Gene Cloning of a New Plasma CC Chemokine, Activating and Attracting Myeloid Cells in Synergy with Other Chemoattractants[†,‡]

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ABSTRACT: Chemokines are important mediators of cell migration during inflammation and normal leukocyte trafficking. Inflammatory chemokines are induced in multiple cell types at sites of infection. Here, we describe a novel bovine CC chemokine, designated regakine-1, that is constitutively present at high concentrations in plasma. Cloning of its gene revealed an expected two intron/three exon organization, with a rather long first intron. In addition to a 21-residue signal peptide, the coding sequence corresponded to a 71-residue secreted protein. However, the natural regakine-1 protein missed the COOH-terminal lysine residue. Regakine-1 has only weak sequence similarity (<50% identical residues) with other animal or human chemokines. Northern blot analysis demonstrated regakine-1 RNA expression in spleen and lung. At physiological concentrations (30−100 ng/mL), natural 7.5 kDa regakine-1 stimulated gelatinase B release from neutrophils and chemoattracted immature myeloid HL-60 cells, as well as mature granulocytes. Regakine-1 was more potent on human myeloid cells than the human plasma CC chemokine hemofiltrate CC chemokine-1 (HCC-1). Moreover, regakine-1 synergized with the bacterial peptide *N*-formylmethionylleucylphenylalanine (fMLP), yielding a 10-fold increase in neutrophil chemotactic response above their additive effect. Regakine-1 did not compete with interleukin-8 (IL-8) for binding to neutrophils, nor did it affect fMLP-induced calcium signaling, suggesting that regakine-1 recognizes a different receptor. In view of its high constitutive plasma concentration, regakine-1 is believed to recruit myeloid cells into the circulation, whereas its synergy with other neutrophil chemoattractants suggests that it also enhances the inflammatory response to infection.

Chemokines constitute a large family of chemotactic cytokines, whose primary structure is characterized by the presence of four conserved cysteine residues. Between these cysteines two disulfide bridges are formed that are important for biological activity. Depending on whether the first two cysteines are adjacent or not, chemokines are subdivided in two predominant groups, the CXC and CC families. In human, the CXC family consists of 16 identified members, whereas more than 20 CC chemokines have been identified (*1*−*3*). As a consequence, a novel official nomenclature system (CCL, CXCL) has been introduced (*4*). Like classical chemoattractants, such as complement factor C5a and bacterial formyl peptides, chemokines activate and attract various leukocytic cell types through binding to G protein-coupled receptors (*4*). The spectrum of target cells for a particular chemokine is determined by the selective expression of one or several receptors. Since a single chemokine can bind to different receptors, these molecules interact with leukocytes in an apparently redundant, but robust network (*5*). Chemokines not only attract inflammatory cells to sites of infection or inflammation, they are also responsible for selective migration (homing) of leukocyte subtypes, such as lymphocytes and dendritic cells, to lymphoid organs, allowing their differentation and maturation. In addition, chemokines interfere with other biological processes such as angiogenesis and hematopoiesis (*3*). Finally, chemokines play an important role in AIDS, since HIV viruses infect mononuclear leukocytes via binding to chemokine receptors (*1*, *3*, *4*).

Although in humans a large number of chemokines have been identified by now, this is certainly not the case for the most important laboratory and domestic animals. For example, in the bovine species the number of characterized chemokines is restricted to 10 molecules, i.e., the CC chemokines monocyte chemotactic protein-1 (MCP-1), MCP-2, RANTES, and eotaxin and the CXC chemokines GROα, GROβ, GROγ, platelet factor-4 (PF-4), granulocyte chemotactic protein-2 (GCP-2), and interleukin-8 (IL-8). Since many infectious diseases in cattle imply leukocyte infiltration, efforts to identify the mediators involved steadily increase. In the past, a novel bovine CXC chemokine, i.e., GCP-2, different from IL-8, has been discovered in our laboratory

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[‡] The regakine-1 gene sequence has been deposited in the EMBL database. The assigned accession number is AJ313203.

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(*6*). In the present study we describe the molecular cloning of a novel CC chemokine, present at high concentrations in plasma. Sequence homology searches revealed that the human equivalent of this bovine chemokine has not yet been identified. Hence, this CC chemokine represents a novel neutrophil chemoattractant predominantly present in the blood circulation.

MATERIALS AND METHODS

Cell Cultures and Chemokines. Natural human IL-8 (CXCL8) and human neutrophil-activating peptide-2 (NAP-2, CXCL7) were purified to homogeneity from monocyte-derived conditioned medium as described previously (*7*). Recombinant intact human HCC-1[1] (CCL14) was purchased from Peprotech (Rocky Hill, NJ), and the bacterial-derived chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMLP) was obtained from Sigma (St. Louis, MO). Human myeloid HL-60 cells were cultured in RPMI 1640 (Bio Whittaker, Verviers, Belgium) enriched with 20% fetal calf serum (FCS; Gibco/Life Technologies, Paisley, U.K.). Human embryonic kidney (HEK) 293 cells transfected with CXCR1 and CXCR2 (*8*) were a gift from Dr. J. M. Wang (Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick, MD). These cells were grown in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker) supplemented with 10% FCS and 800 μg/mL geneticin (Gibco/Life Technologies) to maintain the selection pressure.

