Characterization of Synthetic Human Granulocyte Chemotactic Protein 2: Usage of Chemokine Receptors CXCR1 and CXCR2 and *in Vivo* Inflammatory Properties†

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ABSTRACT: Human granulocyte chemotactic protein 2 (GCP-2) has originally been isolated from cytokine-stimulated osteosarcoma cells as a chemokine coproduced in minute amounts together with interleukin 8. Human GCP-2 (75 residues) was synthesized on a 0.25-mmol scale using Fmoc chemistry. After disulfide bridge formation and purification, monomeric GCP-2 was recovered as a 6-kDa protein; the pure synthetic protein showed a molecular mass of 8076 Da as determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The exact amino acid sequence of synthetic GCP-2 was confirmed by Edman degradation. Synthetic GCP-2 was an equally active (minimal effective concentration of 1−3 nM) chemoattractant for neutrophilic granulocytes as was natural 75-residue GCP-2. At concentrations up to 30 nM, synthetic GCP-2 did not stimulate eosinophil, monocyte, or lymphocyte chemotaxis. GCP-2 induced a dose-dependent increase in $[Ca^{2+}]_i$ in neutrophils, 1 nM being the minimal effective concentration. The GCP-2-induced $[Ca^{2+}]_i$ increase was completely prevented by pertussis toxin. Prestimulation of neutrophils with equimolar concentrations of purified natural IL-8, GROα, GROγ and ENA-78 abolished the $[Ca^{2+}]_i$ increase in response to 1 nM GCP-2. Alternatively, the $[Ca^{2+}]_i$ rise induced by these CXC chemokines was inhibited by pretreatment of neutrophils with GCP-2. GCP-2 stimulated $[Ca^{2+}]_i$ increases in CXCR1- and CXCR2-transfected cells, demonstrating that GCP-2 binds to both IL-8 receptors. Intradermal injection of synthetic GCP-2 resulted in a dose-dependent neutrophil accumulation and plasma extravasation in rabbit skin. To provoke this skin reaction, GCP-2 (10 pmol/site) was nearly as effective as IL-8, indicating that it is an important complementary mediator of the inflammatory response.

The attraction of leukocytes to inflammatory sites is an important phenomenon in host defense. Adhesion molecules (Springer, 1994) and chemotactic cytokines, called chemokines (Baggiolini et al., 1994; Taub & Oppenheim, 1994; Van Damme, 1994), are the key mediators in leukocyte recruitment. Chemokines are divided into two subgroups, depending on whether the first two of four cysteines are separated by one amino acid (CXC chemokines) or not (CC chemokines). A large number of human CC chemokines (e.g. MCP-1, -2, -3, and -4, MIP-1α, MIP-1β, I-309, RANTES, and eotaxin) has already been molecularly and biologically identified (Van Damme et al., 1992; Baggiolini et al., 1994; Taub & Oppenheim, 1994; Kitaura et al., 1996; Uguccioni et al., 1996). CC chemokines attract different types of leukocytes including monocytes, lymphocytes, natural killer cells, dendritic cells, and eosinophilic and basophilic granulocytes (Baggiolini et al., 1994; Taub & Oppenheim, 1994; Proost et al., 1996) and have been recently reported as HIV suppressive factors (Cocchi et al., 1995). Chemokines with chemotactic activity for neutrophilic granulocytes belong to the CXC subfamily. The CXC chemokine most intensively studied *in vitro* and *in vivo* is interleukin 8 (IL-8;[1] Van Damme, 1994). *In vitro*, IL-8 is a more potent neutrophil chemotactic factor than the other human CXC chemokines including granulocyte chemotactic protein 2 (GCP-2; Proost et al., 1993a), epithelial cell-derived neutrophil-activating protein 78 (ENA-78; Walz et al., 1991; Koch et al., 1994), GROα (Richmond et al., 1988; Schröder et al., 1990), GROβ, GROγ (Haskill et al., 1990) and neutrophil-activating protein 2 (NAP-2; Van Damme et al., 1990; Walz & Baggiolini, 1990). CXC chemokines that do not contain the ELR motif in the NH_2-terminal sequence [e.g., platelet factor 4 (PF-4), monokine induced by IFN-γ (MIG), and IFN-γ-inducible protein 10 (IP-10)] are weak or totally inactive as neutrophil chemoattractants. On the

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[1] Abbreviations: IL-8, interleukin 8; GCP, granulocyte chemotactic protein; ENA-78, epithelial cell-derived neutrophil-activating protein 78; NAP-2, neutrophil-activating protein 2; IL-8R, interleukin 8 receptor; CXCR, CXC chemokine receptor; HPLC, high-performance liquid chromatography; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; HBSS, Hanks' balanced salt solution; CGRP, calcitonin gene-related peptide.

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basis of the presence or absence of the ELR motif, CXC chemokines have been identified as angiogenic or angiostatic factors, respectively (Maione et al., 1990; Strieter et al., 1995).

Two seven-transmembrane-segment G-protein-linked receptors for IL-8 were molecularly identified: IL-8 receptor A (IL-8RA) or CXC chemokine receptor 1 (CXCR1), which shows high affinity for IL-8 only, and IL-8RB or CXCR2, which binds IL-8 but also GROα, GROβ, GROγ and NAP-2 with high affinity (Holmes et al., 1991; Murphy & Tiffany, 1991; Lee et al., 1992; Cerretti et al., 1993; Murphy, 1994). Binding of IL-8 to its receptor induces an increase in $[Ca^{2+}]_i$ that can be blocked by pertussis toxin (Thelen et al., 1988).

Chemokines are produced by a variety of cell lines, including tumor cells. GCP-2 has originally been isolated from cytokine-stimulated MG63 osteosarcoma cells, in addition to IL-8 (GCP-1) and other CXC chemokines (GRO, ENA-78, and IP-10). Since very low amounts of GCP-2 were recovered compared to IL-8, the production of natural GCP-2 is expensive and time-consuming. As an alternative, GCP-2 was synthesized by solid-phase peptide synthesis using Fmoc chemistry. Synthetic GCP-2 was purified and biochemically evaluated for correct sequence and folding. Synthetic chemokine was used for further biological characterization of GCP-2 *in vitro* and *in vivo*. From these experiments, it can be concluded that GCP-2 is a neutrophil-specific chemoattractant that binds to CXCR1 and CXCR2 and that it is a potent inflammatory mediator *in vivo*.

