THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 16, Issue of April 21, pp. 11721–11727, 2000

*Printed in U.S.A.*

# Examination of the Function of RANTES, MIP-1α, and MIP-1β following Interaction with Heparin-like Glycosaminoglycans*

(Received for publication, January 21, 2000)

**Simi Ali‡, Adrian C. V. Palmer, Ben Banerjee, Sarah J. Fritchley, and John A. Kirby**

*From the Immunobiology Group, Department of Surgery, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, United Kingdom*

**Chemokines are a group of small proteins that have a variety of functions, including the activation and recruitment of immune cells during episodes of inflammation. In common with many cytokines, it has been observed that chemokines have the potential to bind heparin-like glycosaminoglycan molecules, which are normally expressed on proteoglycan components of the cell surface and extracellular matrix. The significance of this interaction for chemokine activity remains a subject of debate. In this study, Chinese hamster ovary cells were transfected separately with the human chemokine receptors CCR1 and CCR5, and these receptors were shown to induce an intracytoplasmic $Ca^{2+}$ flux and cellular chemotaxis following stimulation with the natural CC chemokine ligands (MIP-1α, RANTES (regulated on activation normal T cell expressed), and MIP-1β). In further experiments, mutant CHO cells, with a defect in normal glycosaminoglycan (GAG) expression, were also transfected with, and shown to express similar levels of, CCR1 and CCR5. Although these receptors were functional, it was found that the mutant cells required exposure to higher concentrations of ligands than the wild-type cells in order to produce the same intracytoplasmic $Ca^{2+}$ flux. Radioligand binding experiments demonstrated that specific chemokine receptors expressed by wild-type cells had a significantly greater affinity for MIP-1α than similar receptors expressed by GAG-deficient mutants. However, there was no significant difference between these cells in their affinity for RANTES or MIP-1β. In conclusion, it has been demonstrated clearly that GAG expression is not necessary for the biological activity of the chemokines MIP-1α, RANTES, or MIP-1β. However, the presence of cell surface GAGs does enhance the activity of low concentrations of these chemokines by a mechanism that appears to involve sequestration onto the cell surface.**

The cell-mediated immune response is critically dependent on patterns of leukocyte migration and activation within target tissues. The vascular endothelium plays a central role in the recruitment of blood-borne cells to the subendothelial tissues during inflammation by facilitating a cascade process involving intravascular arrest of certain cell types and directed extravasation of responsive cells (1–3). It is becoming clear that this process is regulated principally by cytokine molecules termed the chemokines (4).

The chemokines are a family of small chemoattractant proteins that are capable of activating and promoting vectorial migration of a variety of leukocytes. They can be grouped into four subfamilies on the basis of the disposition of conserved cysteine residues; these are termed the C, $CX_3C$, CC, and CXC groups (5–8). The C and $CX_3C$ groups each have only one known member, whereas the CC and CXC groups have many members. The CC (or β-) chemokines, in which the first two conserved cysteine residues are adjacent, are believed to be the most important mediators of mononuclear cell inflammation.

Chemokines bind to receptors of the seven-transmembrane-spanning type and also bind to glycosaminoglycans (GAGs),[1] including heparan sulfate (9, 10). Nine receptors for CC chemokines (CCRs) have been identified (11–13); however, *in vitro* studies have shown some redundancy with more than one chemokine binding to a given receptor and several receptors being able to bind a single chemokine.

Previous *in vivo* and *in vitro* studies have demonstrated clearly that chemokines provide directional cues. However, the mechanism by which the chemokine concentration gradient required for cellular chemotaxis is maintained remains unclear. A gradient of soluble chemokine would not be expected to remain stable *in vivo*, particularly under conditions of blood flow at the endothelial surface, although it is known that the CXC chemokine, IL-8, retains its ability to induce neutrophil migration after adsorption onto a solid phase support (14).

Furthermore, the potential for binding to GAG components of proteoglycans, either at the cell surface or in the extracellular matrix, might allow formation of an immobilized chemokine gradient for presentation to leukocytes. In support of this, Gilat *et al.* (15) have shown that MIP-1β and RANTES bind to an *ex vivo* extracellular matrix preparation in a heparinase-sensitive manner, and that these bound chemokines are capable of stimulating leukocyte activation.