Purification and Identification of Regakine-1. Regakine-1 was isolated from FCS (Gibco/Life Technologies) by subsequent adsorption to silicic acid, heparin−Sepharose affinity chromatography, cation-exchange chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC) as previously described (*9*). The purity of regakine-1 was confirmed by SDS−PAGE on Tris/tricine gels under reducing conditions (*10*). The relative molecular mass markers (Gibco/Life Technologies) were lysozyme (M_r 14 300), bovine trypsin inhibitor (M_r 6200), and the insulin β chain (M_r 3400). The NH_2-terminal amino acid sequence of regakine-1 was determined by Edman degradation on a pulsed liquid-phase protein sequencer (477/120A; PE Biosystems) with on-line detection of phenylthiohydantoin amino acids (*9*). The extended NH_2-terminal sequence (NEEPAGNMRVCCFSSVTRKIPLSLVKNYERTGDKCPQEAVIF...) was obtained by using *o*-phthalaldehyde to minimize background signals (*11*).

The molecular mass of RP-HPLC-purified regakine-1 was determined on an electrospray ion trap mass spectrometer (Esquire; Bruker Daltonik, Bremen, Germany). The protein was diluted 10-fold in 0.1% acetic acid and 50% methanol in ultrapure water and applied to the mass spectrometer by direct infusion at a flow rate of 4 μL/min. Average molecular masses were calculated from the summation of 400 spectra, resulting in an accuracy of $\pm$1.0 mass unit for chemokines.

Isolation of Neutrophilic Granulocytes from Peripheral Blood. Granulocytes were isolated from single blood donations of healthy donors (*12*). Mononuclear and polymorphonuclear cells were separated by density gradient centrifugation on Ficoll−sodium diatrizoate (Lymphoprep, Gibco/Life Technologies). The cell pellet containing granulocytes and erythrocytes was suspended in hydroxyethyl starch (Plasmasteril, Fresenius, Bad Homburg, Germany) and placed at 37 °C for 30 min to remove erythrocytes by sedimentation. Residual erythrocytes were lysed by hypotonic shock (30 s) in bidistilled water. The total granulocyte fraction was used to measure neutrophil activation.

Chemotaxis, Enzyme Release, and Calcium Signaling Assays. Chemotactic activity was determined in the Boyden microchamber assay (Neuroprobe, Cabin John, MD) (*12*). Briefly, samples were diluted in HBSS (Life Technologies) supplemented with 1 mg/mL human serum albumin (Belgian Red Cross). HL-60 cells or neutrophils were suspended in the same buffer at 2 and 1 $\times$ 10^6 cells/mL, respectively. Neutrophil migration through 5 μm pore size poly(vinylpyrrolidone)-free (PVPF) polycarbonate filters (Nuclepore, Pleasanton, CA) was allowed for 45 min at 37 °C. For HL-60 chemotaxis (2 h, 37 °C) fibronectin-coated 5 μm, PVPF polycarbonate filters were used. Migrated cells were fixed and visualized using Hemacolor staining solutions (Merck, Darmstadt, Germany) and were counted microscopically (10 oil immersion fields/well at 500$\times$ magnification). The chemotactic index was calculated by dividing the number of migrated cells toward the chemokine by the number of cells migrated toward the dilution buffer.

As an alternative assay for neutrophil activation, the release of gelatinase B was determined. After chemokine stimulation for 15 min at 37 °C, culture supernatants of freshly isolated neutrophils (3×10^5 cells) were centrifuged to remove cells. Gelatinase B activity was determined by SDS−PAGE zymography as described previously with gelatin as substrate (*6*, *13*). Quantitative determination of gelatinase B activity was achieved by scanning densitometry.

Differences in intracellular calcium concentrations ($[Ca^{2+}]_i$) induced by chemokines were monitored by fluorescence spectrophotometry on a LS50B spectrophotometer (Perkin Elmer, Norwalk, CT) by loading freshly isolated neutrophils with the fluorescent dye fura-2 (Molecular Probes, Leiden, The Netherlands) (*14*).

Receptor Binding Competition Assay. HEK293/CXCR1 or HEK293/CXCR2 cells (2×10^6) suspended in binding buffer (PBS supplemented with 20 mg/mL bovine serum albumin) were incubated with 0.2 ng/mL [^{125}I]-IL-8 and increasing concentrations of unlabeled intact IL-8 or regakine-1. Alternatively, 0.2 ng/mL [^{125}I]-IL-8 was added to freshly isolated human neutrophils together with regakine-1 at 1 μg/mL. Finally, neutrophils were preincubated (30 min at 37 °C) with regakine-1 at 300 ng/mL before addition of labeled IL-8. After incubation on ice for 2 h to allow interaction of chemokines with their receptors, cells were centrifuged and washed three times with binding buffer before determination of the bound radioactivity in a γ counter.

Cloning of the Regakine-1 Gene. On the basis of the regakine-1 protein sequence obtained by NH_2-terminal amino acid sequence analysis and the rather conserved exon/intron structure of CC chemokine genes, two degenerate primers were designed to amplify by PCR the putative second exon of the chemokine gene: 5′-GGNAAYATGMGNGTNGYTG-3′ (forward) and 5′-GCYTCYTGNGGRCAYTTRTC-

[1] Abbreviations: CXCR, CXC chemokine receptor; CI, chemotactic index; ELR, glutamate−leucine−arginine; fMLP, *N*-formylmethionylleucylphenylalanine; HCC, hemofiltrate CC chemokine; IL-8, interleukin-8; MCP, monocyte chemotactic protein; PF-4, platelet factor-4.

