Biochemical and Biological Characterization of Neutrophil Chemotactic Protein, a Novel Rabbit CXC Chemokine from Alveolar Macrophages†

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Received May 17, 2000; Revised Manuscript Received September 12, 2000

ABSTRACT: The role of interleukin-8 (IL-8) and related CXC chemokines has been demonstrated in many human diseases. However, more profound studies, e.g., by blocking the effect of these inflammatory mediators, request animal models and hence the identification of all human counterparts for commonly used laboratory animals. In this study, we describe the identification of a novel neutrophil chemotactic protein (NCP) of the rabbit. Intact and NH_2-terminally truncated NCP forms and IL-8 were isolated from LPS-stimulated rabbit alveolar macrophages and purified to homogeneity by a four-step purification procedure. Determination of the complete primary structure of NCP by mass spectrometry and NH_2-terminal sequencing of natural protein revealed high structural homology with human epithelial cell-derived neutrophil attractant-78 (ENA-78) and granulocyte chemotactic protein-2 (GCP-2), two related ELR^+CXC chemokines. Intact NCP(1-76) was found to be 10-fold less potent than truncated NCP(7,8-76) at inducing neutrophil chemotaxis. NCP(7,8-76) was equally potent as intact rabbit IL-8 at chemoattracting human neutrophils and at inducing calcium fluxes in rabbit neutrophils, 1 ng/mL being the minimal effective concentration. However, like IL-8, NCP failed to induce monocyte or eosinophil migration at 300-fold higher concentrations. IL-8 desensitized the calcium increase induced by NCP and vice versa. Finally, intradermal injection of NCP induced a dose-dependent and significant infiltration of neutrophils in mice skin. It can be concluded that NCP is a novel rabbit CXC chemokine that is, like IL-8, implicated in animal models used to study various human disorders in which neutrophils play an important role.

The migration of leukocytes from the blood vessel into inflamed tissues is essential for host defense. Leukocytes are attracted to inflammatory sites by locally produced chemotactic cytokines or chemokines. This protein family can be divided into four subclasses depending on the positioning of conserved cysteine residues (CXC, CC, C, and CX_3C chemokine subfamily) (*1*−*4*). CXC chemokines that contain the sequence Glu-Leu-Arg (ELR)[1] just in front of the first cysteine (ELR^+CXC chemokines) mainly attract neutrophils. In addition, these ELR^+CXC chemokines have angiogenic properties (*5*). In human, seven ELR^+CXC chemokines [interleukin-8 (IL-8); neutrophil-activating protein-2 (NAP-2); GROα, *β*, and *γ*; epithelial cell-derived neutrophil attractant-78 (ENA-78); and granulocyte chemotactic protein-2 (GCP-2)] and two receptors for these chemokines [CXC chemokine receptor 1 (CXCR1) and CXCR2] have been identified (*6*−*8*). Whereas IL-8 and GCP-2 can use both receptors to activate cells efficiently, the other ELR^+CXC chemokines are only efficacious ligands for CXCR2 (*9*, *10*).

The presence of chemokines in inflammatory and infectious diseases has been studied in great detail. ELR^+CXC chemokines have been detected in body fluids and tissues in a variety of pathological situations (*6*). For example, increased urinary IL-8 levels are present during glomerular diseases (*11*). Synovial fluid and plasma of rheumatoid arthritis patients contain IL-8, ENA-78, and GROα (*12*−*15*). High levels of IL-8 are found in bronchoalveolar lavage fluid of patients with the acute respiratory distress syndrome (ARDS) (*16*, *17*).

To further elucidate the pathophysiological role of ELR^+CXC chemokines, rabbit models are more appropriate than mouse models. Whereas in all probability IL-8 does not exist and only one IL-8 receptor [IL-8 receptor homo-

† This work was supported by the Fund for Scientific Research of Flanders, the Concerted Research Actions of the Regional Government of Flanders, the InterUniversity Attraction Pole Initiative of the Belgian Federal Government, and the Biomed Program of the European Community. P.M. and S.S. are research assistants and P.P. is a senior research assistant of the Fund for Scientific Research of Flanders.

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[1] Abbreviations: ARDS, acute respiratory distress syndrome; $[Ca^{2+}]_i$, intracellular calcium concentration; CI, chemotactic index; CPG, controlled pore glass; CXCR, CXC chemokine receptor; ELR, Glu-Leu-Arg; ENA-78, epithelial cell-derived neutrophil attractant-78; FCS, fetal calf serum; GCP-2, granulocyte chemotactic protein-2; HBSS, Hanks' balanced salt solution; IL-8, interleukin-8; LPS, lipopolysaccharide; NAP-2, neutrophil-activating protein-2; NCP, neutrophil chemotactic protein; PBS, phosphate-buffered saline; PF-4, platelet factor-4; RP-HPLC, reversed phase-high performance liquid chromatography.

10.1021/bi0011227 CCC: $19.00
Published on Web 11/02/2000

logue (IL-8Rh)] is known in the mouse (*18*), the rabbit homologues of human IL-8 and the two IL-8 receptors have been identified (*19*, *20*). Using rabbit models, the role of IL-8 has been studied in several pathologies, e.g., glomerulonephritis (*21*), ischemia-reperfusion-induced injury (*22*, *23*), rheumatoid arthritis (*24*), and ARDS (*25*, *26*). To evaluate other chemokines during pathological conditions in a rabbit model, the homologues of the human chemokines have to be identified. Indeed, in rabbit, only the ELR$^+$CXC chemokines IL-8 and GRO are known. Rabbit permeability factor 1 (RPF1) or IL-8 and RPF2 or GROα were first isolated from inflammatory exudate as oedema-inducing and neutrophil chemotactic factors (*19*, *27*). The complete amino acid sequences of rabbit IL-8 and GROα were revealed by their cDNA cloning from concanavalin A-stimulated spleen cells and lipopolysaccharide (LPS)-stimulated alveolar macrophages, respectively (*28*−*30*). Rabbit GROβ was identified by cloning of the cDNA from LPS-stimulated alveolar macrophages (*29*). Rabbit GROβ chemoattracts rabbit neutrophils both in vitro and in vivo and is detected in the bronchoalveolar lavage fluid of rabbits with bacterial pneumonia (*31*). The cDNA of rabbit GROγ was cloned from aortic endothelial cells treated with minimally modified low-density lipoprotein (MM-LDL) (*32*). This protein plays a role in monocyte binding to MM-LDL-stimulated endothelial cells (*32*). Similar to the human situation, two rabbit IL-8 receptors have been cloned: one receptor that binds human and rabbit IL-8, but not human GROα, NAP-2, or platelet factor-4 (PF-4) (*33*−*36*) and a second receptor that binds the human chemokines IL-8, NAP-2, and GROα (*20*).