MATERIALS AND METHODS

Solid-Phase Peptide Synthesis and Folding of Peptides. GCP-2 was synthesized by solid-phase peptide synthesis using amino acids with Fmoc-protected α-amino groups (Atherton & Sheppard, 1989) on a Model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) using standard FastMoc programs. The side-chain protecting groups used were trityl (Trt) for Asn, Cys, and Gln, *tert*-butyl (tBu) for Ser and Thr, *tert*-butyl ester (OtBu) for Asp and Glu, *tert*-butyloxycarbonyl (Boc) for Lys, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg. The COOH-terminal amino acid was loaded on an HMP [[[4-(hydroxymethyl)phenoxy]methyl]copolystyrene−1% divinylbenzene] resin by a symmetric anhydride binding. After capping of remaining free hydroxyl functions on the resin with benzoic anhydride and removal of the Fmoc group from the amino acid by 20% piperidine, the next HBTU/HOBT [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/hydroxybenzotriazole] preactivated Fmoc-amino acid was added. After each coupling step, the free amino termini of nonreacted peptides were capped by acetic anhydride to avoid elongation of incompletely synthesized peptides. The Fmoc removal, coupling, and capping steps were repeated for each amino acid.

To cleave the peptide from the resin and to remove the side-chain protecting groups, the resin-bound protein was stirred for 90 min in a cleavage mixture containing 0.75 g of crystalline phenol, 250 μL of ethanedithiol (EDT), 500 μL of thioanisole, 500 μL of bidistilled water, and 10 mL of trifluoroacetic acid (TFA) at room temperature (King et al., 1990). The peptide was separated from the resin by filtration through a medium-porosity glass filter and precipitated in cold methyl *tert*-butyl ether (MTBE). The peptide was washed with MTBE, dissolved in bidistilled water, and dried.

Disulfide bridges were formed by incubation of purified unfolded peptide (100 μg/mL) for 90 min at room temperature in 150 mM Tris/HCl, pH 8.7, containing 1 mM EDTA, 0.3 mM oxidized glutathione, 3 mM reduced glutathione and 1 M guanidinium chloride (Jaenicke & Rudolph, 1989). Folded peptide was purified by reversed-phase high-performance liquid chromatography (HPLC).

Purification of Crude and Folded Synthetic Peptides. Crude peptide was dissolved in 0.1% (v/v) TFA and injected on a 100- × 8-mm C-18 Deltapak (30 nm) column (Waters, Millipore Corp., Milford, MA). Peptides were eluted with an acetonitrile gradient [0−80% (v/v) acetonitrile in 0.1% (v/v) TFA; 2 mL/min, 2-mL fractions]. Absorbance at 220 nm was measured as a parameter for protein concentration.

Folded peptides were purified by reversed-phase HPLC on a 220- × 2.1-mm C-8 Aquapore RP-300 column (Applied Biosystems Inc.) and eluted with an acetonitrile gradient [0−80% (v/v) acetonitrile in 0.1% (v/v) TFA; 0.4 mL/min; 0.4-mL fractions]. Absorbance at 220 nm was measured.

Production and Purification of Natural Chemokines. Human peripheral blood mononuclear cells were purified from 75 buffy coats on Ficoll−sodium metrizoate (*vide infra*). The mononuclear cell fraction was incubated in stationary cultures (175 cm^2; Nunc, Roskilde, Denmark) at 5×10^6 cells/mL in serum-free Eagle's minimum essential medium (EMEM; Gibco, Paisley, Scotland; 100 mL) for 2 h at 37 °C. Nonadherent cells were removed by repeated washes with serum-free medium and the cultures were replenished with EMEM supplemented with 2% fetal calf serum (FCS; Gibco). Cultures of adherent mononuclear cells were induced with concanavalin A (10 μg/mL) for 48 h. Chemokines, present in the conditioned medium (1500 mL), were purified by a four-step purification procedure (adsorption to controlled pore glass beads, heparin−Sepharose affinity chromatography, cation-exchange chromatography, and reversed-phase HPLC) as described previously (Wuyts et al., 1994; Proost et al., 1993a). Purified proteins were analyzed for purity and molecular mass by SDS−PAGE under reducing or nonreducing conditions on Tris/tricine gels (Schägger & von Jagow, 1987; Wuyts et al., 1996). The relative molecular mass markers (Bio-Rad Laboratories, Richmond, CA) used were phosphorylase *b* (M_r 92 500), BSA (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400) and the low molecular mass marker (Pierce Chemical Co., Rockford, IL) aprotinin (M_r 6 500). By use of this purification strategy, natural IL-8 (7.5 kDa), ENA-78 (6 kDa), GROα (6 kDa), and GROγ (6 kDa) were isolated from the monocyte-conditioned medium and purified to homogeneity. NH_2-terminal amino acid sequence analysis revealed the identity of the chemokines. Similarly, natural 75-residue GCP-2 was purified from cytokine-stimulated osteosarcoma cells (Proost et al., 1993a,b).

Protein Sequencing and Mass Analysis. The NH_2-terminal sequence of pure proteins was determined by Edman degradation on a pulsed liquid protein sequencer (477A/120A; Applied Biosystems Inc.) with on-line detection of phenylthiohydantoin amino acids. The presence of cysteine residues was obvious from the absence of any detectable signal. For internal sequencing, pure protein was enzymatically digested. Synthetic GCP-2 was incubated for 18 h at 37 °C with endoproteinase Lys-C (Boehringer Mannheim, Mannheim, Germany) in 25 mM Tris/HCl, pH 8.5, containing 1 mM EDTA at an enzyme/substrate ratio of 1/20.

Proteolytic fragments were separated by reversed-phase HPLC on an Aquapore RP-300 column (50 × 1 mm, Applied Biosystems Inc.) and sequenced. Alternatively, pure protein was chemically digested with 75% formic acid for 50 h at 37 °C before Edman degradation.

The molecular mass of synthetic protein was determined by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Sinapinic acid and cytochrome *c* were used as matrix and internal standard, respectively.

Chemotaxis in Vitro. Neutrophilic granulocytes and mononuclear cells were isolated from heparinized peripheral blood from a single donor as previously described (Wuyts et al., 1994). Briefly, erythrocytes were removed by sedimentation for 30 min at 37 °C in hydroxyethylstarch solution (Plasmasteril; Fresenius AG, Bad Homburg, Germany). Mononuclear cells and granulocytes were separated by gradient centrifugation for 30 min at 400*g* on Ficoll−sodium metrizoate (Lymphoprep; Gibco). Remaining erythrocytes in the granulocyte pellet were removed by lysis in bidistilled water for 30 s. Neutrophilic granulocytes were used in the microchamber assay at a concentration of 10^6 cells/mL in Hanks' balanced salt solution (HBSS; Gibco) supplemented with 1 mg/mL human serum albumin (HSA).

To isolate eosinophils from the granulocyte pellet, the cells were incubated 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 more than 90% eosinophils. Eosinophils were used in the microchamber assay at a concentration of 10^6 cells/mL.

Monocytes and lymphocytes were isolated from the mononuclear cell fraction by incubation of the cell suspension with paramagnetic microbeads conjugated with mAb against CD14 (Miltenyi Biotech GmbH) and CD3 (Miltenyi Biotec GmbH), respectively, for 15 min at 4 °C. Cells were passed over a column in a magnetic field. Labeled cells were retained on the column and were eluted with buffer after the column was removed from the magnetic field. Monocytes and lymphocytes (>90% purity) were used in the microchamber assay at concentrations of 2×10^6 and 10×10^6 cells/mL, respectively. The presence of paramagnetic beads did not influence the chemotaxis of the cells (data not shown).

