this synergistic effect is mediated specifically via one of the IL-8 receptors (i.e., CXCR2), the platelet-derived CXCR2 ligand NAP-2 was tested in combination with plasma-derived regakine-1. Regakine-1 alone had a weak but statistically significant neutrophil chemotactic activity (e.g., CI $\pm$ S.E.M. of 6.4 $\pm$ 2.8 and 5.3 $\pm$ 2.4 at 100 and 30 ng/ml, respectively; p = 0.01 versus control buffer) (Fig. 2). Regakine-1 dose-dependently increased the neutrophil chemotactic activity of the chemokine NAP-2 (Fig. 2). Indeed an agonistic concentration (30 or 100 ng/ml) of regakine-1 enhanced the chemotactic index of NAP-2 (30 and 100 ng/ml) 3- to 6-fold above the additive effect of the two agonists. For comparison, it was confirmed that the neutrophil chemotactic activity of IL-8 (5 ng/ml) was also enhanced by regakine-1 (100 ng/ml). It can be deduced that different CXC chemokines binding to CXCR2 (i.e., IL-8 and NAP-2) cooperate with regakine-1 in neutrophil chemotaxis experiments.

Enhanced Inflammatory Response to C5a and fMLP in the Presence of Regakine-1. To verify whether a synergistic effect also exists between regakine-1 and neutrophil-

activating inflammatory mediators other than chemokines, similar chemotaxis experiments were executed with the anaphylatoxin C5a. This potent chemoattractant, found in serum during septic shock, activates neutrophils expressing the unique C5a receptor (Gerard and Gerard, 1991). Natural C5a was purified to homogeneity from human plasma (see *Materials and Methods*). In Fig. 3, it is shown that regakine-1 (30 or 100 ng/ml) significantly enhanced the neutrophil chemotactic response toward C5a (30 or 100 ng/ml). With this combination, a maximal neutrophil chemotactic index of more than 100 was reached, whereas regakine-1 and C5a alone elicited maximal indexes of about 3 and 30, respectively. Similarly, regakine-1 was also capable to synergize with the bacterial peptide fMLP (Fig. 3).

In a separate set of experiments using a different preparation of natural regakine-1, it was verified whether direct addition of regakine-1 to neutrophils or preincubation of the cells with this chemokine before transfer to the chemotaxis assay could either enhance or decrease the chemotactic response toward C5a. Table 2 demonstrates that parallel to simultaneous addition of regakine-1 and C5a to the lower wells of the chemotaxis chamber, (pre)treatment of neutrophils with regakine-1 resulted in an equally significant synergy ($p < 0.01$). The results also indicate that pretreatment (10 min at 37°C) of neutrophils with regakine-1 and subsequent removal of the chemokine by washing did not desensitize but rather increased the sensitivity of the cells for chemotaxis in response to C5a.

Lack of Synergy between C5a and Plasma Chemokines Other Than Regakine-1. In a further attempt to precisely delineate the spectrum of synergy between plasma chemokines and C5a, other chemokines constitutively present in plasma were evaluated for their synergistic capacity in C5a-induced neutrophil chemotaxis (Table 3). CTAP-III (15 ng/ml), NAP-2 (30 ng/ml), and HCC-1 (300 ng/ml) failed to enhance the chemotactic effect of C5a at a concentration (30 ng/ml) that synergized with regakine-1 (Fig. 3). This demonstrates that the neutrophil chemotactic effect of C5a is selectively enhanced by the CC chemokine regakine-1 and not by other plasma CC (HCC-1) or CXC (CTAP-III, NAP-2) chemokines. Indeed, only cumulative chemotactic indexes were reached when each of the three chemokines was combined with C5a (Table 3). Thus, unlike regakine-1, the CXCR2 agonist NAP-2 and the CC chemokine HCC-1, to which no neutrophil chemotactic activity has been ascribed, did not synergize with C5a when combined at concentrations present in the circulation.

The CC Chemokines MCP-3 and MIP-1α/LD78β Lack Synergistic Activity with Neutrophil Chemoattractants, Including Regakine-1. To obtain further evidence for the specificity of the synergy observed with regakine-1, experiments were performed with MCP-3, another CC chemokine with weak neutrophil chemotactic potency (Xu et al., 1995). Table 4 shows that, in contrast to regakine-1 (Fig. 3), biologically active MCP-3 (30 and 100 ng/ml) did not enhance the neutrophil chemotactic index of C5a (10 and 30 ng/ml). In addition, MCP-3 also failed to synergize (Table 4) with fMLP (10 nM) or IL-8 (5 ng/ml), whereas the latter cooperates with regakine-1 in neutrophil chemotaxis (Fig. 2 and 3). These data further point to the unique capacity of regakine-1 to synergize with other neutrophil chemoattractants.