A number of growth factors and proinflammatory cytokines have been shown to bind to GAGs (16); these include acidic and basic fibroblast growth factors (17), several chemokines (18), interferon-γ (19), and several of the interleukins (10). While the functional implications of these interactions are not fully understood, it has been proposed that proteoglycans protect these small molecules from degradation, act as cytokine storage sites, or aid presentation to specific cell-signaling receptors. The presentation hypothesis suggests that chemokines are presented to leukocytes in the form of a solid-phase gradient while bound to immobile GAGs. Hence, it is possible that chemokine-GAG interactions are important not only for receptor presentation but also for target cell activation *in vivo*.

* This work was supported by the Northern Counties Kidney Research Fund, the Wellcome Trust, and the British Heart Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 0191 222 7158; Fax: 0191 222 8514; E-mail: simi.ali@newcastle.ac.uk.

[1] The abbreviations used are: GAG, glycosaminoglycan; CCR, CC chemokine receptor; IL, interleukin; CHO, Chinese hamster ovary; RANTES, regulated on activation normal T cell expressed; CS-A, chondroitin sulfate A.

The relationship between chemokine-GAG interactions and activity has been addressed in only a few studies. Maione *et al.* (20) have presented evidence that a non-GAG binding mutant of the CXC chemokine PF4, in which four basic residues in the C-terminal helix are changed to acidic or neutral residues, retains both *in vitro* anti-angiogenic activity and *in vivo* anti-tumor activity. These results indicate that for PF4, heparin binding capacity and biological activity is not directly related. By contrast, IL-8 with a truncated C-terminal helix fails to bind heparin and has impaired cell activation and receptor binding properties (21). It is known that the heparin binding site on human IL-8 is spatially distinct from the residues involved in receptor binding, which suggests that GAGs may play a role in presenting IL-8 to its receptors (22) in a manner similar to that proposed for basic fibroblast growth factor (23). However, a recent study used a non-heparin-binding mutant of the CC chemokines MIP-1$\alpha$ and MIP-1$\beta$ to demonstrate that GAG binding is not a prerequisite for receptor ligation and signal transduction *in vitro* (24, 25).

In this study, a GAG-deficient Chinese hamster ovary cell mutant (745-CHO) and the corresponding wild-type cell (K1-CHO) were each transfected separately with the human chemokine receptors CCR1 and CCR5. This allowed examination of ligand-induced signaling through a single receptor and direct investigation of the contribution of GAG molecules to receptor affinity and cellular activation.

## EXPERIMENTAL PROCEDURES

*Cell Lines*—Professor Jeffrey Esko (Department of Biochemistry, University of Alabama at Birmingham) kindly supplied the CHO cell lines used in the present study. Wild-type CHO cells (K1-CHO) and the proteoglycan-deficient variant pgsA-745 (745-CHO), which makes little if any cell surface proteoglycan (26), were each maintained in Ham's F-12 medium (Sigma) supplemented with 10% fetal calf serum; the cells were subcultured every 3–4 days.

*Generation of Stable Transfectants*—A DNA fragment encoding the entire open reading frame of human CCR1 was amplified by reverse transcription-coupled PCR using RNA prepared from freshly isolated peripheral blood mononuclear cells from a healthy adult volunteer. The sense and antisense primers used for the amplification were (5′-CCATGGAAACTCCAAACACC-3′ and 5′-CCTACATCAGTGTCTCC-3′). The polymerase chain reaction product was subsequently cloned into the TA cloning vector (Invitrogen) before CCR1 was excised on a *Bam*HI/*Bgl*II restriction fragment and subcloned into the eukaryotic expression vector pSR$\alpha$. The sequence of the resulting construct was verified by DNA sequence analysis. A plasmid containing the full-length human chemokine receptor CCR5 was kindly provided by Dr. M. Parmentier (IRIBHN, Universite libre de Bruxelles, Belgium).

CCR1 and CCR5 transfectants were generated for both the mutant and wild-type CHO cell lines by electroporation. Stable transfectants were selected by culture in medium supplemented with G418 (600 $\mu$g/ml; Calbiochem). Individual G418-resistant colonies were picked and expanded.

*Antibodies and Flow Cytometric Analysis*—The antibodies used for immunofluorescence flow cytometric analysis were 2D7/CCR5 (anti-CCR5, murine monoclonal; Becton Dickinson) and H-52 (anti-CCR1, rabbit polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Control and transfected cells were labeled with an optimal concentration of the appropriate antibody at 4 °C for 20 min, washed, and counterstained with a fluorescein isothiocyanate-conjugated secondary antibody for a further 20 min. The stained cells were analyzed by flow cytometry (FACSort; Becton Dickinson). Data analysis was performed using Lysis II software (Becton Dickinson). An irrelevant, isotype-matched antibody (IgG1, X931; Dako) was used as a negative control in each labeling experiment.