Chemotaxis assays were performed in a 48-well microchamber (Neuro Probe Inc., Cabin John, MD) as described previously (Wuyts et al., 1994). The lower compartment, containing test sample or control, was separated from the upper compartment, containing cells, by a 5-μm pore size polycarbonate filter [Nuclepore, Pleasanton, CA; polyvinylpyrrolidone- (PVP-) free for neutrophils and eosinophils, PVP-treated for monocytes, and coated with fibronectin for lymphocytes (Taub et al., 1995)]. The incubation times were 45 min, 60 min, 120 min, and 4 h for neutrophils, eosinophils, monocytes, and lymphocytes, respectively. Monocyte chemotactic protein 3 (MCP-3) was used as a positive control for eosinophil, monocyte, and lymphocyte chemotaxis. The chemotactic activity was expressed as a chemotactic index, being the number of cells migrated to the sample divided by the number of cells migrated to the negative control.

Measurement of Intracellular Calcium Concentration. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were measured using the fluorescent indicator fura-2 as described by Grynkiewicz et al. (1985). Purified neutrophils (10^7/mL) were incubated for 30 min at 37 °C with 2.5 μM fura-2/AM (Molecular Probes Europe BV, Leiden, The Netherlands) and 0.01% Pluronic F-127 (Sigma, St. Louis, MO) in EMEM supplemented with 0.5% FCS. After incubation, cells were washed twice and resuspended (10^6/mL) in HBSS (1 mM Ca^{2+}) supplemented with 0.1% FCS and buffered at pH 7.4 with 10 mM Hepes/NaOH. Cells were allowed to equilibrate at 37 °C for 10 min before fura-2 fluorescence was measured in a Perkin-Elmer LS50B luminescence spectrophotometer fitted with a water-thermostatable stirred 4-position cuvette holder (Perkin-Elmer, Norwalk, CT). Excitation wavelengths used were 340 and 380 nm; the emission was measured at 510 nm. $[Ca^{2+}]_i$ was calculated using the Grynkiewicz equation. R_{max} was obtained after lysis of the cells with 50 μM digitonin; R_{min} was determined by addition of 10 mM EGTA to the lysed cells after the pH was adjusted to 8.5 with 20 mM Tris. The K_d used for calibration was 224 nM.

To perform desensitization experiments, neutrophils were first stimulated with buffer (control) or chemokine at different concentrations. As a second stimulus, chemokines were used at a concentration inducing a significant increase in $[Ca^{2+}]_i$ after prestimulation with buffer. The second stimulus was added 2 min after the first stimulus. The percentage inhibition of the $[Ca^{2+}]_i$ increase in response to the second stimulus by prestimulation of the cells was calculated.

In some experiments, neutrophils were incubated with different concentrations of pertussis toxin (Sigma) or cholera toxin (Calbiochem, La Jolla, CA) for 90 min at 37 °C. After incubation, cells were washed twice and loaded with fura-2/AM.

Calcium measurements were also performed with 293 human embryonic kidney cells, transfected with CXCR1, CXCR2, or vector alone (Ben-Baruch et al., 1995). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FCS and 800 μg/mL geneticin (Gibco) to maintain the transfected characteristics. For calcium measurements, cells were used on the second or third day after subcultivation (split ratio 1/4). The cells were trypsinized, washed, and loaded with fura-2/AM in DMEM with 10% FCS as described for neutrophils.

Neutrophil Accumulation and Edema Formation in Rabbit Skin. To perform *in vivo* experiments, male New Zealand white rabbits (2.5−3 kg; Penet Farm, Moorsel, Belgium) were used. From previous studies it is known that human cytokines and chemokines are active in the inflammatory rabbit model (Rampart et al., 1989). Neutrophils were purified from 72 mL of citrated rabbit blood by Percoll−plasma gradient centrifugation (Percoll 63% and 80%; Pharmacia, Uppsala, Sweden) after sedimentation of erythrocytes with hydroxyethylstarch. Neutrophils were collected from the 63%/80% interface and incubated with $^{111}InCl_3$ (50−200 μCi; Amersham International, Buckinghamshire, U.K.) chelated to 2-mercaptopyridine-*N*-oxide (MERC, 40 μg/mL; Sigma) for 15 min at room temperature (Rampart & Williams, 1988). The labeled cells were washed twice and resuspended in autologous citrated plasma. ^{111}In-labeled neutrophils and ^{125}I-human serum albumin [5 μCi (Amersham International) mixed with 2 mL of a 2.5% Evans blue solution (Sigma)] were injected intravenously in a recipient rabbit. After 15 min, chemoattractants (100 μL/site) or bradykinin (BK; Sigma) were injected intradermally in the back skin of rabbits according to a balanced site pattern, each agent having 6 injection sites/rabbit. Neutrophil infiltration

and plasma leakage in rabbit skin are based on a synergism between an increase in local blood flow on the arteriolar side and an increase in vascular permeability on the venular side. Therefore, all agents were injected together with 10 pmol of calcitonin gene-related peptide (CGRP; Sigma) per site as vasodilator to increase the basal blood flow. After 60 min (Rampart et al., 1989; Van Osselaer et al., 1991), the rabbit was killed by an overdose of sodium pentobarbitone (Abbott, Paris, France), the back skin was removed, and injection sites were excised. Skin samples were counted in a Packard 5005 Cobra γ-counter with automatic spillover correction (Parkland, Warrenville, IL). Neutrophil accumulation is expressed as the number of labeled neutrophils per injection site determined by comparing skin sample ^{111}In counts with ^{111}In counts per cell in the preparation prior to intravenous injection. Plasma exudation is expressed in terms of equivalents of microliters of plasma per site by dividing skin sample ^{125}I counts by ^{125}I counts in 1 μL of plasma.

RESULTS

(1) Synthesis, Purification, and Folding of Human GCP-2. On the basis of the known amino acid sequence (Proost et al., 1993b), human GCP-2 was synthesized at a 0.25-mmol scale using Fmoc chemistry and amino acids with appropriate side-chain protecting groups. High coupling and deprotection yields were essential to generate a peptide with 75 residues. Assuming that every combined coupling and deprotection step was performed with a yield of 99%, maximally 47.5% of the generated protein would be completely assembled. Whenever troublesome coupling reactions were expected, double coupling steps were programmed (cycles 9−13 and 32−35). However, despite double coupling of Lys at cycle 12, low efficiency was reached for this step. In addition, poor single coupling was obtained for Asn at cycle 53 and for Cys at cycle 63, resulting in a relatively lower overall yield than expected. After each coupling step, the remaining free NH_2-termini were capped with acetic anhydride to avoid elongation of incomplete peptides.