In this study, we describe the purification and identification of a novel rabbit ELR$^+$CXC chemokine from conditioned medium of stimulated alveolar macrophages. Determination of the primary structure of the protein revealed that this novel chemokine shows highest homology with human ENA-78 and GCP-2. The activities of different isoforms of this novel chemokine were compared with those of natural rabbit IL-8 both in vitro and in vivo.

MATERIALS AND METHODS

Production and Purification of Rabbit Chemokines. Alveolar macrophages were isolated from rabbit lungs by rinsing with saline. The macrophages were washed and resuspended at 10^6 cells/mL in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) containing 10% fetal calf serum (FCS; Life Technologies, Paisley, Scotland) and supplemented with LPS at 10 μg/mL for 24 h. Chemokines present in the conditioned medium (4.2 L) were first concentrated and partially purified by adsorption to controlled pore glass beads (CPG-10-350; Serva, Heidelberg, Germany) (*37*). After dialysis against 50 mM Tris-HCl and 50 mM NaCl, pH 7.4, the CPG eluate was loaded onto a heparin-Sepharose column (40 × 1.6 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), and proteins were eluted with a linear NaCl gradient (0.05−2 M; 20 mL/h; 5-mL fractions). The protein concentration in the fractions was determined by a Coomassie blue G-250 binding assay (*38*) using the Bio-Rad commercial kit (Bio-Rad Laboratories, Hercules, CA). Fractions containing neutrophil chemotactic activity were dialyzed against 50 mM formate and 0.01% Tween 20, pH 4.0, and loaded on a Mono S cation-exchange column (50 × 5 mm; Amersham Pharmacia Biotech). Proteins were eluted with a linear NaCl gradient (0−1 M; 1 mL/min; 1-mL fractions), and absorbance at 220 nm was measured as a parameter for protein concentration. Finally, neutrophil chemotactic proteins were purified to homogeneity by reversed phase-high performance liquid chromatography (RP-HPLC) on a 220 × 2.1 mm C_8 Aquapore RP-300 column (PE Biosystems, Foster City, CA) equilibrated with 0.1% trifluoroacetic acid in water. Proteins were eluted with an acetonitrile gradient (0−80%; 0.4 mL/min; 0.4-mL fractions), and absorbance at 220 nm was measured (*37*).

Purified proteins were analyzed for relative molecular mass and purity by SDS−PAGE under reducing conditions on Tris/tricine gels (*39*). The relative molecular mass markers used were carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400) (Bio-Rad Laboratories) and the low molecular mass marker aprotinin (M_r 6500) (Pierce Chemical Co., Rockford, IL).

ELISA. Rabbit IL-8 was detected with a classical sandwich ELISA. Polyclonal anti-rabbit IL-8, prepared by repeated injection of rabbit IL-8 in goat, was used as coating antibody. Monoclonal mouse anti-human IL-8 (R&D Systems, Abingdon, UK), which cross-reacts with rabbit IL-8, was used as capturing antibody. Rabbit IL-8, purified from conditioned medium of stimulated alveolar macrophages, was used as standard. The detection was performed with peroxidase-labeled goat anti-mouse mAb and 3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate (TMB; Aldrich Chemical Co., Milwaukee, WI). The detection limit for IL-8 was 0.5 ng/mL.

Amino Acid Sequence Analysis of Rabbit Chemokines. The NH_2-terminal amino acid sequence of pure proteins was determined by Edman degradation on a pulsed liquid-phase protein sequencer (477A/120A; PE Biosystems) with on-line detection of phenylthiohydantoin amino acids (*37*). For internal sequencing, pure protein was enzymatically digested by incubation for 18 h with endoproteinase Lys-C (37 °C, 25 mM Tris-HCl buffer, pH 8.5, and 1 mM EDTA; Boehringer Mannheim, Mannheim, Germany) or with endoproteinase Glu-C (25 °C, 25 mM ammonium carbonate buffer, pH 7.8; Boehringer Mannheim) at an enzyme/substrate ratio of 1/20. Proteolytic fragments were separated by RP-HPLC on a C_8 Aquapore RP-300 column (50 × 1 mm, PE Biosystems) and sequenced. Alternatively, protein was chemically digested with 75% formic acid at 37 °C for 50 h before Edman degradation. To obtain only the sequence of the COOH-terminal part, the original NH_2-terminus of the protein was blocked with *o*-phthalaldehyde (Fluoropa; Pierce Chemical Co.). The presence of cysteine residues was obvious from the absence of any detectable signal (*37*) and was confirmed by mass spectrometry.

Mass Spectrometry. The molecular mass of proteins or proteolytic fragments was determined on an electrospray ion trap mass spectrometer (Esquire; Bruker Daltonik, Bremen, Germany). RP-HPLC-purified proteins were diluted 10-fold in methanol/water (1/1) including 0.1% acetic acid and applied to the mass spectrometer by direct infusion at a flow rate of 4 μL/min. Average relative molecular masses were calculated from the summation of 100 spectra, resulting in an accuracy of ±1.0 mass unit for chemokines. Partial sequences of proteolytic fragments were obtained after MS/MS analysis.