*Chemotaxis Assay*—Chemotaxis assays were performed using CCR-transfected and untransfected K1-CHO cells. MIP-1$\alpha$ was obtained from R & D Systems (Abingdon, Oxon, UK), dissolved at 10 $\mu$g/ml in sterile phosphate-buffered saline and diluted to between 1 and 10 nM in HEPES-buffered RPMI 1640 supplemented with 1% bovine serum albumin. This chemokine was placed in the lower well of a 24-well transwell system (Falcon). The CHO cells were suspended at $2 \times 10^6$/ml in RPMI 1640 containing 1% bovine serum albumin and added to the upper chamber in a volume of 50 $\mu$l. The two chambers were separated by a polycarbonate filter with 8-$\mu$m pores, which was precoated with type IV collagen. The chamber was incubated for 5 h at 37 °C in 5% $CO_2$. After this time, the filter was stripped to remove cells from the upper surface, and the cells on the underside were fixed with methanol and stained with hemotoxylin. Quantification of the chemotaxis was performed by counting the mean number of migrant cells per high power field. All assays were performed in triplicate.

*$Ca^{2+}$ Mobilization by Chemokines*—Wild-type and mutant CHO transfectants were grown on 10-mm coverslips in 24-well tissue culture plates until confluent. The coverslips were then removed and transferred in Petri dishes, and the cells were loaded with Fura-2 (Sigma) by incubation for 15 min at 37 °C in 1 ml of medium containing 3 $\mu$g/ml of the acetoxymethylester form of the dye (diluted from 1 mg/ml stock in dimethyl sulfoxide). The cells were then washed with fresh medium to remove excess dye before the coverslips were mounted in a superfusion chamber on the stage of a microspectrofluorimeter based on a Nikon Diaphot epifluorescence microscope fitted with an excitation filter changer and multipoint imaging system. Up to 16 single cells were monitored per experiment. Cells were temperature-equilibrated for 5 min and superfused with balanced salt solution (140 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 2.5 mM $CaCl_2$, 5 mM glucose, 5 mM HEPES, pH 7.4) at a rate of approximately 5 ml/min. During this time, the basal fluorescence spectrum was observed.

Chemokines were added to the chamber at concentrations between 0.5 and 100 nM, and the fluorescence was monitored. The results were collected every 1.5 s as the ratio of the fluorescence signal at 500 nm following excitation at 340 nm to that at 380 nm. ATP (1 $\mu$M; Sigma) was used at the end of each experiment for positive control. Each experiment was repeated three times on different days with similar results for each clone.

*Radioligand Binding Assays*—Before assay each of the transfectant cell lines was seeded in flat-profile 96-well plates at 5000 cells/well in Ham's F-12 medium containing 10% fetal calf serum and allowed to attach for 48 h. After this period, the cells were rinsed with phosphate-buffered saline, and the medium was removed by aspiration. Cold ligand competition assays were performed in a total volume of 150 $\mu$l (Hanks' balanced salt solution, 10 mM HEPES, 0.1% bovine serum albumin) using 100 pM $^{125}$I-MIP-1$\alpha$, $^{125}$I-RANTES, or $^{125}$I-MIP-1$\beta$ and a variable concentration of the corresponding unlabeled chemokines. After incubation of the cells at 37 °C for 90 min, the plate was washed with Hanks' balanced salt solution containing 10 mM HEPES and 0.5 M NaCl to remove the unbound $^{125}$I-chemokines. The cells were then lysed by incubation at 37 °C for 2 h in a solution containing 1% SDS and 1 M NaOH. The lysed cells were transferred to test tubes, and the radioactivity was measured in a $\gamma$-counter. Radioligand binding parameters were calculated using Prism 3 software (GraphPad).

The effect of the soluble GAGs, heparin, heparan sulfate, and CS-A on chemokine binding was investigated by the addition of these molecules at concentrations between 0 and 250 $\mu$g/ml at the time of radioligand binding.

*Statistical Analysis*—All results are expressed as mean ± S.D. (or S.E.) of the corresponding replicates. The significance of changes in receptor affinity by various ligands was assessed by the application of Student's *t* test. Data were stored and analyzed using Prism 3 software.