To verify cooperation of regakine-1 with other neutrophil-attracting CC chemokines, regakine-1 was combined with MCP-3 and MIP-1α/LD78β (Table 5). It was found that the low neutrophil chemotactic index obtained with MCP-3 (30 and 100 ng/ml) or MIP-1α/LD78β (30 and 100 ng/ml) was not augmented in a synergistic way in the presence of active regakine-1 concentrations (30 and 100 ng/ml). Instead additive effects were observed between MCP-3 or MIP-1α and

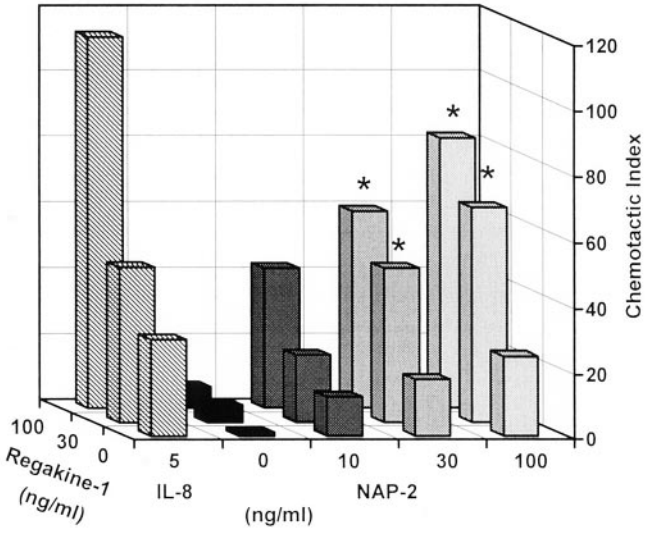

Fig. 2. Regakine-1 synergizes with the CXC chemokine NAP-2 in neutrophil chemotaxis. NAP-2 (▨) and IL-8 (▧) were combined with different concentrations of regakine-1 in the lower compartment of the microchamber to measure human neutrophil chemotaxis. The chemotactic response is expressed as the mean CI ± S.E.M., derived from two to five (NAP-2) or two to seven (IL-8) independent experiments. On average, the S.E.M. did not exceed 30% of the mean CI and is not shown for clarity. Statistically significant differences in chemotactic indexes between NAP-2 alone or in combination with regakine-1, determined by the Mann-Whitney test, are indicated by ⋆ ($p < 0.05$).

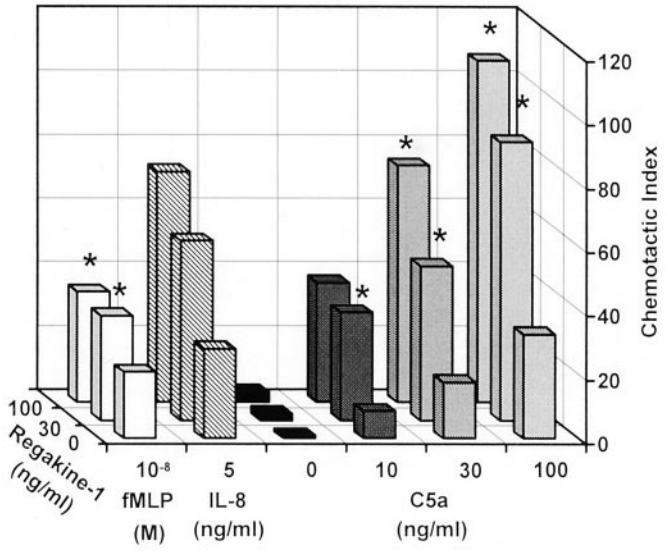

Fig. 3. Regakine-1 cooperates with C5a in neutrophil chemotaxis. C5a (▨), fMLP (□) or IL-8 (▧) were combined with different concentrations of regakine-1 in the lower compartment of the microchamber to measure human neutrophil chemotaxis. The chemotactic response is expressed as the mean CI ± S.E.M., derived from four independent experiments. On average, the S.E.M. did not exceed 30% of the mean CI and is not shown for clarity. Statistically significant differences in chemotactic indexes between C5a, fMLP, or IL-8 alone or combined with regakine-1, determined by the Mann-Whitney test, are indicated by ⋆ ($p < 0.05$).

TABLE 2

Lack of C5a receptor desensitization by regakine-1

Regakine-1, C5a, or both were added to the lower compartment of the microchamber to measure human neutrophil chemotaxis. Human neutrophils were added to the upper compartment of the microchamber, either directly with regakine-1 (no preincubation) or after 10 min of preincubation (at 37° C) and subsequent removal of regakine-1 (by washing twice). C5a or buffer was always added to the lower compartment of the microchamber. The results are expressed as the mean CI ± S.E.M., derived from three (lower wells) or six (upper wells) independent experiments. Statistically significant differences between C5a alone or in combination with regakine-1 were determined by the Mann-Whitney test.

		Migration of Neutrophils		
		Lower Wells (Chemotaxis)	Upper Wells	
Regakine-1	C5a		No Preincubation	Preincubation
ng/ml				
100	0	2.9 ± 1.2	2.1 ± 0.6	2.5 ± 0.6
0	100	14.9 ± 1.1		
100	100	22.2 ± 0.6*	24.1 ± 1.6**	23.8 ± 1.0**

* $p < 0.05$; ** $p < 0.01$.

regakine-1. This demonstrates that the synergistic effect of regakine-1 on neutrophils does not extend to other weak neutrophil-attracting CC chemokines, which can bind to CCR1, CCR2, CCR3, or CCR5. Because CCR1 has been shown to be functionally present on neutrophils (Crisman et al., 1999; Zhang et al., 1999) and mediates the effect of MIP-1α/LD78β (Struyf et al., 2001b), this receptor is probably not implicated in the synergistic action of regakine-1.