3′ (backward). These primers yielded a 116 bp fragment when PCR was performed on a λ phage bovine genomic library (Clontech Laboratories, Palo Alto, CA). The amplified fragment was subcloned in the pGEM-T vector (Promega Corp., Madison, WI). Sequence analysis using the dideoxynucleotide termination method on an automated laser fluoresence sequencer (ALF, Amersham Pharmacia Biotech, Rainham, U.K.) confirmed that the primers amplified the second exon of the regakine-1 gene. Consecutively, the cloned fragment was used to screen the same bovine genomic library. The probe was labeled with [^{32}P]dCTP by random priming (Megaprime DNA labeling system; Amersham Pharmacia Biotech) and purified on a Chroma Spin column (Clontech). Plaque screening was performed following standard protocols (*15*). Both strands of the gene were sequenced from a 7000 bp *Sac*I fragment by primer walking. The sequence was analyzed for homologies with the BLAST network service at the National Center for Biotechnology Information (NCBI, Bethesda, MD).

Northern Blot Analysis. Poly(A)$^+$ RNA isolated from bovine heart, lung, spleen, and liver was purchased from Clontech and prepared for Northern analysis using a kit, following the manufacturers instructions (NorthernMax-Gly; Ambion, Austin, TX). Two micrograms of poly(A)$^+$ RNA from each tissue was loaded into individual lanes of a 1% agarose gel. Electrophoresis was performed, and the separated RNA was blotted onto a nylon membrane (Hybond XL, Amersham Pharmacia Biotech). The membrane was then hybridized with a [^{32}P]dCTP labeled 660 bp *Hin*dIII restriction fragment containing the second and third exon of the regakine-1 gene. The blot was hybridized at 42 °C for 2 h, followed by washes at room temperature, at 42 °C, and at 50 °C. To control the amount of the RNA samples and their processing, the blot was stripped and rehybridized with a cDNA probe to detect constitutively expressed elongation factor-1α (EF-1α) RNA (*16*).

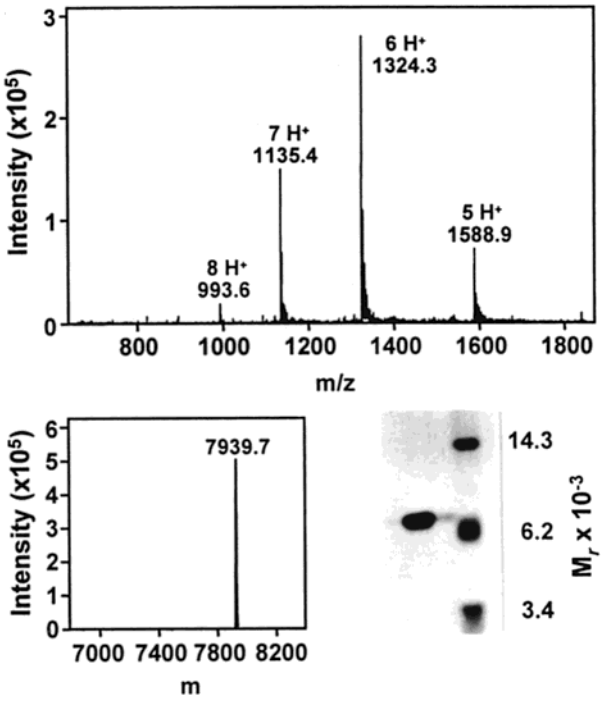

FIGURE 1: Biochemical analysis of natural regakine-1. Regakine-1 was isolated from newborn calf serum in a four-step procedure including adsorption to silicic acid, heparin affinity chromatography, cation-exchange chromatography, and HPLC. After the final purification step, regakine-1 (100 ng) was subjected to SDS−PAGE on Tris/tricine gels under reducing conditions, and proteins were visualized by silver staining. The relative molecular mass markers are indicated in Materials and Methods. The molecular mass of natural pure regakine-1 was determined by electrospray ion trap mass spectrometry. An unprocessed (upper panel) and charge-deconvoluted (lower panel) spectrum is shown. In the unprocessed spectrum, the *m*/*z* values for the differently charged ions are indicated, as are the number of protons (H^+) they carry. The average relative molecular mass (with an accuracy of $\pm$1.0) is calculated from the sum of 400 spectra.

RESULTS

Characterization of Regakine-1 as a Neutrophil Chemoattractant. During routine purification of human and mouse chemokines from in vitro cultured cell lines, a predominant low molecular mass protein was constantly recovered. Upon chromatography this 7.5 kDa protein recurrently eluted at a fixed position, irrespective of the animal cell line used as a source. A more detailed investigation revealed that the protein involved was in fact derived from the bovine serum added to grow the human and mouse cells in vitro. The purity of the serum-derived protein was confirmed by SDS−PAGE and mass spectrometry (Figure 1). Its average relative molecular mass as determined by mass spectrometry was 7939.7 $\pm$ 1.0. NH_2-terminal sequence analysis (Figure 4) demonstrated that the 7.5 kDa protein corresponded to a novel bovine CC chemokine, tentatively designated regakine-1. The true origin of regakine-1 was demonstrated by isolating the same molecule from commercially available bovine serum used for animal cell culture or from bovine plasma. When bovine serum was used as a source, sufficient quantities of regakine-1 could be purified to homogeneity, allowing biological characterization of this new CC chemokine.