## RESULTS

The K1-CHO and 745-CHO cell lines formed monolayers in culture and showed cobblestone morphology typical of epithelioid cells. The plasmids pSR$\alpha$-CCR1 and -CCR5 were transfected separately into the K1-CHO and 745-CHO cell lines. Following selection for G418 resistance, at least 20 colonies from each transfection of each cell line were picked for expansion. Clones were examined by immunofluorescence flow cytometry to compare surface expression of CCR1 or CCR5; those expressing approximately equal levels of each receptor were chosen for further study. Neither CCR1 nor CCR5 was detectable in mock-transfected cell lines. Representative immunofluorescence results for CCR5 expression are shown in Fig. 1; in each case, the median fluorescence for the transfected cells was similar. Similar data (not shown) was collected for the CCR1 transfectants.

*Ligand-induced Migration of CCR1- and CCR5-transfected Cells*—This assay was performed to determine whether the CCR-transfected CHO cells contained all the intracellular mol-

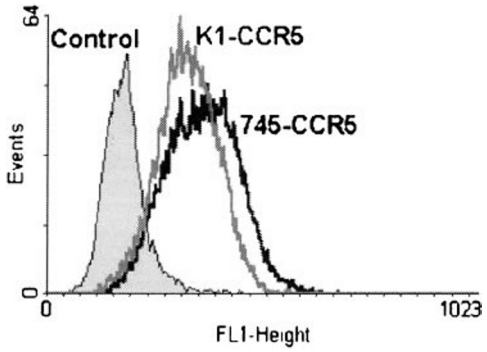


FIG. 1. **Representative flow cytometric results showing the expression of CCR5 on the surface of K1-CHO (K1-CCR5) and 745-CHO (745-CCR5) transfectants.** These lines were selected for further study on the basis of similar fluorescence intensity, which indicates approximately equal levels of receptor expression.

ecules required for chemokine-receptor signal transduction. The results showed that both K1-CCR1 and K1-CCR5 cells were able to migrate in response to chemotactic stimulation by MIP-1$\alpha$ and RANTES or MIP-1$\alpha$, RANTES, and MIP-1$\beta$, respectively. Wild-type untransfected cells were unresponsive to chemokine stimulation in this assay. These experiments were carried out with agonist concentrations between 0.01 and 100 nM; limited migration occurred at 1 nM, but it reached a maximal level at 10 nM. Representative results for K1-CCR1- and K1-CCR5-expressing cells are shown in Fig. 2.

Having established that K1-CHO transfectants have all the components necessary for chemokine-mediated intracellular signal transduction, a series of chemotaxis experiments was performed to compare the chemokine sensitivity of CCR-expressing wild-type cells (K1-CHO) with that of the 745-CHO transfectants. At an agonist concentration of 10 nM, the number of cells (745-CCR1 and 745-CCR5) migrating in response to chemotactic stimulation were significantly less than number of migrating wild-type transfectants (Fig. 2). 745-CCR5 and 745-CCR1 did not yield a typical bell-shaped dose-response curve, and the number of migrating cells was still increasing at agonist concentrations of 100 nM (data not shown).

*Calcium Mobilization by K1-CHO Cells Expressing CCR1 and CCR5*—A series of experiments was performed to determine whether the transfected CCR1 and CCR5 receptors could induce an intracellular $Ca^{2+}$ flux following stimulation by MIP-1$\alpha$ at 100 nM. Control cells, which had been transfected with vector alone, did not respond to MIP-1$\alpha$ (Fig. 3*a*). However, it was found that both the K1-CCR1 and K1-CCR5 cells showed a rapid and transient increase in intracellular $Ca^{2+}$ following stimulation with this chemokine (Fig. 3, *b* and *c*). All of these cells showed a positive control response following stimulation with ATP.

*Calcium Mobilization by Mutant 745-CHO Expressing CCR1 and CCR5*—Following stimulation with a high concentration (100 nM) of MIP-1$\alpha$, both the K1-CCR1 and the 745-CCR1 cells showed a similar $Ca^{2+}$ flux (Fig. 4*a*); similar results were observed for K1-CCR5 and 745-CCR5 cells (Fig. 4*b*). However, examination of the MIP-1$\alpha$ dose-response curve for both CCR1 and CCR5 transfectants (Fig. 4) showed an $EC_{50}$ for K1-CCR1 and 745-CCR1 of 1.5 and 8 nM, whereas the corresponding $EC_{50}$ values for the K1-CCR5 and 745-CCR5 were 1.10 and 10.0 nM, respectively.