Regakine-1 Does Not Compete for fMLP or C5a Binding Sites on Neutrophils and Receptor Transfectants. Because regakine-1 synergized with both fMLP and C5a, it was verified whether regakine-1 acts on the receptors of these chemoattractants on neutrophils or receptor-transfected cell lines. Regakine-1, in contrast to unlabeled fMLP, did not displace [^{3}H]fMLP from RBL cells transfected with the high affinity fMLP receptor at concentrations as high as 750 ng/ml (Fig. 4, A and B). In addition, 1 nM of fMLP inhibited binding of [^{3}H]fMLP to freshly isolated neutrophils for more than 80%, whereas regakine-1 at 750 ng/ml did not (≤ 10% reduction) affect [^{3}H]fMLP binding (data not shown). Similarly, it was found that different concentrations of regakine-1 were unable to displace ^{125}I-C5a from neutrophils, whereas unlabeled recombinant C5a was dose-dependently preventing the binding of ^{125}I-C5a (Fig. 4, C and D).

The Synergistic Activity of Regakine-1 Does Not Reside in Its NH_2-Terminal Region. For many CXC and CC chemokines, the NH_2-terminal region determines their binding capacity to G protein-coupled receptors. Post-translational processing of chemokines by proteases resulted in either reduced (e.g., MCP-1, MCP-2, eotaxin) or enhanced (IL-8, NAP-2, HCC-1, MIP-1α/LD78β) chemotactic potencies (Detheux et al., 2000; Van Damme et al., 1999; Van den Steen et al., 2000; Struyf et al., 2001a). Differently truncated isoforms of regakine-1 lacking two, four, or eight residues at the NH_2 terminus were therefore chemically synthesized and compared for their synergistic effect with C5a on neutrophil

TABLE 3

Regakine-1, but not other plasma chemokines (HCC-1, NAP-2, CTAP-III), synergizes with C5a in neutrophil chemotaxis

HCC-1, NAP-2 or CTAP-III were combined with buffer or 30 ng/ml of C5a in the lower compartment of the microchamber to measure human neutrophil chemotaxis. The chemotactic response is expressed as the CI ± S.E.M., derived from 3 independent experiments.

Chemokine	C5a	CI ± S.E.M.
	ng/ml	
Buffer	30	25.4 ± 11.6
HCC-1 (30)	0	1.0 ± 0.0
HCC-1 (300)	0	1.2 ± 0.2
NAP-2 (30)	0	9.3 ± 1.0
CTAP-III (15)	0	1.0 ± 0.0
HCC-1 (30)	30	27.4 ± 15.5
HCC-1 (300)	30	13.6 ± 9.3
NAP-2 (30)	30	27.2 ± 3.4
CTAP-III (15)	30	31.7 ± 14.8

TABLE 4

MCP-3 does not synergize with IL-8, C5a, and fMLP to chemoattract neutrophils

IL-8, C5a, or fMLP were combined with different concentrations of MCP-3 or buffer in the lower compartment of the microchamber to measure human neutrophil chemotaxis. The chemotactic response is expressed as the mean CI ± S.E.M., derived from three or four (*n*) independent experiments.

Chemoattractant	MCP-3	CI ± S.E.M.
	ng/ml	
Buffer	30	2.8 ± 0.6 (4)
Buffer	100	2.5 ± 1.3 (4)
IL-8 (5 ng/ml)	0	8.5 ± 1.3 (4)
IL-8 (5 ng/ml)	30	11.7 ± 2.2 (4)
fMLP (10 nM)	0	10.7 ± 0.8 (3)
fMLP (10 nM)	30	12.8 ± 0.9 (3)
fMLP (10 nM)	100	12.1 ± 0.1 (3)
C5a (10 ng/ml)	0	9.9 ± 3.2 (3)
C5a (10 ng/ml)	30	9.9 ± 1.0 (3)
C5a (10 ng/ml)	100	11.3 ± 1.7 (3)
C5a (30 ng/ml)	0	20.8 ± 5.8 (3)
C5a (30 ng/ml)	30	19.6 ± 2.7 (3)
C5a (30 ng/ml)	100	22.0 ± 4.3 (3)

TABLE 5

Regakine-1 fails to synergize with other neutrophil chemoattracting CC chemokines in neutrophil chemotaxis

MCP-3 or MIP-1α/LD78β were combined with different concentrations of regakine-1 in the lower compartment of the microchamber to measure human neutrophil chemotaxis. The chemotactic response is expressed as the mean CI ± S.E.M., derived from four independent experiments.