After cleavage of the peptide from the resin and removal of side-chain protecting groups, raw peptide was dissolved in 0.1% TFA and injected on a C-18 Deltapak column (data not shown). The protein eluting at 40% acetonitrile was identified as intact GCP-2 by NH_2-terminal sequence analysis and by mass determination using MALDI-MS (8072 ± 16 Da; theoretical mass is 8074 Da). On SDS−PAGE (reducing conditions), the purified protein corresponded to a band of 6.5 kDa (data not shown).

Disulfide bridge formation was carried out by incubation of the purified raw peptide with oxidized and reduced glutathione (0.3 and 3 mM, respectively) and guanidinium chloride (1 M). Subsequent C-8 reversed-phase HPLC (Figure 1A) yielded pure monomeric GCP-2 as determined by SDS−PAGE analysis under reducing (Figure 1B) and nonreducing (Figure 1C) conditions. The apparent molecular masses of the synthetic protein and natural GCP-2 were identical, under both reducing and nonreducing conditions (6 kDa). Upon HPLC, synthetic GCP-2 eluted at the same acetonitrile concentration (33%) as natural GCP-2. The molecular mass of the pure folded synthetic GCP-2 (8076 ± 16 Da) was determined by MALDI-MS and corresponded well with the theoretical molecular mass of 8070 Da. The amino acid sequence of synthetic GCP-2 was confirmed by

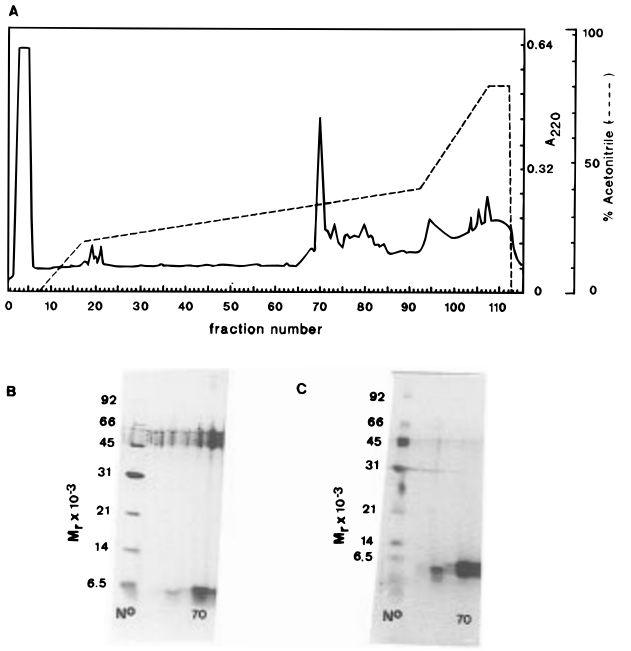

FIGURE 1: Reversed-phase HPLC purification of folded synthetic GCP-2 and analysis by SDS−PAGE. (A) Folded synthetic protein was injected on a 220- × 2.1-mm C-8 Aquapore RP-300 column and eluted with an acetonitrile gradient. Absorbance was measured at 220 nm. (B, C) Fractions derived from the C-8 Aquapore RP-300 column were run on SDS−PAGE under reducing (B) and nonreducing (C) conditions. Proteins were visualized by silver staining. Relative molecular mass markers used are as indicated in Materials and Methods.

Edman degradation (Figure 2). For internal sequencing, GCP-2 was chemically digested with formic acid. Alternatively, protein was enzymatically cleaved with endoproteinase Lys-C. Peptide fragments were purified by reversed-phase HPLC and sequenced. Alignment of four stretches of amino acid sequence allowed us to confirm the authentic primary structure of human GCP-2. The availability of milligrams of synthetic GCP-2 with the expected biochemical properties allowed for its more detailed biological characterization *in vitro* and *in vivo*.

(2) Chemotactic Activity of Synthetic and Natural GCP-2 on Various Leukocyte Subpopulations. The neutrophil chemotactic potency of synthetic and natural GCP-2 were first compared in the microchamber chemotaxis assay with that of natural IL-8. As shown in Table 1, synthetic GCP-2 and natural GCP-2 (75 amino acids) were equally active, the minimal effective concentration being 1−3 nM, but both GCP-2 sources were less potent than natural IL-8 (chemotactic index of 15.6 ± 4.8 and 4.2 ± 1.4 at 3 and 0.3 nM, respectively). Synthetic GCP-2 was therefore used for further biological characterization of the chemokine.

In order to evaluate its cell specificity, GCP-2 was tested for eosinophil, monocyte, and lymphocyte chemotactic activity in the microchamber chemotaxis assay (Table 1). Monocyte chemotactic protein 3 (MCP-3) was used as a positive control in these assays. Concentrations up to 30 nM GCP-2 did not induce chemotaxis of eosinophilic granulocytes, nor was GCP-2 chemotactic for freshly isolated monocytes or lymphocytes, whereas MCP-3 was very effective on these cells at 3 nM. This indicates that GCP-2 is a specific chemoattractant for neutrophilic granulocytes.

(3) Induction of Intracellular Calcium Increase in Neutrophils by GCP-2 and Desensitization by Other CXC Chemokines. On neutrophils, GCP-2 induced a transient rise

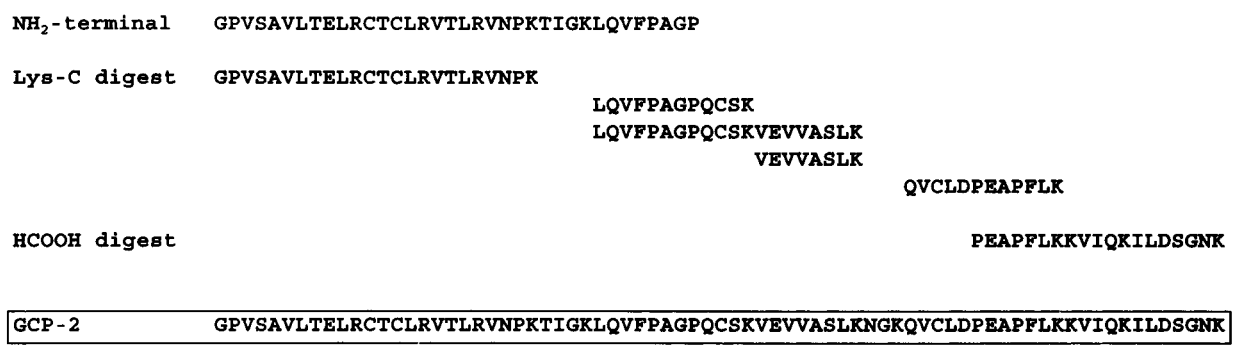

FIGURE 2: Amino acid sequence analysis of folded synthetic GCP-2. The NH_2-terminal amino acid sequence of synthetic GCP-2 was determined by Edman degradation. To confirm the complete sequence, GCP-2 was chemically digested with formic acid and enzymatically cleaved with endoproteinase Lys-C. The amino acid sequences of internal fragments are aligned.