Cell Preparation. Human neutrophils were isolated from fresh heparinized peripheral blood from single human donors. Rabbit neutrophils were isolated from heparinized peripheral blood from single blood donations (chemotaxis) or from blood, obtained by cardiac punctures from anesthesized rabbits (calcium assay). Mononuclear cells were separated from the granulocytes and erythrocytes by gradient centrifugation on Ficoll-sodium diatrizoate (Lymphoprep; Life Technologies) or on Nycodenz solution (Nycoprep; Life Technologies) for human and rabbit leukocytes, respectively. The pellet containing erythrocytes and granulocytes was mixed with 1 vol of phosphate-buffered saline (PBS; Life Technologies) and 1 vol of hydroxyethyl starch solution (Plasmasteril; Fresenius AG, Bad Homburg, Germany) and incubated for 30 min at 37 °C to allow sedimentation of the erythrocytes. After centrifugation of the supernatant, the remaining erythrocytes in the granulocyte pellet were removed by lysis in bidistilled water for 30 s. Cells were washed and resuspended in Hanks' balanced salt solution (HBSS; Life Technologies) supplemented with human serum albumin (1 mg/mL) (chemotaxis) or in Eagle's minimum essential medium with Earle's salts (Life Technologies) supplemented with 2% FCS (calcium experiments) (*37*).

Human eosinophils were isolated from the granulocyte pellet after density gradient centrifugation of peripheral blood by incubation of the granulocytes for 30 min at 4 °C with paramagnetic microbeads conjugated with mAb against CD16 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cell suspension was passed over a column placed in a strong magnetic field (VarioMACS; Miltenyi Biotec GmbH). The cells that passed through the column were for more than 90% eosinophils.

Rabbit mononuclear cells were used for monocyte chemotaxis experiments. The mononuclear cell fraction, obtained after density gradient centrifugation, was washed and resuspended in HBSS supplemented with human serum albumin (1 mg/mL).

Chemotaxis. The chemotactic activity of rabbit chemokines for neutrophilic granulocytes, eosinophils, or monocytes was determined in the 48-well microchamber assay as described previously (*37*). Briefly, the lower compartments of the chamber were filled with test samples or controls, the upper compartments were filled with neutrophils (1×10^6 cells/mL), eosinophils (2×10^6 cells/mL), or mononuclear cells (2×10^6 cells/mL). The two compartments were separated by a 5-μm pore-size polycarbonate filter (polyvinylpyrrolidone) (PVP)-free for neutrophils and eosinophils, PVP-coated for monocytes; Nuclepore, Acton, MA). After incubation at 37 °C during 45 min, 1 h, or 2 h for neutrophils, eosinophils, and monocytes, respectively, migrated cells were fixed, stained, and counted. Human synthetic MCP-3 and human eotaxin (R&D Systems) were used as positive controls for monocyte and eosinophil chemotaxis, respectively. The chemotactic activity is expressed as the chemotactic index (CI), being the number of cells migrated to the test sample divided by the number of cells migrated to the negative control.

Measurement of Intracellular Calcium Concentration. The induction of an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) was measured using the fluorescent Ca^{2+}-indicator fura-2 as described previously (*37*). Neutrophils (10^7 cells/mL) were loaded with 2.5 μM fura-2/AM (Molecular Probes Europe BV, Leiden, The Netherlands) at 37 °C for 30 min. After incubation, cells were washed twice and resuspended (10^6 cells/mL) in HBSS (1 mM Ca^{2+}) supplemented with 0.1% FCS and buffered at pH 7.4 with 10 mM Hepes/NaOH. Fura-2 fluorescence was measured in a PerkinElmer LS50B luminescence spectrophotometer fitted with a water-thermostatable stirred cuvette holder at 37 °C (PerkinElmer, Norwalk, CT). Excitation wavelengths used were 340 and 380 nm, emission was measured at 510 nm. The $[Ca^{2+}]_i$ was calculated using the Grynkiewicz equation (*40*). The K_d used for calibration was 224 nM. For desensitization experiments, cells were first stimulated with buffer (control) or with chemokines at different concentrations. As a second stimulus, added 2 min after the first stimulus, a chemokine concentration was used that induced a significant calcium increase after prestimulation with buffer. The percentage inhibition of the response induced by the second stimulus after prestimulation of the cells is a measure for chemokine desensitization.

Inflammatory Properties of Chemokines In Vivo. To evaluate the effects of chemokines in vivo, C57BL/6 mice were shaved on the abdomen, and chemokines, diluted in 0.9% NaCl, or dilution medium, were injected intradermally (50 μL/site). After 2 h, mice were sacrificed, and injection sites were excised. Skin biopsies were fixed for 24 h in Bouin's fixative. Standard paraffin embedding, sectioning, and staining with hematoxylin-eosin were performed, followed by microscopic examination of the sections at a magnification of $\times$400. The granulocytes were counted in 20 fields for each injection site. Results are expressed as the mean number of granulocytes per field.

Statistical Analysis. Statistical analysis was performed using the Mann−Whitney *U* test.

RESULTS

Production and Purification of Neutrophil Chemotactic Factors from Rabbit Alveolar Macrophages. Rabbit alveolar macrophages were stimulated with LPS for chemokine production. The conditioned medium, concentrated and partially purified by adsorption to CPG, was subjected to heparin-Sepharose affinity chromatography. Two peaks of neutrophil chemotactic activity eluted from the column, a first peak at 0.45−0.65 M NaCl (fractions 9−11) and a second peak at 0.85−1.10 M NaCl (fractions 13−16), respectively (Figure 1A). The first peak of biological activity completely coincided with the bulk of protein and with the peak of IL-8 immunoreactivity. Further purification of this first peak by cation-exchange chromatography and RP-HPLC yielded pure rabbit IL-8 (Figure 2A) as determined by NH_2-terminal amino acid sequence analysis (AVLTRIGTELRCQC...) and mass spectrometry (determined M_r of 9093.6 versus theoretical M_r of 9093.7). In addition to intact IL-8, minor amounts of NH_2-terminally truncated IL-8 forms, missing 1−6 amino acids, were isolated (data not shown). The second peak of neutrophil chemotactic activity, containing only a low amount of IL-8 immunoreactivity, was also further purified by cation-exchange chromatography at pH 4.0 (Figure 1B). Two peaks of neutrophil chemotactic activity, corresponding to 6−10-kDa proteins on SDS−PAGE, eluted from the cation exchanger at 0.45−0.65 M NaCl (fractions 37−44) and at 0.75−0.85 M NaCl (fractions