Chemokine	Regakine-1	CI ± S.E.M.
	ng/ml	
Buffer	30	5.0 ± 1.0
Buffer	100	5.5 ± 2.4
MCP-3 (30)	0	2.0 ± 0.7
MCP-3 (30)	30	3.9 ± 1.2
MCP-3 (30)	100	5.3 ± 1.5
MCP-3 (100)	0	2.3 ± 0.4
MCP-3 (100)	30	5.2 ± 1.8
MCP-3 (100)	100	7.3 ± 4.4
MIP-1α/LD78β (30)	0	2.3 ± 0.6
MIP-1α/LD78β (30)	30	4.7 ± 1.1
MIP-1α/LD78β (30)	100	6.1 ± 1.2
MIP-1α/LD78β (100)	0	1.9 ± 0.3
MIP-1α/LD78β (100)	30	2.7 ± 4.3
MIP-1α/LD78β (100)	100	6.8 ± 1.9

chemotaxis. It was found that regardless of the length of their NH_2-terminal region, all regakine-1 isoforms were equipotent at enhancing the chemotactic response of neutrophils to C5a (Fig. 5). For comparison, it was shown (Table 3) that both NAP-2 and its precursor CTAP-III did not synergize with C5a, whereas NAP-2 did cooperate with regakine-1 to chemoattract neutrophils (Fig. 2)

The Effect of Protein Kinase Inhibitors on the Synergy between C5a and Regakine-1. To determine whether protein kinases mediate the chemotactic activity of regakine-1 and its enhancing effect on neutrophil responses to C5a, neutrophils were treated with different concentrations of either PD98059 (a p44/42 mitogen-activated protein kinase inhibitor), wortmannin (a phosphatidylinositol-3-kinase inhibitor) or staurosporin (a protein kinase A and C inhibitor) before transfer to the upper wells of the Boyden chamber. Figure 6 illustrates that the chemotactic response to 30 ng/ml C5a or 30 ng/ml C5a in combination with 100 ng/ml regakine-1 was not inhibited by any of these inhibitors. Nevertheless, a significant inhibition of 5 ng/ml IL-8–induced chemotaxis was observed with 50 μM PD98059. On the other hand, wortmannin and staurosporin (100 nM) did not inhibit IL-8–induced neutrophil chemotaxis.

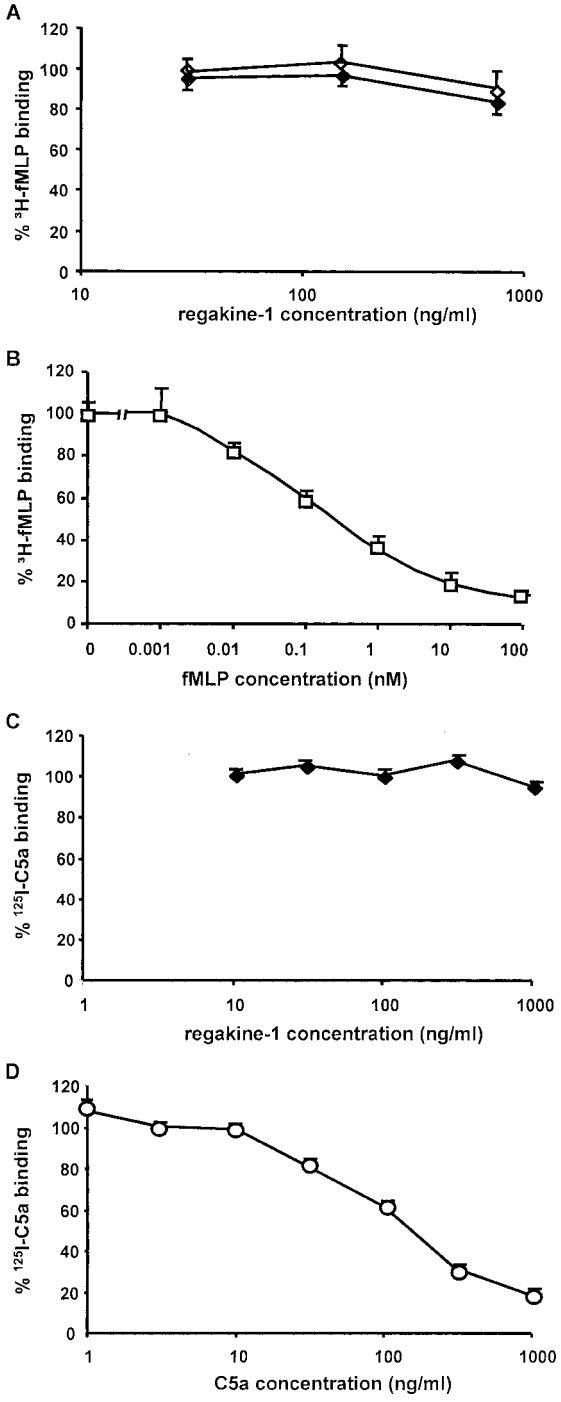

Fig. 4. Regakine-1 fails to compete for [^{3}H]fMLP binding or ^{125}I-C5a binding. RBL cells transfected with the high affinity fMLP receptor were incubated with 30 nM [^{3}H]fMLP and varying concentrations of regakine-1 (A: ♦, natural regakine-1; ◇, synthetic regakine-1) or unlabeled fMLP (B: □). Results are expressed as the percentage of [^{3}H]fMLP specific binding (mean ± S.E.M. of two to four independent experiments). Neutrophils were incubated with 0.04 nM ^{125}I-C5a and different concentrations of natural regakine-1 (C: ♦) or unlabeled recombinant C5a (D: ○). Results are expressed as the percentage of ^{125}I-C5a specific binding (mean ± S.E.M. of two to five independent experiments).