Table I shows that significant differences in $EC_{50}$ were also observed for these cells following stimulation with RANTES (CCR1 transfectants) or both RANTES and MIP-1$\beta$ (CCR5 transfectants). These data show that the expression of GAGs is not a prerequisite for CCR1 or CCR5 signaling *in vitro*. However, the presence of GAG molecules does appear to increase

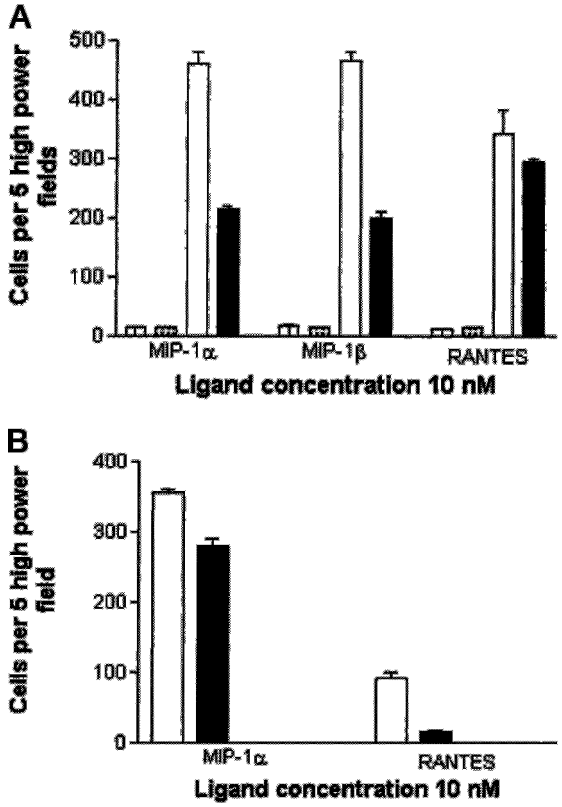


FIG. 2**. Chemotactic migration of K1-CHO cells, 745-CHO cells, and their corresponding CCR1 and CCR5 transfectants following stimulation by 10 nM MIP-1$\alpha$, MIP-1$\beta$, and RANTES.** *a*, chemotaxis by CCR5 transfectants: K1-CCR5 (*open bars*) and 745-CCR5 (*filled bars*). Control migration by untransfected K1-CHO cells (*striped bars*) and 745-CHO (*dotted bars*) was also measured. *b*, chemotaxis by CCR1 transfectants: K1-CCR1 (*unfilled bars*) and 745-CCR1 (*filled bars*). This assay was performed in triplicate, and the number of migrating cells in five high power fields ($\times$ 400) was counted for each membrane. The mean ($\pm$ S.D.) is shown.

the sensitivity of the transfectants to chemokine stimulation by between 4- and 9-fold.

*Effect of Heparin on $Ca^{2+}$ Mobilization by MIP-1$\alpha$*—A series of experiments was performed to determine whether heparin can directly antagonize the activity of MIP-1$\alpha$. It was found that pretreatment of MIP-1$\alpha$ (30 nM) with heparin (250 $\mu$g/ml) resulted in 64 and 66% (mean values from at least 16 separate cells) blockade of the potential chemokine-mediated $Ca^{2+}$ mobilization in the CCR1- and CCR5-expressing K1-CHO transfectants, respectively (Fig. 5). Heparin alone had no effect on the intracellular concentration of $Ca^{2+}$ (results not shown).

*Effect of Heparin-like and Other GAGs on Binding of RANTES to Its Receptor CCR5 in K1 and 745-CHO Transfectants*—In view of the significant blockade of the chemokine-mediated $Ca^{2+}$ mobilization by heparin, the effect of heparin, heparan sulfate, and CS-A on binding of $^{125}$I-RANTES to its receptor CCR5 was also studied. Fig. 6 compares the antagonistic properties of weight-matched doses of a range of GAG molecules (50–250 $\mu$g/ml). These results indicate that CS-A had no effect on the RANTES binding to its specific receptor in both K1-CCR5 and 745-CCR5 transfectants ($p > 0.07$). However, both heparin and heparan sulfate produced a significant inhibition of the binding of RANTES to K1-CCR5 (Fig. 6*a*) and 745-CCR5 (Fig. 6*b*) transfectants. Heparan sulfate also inhibited RANTES binding to K1-CCR1 cells at a concentration of 100 $\mu$g/ml ($p < 0.05$), while heparin was inhibitory at 50 $\mu$g/ml ($p < 0.05$).