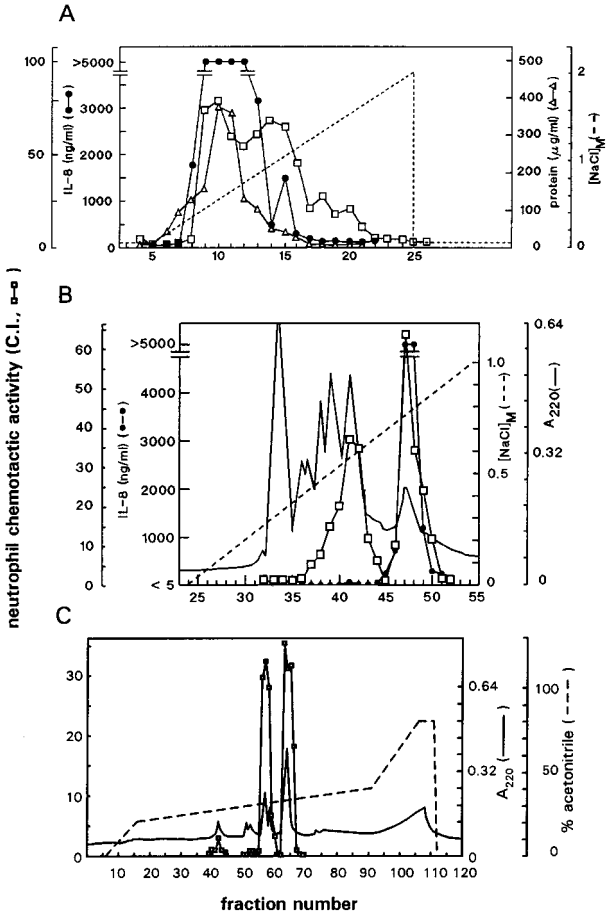
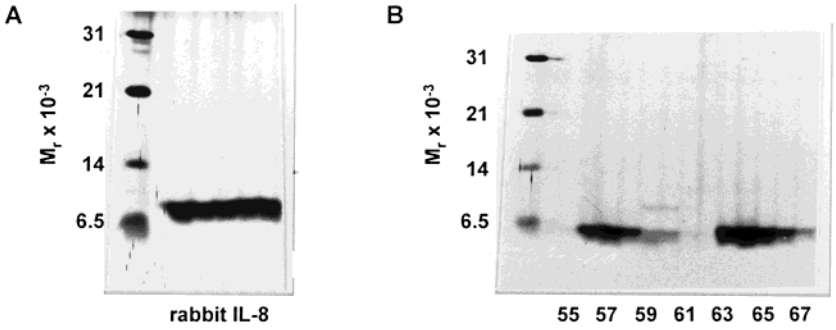


FIGURE 1: Purification of neutrophil chemotactic factors from conditioned medium of rabbit alveolar macrophages. (A) Supernatant of stimulated alveolar macrophages was first concentrated and partially purified by adsorption to CPG. The CPG eluate was loaded on a heparin-Sepharose column. Proteins were eluted with a linear NaCl gradient. The protein and IL-8 concentrations in the fractions were determined by a Coomassie blue G-250 binding assay and ELISA, respectively. Fractions were tested for neutrophil chemotactic activity (dilution 1/300) in the microchamber chemotaxis assay, using human neutrophils. (B) The second peak of neutrophil chemotactic activity that eluted from the heparin-Sepharose column (fractions 13−16) was further purified by cation-exchange chromatography. Proteins were eluted with a linear NaCl gradient. Absorbance at 220 nm was measured. Fractions were analyzed for IL-8 immunoreactivity (ELISA) and neutrophil chemotactic activity (dilution 1/300; microchamber chemotaxis assay). (C) The first peak of neutrophil chemotactic activity (fractions 41 and 42) eluted from the cation-exchange column was subjected to C_8 RP-HPLC. Proteins were eluted with an acetonitrile gradient. The absorbance at 220 nm was measured, and fractions were analyzed for neutrophil chemotactic activity in the microchamber chemotaxis assay (dilution 1/300).

46−50), respectively (Figure 1B). The 0.8 M NaCl peak of biological activity fully corresponded to the peak of IL-8 immunoreactivity and eluted at the same position as the bulk of IL-8. The 0.55 M NaCl peak of neutrophil chemotactic activity from the cation-exchange column was further purified by C_8 RP-HPLC. This yielded two major peaks of neutrophil chemotactic activity eluting at 32% (fractions 56−60) and 34% (fractions 63−66) acetonitrile, respectively (Figure 1C) and corresponding to 6-kDa protein bands on SDS−PAGE (Figure 2B). No IL-8 immunoreactivity was detected in these fractions (data not shown). NH_2-terminal amino acid sequence analysis of the protein corresponding to the first HPLC peak containing neutrophil chemotactic activity revealed a novel chemokine. This protein contained in its NH_2-terminal sequence the Glu-Leu-Arg- and Cys-Xaa-Cys- motifs, typical for neutrophil-activating chemokines (Figure 2C). Four isoforms, each differing one amino acid in length at the NH_2-terminus, were present, as revealed by amino acid sequence analysis and electrospray mass spectrometry (Figure 2C). The second HPLC peak of activity contained the same protein as the first peak but showed an extension of another four additional amino acids at the NH_2-terminus, indicating that this novel rabbit chemokine occurs in several NH_2-terminally processed isoforms (Figure 2C).

Complete Protein Sequence Analysis of the Novel Rabbit Neutrophil Chemotactic Protein (NCP). Extended NH_2-terminal amino acid sequence analysis of this novel rabbit chemokine revealed the identity of the first 40 amino acids (Figure 3). To determine the internal sequence, 5 μg of chemokine was enzymatically digested with the endoproteinases Lys-C or Glu-C. Peptide fragments were separated by RP-HPLC and sequenced. Alternatively, 5 μg of chemokine was chemically cleaved by formic acid, and the sequence of the COOH-terminal part was determined. This resulted in the identification of the complete primary structure of this novel chemokine, which was not identical to that of any known protein (Figure 3). Mass spectrometrical analysis confirmed that the primary structure of the novel protein was indeed elucidated completely and confirmed the presence of the indirectly determined four cysteine residues and a maximal length of 76 residues (determined M_r of 8086.0 versus theoretical M_r of 8086.7). The protein shows highest homology with human ENA-78 (82%) (*41*) and GCP-2 (72%) (*42*) and is therefore considered to be a rabbit equivalent of these two highly related human ELR$^+$CXC chemokines (Figure 3 and Table 1). However, because this novel rabbit chemokine does only contain maximally 76 amino acids (as experimentally determined on the mature natural protein) instead of 78 amino acids for ENA-78 and was isolated from alveolar macrophages instead of epithelial cells, we designate this chemokine "neutrophil chemotactic protein (NCP)".