Discussion

Within the family of chemotactic cytokines, the classification as CXC or CC chemokine is based on biochemical structure rather than on functional properties. Consequently, inducible inflammatory and constitutive homeostatic chemokines belong to both the CXC and CC subfamilies, defined by the positioning of conserved cysteine residues in their sequence. The corresponding receptors were designated CXCR and CCR. Several of these receptors have multiple ligands, and one chemokine is often capable of binding to different receptors (Wuyts et al., 1999; Murphy et al., 2000; Rossi and Zlotnik, 2000). Only a few chemokines are constitutively present in normal plasma. These include the platelet-derived CXC chemokines platelet factor-4 and β-thromboglobulin, known for more than 2 decades (Deuel et al., 1977; Begg et al., 1978), and the recently discovered CC

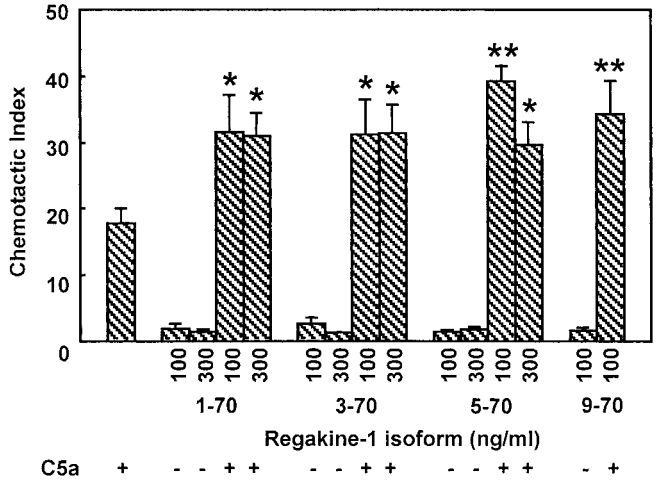

Fig. 5. Synergistic effect between NH_2-terminally truncated forms of regakine-1 and C5a. Synthetic intact regakine-1(1–70) and the truncated isoforms regakine-1(3–70), regakine-1(5–70) and regakine-1(9–70), missing two, four, or eight amino acids, respectively, at the NH_2 terminus, were combined with C5a (30 ng/ml) in the microchamber assay to measure neutrophil chemotaxis. Results represent the mean CI (± S.E.M.) of six or seven independent experiments. Statistically significant differences in chemotactic indexes between C5a alone or combined with regakine-1, determined by the Mann-Whitney test, are indicated by ⋆ ($p < 0.05$) and ⋆⋆ ($p < 0.01$).

chemokines HCC-1 and regakine-1 (Schulz-Knappe et al., 1996; Struyf et al., 2001b). For platelet factor-4 and regakine-1, no receptors have been identified, whereas for β-thromboglobulin and HCC-1, post-translational processing by proteases is essential to become functionally active as the CXCR2 ligand NAP-2 (Walz et al., 1989) and the CCR1, -3, -5 ligand HCC-1(9–74) (Detheux et al., 2000), respectively. Such increase in biological potency by NH_2-terminal processing is a common phenomenon for other CXCR2 ligands [e.g., IL-8 after cleavage by gelatinase B (Van den Steen et al., 2000)], as well as for other CCR1 ligands such as MIP-1α/LD78β (Struyf et al., 2001a) after processing by CD26/dipeptidyl peptidase IV (CD26/DPP IV). However, for most CC chemokines, removal of the NH_2-terminal dipeptide by CD26/DPP IV results in a partial or complete loss of receptor recognition and hence biological activity (Van Damme et al., 1999).