A study of basic fibroblast growth factor has shown that a

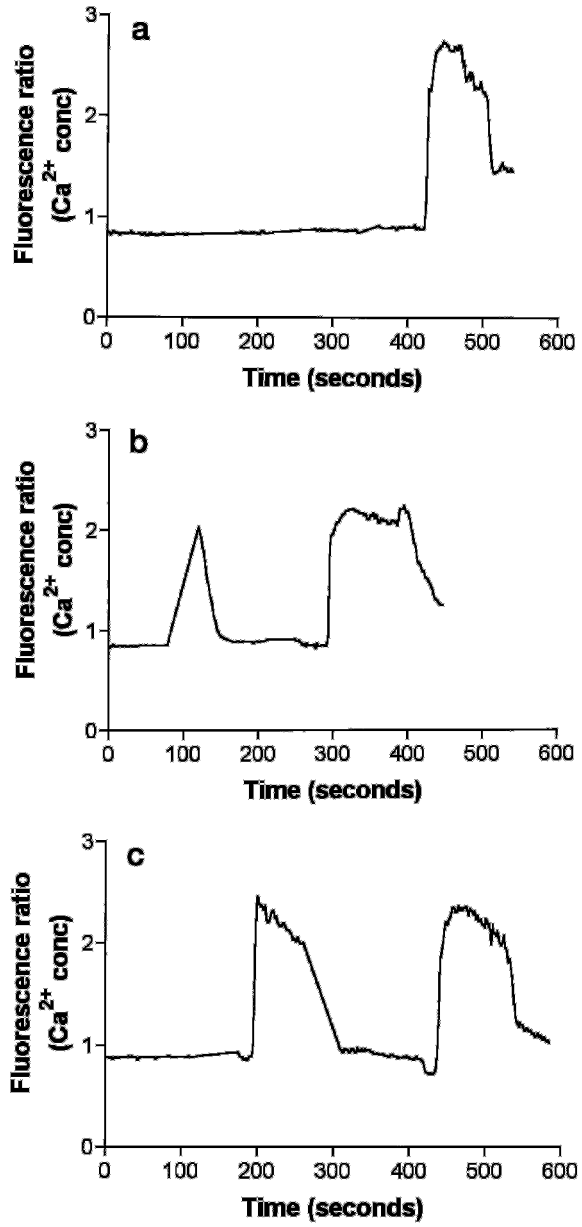


FIG. 3. **Representative changes in the intracellular [$Ca^{2+}$] concentration following MIP-1$\alpha$ (100 nM) and control (ATP, 1 $\mu$M) stimulation of untransfected K1-CHO cells (*a*), K1-CHO transfected with CCR1 (*b*), and K1-CHO transfected with CCR5 (*c*).** The single peak in *a* and the second peak in *b* and *c* are the result of positive control stimulation with ATP; the first peak in *b* and *c* is the result of stimulation with MIP-1$\alpha$. In each case, the results show for 16 cells the mean ratio of the fluorescence signal at 500 nm following excitation at 340 nm to that following excitation at 380 nm.