Preliminary chemotaxis assays with leukocytic cell lines, routinely used in the laboratory, such as monocytic THP-1 and lymphocytic SUP-T1 cells, indicated that regakine-1 was not a potent (still inactive at 300 ng/mL) chemoattractant for mononuclear cells (data not shown). However, on immature myeloid HL-60 cells, regakine-1 dose-dependently induced chemotaxis, 30 ng/mL resulting in a maximal response (Figure 2A). For comparison, its chemotactic effect on HL-60 cells was found to be more pronounced than that of the human hemofiltrate CC chemokine HCC-1 (Figure 2A), another plasma-derived CC chemokine to which weak growth activity for myeloid progenitors has been ascribed (*17*). Since promyelocytic HL-60 cells can differentiate into granulocytes, the effect of regakine-1 was also evaluated on freshly isolated human peripheral blood neutrophils. Figure 2B shows that regakine-1 had a dose-dependent chemotactic effect on neutrophils, which was superior to that of human HCC-1. The neutrophil-activating potential of regakine-1 was confirmed using bovine neutrophils as well (data not shown). Furthermore, regakine-1 was capable of inducing release of significant gelatinase B activity from human neutrophils at 170 ng/mL (Figure 2C). However, the human CXC chemokine IL-8 was 30−100-fold more potent as a degranulator.

Synergy between Regakine-1 and fMLP. To obtain an insight in receptor usage, regakine-1 was used to desensitize the chemotactic response of human neutrophils to IL-8. No

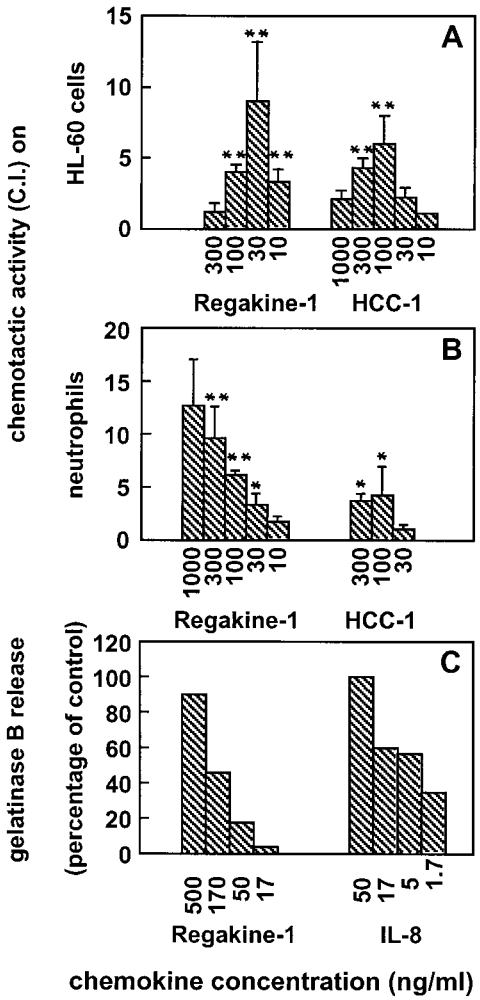

FIGURE 2: Biological activities of regakine-1. The chemotactic potency of bovine regakine-1 and human HCC-1 was compared on the human myeloid leukemia cell line HL-60 (A) and on freshly isolated human neutrophils (B) in the Boyden chamber assay. The results were expressed as the mean chemotactic index (CI) ± SEM of at least five independent experiments. Asterisks indicate significant (*, $p < 0.05$; **, $p < 0.01$, determined by the Mann−Whitney U test) increases in CI above buffer controls. Panel C shows the amount of gelatinase B released by freshly isolated human neutrophils (two independent experiments) after stimulation with human IL-8 and bovine regakine-1 at various concentrations. Released gelatinase activity in the cell supernatants was determined by gelatin zymography and quantified by scanning densitometry. After subtraction of background levels, the results were expressed relative to the release obtained in response to the highest used dose of IL-8.

inhibitory but rather a stimulatory activity on the chemotactic response of human IL-8 was observed with 300 ng/mL regakine-1 (data not shown). Similarly, regakine-1 synergized with the human CXC chemokine neutrophil-activating peptide-2 (NAP-2) in chemotaxis assays with human neutrophils (Table 1). It was then verified whether regakine-1 affected the chemotactic response to more distantly related chemoattractants such as fMLP. The CC chemokine was able to dose-dependently enhance the neutrophil chemotactic response of fMLP (at 10^{-8} or 10^{-9} M), significant increases being obtained with 30 and 100 ng/mL chemokine (Figure 3). At an optimal combination (100 ng/mL regakine-1 and 10^{-9} M fMLP) a chemotactic index was reached which was 10-fold higher than the additive effect of the two molecules tested separately.

Table 1: Regakine-1 Enhances the Neutrophil Chemotactic Response toward NAP-2

NAP-2[a] (ng/mL)	regakine-1[a] (ng/mL)	chemotactic index			
		expt 1	expt 2	expt 3	mean ± SEM
30	0	2.0	22.1	14.2	12.8 ± 5.8
30	30	3.4	47.3	51.8	34.2 ± 15.4
30	100	93.2	74.1	77.0	81.4 ± 5.9
0	30	2.0	2.3	1.8	2.0 ± 0.1
0	100	2.0	1.5	1.0	1.5 ± 0.3

[a] The human CXC chemokine NAP-2, the bovine CC chemokine regakine-1, or a combination of both chemokines was added to the lower wells of the microchamber.