Comparison of the Chemotactic Potency of Rabbit NCP Isoforms and of Rabbit IL-8. Intact and NH_2-terminally truncated forms of NCP (Figure 2C), indicated as NCP(1-76) (RP-HPLC fraction 63) and NCP(7,8-76) (fraction 56), were evaluated in parallel with natural intact rabbit IL-8 for their bioactivity on human neutrophilic granulocytes in the microchamber chemotaxis assay (Figure 4A). Both isoforms of rabbit NCP showed significant neutrophil chemotactic activity. However, NCP(7,8-76) was 10-fold more potent at inducing neutrophil chemotaxis than NCP(1-76), the minimal effective concentrations being 1.3 and 15 ng/mL, respectively (Figure 4A). NCP(7,8-76) was at least as potent as intact rabbit IL-8 which was chemotactic from 3 ng/mL onward. To confirm the potency of these rabbit CXC chemokines, chemotaxis assays were also performed using rabbit neutrophils (Figure 4B). On rabbit neutrophils, rabbit NCP(7,8-76) was again 10 times more potent than NCP(1-76) (minimal effective concentrations of 13 and 150 ng/mL, respectively) (Figure 4B). The most active chemokine on rabbit neutrophils seemed to be rabbit IL-8, which was active from 3 ng/mL onward (Figure 4B). Indeed, NCP was 10 times less potent on rabbit neutrophils than on human



C

HPLC-fraction	determined NH_2-terminal amino acid sequence (relative amount)		determined M_r	corresponding NH_2-terminus
56	RELRXVXLSTTAIHPKMIAR...	(61%)	7493.8	RELRCVC...
	VRELRXVXLSTTAIHPKMIAR...	(39%)	7592.0	VRELRCVC...
57	nd		7591.9	VRELRCVC...
			7690.4	VVRELRCVC...
			7762.5	AVVRELRCVC...
63	GPVAAVVRELRXVXLSTTA...	(100%)	8086.0	GPVAAVVRELRCVC...
64	nd		8086.0	GPVAAVVRELRCVC...

FIGURE 2: Identification of neutrophil chemotactic proteins from rabbit alveolar macrophages. (A) IL-8 immunoreactivity present in the conditioned medium of LPS-stimulated alveolar macrophages was purified by adsorption to CPG, heparin-Sepharose affinity chromatography, cation-exchange chromatography, and RP-HPLC. HPLC-fractions containing IL-8 immunoreactivity were analyzed by SDS−PAGE on Tris/tricine gels under reducing conditions (1 μL/lane). Proteins were visualized by silver staining. Molecular mass markers are as indicated in Materials and Methods. Protein bands were identified as intact rabbit IL-8 by NH_2-terminal amino acid sequence analysis and electrospray ion trap mass spectrometry. (B) The peak of neutrophil chemotactic activity eluting from the cation-exchange column at 0.55 M NaCl (Figure 1B) was purified by RP-HPLC. HPLC-fractions containing neutrophil chemotactic activity (Figure 1C) were analyzed by SDS−PAGE on Tris/tricine gels under reducing conditions (fractions 55−67, 4 μL/lane). (C) The NH_2-terminal amino acid sequence of pure neutrophil chemotactic protein (NCP) (panel B) was determined by Edman degradation. The presence of cysteines was obvious from the absence of any detectable signal (X). The M_r was determined by electrospray ion trap mass spectrometry.

		Theoretical M_r	Determined M_r
NH_2-terminal sequence			
	GPVAAVVRELRXVXLSTT AXXP		
	VRELRXVXLSTT AIHPKMIARLQVIAAGPQXSKV		
	RELRXVXLSTT AIHPKMIARLQVIAAGPQXSKV		
Lys-C digest			
	GPVAAVVRELRCVCLSTT AIHPK	2420.9	2420.7
	VXLSTT AIHPX		
	MIARLQVIAAGPQCSK	1686.1	1686.9
	VEVVASLK	844.0	844.0
	MIARLQVIAAGPQCSXVEVVASXX	2512.1	2511.8
	EICLDPEAPLIK	1340.6	1339.9
Glu-C digest			
	GPVAAVVRE	897.0	897.9
	LRCVCLSTT AIHPKMIARLQVIAAGPQCSKVE	3438.2	3438.2
	VVASLKNGKEICLDPEAPLIKKAIQKILE	3161.8	3162.0
Formic acid digest			
	PEAPLIKKAIQKILESGNKEN	2320.7	2320.9
rabNCP	GPVAAVVRELRCVCLSTT AIHPKMIARLQVIAAGPQCSKVEVVASLKNGKEICLDPEAPLIKKAIQKILESGNKEN	8086.7	8086.0
huENA-78	AGPAAAVLRELRCVCLQTTQGVHPKMISNLQVFAIGPQCSKVEVVASLKNGKEICLDPEAPFLKKVIQKILDGGNKEN		
huGCP-2	GPVSAVLTELRCTCLRVTLRVNPKTIGKLQVFPAGPQCSKVEVVASLKNGKQVCLDPEAPFLKKVIQKILDSGNKKN		

FIGURE 3: Complete amino acid sequence analysis of neutrophil chemotactic protein (NCP). The NH_2-terminal amino acid sequence of the NCP isolated from alveolar macrophages (Figure 2B,C) was identified by sequence analysis of the intact protein. Internal amino acids were identified after digestion of intact protein with endoproteinase Lys-C or Glu-C and subsequent sequencing of the RP-HPLC-purified fragments. The COOH-terminal sequence was determined after formic acid digestion. The presence of cysteine residues was obvious from the absence of a detectable signal and was confirmed by mass spectrometry. Probable amino acids are in italic; unidentified amino acids are indicated with X. The M_r of proteolytic fragments was determined by electrospray ion trap mass spectrometry. Residues that were confirmed by MS/MS analysis of proteolytic fragments are underlined. The complete amino acid sequence is aligned with that of human ENA-78 and GCP-2.

neutrophils, whereas IL-8 was equally potent on both cell types.