Table 1: Comparison of the Chemotactic Potency of Synthetic and Natural GCP-2 on Neutrophilic Granulocytes and Lack of Chemotactic Activity of GCP-2 for Eosinophilic Granulocytes, Monocytes, and Lymphocytes

		chemotactic index (mean ± SEM)			
chemokine	concn (nM)	neutrophils	eosinophils	monocytes	lymphocytes
natural GCP-2	3	8.6 ± 2.3 (4)[a]	ND[b]	ND	ND
	1	3.2 ± 1.0 (4)	ND	ND	ND
	0.3	2.3 ± 0.4 (4)	ND	ND	ND
synthetic GCP-2	30	54.5 ± 6.8 (4)	1.5 ± 0.1 (2)	0.8 ± 0.2 (6)	2.0 ± 0.6 (4)
	10	49.3 ± 3.6 (4)	1.0 ± 0.2 (2)	0.7 ± 0.3 (6)	0.8 ± 0.3 (4)
	3	12.8 ± 1.2 (4)	0.8 ± 0.6 (2)	0.7 ± 0.3 (6)	0.3 ± 0.2 (4)
	1	3.8 ± 1.9 (4)	ND	0.1 ± 0.1 (2)	ND
synthetic MCP-3	30	ND	48.5 ± 8.3 (5)	ND	ND
	10	ND	22.7 ± 3.4 (5)	8.8 ± 1.5 (6)	11.8 ± 2.4 (4)
	3	ND	10.4 ± 2.4 (5)	ND	ND
	1	ND	ND	4.6 ± 1.1 (6)	5.3 ± 0.9 (4)
	0.1	ND	ND	1.0 ± 0.3 (4)	2.6 ± 0.8 (4)

[a]Numbers in parentheses represent the number of individual experiments. Within each experiment, the chemotactic index is calculated from triplicate cultures. [b]ND, not determined.

in $[Ca^{2+}]_i$ in a dose-dependent manner, the minimal effective concentration being 1 nM (Figure 3). Synthetic GCP-2 was less potent than natural IL-8 and GROα (minimal effective concentrations of 0.3 and 0.1 nM, respectively) but equally as potent as ENA-78 and GROγ.

The increase in $[Ca^{2+}]_i$ induced by 10 nM GCP-2 was completely prevented by pertussis toxin at 0.5 μg/mL, whereas 2 μg/mL was necessary to block the response to 1.5 nM IL-8. In contrast, cholera toxin at 2 μg/mL did not inhibit the $[Ca^{2+}]_i$ increase induced by IL-8 or GCP-2 (Figure 4). These data indicate that GCP-2 acts on neutrophils through pertussis toxin-sensitive G-protein coupled receptors.

Calcium changes were also measured in desensitization experiments using different CXC chemokines (Table 2). The $[Ca^{2+}]_i$ increase in response to 1 nM GCP-2 was abolished or strongly reduced after prestimulation of neutrophils with equimolar concentrations of homogeneous natural IL-8 (100% inhibition), GROα (100%), GROγ (84%), ENA-78 (68%) and synthetic GCP-2 (90%). Alternatively, pretreatment of neutrophils with 3 nM GCP-2 was sufficient to completely abolish the $[Ca^{2+}]_i$ increase induced by effective concentrations of ENA-78 (1 nM), GROα (0.1 nM) and GROγ (1 nM), whereas for IL-8 (0.3 nM), 10 nM GCP-2 was necessary. These data indicate that GCP-2 shares its receptor(s) or signal transduction pathway(s) with those used by other CXC chemokines.

(4) Human GCP-2 Induces Intracellular Calcium Increase in CXCR1- and CXCR2-Transfected Cells. The chemokines GCP-2 and IL-8 were further compared with regard to calcium mobilization in 293 cells transfected with the chemokine receptor CXCR1 or CXCR2 or with vector alone. As expected, IL-8 and GCP-2 induced a significant increase in $[Ca^{2+}]_i$ in CXCR2-transfected cells (minimal effective concentrations of 1.5 and 3 nM, respectively; Table 3). After stimulation of CXCR1-transfected cells with IL-8, a rise in $[Ca^{2+}]_i$ was observed from 0.5 nM onward. GCP-2 induced a comparable increase in $[Ca^{2+}]_i$ at 3 nM (Table 3). Figure 5 shows the rise in $[Ca^{2+}]_i$ after stimulation of CXCR1- and CXCR2-transfected cells with 5 nM IL-8 or 10 nM GCP-2. In contrast, FMLP, even at 10 or 100 nM, did not induce a $[Ca^{2+}]_i$ rise in CXCR1- or CXCR2-transfected cells. IL-8 and GCP-2 had no effect on $[Ca^{2+}]_i$ in cells transfected with vector alone. From these data it can be concluded that GCP-2 not only uses CXCR2 to activate neutrophils but also binds to CXCR1.

(5) GCP-2 Induces Neutrophil Accumulation and Plasma Extravasation in Rabbit Skin. To investigate its inflammatory properties *in vivo*, synthetic GCP-2 was injected intradermally in rabbit skin and compared with other inflammatory agents. Neutrophil infiltration and plasma leakage in response to chemokines, bradykinin, and the vasodilator CGRP were measured by the intradermal accumulation of intravenously injected ^{111}In-labeled neutrophils and ^{125}I-albumin, respectively (Figure 6). Injection of CGRP alone (10 pmol/site) did not induce plasma leakage nor neutrophil accumulation. Bradykinin (BK; 100 pmol/site) plus CGRP induced plasma leakage but no neutrophil infiltration. Intradermal injection of synthetic GCP-2 or natural IL-8 in the presence of CGRP into rabbits caused plasma leakage and neutrophil accumulation in a dose-dependent manner, the minimal effective concentrations for GCP-2 and IL-8 being 10 and 5 pmol/site, respectively

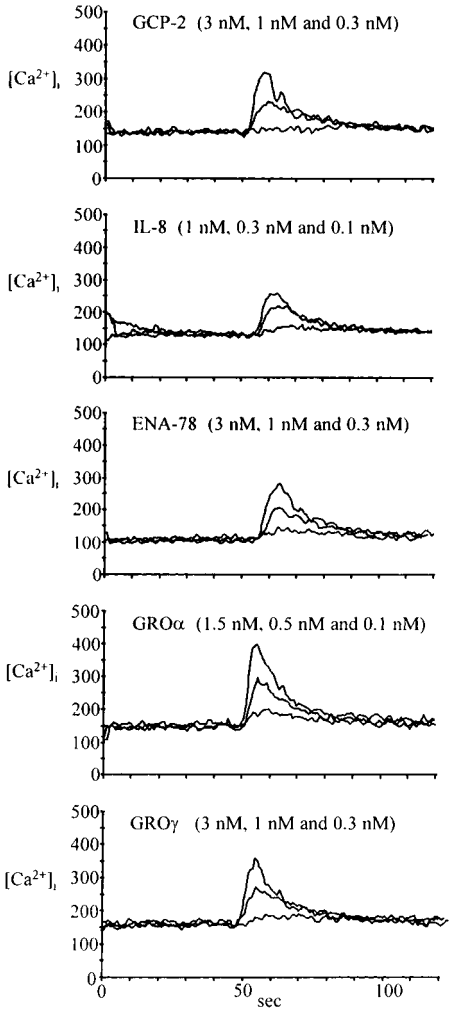

FIGURE 3: GCP-2 induces an increase of $[Ca^{2+}]_i$ in neutrophilic granulocytes. Neutrophils, loaded with fura-2/AM, were stimulated with different concentrations (high to low concentration from top to bottom for each panel) of GCP-2, IL-8, ENA-78, GROα, or GROγ. $[Ca^{2+}]_i$ (nanomolar) was determined by the method of Grynkiewicz et al. (1985) as described in Materials and Methods. Data shown are derived from one experiment out of three.