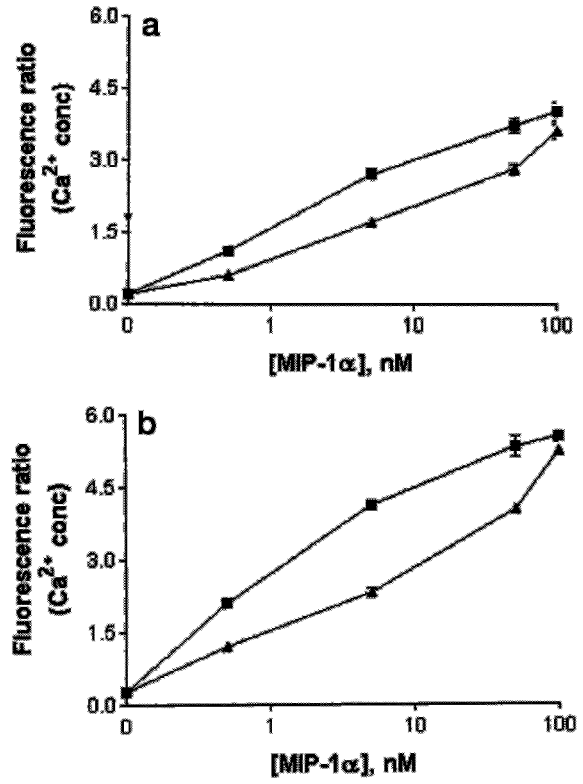


FIG. 4. **Dose-response curve showing changes in the maximal intracellular [$Ca^{2+}$] measured in K1-CHO (■) and 745-CHO (▲) cells transfected with CCR1 (*a*) or CCR5 (*b*) following stimulation with MIP-1$\alpha$.** The mean values from three separate experiments are plotted as a ratio of the fluorescence emission at 500 nm following excitation at 340 nm to that following excitation at 380 nm. The calculated $EC_{50}$ for K1-CCR1 and K1-CCR5 was 1.5 and 1.1 nM, respectively, whereas the $EC_{50}$ for 745-CCR1 and 745-CCR5 was 8.0 and 10.0 nM.

TABLE I

*Dose-response curves showing changes in the intracellular $Ca^{2+}$ concentration were measured for each transfectant following chemokine stimulation*

The calculated $EC_{50}$ values are shown.

| Chemokines | K1-CCR1 | 745-CCR1 | K1-CCR5 | 745-CCR5 |
|---|---|---|---|---|
| | | *nM* | | |
| MIP-1$\alpha$ | 1.5 | 8 | 1.1 | 10 |
| RANTES | 2 | 8 | 1.5 | 7 |
| MIP-1$\beta$ | ND[a] | ND | 3 | 7.5 |

[a] ND, not determined.

low concentration (40 ng/ml) of free heparin can reconstitute ligand binding to GAG-deficient cells (27). As a consequence of this report, an additional series of experiments was performed to examine this possibility for chemokines. The binding of $^{125}$I-RANTES or $^{125}$I-MIP-1$\alpha$ to K1-CCR1, K1-CCR5, 745-CCR5, or 745-CCR1 cells was examined in the presence of low concentrations of soluble heparin (20–320 ng/ml). However, there was no potentiating effect of this range of concentrations of heparin on the high affinity binding of these chemokines to their receptors on either GAG-deficient 745-CHO transfectant or the wild-type transfectants. A similar study of the $Ca^{2+}$ flux induced by these chemokines in mutant CHO transfectants also failed to demonstrate a significant increase in biological activity (data not shown).

*Effect of Cell Surface GAGs on the Affinity of Chemokine Binding to Cells Transfected with CCR1 and CCR5 Receptors*—To determine whether the presence of GAG molecules on the cell surface can alter the apparent affinity for ligands of CCR1 or CCR5, a series of radioligand binding assays were performed using $^{125}$I-MIP-1$\alpha$, $^{125}$I-RANTES, and $^{125}$I-MIP-1$\beta$. An initial experiment demonstrated that incubation of untransfected K1-CHO or 745-CHO with a high concentration (5 nM) of $^{125}$I-MIP-1$\alpha$ resulted in approximately 3 times more binding to the wild-type cells than to a similar number of GAG-deficient mutant cells ($p < 0.01$). Detailed radioligand binding experiments were then performed using the full panel of transfected cell lines.

Representative homologous cold-ligand competition curves for $^{125}$I-MIP-1$\alpha$ are shown in Fig. 7. In all cases, the data were consistent with a single class of receptors, but it is possible for additional low affinity sites to exist beyond the range of this assay. The $K_d$ value for K1-CCR1 and K1-CCR5 was found to be 116 ± 26 and 196 ± 82 pM (mean ± S.D., $n = 3$), whereas

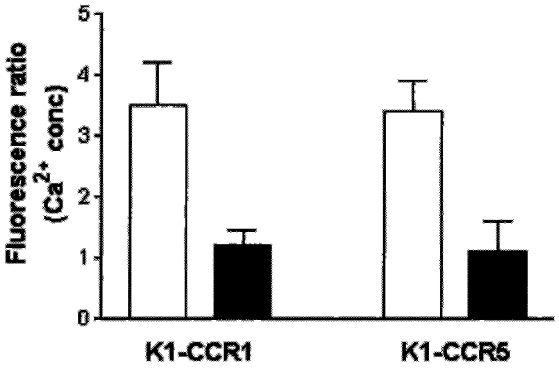


FIG. 5. **Effect of heparin on MIP-1$\alpha$-induced $Ca^{2+}$ mobilization by K1-CCR1 and K1-CCR5 transfectants.** The *open bars* show the response to MIP-1$\alpha$, while the *solid bars* indicate the response to a mixture of heparin and MIP-1$\alpha$. MIP-1$\alpha$ at 30 nM concentration was pretreated with heparin (250 $\mu$g/ml) for 1 h at 4 °C. The representative data are presented as the mean ± S.D. for at least 16 different cells and show the ratio of the fluorescence emission at 500 nm following excitation at 340 nm to that following excitation at 380 nm.