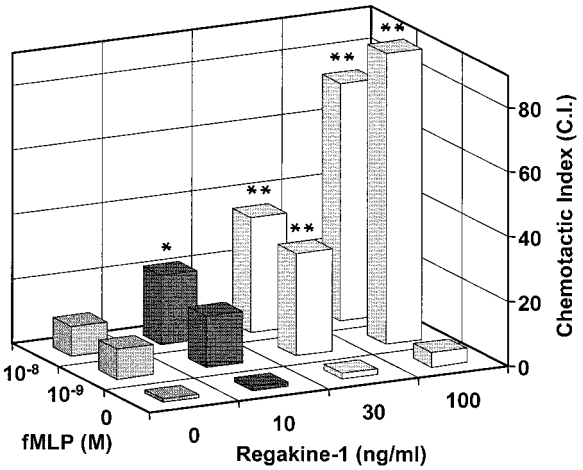

FIGURE 3: Synergistic effect of regakine-1 on neutrophil chemotaxis toward fMLP. The neutrophil chemoattractant fMLP and pure natural bovine regakine-1 were combined in the lower compartment of the microchamber to measure human neutrophil chemotaxis. The mean chemotactic indexes (CI) are derived from five independent experiments. Statistical significant increases above the additive effect of the individual chemoattractants, determined by the Mann−Whitney U test, are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$).

Further, it was verified whether regakine-1 interfered with chemokine binding to human neutrophils. First, it was found that regakine-1, at a concentration as high as 1 μg/mL added together with 0.2 ng/mL human [^{125}I]-IL-8 to neutrophils, did not displace labeled IL-8 (data not shown). In addition, [^{125}I]-IL-8 binding to neutrophils was not enhanced or decreased after preincubation for 30 min with regakine-1 (300 ng/mL), excluding the possibility that regakine-1 upregulates IL-8 receptors (CXCR1 and CXCR2) on neutrophils (data not shown).

Regakine-1 did not induce an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) in CXCR1 or CXCR2 transfectants (data not shown), a finding that is in agreement with its lack of competition for IL-8 binding to neutrophils. However, regakine-1 (1 μg/mL) by itself also failed to induce significant $[Ca^{2+}]_i$ increases in freshly isolated human neutrophils, whereas human IL-8 was capable of doing so at 3 ng/mL (data not shown). For comparison, at 500 ng/mL human HCC-1 also failed to induce calcium mobilization in neutrophils (data not shown), despite the fact that it was chemotactic for these cells at 100 ng/mL (Figure 2B). In addition, regakine-1 (300 ng/mL) could not desensitize the calcium response in human neutrophils to either IL-8 or fLMP. The apparent discrepancy between calcium signaling and neutrophil chemotactic responses observed with

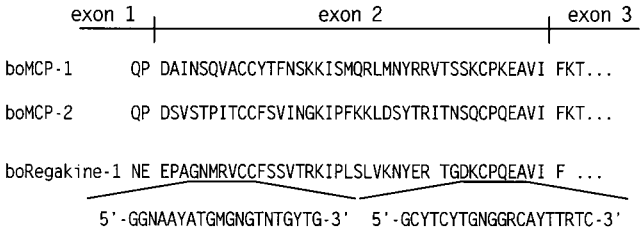

FIGURE 4: Design of a degenerate primer set for the amplification of the putative second exon of the regakine-1 gene. The NH_2-terminal amino acid sequence of regakine-1 was aligned to the bovine MCP-1 and MCP-2 sequences to allow prediction of the exon distribution of the regakine-1 gene. A set of degenerate primers was chosen on the bounderies of the putative second exon. In the primer sequences the consensus code for nucleotides is used: N = A, T, G, C; Y = C, T; R = A, G; M = A, C.

regakine-1 is a phenomenon previously reported for other chemokines, e.g., MCP-3 which induces no calcium signal through its receptor CCR5 (*18*).

Cloning of the Regakine-1 Gene. To clone the regakine-1 gene, degenerate primers were designed, on the basis of the obtained protein sequence, to perform PCR (Figure 4). It was assumed that the three exon/two intron structure of other CC chemokine genes would be preserved in the regakine-1 gene, and primers were chosen on the putative bounderies of exon 2 to maximize the length of the PCR product. The forward primer covered seven amino acids, including the first two adjacent cysteine residues. The backward primer was chosen to contain the third cysteine residue that is localized in front of the AVIF motif, four consecutive amino acids that are conserved in different species among the MCP group of CC chemokines.

A fragment of the expected size (116 bp) was amplified by PCR from a bovine genomic library and was cloned. Sequence analysis confirmed that the fragment corresponded to the second exon of regakine-1. Subsequently, this fragment was used as a probe to screen the genomic library by phage hybridization. A positive phage clone was isolated, and sequence analysis revealed the presence of the second exon as well as of the whole coding region of the regakine-1 protein. About 6.7 kb of the gene have been sequenced (Figure 5). The isolated gene sequence perfectly encoded the NH_2-terminal amino acid sequence obtained by sequencing the regakine-1 protein. The molecular mass of natural regakine-1 corresponded to the theoretical molecular weight of the protein deduced from the coding sequence of the regakine-1 gene minus the COOH-terminal lysine. The regakine-1 gene has an exon/intron organization that is highly similar to that of other CC chemokine genes. Three exons are separated by two intron sequences, a rather large first intron of 5198 bp and a second intron of 227 bp. The 5′ and 3′ ends of the introns conform to the GT/AG consensus sequence of eukaryotic splice junctions. The first intron contains different repeats, including a short interspersed nuclear element or SINE, a $(TGC)_6$ microsatellite, and direct repeats (data not shown). The methionine residue at nucleotide position 479 in the first exon was predicted as the translation initiation position by the CBS prediction server NetStart (*19*; Center for Biological Sequence Analysis, Copenhagen, www.cbs.dtu.dk). This translation start agrees with the consensus sequence for translation initiation by Kozak et al. (*20*) in that at the −3 position an adenosine is present and that the region 5′ to the ATG start is deficient in thymidines. The first exon comprises the coding sequence for the signal peptide and the first two amino acids of the mature protein. The putative signal peptide counts 21 amino acids, and the cleavage site is confirmed by the SignalP prediction program at the CBS server (*21*) and by NH_2-terminal amino acid sequence analysis on natural regakine-1. The codons for amino acids 3−41 are located in the second exon. The third exon carries the codons for the COOH-terminal part (amino acids 42−71) of regakine-1 and a 3′ untranslated region.