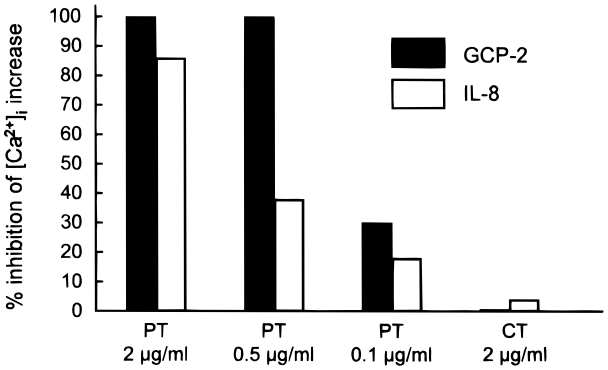

FIGURE 4: Effect of pertussis toxin and cholera toxin on the induction of $[Ca^{2+}]_i$ increase by GCP-2 in neutrophils. Neutrophils were incubated with different concentrations of pertussis toxin or with cholera toxin (2 μg/mL) and loaded with fura-2/AM. Changes in $[Ca^{2+}]_i$ after stimulation of neutrophils with IL-8 and GCP-2 were determined. Results are expressed as the percentage inhibition of the response induced by IL-8 or GCP-2 in cells incubated with buffer. Data represent the mean of two independent experiments.

(Figure 6). Thus, it seems that GCP-2 is almost as potent as IL-8 in stimulating the local inflammatory response *in vivo*.

Table 2: Cross-Desensitization between GCP-2 and CXC Chemokines

first stimulus	concn (nM)	second stimulus	concn (nM)	% inhibition of response to second stimulus[a]	
(A) $[Ca^{2+}]_i$ Increase in Neutrophils Induced by GCP-2 Is Inhibited by IL-8, ENA-78, GROα, and GROγ Pretreatment					
GCP-2	3	GCP-2	1	100	100
	1			81	100
	0.3			22	40
IL-8	1	GCP-2	1	100	100
	0.3			73	51
	0.1			10	0
ENA-78	3	GCP-2	1	94	100
	1			77	59
	0.3			41	18
GROα	1.5	GCP-2	1	100	ND[b]
	0.5			100	77
	0.1			54	58
GROγ	3	GCP-2	1	100	ND
	1			100	69
	0.3			27	44
	0.1			ND	31
(B) GCP-2 Abolishes the $[Ca^{2+}]_i$ Increase Induced by Other CXC Chemokines					
GCP-2	3	GCP-2	1	100	100
	1			81	100
	0.3			22	40
GCP-2	10	IL-8	0.3	100	100
	3			75	74
	1			7	40
GCP-2	10	IL-8	1	68	ND
	3			26	ND
	1			13	ND
GCP-2	3	ENA-78	1	100	100
	1			60	34
	0.3			35	38
GCP-2	3	GROα	0.1	100	100
	1			28	76
	0.3			22	5
GCP-2	3	GROγ	1	100	90
	1			31	28
	0.3			0	2

[a]Data from two independent experiments are shown. [b]ND, not determined.

Table 3: Induction of an Increase in $[Ca^{2+}]_i$ by GCP-2 in CXCR1- and CXCR2-Transfected 293 Cells

		$[Ca^{2+}]_i$ increase [nM; shown as X ± SEM (*n*)]	
chemokine	dose (nM)	CXCR1-transfected cells	CXCR2-transfected cells
IL-8	5	82 ± 7 (6)[a]	94 ± 19 (4)
	1.5	64 ± 24 (4)	68 ± 17 (3)
	0.5	37 ± 19 (4)	<15 (1)
	0.1	<15 (1)	ND[b]
GCP-2	10	68 ± 7 (5)	107 ± 22 (4)
	3	42 ± 22 (4)	61 ± 8 (2)
	1	<15 (3)	<15 (1)

[a]Results from independent experiments (*n*) on weakly subcultured 293 cells transfected with CXCR1 and CXCR2. Vector-transfected cells yielded on average <15 nM $[Ca^{2+}]_i$ increase after stimulation with IL-8 (5 nM) and GCP-2 (10 nM). [b]ND, not determined.

DISCUSSION

The CXC chemokine GCP-2, recently isolated from cytokine-stimulated human osteosarcoma cells, contains the ELR motif, is chemotactic for neutrophils (Proost et al., 1993a), and is angiogenic (Strieter et al., 1995). Different NH_2-terminal forms of GCP-2 were purified from the conditioned media of these cells. No difference in neutrophil

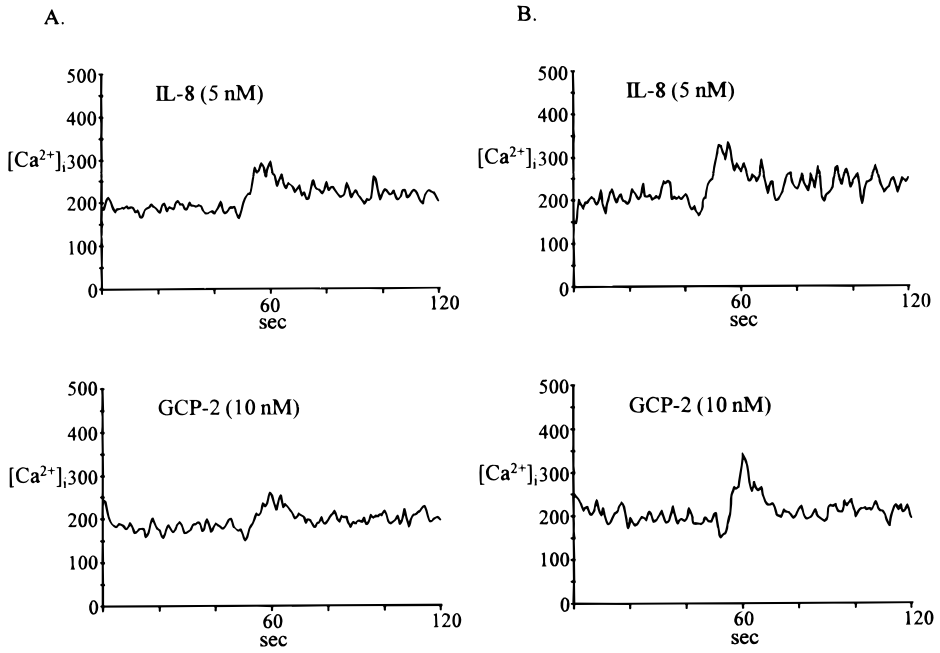

FIGURE 5: GCP-2 induces an increase of $[Ca^{2+}]_i$ in CXCR1- and CXCR-2 transfected cells. CXCR1- (panel A) and CXCR2- (panel B) transfected cells, loaded with fura-2/AM, were stimulated with GCP-2 or IL-8. $[Ca^{2+}]_i$ (nanomolar) was determined by the method of Grynkiewicz et al. (1985) as described in Materials and Methods. Data shown are derived from one experiment out of four.