Furthermore, the cell specificity of NCP toward neutrophils was verified. The chemotactic activity of NCP was evaluated on rabbit mononuclear cells, rabbit monocytes, and human eosinophils (Table 2). At 10 ng/mL, human MCP-3 induced migration of mononuclear cells and monocytes after 2 h in the microchamber assay, whereas no chemotactic activity

Table 1: Homology (% Identical Amino Acids) between Human and Rabbit ELR$^+$CXC Chemokines

	huIL-8	huGROα	huGROβ	huGROγ	huENA-78	huGCP-2	huNAP-2	rabIL-8	rabGROα	rabGROβ	rabGROγ
huIL-8	100										
huGROα	42	100									
huGROβ	41	88	100								
huGROγ	40	85	84	100							
huENA-78	34	49	48	51	100						
huGCP-2	30	44	41	44	77	100					
huNAP-2	48	59	55	52	51	44	100				
rabIL-8	82	40	38	38	35	32	48	100			
rabGROα	41	70	77	75	52	45	55	42	100		
rabGROβ	41	68	78	70	55	45	55	41	93	100	
rabGROγ	38	71	74	74	51	42	52	37	70	67	100
rabNCP	35	49	46	50	82	72	54	36	49	50	50

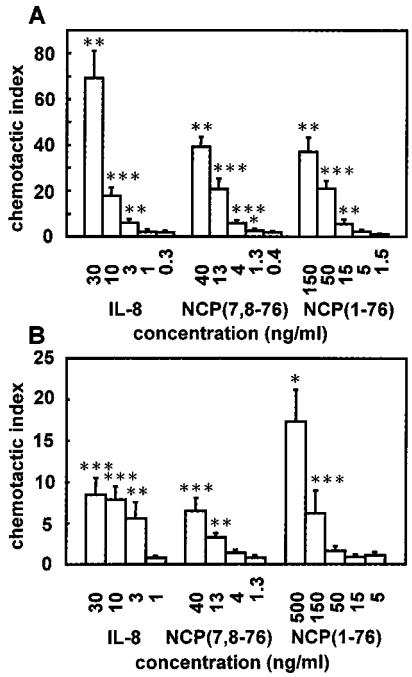


FIGURE 4: Comparison of the neutrophil chemotactic activity of rabbit IL-8 and different NCP isoforms. The potency of rabbit IL-8, NCP(7,8-76), and NCP(1-76) to induce neutrophil chemotaxis was compared in the microchamber chemotaxis assay using human neutrophils (A) or rabbit neutrophils (B). Data (mean ± SEM) are derived from 4 to 9 (human) and from 3 to 11 (rabbit) independent experiments. In each experiment, samples were tested in triplicate. Asterisks indicate the significance of the chemotactic activity of the chemokine as compared to negative control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

on these cells was observed for rabbit IL-8 or NCP at more than 30-fold higher concentrations. Eotaxin was found to attract eosinophils from 30 ng/mL onward, but no response was observed with NCP or IL-8 at 10 times higher concentrations (Table 2).

Calcium Mobilization in Rabbit Neutrophils by NCP Isoforms and IL-8. The ability of rabbit NCP(7,8-76) and NCP(1-76) to induce an increase in $[Ca^{2+}]_i$ in rabbit neutrophils was evaluated in parallel with rabbit IL-8 (Figure 5). Although IL-8 was more active at attracting rabbit neutrophils as compared to NCP(7,8-76), these chemokines were equally potent at inducing an increase in the $[Ca^{2+}]_i$, the minimal effective concentration being 1.3 ng/mL (Figure 5). As observed in the chemotaxis assay, NCP(1-76) (minimal effective concentration of 5 ng/mL) was again less potent than truncated NCP. For all these chemokine forms, the minimal effective concentration to induce a calcium increase was lower than the concentration necessary to attract rabbit neutrophils.

In desensitization experiments, 13 ng/mL of rabbit IL-8 completely inhibited the calcium increase induced by 5 ng/mL of NCP(1-76) and by 1.3 ng/mL of NCP(7,8-76) (Table 3). Furthermore, a 10- and 40-fold excess of rabbit NCP-(7,8-76) and NCP(1-76), respectively, abolished the calcium rise stimulated by 1.3 ng/mL of IL-8. These data confirm the higher potency of NCP(7,8-76) as compared to NCP(1-76) as observed in chemotaxis experiments and indicate that rabbit IL-8 and NCP share receptors and/or signal transduction pathways.

Inflammatory Properties of Rabbit NCP and IL-8 In Vivo. To confirm the neutrophil chemotactic activity of rabbit NCP and IL-8 observed in the in vitro chemotaxis assay, rabbit IL-8 (300 ng or 100 ng/site), truncated NCP (1000, 300, or 100 ng/site) or 0.9% NaCl (negative control) were injected intradermally in mice (Figure 6). Both chemokines induced neutrophil accumulation but no mononuclear cell infiltration 2 h after injection. In accordance with the in vitro chemotaxis results, IL-8 was 3-fold more potent than NCP.