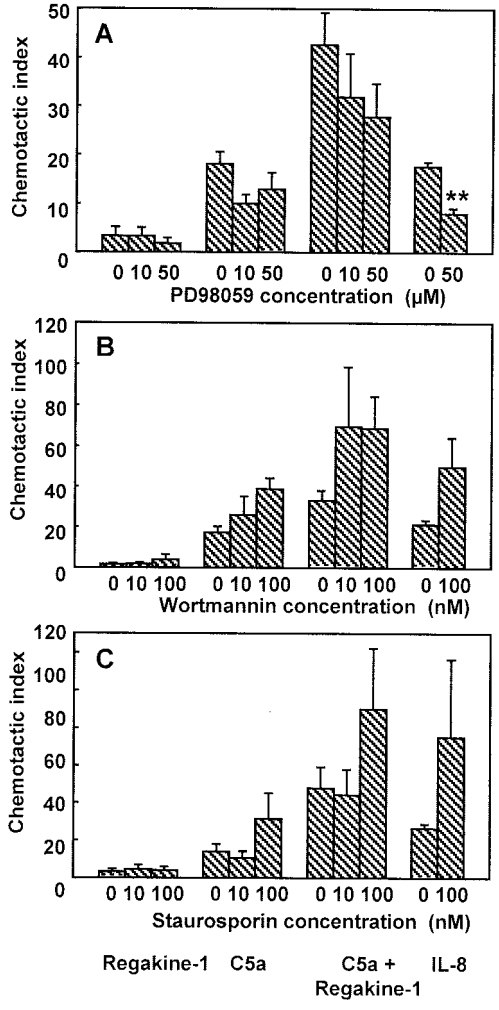

Fig. 6. The synergy between regakine-1 and C5a in neutrophil chemotaxis is not affected by protein kinase inhibitors. Regakine-1, C5a, a combination of both, or IL-8 was added to the lower compartment of the chemotaxis microchamber. The combination of C5a and regakine-1 yielded a statistically significant synergy in neutrophil chemotaxis (not indicated). Neutrophils were treated with PD98059 at 10 and 50 μM (A; $n = 5$), wortmannin at 10 and 100 nM (B; $n = 9$), staurosporin at 10 and 100 nM (C; $n = 8$), or were left untreated before transfer to the upper wells of the microchamber. The chemotactic indexes were calculated using the appropriate controls (individual protein kinase inhibitor treated versus untreated control cells) and are expressed as the mean CI ± S.E.M. Statistically significant differences between chemotactic responses in the presence or absence of protein kinase inhibitors were calculated with the Mann-Whitney test and are indicated by ⋆⋆ ($p < 0.01$).

In this study, we have investigated the impact of NH_2-terminal truncation of regakine-1 on its unique property to attract neutrophils and to synergize with other proinflammatory mediators. Indeed, it was found that at a physiological concentration (30 ng/ml), regakine-1 synergized with the selective CXCR2 agonist NAP-2. Furthermore, significantly enhanced neutrophil chemotaxis was observed when regakine-1 was combined with the classical chemoattractant C5a at a concentration (30 ng/ml) detected in the blood circulation during an inflammatory response (Hogasen et al., 1995). Deletion of the two, four, or eight NH_2-terminal residues of regakine-1 (in front of the CC motif) neither enhanced the neutrophil chemotactic potency of regakine-1 nor altered its synergy with C5a. The relevance of this synergistic effect between regakine-1 and other chemoattractants binding to G protein-coupled receptors, including also the bacterial peptide fMLP, was endorsed by the finding that other CC chemokines with neutrophil chemotactic activity, such as MCP-3, did not possess the characteristic of regakine-1 to cooperate with C5a, fMLP, or IL-8 on neutrophils. The weak neutrophil agonists (MCP-3, MIP-1α/LD78β) did also not enhance the neutrophil chemotactic potency of regakine-1. Furthermore, other CXC (CTAP-III) and CC (intact HCC-1) plasma chemokines with poor chemokine receptor binding capacity and hence chemotactic activity failed to synergize with regakine-1.

It is expected that the synergy between regakine-1 and IL-8, NAP-2, C5a, or fMLP is dependent on the binding of these latter agonists to their known G protein-coupled receptors. To unravel whether the synergy depended on regakine-1 binding to the same receptor(s), binding competition experiments were executed. It was found that regakine-1 was unable to displace labeled C5a or fMLP from binding to neutrophils or cells transfected with the corresponding receptor. Furthermore, in an attempt to desensitize neutrophils by pretreatment with regakine-1, it was found that no inhibition of the calcium flux and chemotaxis induced by other chemokines, fMLP or C5a, could be obtained. Moreover, regakine-1 on its own was unable to induce a calcium signal. In this respect, regakine-1 behaved differently than other CC chemokines such as MIP-1α, which induced a weak but consistent calcium flux in neutrophils (McColl et al., 1993). This effect was even more pronounced after NH_2-terminal truncation of MIP-1α/LD78β by CD26/DPP IV (Struyf et al., 2001a). Different biological responses were observed with leukotactin-1, another CC chemokine that chemoattracts neutrophils. In contrast to regakine-1 and MIP-1α, leukotactin-1 induced a potent calcium signal in neutrophils despite the fact that it shares CCR1, the functional receptor on these cells, with MIP-1α/LD78β (Zhang et al., 1999). The lack of calcium signaling observed with regakine-1 is not unique, in that other CC chemokines and NH_2-terminally truncated chemokine isoforms that still bind to their receptor have been reported to induce weak or marginal calcium signals (Pettit and Fay, 1998; Blanpain et al., 1999; Van Damme et al., 1999). These NH_2-terminally processed chemokines are devoid of any chemotactic activity and function as natural inhibitors of the intact chemokines. Intact regakine-1 induced the release of gelatinase B from neutrophils and enhanced the shape change induced by C5a, confirming the role of regakine-1 in inflammation (data not shown). Finally, protein kinase inhibitors, such as PD98059, wortmannin, and staurosporin, had no effect on the synergy between regakine-1 and C5a. In all probability, protein

kinase signaling is not the major pathway mediating the synergy between regakine-1 and C5a. However, the literature on the effect of protein kinase inhibitors in neutrophil chemotaxis is rather contradictory (Thelen et al., 1995; Knall et al., 1997; Zu et al., 1998; Nagata et al., 2001).