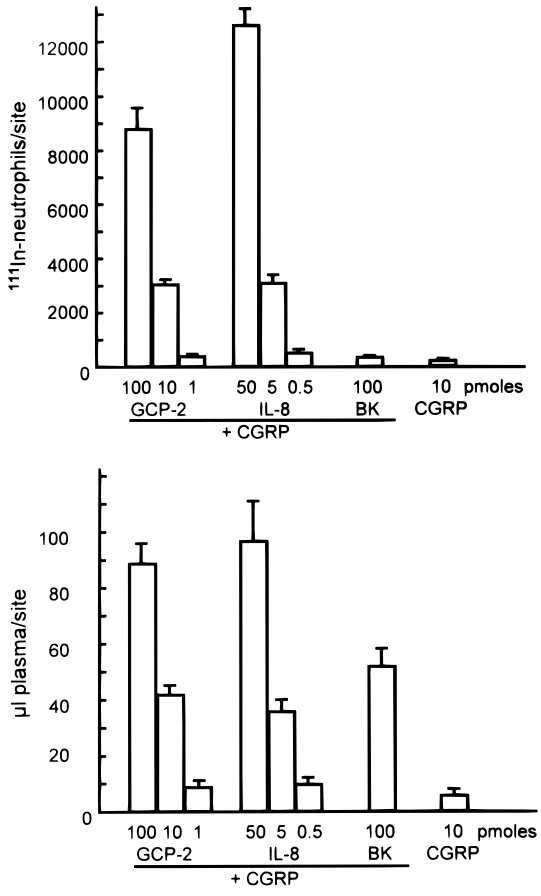

FIGURE 6: GCP-2 induces neutrophil accumulation and plasma extravasation in rabbit skin. Rabbits were injected intradermally with different concentrations of GCP-2 and IL-8. Neutrophil accumulation and plasma exudation were evaluated after 60 min and are expressed as the number of labeled neutrophils per injection site and the plasma volume (microliters) per site, respectively. Data represent the mean ± SEM of six replicates in one rabbit.

chemotactic activity was observed between these forms (Proost et al., 1993b), whereas NH_2-terminal cleavage of βTG and IL-8 (Van Damme et al., 1990; Van Damme, 1994; Walz & Baggiolini, 1990) and also of mouse GCP-2 (Wuyts et al., 1996) resulted in increased specific biological activity. In contrast, modification of the NH_2-terminus of some chemokines, e.g., monocyte chemotactic protein 1 (MCP-1) provokes complete loss of bioactivity (Gong et al., 1995).

Only a few micrograms of natural GCP-2 could be isolated from liters of conditioned media from cultured cells. As a consequence, the biological characterization of GCP-2 has only been partially accomplished. As a fast alternative for the time-consuming and expensive production and purification system for natural GCP-2, the protein was synthesized by Fmoc chemistry based upon the known amino acid sequence (Proost et al., 1993b). The elution profile on HPLC, the amino acid sequence, and the apparent molecular weight under reducing and nonreducing conditions were identical for both natural and synthetic GCP-2. The molecular mass of the synthetic protein as determined by MALDI-MS corresponded to the theoretical molecular mass of 8070 Da. Natural and synthetic GCP-2 were also equally active in the neutrophil chemotaxis assay.

Two receptors for IL-8 have been cloned: IL-8RA or CXCR1 and IL-8RB or CXCR2 (Holmes et al., 1991; Murphy & Tiffany, 1991). These receptors show 77% structural identity and belong to the family of G-protein-coupled receptors that contain seven membrane-spanning domains. Binding of IL-8 to its receptor induces an increase in $[Ca^{2+}]_i$ that can be blocked by pertussis toxin (Thelen et al., 1988). CXCR1 shows high affinity for IL-8 only, whereas CXCR2 binds IL-8, GRO, and NAP-2 with high affinity (Lee et al., 1992; Cerretti et al., 1993). Our experiments demonstrate that GCP-2 also activates neutrophils through binding to pertussis toxin-sensitive G-protein-coupled receptors. Desensitization experiments indicate that GCP-2 shares its receptor(s) or signal transduction pathway(s) on neutrophils with the other CXC chemokines. The increase in $[Ca^{2+}]_i$ in response to 1 nM GCP-2 was blocked by equimolar concentrations of IL-8, ENA-78, GROα and GROγ. Alternatively, the response of neutrophils to these latter chemokines could be inhibited by prestimulation with

GCP-2. Both IL-8 and GCP-2 induced an $[Ca^{2+}]_i$ increase in 293 cells transfected with CXCR2 but also in cells transfected with CXCR1. In contrast to published results with the CXC chemokines GRO and NAP-2 (Lee et al., 1992; Cerretti et al., 1993), GCP-2 seems to efficiently use both CXCR1 and CXCR2. This might explain why GCP-2 efficiently activates neutrophils both *in vitro* and *in vivo*.

Both IL-8 receptors are expressed on neutrophils, monocytes, a subset of NK cells, and a subset of T cells. CXCR1 and CXCR2 were not detected on eosinophils or B cells (Chuntharapai et al., 1994; Morohashi et al., 1995). The absence of CXCR on eosinophils is in accordance with the lack of eosinophil chemotactic activity of GCP-2. Although both CXCR1 and CXCR2 are expressed on mononuclear cells, no chemotactic activity for monocytes or lymphocytes was detectable with GCP-2, indicating that this chemokine is a neutrophil-specific chemoattractant.

Intradermal injection of human GCP-2 at 10 pmol/site induced neutrophil accumulation and plasma leakage in rabbit skin, comparable to the effect observed with human IL-8, whereas NAP-2 was less potent than IL-8 (Van Osselaer et al., 1991). Intravenous injection of IL-8 or NAP-2 resulted in an immediate leukopenia followed by a profound neutrophilia which is accompanied by the release of immature cells from the bone-marrow reservoir (Jagels & Hugli, 1992; Van Damme, 1994; Laterveer et al., 1995). The potent *in vivo* effect of human GCP-2 is reminiscent of that found for mouse GCP-2. In view of the absence of a murine counterpart for IL-8, murine GCP-2 exerts an important role as neutrophil chemotactic and activating factor in the mouse (Wuyts et al., 1996). Synthetic GCP-2 is currently also used to generate specific monoclonal and polyclonal antibodies, necessary to develop specific immunoassays. These tests will allow us to study the gene regulation of GCP-2 and to measure the levels of this protein in body fluids during disease states.