Regakine-1 did not show sufficient similarity in amino acid sequence with any known human (Table 2) or mouse chemokine, to be considered as the bovine homologue of one of these. Indeed, bovine regakine-1 was found to have the highest similarity (<50% identical residues) with human eotaxin, whereas for a number of other bovine CC and CXC chemokines, the human equivalent has 65−82% identical amino acids (Table 2).

Northern Blot Analysis. To evaluate the steady-state expression of regakine-1, poly(A)$^+$ RNA preparations from different bovine tissues were separated, blotted, and hybridized with a regakine-1-specific DNA probe (Figure 6). Regakine-1 RNA is well expressed in bovine spleen and lung tissue but not in the liver, suggesting that its presence in serum originates from spleen and lung. Rehybridization of the Northern blot with a probe for the housekeeping gene EF-1α showed that the absence of regakine-1 RNA in the liver was not caused by degradation of the liver RNA or sample processing.

DISCUSSION

More than a decade ago, the prototype neutrophil chemoattractant interleukin-8 was discovered with the help of in vitro chemotaxis and in vivo migration assays (*7*). Subsequently, a number of proinflammatory chemokines attracting other leukocytic cell types to sites of inflammation have been isolated on the basis of the same strategy, e.g., the eosinophil chemoattractant eotaxin (*22*). More recently, an additional number of chemokines have been identified through bioinformatics using expressed sequence tags (*1*). These "second generation" chemokines are implicated in the physiological trafficking of leukocytes, rather than in the inflammatory response. In this study, a novel bovine CC chemokine is described that has been isolated from newborn calf serum used for animal cell culture. The corresponding gene was subsequently cloned from a bovine genomic library, using degenerate primers designed on the protein sequence. This plasma-derived chemoattractant, designated regakine-1, has less than 50% amino acid sequence similarity with any currently known human or mouse chemokine. This is in contrast with other bovine chemokines that have 65−82% amino acid sequence similarity with their corresponding human counterpart (Table 2). Therefore, it must be concluded that regakine-1 represents a new member of the CC chemokine family. We can, however, not exclude that a human or mouse structural homologue does not exist.

Natural regakine-1 was purified to homogeneity as a 7.5 kDa protein from fetal or newborn calf serum (Figure 1). Molecular cloning of its gene revealed a putative protein of 71 amino acids, in addition to a predicted signal peptide of

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GTGCTCGGGGTGAACTTGCACTCACCCCCACATCCCTCTCTGGTGTCCAGCCAGAGCTGC      60
TGATCCTGAGACAGTCACCCAGCCTCTGAGCTCACGGTCCACCCCTTAGGTCGGGCTCTC     120
CGAGGGGGCTTAACCAGCCTCCCAGGGGAGACCAGGAAAGGGGGGCCATTTCTGACATAG     180
CTCCTGGTCTTCAGGCCTCCCCCACCACCACCCTCAAGACATCAGCCAAACGTGAAAGCC     240
AGGGCCACTCTCTGCCTTCCTCTTTTCTTCGTCTAAATTGACTGAGTTCTCATAATTTAC     300
ATATGTCATTGGTCACAATTCCAAAGAATCAAAACTGCAAGCTTAAATTTTTGGATCTTA     360
TGACTTCCTGAGCAGCAACTTTTTATAAAGGAGGCTCAGAGCCGAGATTTCAAGGAGCCA     420
GAGAACCCAGGAGTCCTCAGCCCAGCCCTGTTCCTGTTGCTGGTCGGCACCCAGAACGAT     480
                                                          Me
GAGGGTCAGCCTGGCTGCCCTGGCCTTCCTCCTCACTCTTGCCGTCCTGCACTCGGAGGC
tArgValSerLeuAlaAlaLeuAlaPheLeuLeuThrLeuAlaValLeuHisSerGluAl
CAATGAAGGTGAGCCTGCTACTCCCACTGCTCTGAACAAAGAGAACACAGGGGCTGGAGA     600
aAsnGluG
CAGAAGGTGGGGGTGGGCAGGGTGTGGGGCTGCAGTGAGGAGCTGTGGGTGGTCTGGAAA // 660