DISCUSSION

Proinflammatory ELR$^+$CXC chemokines have been shown to be involved in many pathologies. IL-8 concentrations were found to be increased in urine from patients with glomerular diseases and in bronchoalveolar lavage fluid from patients with ARDS (*11*, *16*, *17*). IL-8, ENA-78, and GROα have been detected in synovial fluid and plasma of rheumatoid arthritis patients (*12*−*15*). Using rabbit models, the role of some CXC chemokines, mainly IL-8, has been studied in different pathologies. IL-8 is important for neutrophil recruitment and the control of bacterial transepithelial translocation in a rabbit model of shigellosis, but it also induces severe epithelial destruction (*43*). IL-8 is involved in the impairment of renal functions in experimental acute immune complex-mediated glomerulonephritis by recruiting and activating neutrophils (*21*). Lung reperfusion injury as well as cerebral reperfusion injury in the rabbit are attenuated by anti-IL-8 (*22*, *23*). IL-8 is also involved in neutrophil recruitment in endotoxin-induced pleurisy (*44*). Furthermore, IL-8 plays a role in endotoxemia-induced ARDS-like lung injury (*25*, *26*) as well as acid aspiration-induced lung injury (*45*). Injection of rabbit IL-8 into rabbit knee joints induces neutrophil influx and cartilage destruction, indicating a role for IL-8 in this disease (*46*). In LPS- or IL-1α-induced acute experimental arthritis, anti-IL-8 diminishes the infiltration of neutrophils into joints and provides protection from tissue damage (*24*).

Table 2: Lack of Chemotactic Activity of Rabbit IL-8 and NCP for Mononuclear Cells, Monocytes, and Eosinophils

		chemotactic index ± SEM (*n*)[a]		
chemokine	concentration (ng/mL)	mononuclear cells	monocytes[b]	eosinophils
huMCP-3	100	4.6 ± 0.6 (8)	7.0 ± 1.2 (3)	ND[c]
	30	4.7 ± 1.0 (8)	6.9 ± 0.7 (3)	ND
	10	2.8 ± 0.6 (8)	3.5 ± 0.7 (3)	ND
	3	1.6 ± 0.4 (3)	2.2 ± 0.5 (3)	ND
huEotaxin	300	ND	ND	53.3 ± 11.5 (3)
	100	ND	ND	32.8 ± 3.1 (3)
	30	ND	ND	12.5 ± 7.8 (3)
	10	ND	ND	0.5 ± 0.4 (3)
rabIL-8	1000	ND	ND	1.4 ± 0.0 (2)
	300	1.2 ± 0.2 (3)	1.2 ± 0.3 (3)	1.2 ± 0.2 (2)
	100	1.2 ± 0.3 (5)	1.3 ± 0.3 (3)	0.8 ± 0.4 (2)
	30	1.5 ± 0.8 (5)	1.0 ± 0.6 (3)	ND
	10	0.7 ± 0.2 (2)	ND	ND
rabNCP(7,8-76)	400	1.7 ± 0.5 (5)	0.8 ± 0.2 (3)	1.4 ± 0.3 (3)
	130	1.8 ± 0.8 (5)	0.5 ± 0.1 (3)	1.6 ± 0.2 (3)
	40	1.3 ± 0.4 (5)	0.9 ± 0.3 (3)	1.0 ± 0.3 (3)
	13	1.6 ± 0.4 (5)	0.7 ± 0.2 (3)	0.4 ± 0.1 (2)
rabNCP(1-76)	500	1.1 ± 0.1 (3)	2.0 ± 0.8 (3)	1.6 ± 0.2 (2)
	150	1.5 ± 0.5 (3)	1.3 ± 0.5 (3)	1.3 ± 0.4 (2)
	50	1.3 ± 0.3 (6)	0.8 ± 0.6 (3)	1.4 ± 0.2 (2)
	15	1.0 ± 0.2 (3)	ND	ND

[a] *n* is the number of independent experiments; in each experiment, samples were tested in triplicate. [b] Mononuclear cells were used as source for monocytes; only migrated monocytes were counted. [c] ND, not determined.

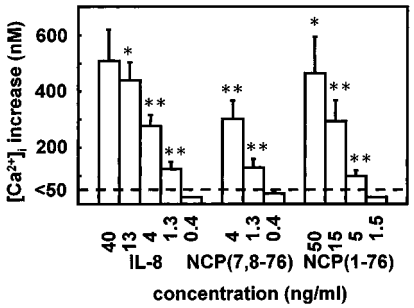


FIGURE 5: Calcium mobilization in neutrophils by rabbit NCP isoforms and IL-8. The potency of rabbit IL-8, NCP(7,8-76), and NCP(1-76) to induce a calcium rise in rabbit neutrophils was compared. Data (mean ± SEM) are derived from 2 to 5 independent experiments. The detection limit for the increase in $[Ca^{2+}]_i$ (50 nM) is indicated by a dotted line. *, $p < 0.05$; **, $p < 0.01$.

In addition to IL-8, also GROα plays a role in neutrophil recruitment in LPS-induced arthritis in the rabbit. However, these two chemokines together account for only 70% of neutrophil infiltration in this model, indicating that also other chemoattractants are involved (*47*).

To study the role of chemokines in different pathologies using rabbit models, the existing rabbit homologues of all the human chemokines have to be identified. Although seven human ELR$^+$CXC chemokines have been identified, only four rabbit ELR$^+$CXC chemokines are known at present i.e., IL-8 (*19*, *28*), GROα (*27*, *29*), GRO*β* (*29*, *31*), and GRO*γ* (*32*). In this study, we isolated a novel rabbit chemokine from supernatant of stimulated alveolar macrophages. This chemokine shows highest homology with human ENA-78 and GCP-2 (Table 1). However, because the natural rabbit chemokine did contain maximally only 76 instead of 78 amino acids (ENA-78) or 77 amino acids (GCP-2) and was not originally isolated from epithelial cells (ENA-78) or tumor cells (GCP-2) but from alveolar macrophages, the chemokine was designated neutrophil chemotactic protein (NCP). Furthermore, NCP did not attract monocytes or eosinophils in vitro, confirming the cell specificity of this ELR$^+$CXC chemokine. Although the amount of NCP isolated from LPS-stimulated rabbit alveolar macrophages was lower than that of IL-8, substantial amounts of both chemokines could be recovered from 4.2 L of conditioned medium (about 100 versus 500 *μ*g). Different NH_2-terminally truncated forms of both IL-8 and NCP were isolated from the stimulated macrophages. NH_2-terminal truncation has already been described for the human ELR$^+$CXC chemokines IL-8, GROα, GRO*γ*, GCP-2, and ENA-78 (*48*) and for murine GCP-2 (*18*) as well as for ELR$^-$CXC chemokines and CC chemokines (*49*). For ELR$^+$CXC chemokines, the inflammatory activity is increased by NH_2-terminal truncation except for human GCP-2, which shows no alteration in activity. In contrast, the chemotactic activity of ELR$^-$CXC chemokines such as stromal cell-derived factor-1 (SDF-1) and of CC chemokines such as eotaxin and RANTES is decreased by truncation, whereas the inhibitory effect on HIV-1 infection may be decreased (SDF-1) or increased (RANTES) (*49*). Concordant with human ELR$^+$CXC chemokines, the truncated forms of rabbit NCP were also more potent at inducing chemotaxis of neutrophils as compared to NH_2-terminally intact NCP. Although on human neutrophils, natural rabbit NCP was found to be at least equally potent as rabbit IL-8, the latter is the most active chemokine on rabbit neutrophils. This example indicates the importance of testing chemokines in an homologous system. In human neutrophils, human IL-8 is more potent than ENA-78 and GCP-2 at inducing an increase in $[Ca^{2+}]_i$ (*42*), whereas NCP-(7,8-76) is equally potent as rabbit IL-8 at inducing a calcium rise in rabbit neutrophils. However, this difference may in part be explained by the fact that NH_2-terminally intact rabbit IL-8 was used in all experiments. Finally, we have shown that both rabbit IL-8 and NCP chemoattract neutrophils in vivo, IL-8 being slightly more potent than NCP.