Taken together, our findings demonstrate that regakine-1, a CC chemokine constitutively present in plasma, but not other plasma or tissue chemokines, synergizes with various unrelated chemoattractants that bind G protein-coupled receptors. This is indicative of its unique role as amplifier of the inflammatory response. Synergistic effects in vitro and in vivo have been described between functionally related cytokines such as IL-1 and tumor necrosis factor-α (Movat et al., 1987; Okusawa et al., 1988) and for IL-1 and interferon-γ (Struyf et al., 1998). Although for these cytokines, enhanced production of second messengers, including chemokines, is implicated, the precise molecular mechanisms remain partially unknown. For regakine-1, one can speculate that the synergy occurs at the extracellular level. The fact that regakine-1 does not interfere with binding to the G protein-coupled receptor of the cooperative chemoattractants implies a separate regakine-1 receptor. In addition, because chemokine receptor heterodimerization (e.g., between CCR2 and CCR5) has been demonstrated to increase the sensitivity and dynamics of the chemokine response (Mellado et al., 2001), this phenomenon might also be applicable to regakine-1. The optimal concentrations for synergy between regakine-1 and C5a are physiological, which suggests that this mechanism effectively occurs in the in vivo situation. Blockade of regakine-1 in the plasma can therefore be considered to downmodulate systemic inflammatory diseases, such as septic shock.

Acknowledgments

We thank Dr. J. M. Wang, National Cancer Institute, for providing FPR/RBL cells and the members of the Laboratory of Clinical Immunology of the University of Leuven and the members of the Blood Transfusion Center of Antwerp for providing buffy coats. The technical assistance of R. Conings and the critical comments by Prof. G. Opdenakker are greatly appreciated.

References

Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, Buechi L, Walz A, Richmond A, and Strieter RM (2000) The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol* **165:**5269–5277.

Ali J, Richardson RM, Tomhave ED, Didsbury JR, and Snyderman R (1993) Differences in phosphorylation of formylpeptide and C5a chemoattractant receptors correlate with differences in desensitization. *J Biol Chem* **268:**24247–24254.

Begg GS, Pepper DS, Chesterman CN, and Morgan FJ (1978) Complete covalent structure of human β-thromboglobulin. *Biochemistry* **17:**1739–1744.

Blanpain C, Migeotte I, Lee B, Vakili J, Doranz BJ, Govaerts C, Vassart G, Doms RW, and Parmentier M (1999) CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. *Blood* **94:**1899–1905.

Crisman JM, Elder PJ, Wilkie NM, and Kolattukudy PE (1999) Identification of amino acids involved in the binding of hMIP-1α to CC-CKR1, a MIP-1α receptor found on neutrophils. *Mol Cell Biochem* **195:**245–256.

Detheux M, Ständker L, Vakili J, Münch J, Forssmann U, Adermann K, Pöhlmann S, Vassart G, Kirchhoff F, Parmentier M, et al. (2000) Natural proteolytic processing of hemofiltrate CC chemokine 1 generates a potent CC chemokine receptor CCR1 and CCR5 agonist with anti-HIV properties. *J Exp Med* **192:**1501–1508.

Deuel TF, Keim PS, Farmer M, and Heinrikson RL (1977) Amino acid sequence of human platelet factor 4. *Proc Natl Acad Sci USA* **74:**2256–2258.

Devalaraja RM, Nanney LB, Du J, Qian Q, Yu Y, Devalaraja MN, and Richmond A (2000) Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol* **115:** 234–344.

Gerard NP and Gerard C (1991) The chemotactic receptor for human C5a anaphylatoxin. *Nature (Lond)* **349:**614–617.

Hogasen K, Mollnes TE, Harboe M, Gotze O, Hammer HB, and Oppermann M (1995) Terminal complement pathway activation and low lysis inhibitors in rheumatoid arthritis synovial fluid. *J Rheumatol* **22:**24–28.

Knall C, Worthen GS, and Johnson GL (1997) Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc Natl Acad Sci USA* **94:**3052–3057.

Le Y, Gong W, Li B, Dunlop NM, Shen W, Su SB, Ye RD, and Wang JM (1999) Utilization of two seven-transmembrane, G-protein-coupled receptors, formyl peptide receptor-like 1 and formyl peptide receptor, by the synthetic hexapeptide WKYMVm for human phagocyte activation. *J Immunol* **163:**6777–6784.

McColl SR, Hachicha M, Levasseur S, Neote K, and Schall TJ (1993) Uncoupling of early signal transduction events from effector function in human peripheral blood neutrophils in response to recombinant macrophage inflammatory proteins-1α and -1β. *J Immunol* **150:**4550–4560.