CATCTCCTGTCTCCCTCCCCTCCTCAGGGGCTGCGGGGAATGCAGATAGAGGTCTAAGCT    5700
TTCTCATGGAGAAGCTTCCAGCATCTTACAGTCCTCTACCTTGCAGAACCAGCTGGTAAC    5760
                                                    luProAlaGlyAsn
ATGAGAGTCTGCTGTTTCTCCTCGGTAACAAGGAAAATCCCACTCTCTTTGGTGAAAAAT
MetArgValCysCysPheSerSerValThrArgLysIleProLeuSerLeuValLysAsn
TATGAAAGGACCGGTGACAAGTGCCCCCAGGAAGCGGTCATGTAAGTACAGCCCCCCCAA    5880
TyrGluArgThrGlyAspLysCysProGlnGluAlaValIl
CAATGGAGAGGGTGCAATCTCAAGAACCAGTAGGGCTGGGAGAGGGAAGGGGGCGGGACT    5940
CTCTAGGAACAGTGACAAGAGCGGGGTGGGGTGGGGGTGACACAGGAGGCGCTGCTGGTG    6000
GGGGTCGGGGGAGAGGCTGGAGTGGTGGGACGGACTCTTCTTTGCCTGCTGACTCTCCAT    6060
TTTTCATTTTCTCCTTCTTGCTCCTCAGCTTCCAGACCAGAAGTGGGCGATCCATCTGTG    6120
                                ePheGlnThrArgSerGlyArgSerIleCysA
CCAACCCAGGCCAGGCCTGGGTACAGAAGTACATCGAATACCTGGACCAAATGTCCAAGT    6180
laAsnProGlyGlnAlaTrpValGlnLysTyrIleGluTyrLeuAspGlnMetSerLys
GAGCTGGGAACAGTGGGACCTTCACAGTCGAGGGACCATGAGAAGAAGCCACAGAGCCAC    6240
CTCCCCTCCCCAACCAGCTCCCTCACCCCAGATGGGCCCTGGGCGAGTCCTGGCCCGAAT    6300
GAAAGCCCGTGCTCGCGTTTCGCGTTTCCGTGCTCCTGCTCTCATGGTCTGCGCTCTCCC    6360
GAAGCTTTGCCGTGAAGCTCCGCCTCCTGGGGCCAGAGGAGACTGTGGCCTCCGGACAGC    6420
ATCTGTCGTCCCCTTGCCCGCGCTCTGGTCTTGAAATAAATCCGTGCTGCAGAAAGGGAC    6480
TGGTGGCTTAAATTGGTTCTCCCACAGACAGCCTGGTCATTTCTTTGATTTTACAGACAC    6540
TTCTATGGTATTTACTGCGTATCCGGGGCTTGTAAACATTTCAATAGGAGTAGATGTAAA    6600
TAGTGGGATGATGGAGGCGTAGTTAGGACCAGGAGGAGGACGAACAGTAACCCCAGTCAG    6660
GGTGCTTAGACAAGGCGGACAGTGTGAATGCAGACGACCGTGGGGAGACCCTCTGTCTGC    6720
ATGTGCAGA
```

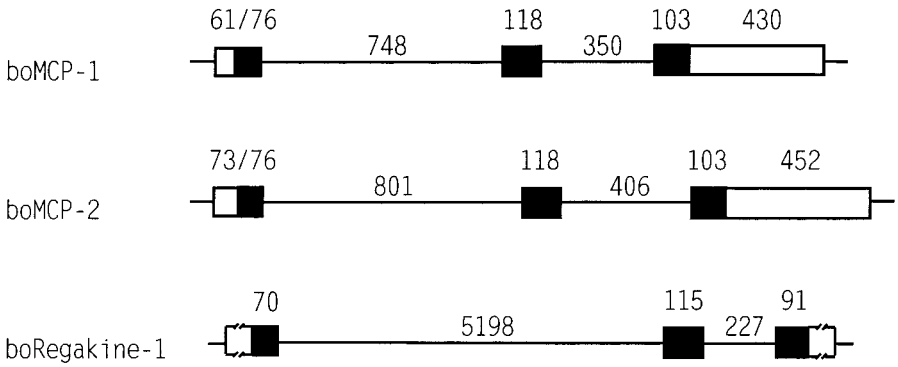

FIGURE 5: Gene structure of regakine-1. In the upper panel, the gene sequence of regakine-1 is shown with the coding DNA and the corresponding protein sequence in bold. The rather large first intron sequence is interrupted. The putative TATA box and polyadenylation sequences are underlined. The regakine-1 gene scheme (lower panel) shows the length (indicated in base pairs) of introns (straight lines) and exons (black boxes for the coding sequences). Because the cDNA sequence of regakine-1 is not available and the start and stop sites of transcription are not known, the 5′- and 3′-end untranslated regions are interrupted. The accession number for the regakine-1 gene sequence in the EMBL Nucleotide Sequence Database is AJ313203.

21 residues (Figure 5). However, NH_2-terminal sequence analysis and mass spectrometry on the purified protein allowed to conclude that natural regakine-1 (M_r of 7940) starts with an asparagine residue and is missing the COOH-terminal lysine and thus contains only 70 residues. The regakine-1 gene is the third bovine CC chemokine gene described so far, following reports on the bovine MCP-1 and MCP-2 genes (*23*, *24*). Similar to other CC chemokines, this gene consists of three exons and two introns. The 500 bp stretch upstream of the start codon contains a TATA box, whereas at the 3′ end of the gene a putative polyadenylation signal, AATAAA, was identified. Compared to these genes

Table 2: Sequence Similarity between Bovine Chemokines and Their Human Counterparts

chemokine		
bovine	human	% identical residues
MCP-1	MCP-1 (CCL2)	72
MCP-2	MCP-2 (CCL8)	67
RANTES	RANTES (CCL5)	82
eotaxin	eotaxin (CCL11)	65
IL-8	IL-8 (CXCL8)	74
GROα	GROα (CXCL1)	74
GROβ	GROβ (CXCL2)	75
GROγ	GROγ (CXCL3)	79
GCP-2	GCP-2 (CXCL6)	67
PF-4	PF-4 (CXCL4)	70
regakine-1	eotaxin (CCL11)[a]	49

[a] Human eotaxin is the CC chemokine with the highest similarity to regakine-1.

(e.g., 3.3 kb for bovine MCP-2), the regakine-1 gene is rather large, due to an extended first intron of 5198 bp. In addition to the three identified bovine CC chemokine genes, the cDNAs of bovine RANTES (*25*) and eotaxin (Genbank Accession Number AJ132003) have been cloned. Furthermore, four CXC chemokine genes (IL-8, GROα, GROβ, and GROγ) are known, three of which are located on chromosome 6 of the bovine genome (*26*).

Human CXC chemokines are chemotactic for neutrophils or lymphocytes, depending on whether their primary structure is characterized by the presence or absence of the glutamate−leucine−arginine sequence (ELR motif), respectively. Members of the CC chemokine family attract all types of leukocytes including monocytes, dendritic cells, lymphocytes, NK cells, eosinophils, basophils, and to a lesser extent also neutrophils (*1*, *2*). At physiological bovine plasma concentrations, regakine-1 was found to optimally stimulate migration of immature human myeloid cells. In addition, it was capable of inducing chemotaxis and gelatinase B release from mature neutrophils, freshly isolated from human peripheral blood (Figure 2). MCP-3, another CC chemokine, has been shown to exert effects on neutrophils including chemotaxis (*27*). Despite its weaker specific activity in neutrophil degranulation