In the human system, two receptors for ELR$^+$CXC chemokines, CXCR1 and CXCR2, have been identified. IL-8 and GCP-2 can efficiently activate neutrophils by binding

Table 3: Desensitization of the NCP-Induced Calcium Increase in Rabbit Neutrophils by IL-8 and Vice Versa

first stimulus	concn (ng/mL)	second stimulus	concn (ng/mL)	increase in $[Ca^{2+}]_i$ in response to second stimulus, mean (n)[a]	% inhibition of response to second stimulus, mean ± SEM[b]
buffer		NCP(1-76)	5	164 (2)	
IL-8	13		5	<40 (2)	100
	4		5	76 (2)	78 ± 22
	1.3		5	142 (2)	12 ± 7
	0.4		5	139 (2)	11 ± 19
buffer		NCP(7,8-76)	1.3	196 (3)	
IL-8	13		1.3	<40 (2)	100
	4		1.3	139 (3)	37 ± 20
	1.3		1.3	219 (3)	3 ± 18
	0.4		1.3	240 (3)	−13 ± 11
buffer		IL-8	1.3	204 (3)	
NCP(1-76)	50		1.3	<40 (2)	100
	15		1.3	69 (3)	53 ± 18
	5		1.3	157 (3)	16 ± 10
	1.5		1.3	134 (2)	−20 ± 8
NCP(7,8-76)	13		1.3	<40 (3)	100
	4		1.3	46 (3)	80 ± 20
	1.3		1.3	156 (3)	1 ± 14
	0.4		1.3	107 (2)	5 ± 1

[a] n is the number of independent experiments. [b] The mean of the percentages of inhibition in independent experiments.

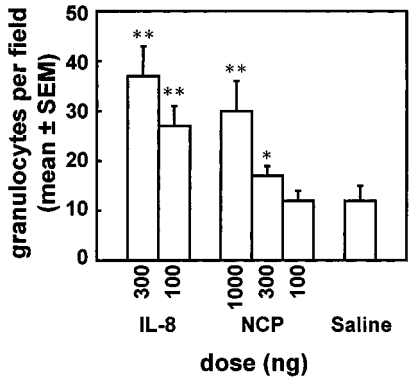


FIGURE 6: In vivo inflammatory properties of rabbit NCP and IL-8. Different amounts of rabbit IL-8 and NH_2-terminally truncated NCP (in 0.9% NaCl) were injected intradermally in mice. Saline (0.9% NaCl) was injected as a negative control. After 2 h, the mice were sacrificed to prepare skin sections for histopathology analysis. Granulocytes were counted in 20 microscopic fields (2−4 sections) per injection site. Data are derived from three mice, injected each with IL-8, NCP, and saline. Results are expressed as the mean number of granulocytes per field ± SEM. Asterisks indicate a significant effect of chemokine as compared to 0.9% NaCl. *, $p < 0.05$; **, $p < 0.01$.

to both CXCR1 and CXCR2, whereas the other ELR$^+$CXC chemokines activate cells predominantly by binding to CXCR2 (*9*, *10*). Similarly, two receptors for ELR$^+$CXC chemokines have been identified in the rabbit: rabbit CXCR1 binds human and rabbit IL-8 but no other human chemokines; rabbit CXCR2 binds human IL-8, NAP-2, and GROα (*20*, *33*−*36*). Except for the binding of rabbit IL-8 to rabbit CXCR1, no binding data are available for binding of rabbit chemokines to these receptors. It is expected that rabbit IL-8 will activate rabbit neutrophils by binding to both rabbit CXCR1 and CXCR2, as described for human IL-8. Our observation that the difference in potency between rabbit IL-8 and NCP on human cells is much lower than on rabbit neutrophils can be explained by the fact that rabbit IL-8 does not bind human CXCR1 (*50*) and thus shows a defective receptor recognition pattern on human neutrophils.

In conclusion, natural forms of a novel rabbit chemokine were isolated from stimulated alveolar macrophages, in parallel with IL-8. The complete primary structure of this neutrophil attracting ELR$^+$CXC chemokine was determined, and the protein was designated NCP. NCP shows highest homology with human ENA-78 and GCP-2. As in humans, the truncated forms of this chemokine are more potent than intact NCP at activating neutrophils and become almost as potent as rabbit IL-8. The identification of this rabbit chemokine will allow its study in rabbit models and to better understand the role of ENA-78 and GCP-2 in human pathology.

ACKNOWLEDGMENT

We thank Hermine Fret (University of Antwerp), René Conings, Jean-Pierre Lenaerts, and Willy Put (University of Leuven) for technical assistance. The technical and scientific advice of Nancy Van Osselaer (University of Antwerp) is greatly acknowledged.

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BI0011227