Mellado M, Rodriguez-Frade JM, Vila-Coro AJ, Fernandez S, Martin de Ana A, Jones DR, Toran JL, and Martinez-A C (2001) Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *EMBO (Eur Mol Biol Organ) J* **20:**2497–2507.

Movat HZ, Burrowes CE, Cybulsky MI, and Dinarello CA (1987) Acute inflammation and a Shwartzman-like reaction induced by interleukin-1 and tumor necrosis factor. Synergistic action of the cytokines in the induction of inflammation and microvascular injury. *Am J Pathol* **129:**463–476.

Murphy PM, Baggiolini M, Charo IF, Hébert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, and Power CA (2000) International Union of Pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol, Rev* **52:**145–176.

Nagata T, Kansha M, Irita K, and Takahashi S (2001) Propofol inhibits fMLP-stimulated phosphorylation of p42 mitogen-activated protein kinase and chemotaxis in human neutrophils. *Br J Anaesth* **86:**853–858.

Okusawa S, Gelfand JA, Ikejima T, Connolly RJ, and Dinarello CA (1988) Interleukin-1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *J Clin Invest* **81:**1162–1172.

Pettit EJ and Fay FS (1998) Cytosolic free calcium and the cytoskeleton in the control of leukocyte chemotaxis. *Physiol Rev* **78:**949–967.

Rossi D and Zlotnik A (2000) The biology of chemokines and their receptors. *Annu Rev Immunol* **18:**217–242.

Schulz-Knappe P, Magert HJ, Dewald B, Meyer M, Cetin Y, Kubbies M, Tomeczkowski J, Kirchhoff K, Raida M, Adermann K, et al. (1996) HCC-1, a novel chemokine from human plasma. *J Exp Med* **183:**295–299.

Struyf S, Menten P, Lenaerts JP, Put W, D'Haese A, De Clercq E, Schols D, Proost P, and Van Damme J (2001a) Diverging binding capacities of natural LD78β isoforms of macrophage inflammatory protein-1α to the CC chemokine receptors 1, 3 and 5 affect their anti-HIV-1 activity and chemotactic potencies for neutrophils and eosinophils. *Eur J Immunol* **31:**2170–2178.

Struyf S, Proost P, Lenaerts JP, Stoops G, Wuyts A, and Van Damme J (2001b) Identification of a blood-derived chemoattractant for neutrophils and lymphocytes as a novel CC chemokine, regakine-1. *Blood* **97:**2197–2204.

Struyf S, Van Coillie E, Paemen L, Put W, Lenaerts JP, Proost P, Opdenakker G, and Van Damme J (1998) Synergistic induction of MCP-1 and -2 by IL-1β and interferons in fibroblasts and epithelial cells. *J Leukocyte Biol* **63:**364–372.

Thelen M, Uguccioni M, and Bosiger J (1995) PI3-kinase-dependent and independent chemotaxis of human neutrophil leukocytes. *Biochem Biophys Res Commun* **217:** 1255–1262.

Van Damme J, Struyf S, Wuyts A, Van Coillie E, Menten P, Schols D, Sozzani S, De Meester I, and Proost P (1999) The role of CD26/DPP IV in chemokine processing. *Chem Immunol* **72:**42–56.

Van Damme J, Van Beeumen J, Conings R, Decock B, and Billiau A (1989) Purification of granulocyte chemotactic peptide/interleukin-8 reveals N-terminal sequence heterogeneity similar to that of β-thromboglobulin. *Eur J Biochem* **181:** 337–344.

Van den Steen PE, Proost P, Wuyts A, Van Damme J, and Opdenakker G (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4 and GRO-α and leaves RANTES and MCP-2 intact. *Blood* **96:**2673–2681.

Walz A, Dewald B, von Tscharner V, and Baggiolini M (1989) Effects of the neutrophil-activating peptide NAP-2, platelet basic protein, connective tissue-activating peptide III and platelet factor 4 on human neutrophils. *J Exp Med* **170:**1745–1750.

Wuyts A, Struyf S, Proost P, and Van Damme J (1999) Chemokines, in *The Cytokine Network and Immune Functions* (Thèze J ed) pp 125–145, Oxford University Press, Oxford.

Xu LL, McVicar DW, Ben-Baruch A, Kuhns DB, Johnston J, Oppenheim JJ, and Wang JM (1995) Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors: binding and signaling of MCP3 through shared as well as unique receptors on monocytes and neutrophils. *Eur J Immunol* **25:** 2612–2617.

Zhang S, Youn BS, Gao JL, Murphy PM, and Kwon BS (1999) Differential effects of leukotactin-1 and macrophage inflammatory protein-1α on neutrophils mediated by CCR1. *J Immunol* **162:**4938–4942.

Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, Huang CK, Sha'afi RI (1998) p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or fMLP stimulation. *J Immunol* **160:**1982–1989.

Address correspondence to: Prof. Jo Van Damme, Laboratory of Molecular Immunology Rega Institute, Minderbroedersstraat 10 B-3000 Leuven, Belgium. E-mail: jozef.vandamme@rega.kuleuven.ac.be