and chemotaxis assays compared to the prototypic CXC chemokine IL-8, regakine-1 was a more potent neutrophil chemoattractant than the hemofiltrate CC chemokine HCC-1. In addition, regakine-1 enhanced the chemotactic response of human neutrophils when combined with the CXC chemokines IL-8 (data not shown) and NAP-2 (Table 1) or the bacterial peptide fMLP (Figure 3). This capacity to synergize resulted in a 10-fold higher chemotactic activity than the additive effects of regakine-1 and fMLP. The exact mechanism of this phenomenon remains to be resolved. Regakine-1 did not displace IL-8 from its receptors on neutrophils, nor did it prevent binding of IL-8 after regakine-1 pretreatment. Such pretreatment (priming) of neutrophils with regakine-1 did not alter the calcium signal elicited by IL-8 or fMLP (data not shown). Preincubation of neutrophils with IL-8 had also no effect on fMLP-elicited calcium signaling (*28*). Nevertheless, for the oxidative burst elicited in neutrophils by fMLP, increasing effects have been described for the ELR$^+$-CXC chemokines IL-8, GROα, or epithelial cell-derived neutrophil-activating protein-78 (ENA-78), ligands of CXCR1 and/or CXCR2 (*28*−*30*). Furthermore, only preincubation of neutrophils with the ELR$^-$-CXC chemokine PF-4 that does not recognize CXCR1 or CXCR2 induced the secretion of myeloperoxidase in response to fMLP (*31*). Another well-described priming agent potentiating neutrophil reactivity is the colony stimulating factor for granulocytes and monocytes, GM-CSF (*32*). This priming action of GM-CSF on the superoxide generation by fMLP on neutrophils is mediated by several kinases that activate the cytosolic NADPH oxidase after phosphorylation (*33*). Finally, the CXC chemokine GROα was able to prime IL-8-induced neutrophil chemotaxis (*34*). It must, however, be noticed that the reports mentioned above required priming of the neutrophils (during at least 10 min at 37 °C), whereas the synergy observed between regakine-1 and fMLP (Figure 3) in chemotaxis was also obtained by direct coapplication in the assay. It can at present not be concluded that a common cellular pathway is responsible for the priming effects of various cytokines on neutrophil activation. In some cases effects at the receptor level have been reported (*29*, *32*), whereas changes in signal transduction are demonstrated in other studies (*33*). Taken together, it is difficult to predict the exact molecular pathway to explain the synergy observed between regakine-1 and other neutrophil chemoattractants.

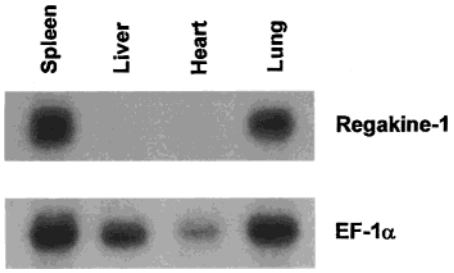

FIGURE 6: Tissue-specific expression of regakine-1 RNA. Poly-(A)$^+$ RNA preparations (Clontech) from various bovine tissues were probed after Northern blotting with a regakine-1 gene segment (upper panel) and EF-1α cDNA (lower panel).

Regakine-1 RNA was found to be expressed in lung and spleen but not in liver (Figure 6), whereas high constitutive protein levels are present in plasma. The hemofiltrate CC chemokine HCC-1, originally isolated from patients with chronic renal failure and also detectable at high concentrations in normal plasma, is predominantly expressed in spleen and heart tissue but not in kidney and brain (*17*). For both chemokines, the exact cellular source remains to be determined. Plasma-derived CXC chemokines, such as PF-4 and neutrophil-activating protein-2 (NAP-2), are solely released from activated platelets, whereas most inflammatory chemokines are inducible in multiple cell types of epithelial, mesenchymal, or hematopoietic origin. The presence of chemokines in the blood circulation under physiological conditions, rather than in inflammatory conditions, implicates a diverging role of these chemokines in normal versus pathological situations. During an inflammatory response to infection within the vascular compartment, the platelet-derived neutrophil chemoattractant NAP-2 may contribute to neutrophil activation and trapping in the microvasculature, e.g., during the adult respiratory distress syndrome, leading to tissue damage (*35*). In contrast, constitutively expressed chemokines, such as regakine-1, could rather be implicated in the recruitment of neutrophils from the bone marrow to the blood circulation. In this respect, the significant chemotactic potency of regakine-1 on immature myeloid cells

represents an important finding. In addition, constitutive regakine-1 can enhance the inflammatory response after infection, through synergy with exogenous (microbial) or endogenous (chemokines) neutrophil chemoattractants. Further research in these directions will allow a better understanding of the molecular mechanisms implicated in the biological activities of this novel chemokine.

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