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REFERENCES

Atherton, E., & Sheppard, R. C. (1989) *Solid phase peptide synthesis: a practical approach*, IRL Press, Oxford, England.

Baggiolini, M., Dewald, B., & Moser, B. (1994) *Adv. Immunol. 55*, 97−179.

Ben-Baruch, A., Bengali, K. M., Biragyn, A., Johnston, J. J., Wang, J.-M., Kim, J., Chuntharapai, A., Michiel, D. F., Oppenheim, J. J., & Kelvin, D. J. (1995) *J. Biol. Chem. 270*, 9121−9128.

Cerretti, D. P., Kozlosky, C. J., Vanden Bos, T., Nelson, N., Gearing, D. P., & Beckmann, M. P. (1993) *Mol. Immunol. 30*, 359−367.

Chuntharapai, A., Lee, J., Hébert, C. A., & Kim, K. J. (1994) *J. Immunol. 153*, 5682−5688.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., & Lusso, P. (1995) *Science 270*, 1811−1814.

Gong, J.-H., & Clark-Lewis, I. (1995) *J. Exp. Med. 181*, 631−640.

Grynkiewicz, G., Poenie, M., & Tsien, R. Y. (1985) *J. Biol. Chem. 260*, 3440−3450.

Haskill, S., Peace, A., Morris, J., Sporn, S. A., Anisowicz, A., Lee, S. W., Smith, T., Martin, G., Ralph, P., & Sager, R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7732−7736.

Holmes, W. E., Lee, J., Kuang, W.-J., Rice, G. C., & Wood, W. I. (1991) *Science 253*, 1278−1280.

Jaenicke, R., & Rudolph, R. (1989) in *Protein Structure, a practical approach* (Creighton, T. E., Ed.) pp 206−211, IRL Press, Oxford, England.

Jagels, M. A., & Hugli, T. E. (1992) *J. Immunol. 148*, 1119−1128.

King, D. S., Fields, C. G., & Fields, G. B. (1990) *Int. J. Pept. Protein Res. 36*, 255−266.

Kitaura, M., Nakajima, T., Imai, T., Harada, S., Combadiere, C., Tiffany, H. L., Murphy, P. M., & Yoshie, O. (1996) *J. Biol. Chem. 271*, 7725−7730.

Koch, A. E., Kunkel, S. L., Harlow, L. A., Mazarakis, D. D., Haines, G. K., Burdick, M. D., Pope, R. M., Walz, A., & Strieter, R. M. (1994) *J. Clin. Invest. 94*, 1012−1018.

Laterveer, L., Lindley, I. J. D., Hamilton, M. S., Willemze, R., & Fibbe, W. E. (1995) *Blood 85*, 2269−2275.

Lee, J., Horuk, R., Rice, G. C., Bennett, G. L., Camerato, T., & Wood, W. I. (1992) *J. Biol. Chem. 267*, 16283−16287.

Maione, T. E., Gray, S. G., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., & Sharpe, R. J. (1990) *Science 247*, 77−79.

Morohashi, H., Miyawaki, T., Nomura, H., Kuno, K., Murakami, S., Matsushima, K., & Mukaida, N. (1995) *J. Leukocyte Biol. 57*, 180−187.

Murphy, P. M. (1994) *Annu. Rev. Immunol. 12*, 593−633.

Murphy, P. M., & Tiffany, H. L. (1991) *Science 253*, 1280−1283.

Proost, P., De Wolf-Peeters, C., Conings, R., Opdenakker, G., Billiau, A., & Van Damme, J. (1993a) *J. Immunol. 150*, 1000−1010.

Proost, P., Wuyts, A., Conings, R., Lenaerts, J.-P., Billiau, A., Opdenakker, G., & Van Damme, J. (1993b) *Biochemistry 32*, 10170−10177.

Proost, P., Wuyts, A., & Van Damme, J. (1996) *J. Leukocyte Biol. 59*, 67−74.

Rampart, M., & Williams, T. J. (1988) *Br. J. Pharmacol.* 94, 1143−1148.

Rampart, M., Van Damme, J., Zonnekeyn, L., & Herman, A. G. (1989) *Am. J. Pathol. 135*, 21−25.

Richmond, A., Balentien, E., Thomas, H. G., Flaggs, G., Barton, D. E., Spiess, J., Bordoni, R., Francke, U., & Derynck, R. (1988) *EMBO J. 7*, 2025−2033.

Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.

Schröder, J.-M., Persoon, N. L. M., & Christophers, E. (1990) *J. Exp. Med. 171*, 1091−1100.

Springer, T. A. (1994) *Cell 76*, 301−314.

Strieter, R. M., Polverini, P. J., Arenberg, D. A., Walz, A., Opdenakker, G., Van Damme, J., & Kunkel, S. L. (1995) *J. Leukocyte Biol. 57*, 752−762.

Taub, D. D., & Oppenheim, J. J. (1994) *Ther. Immunol. 1*, 229−246.

Taub, D. D., Key, M. L., Clark, D., & Turcovski-Corrales, S. M. (1995) *J. Immunol. Methods 184*, 187−198.

Thelen, M., Peveri, P., Kernen, P., Von Tscharner, V., Walz, A., & Baggiolini, M. (1988) *FASEB J. 2*, 2702−2706.

Uguccioni, M., Loetscher, P., Forssmann, U., Dewald, B., Li, H., Lima, S. H., Li, Y., Kreider, B., Garotta, G., Thelen, M., & Baggiolini, M. (1996) *J. Exp. Med. 183*, 2379−2384.

Van Damme, J. (1994) in *The Cytokine Handbook* (Thomson, A. W., Ed.) pp 185−208, Academic Press, London, England.

Van Damme, J., Rampart, M., Conings, R., Decock, B., Van Osselaer, N., Willems, J., & Billiau, A. (1990) *Eur. J. Immunol. 20*, 2113−2118.

Van Damme, J., Proost, P., Lenaerts, J.-P., & Opdenakker, G. (1992) *J. Exp. Med. 176*, 59−65.

Van Osselaer, N., Van Damme, J., Rampart, M., & Herman, A. G. (1991) *Am. J. Pathol. 138*, 23−27.

Walz, A., & Baggiolini, M. (1990) *J. Exp. Med. 171*, 449−454.

Walz, A., Burgener, R., Car, B., Baggiolini, M., Kunkel, S. L., & Strieter, R. M. (1991) *J. Exp. Med. 174*, 1355−1362.

Wuyts, A., Proost, P., Put, W., Lenaerts, J.-P., Paemen, L., & Van Damme, J. (1994) *J. Immunol. Methods 174*, 237−247.

Wuyts, A., Haelens, A., Proost, P., Lenaerts, J.-P., Conings, R., Opdenakker, G., & Van Damme, J. (1996) *J. Immunol. 157*, 1736−1743.

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