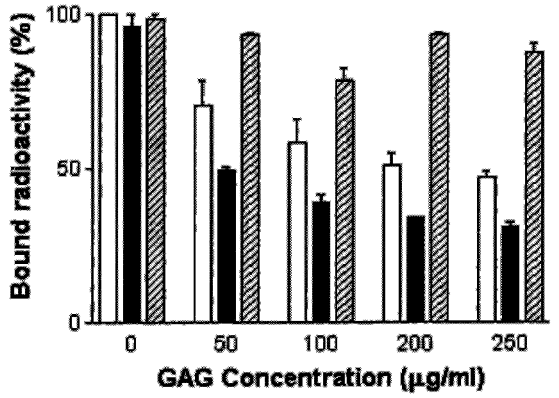


FIG. 6. **Results of a representative experiment showing the effect of increasing concentrations of heparin (*open columns*), heparan sulfate (*filled columns*), and CS-A (*shaded columns*) on binding of $^{125}$I-RANTES to K1-CCR5 cells (*a*) and 745-CCR5 cells (*b*).** *Error bars* indicate the S.E.

745-CCR1 and 745-CC5 had $K_d$ values of 688 ± 364 and 5090 ± 1700 pM, respectively.

A further series of experiments was performed to assess the effect of GAGs on the binding affinities of RANTES and MIP-1$\beta$ to these receptors (Table II). The $K_d$ values measured for RANTES binding to CCR1 and CCR5 receptor on K1-CHO cells were not significantly different from those on 745-CCR1 and 745-CCR5 ($p = 0.17$ for K1-CCR1 and 745-CCR1, and $p = 0.14$ for K1-CCR5 and 745-CCR5) cells. MIP-1$\beta$, which is a ligand for CCR5, also did not demonstrate a significant difference in binding affinities between K1-CCR5 and 745-CCR5 cells ($p > 0.09$).

These data show that GAGs increase the affinity for MIP-1$\alpha$ of both CCR1 and CCR5; the increase for CCR1 was 6-fold, while that for CCR5 was 26-fold ($p < 0.05$). In all cases, the maximal specific ligand binding was similar (data not shown), indicating that all the transfected cells expressed between $6.8 \times 10^5$ and $1.2 \times 10^6$ receptors per cell. These results were consistent with the similarity between the immunofluorescence profiles (Fig. 1).

## DISCUSSION

This series of experiments was performed to define the role played by GAGs in a cellular response elicited by specific stimulation of the receptors CCR1 and CCR5 by their respective ligands. The system chosen for study employed CHO cells, which were shown in this investigation and others (28) to be

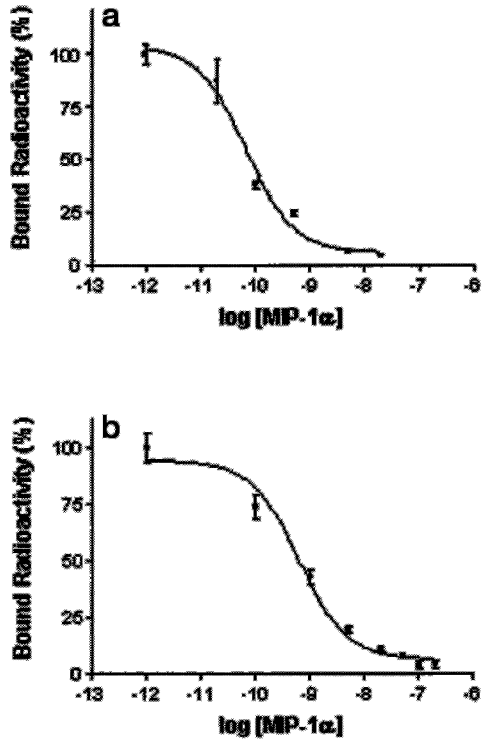


FIG. 7. **Representative homologous cold competition curves showing changes in the amount of $^{125}$I-MIP-1$\alpha$ bound by CCR5-transfected K1-CHO (*a*) and 745-CHO cells (*b*).** The $K_d$ values for this experiment were calculated as 90 pM and 2.2 nM, respectively.

TABLE II
*Binding affinities for RANTES and MIP-1$\beta$ to CCR1- and CCR5-transfected K1-CHO and 745-CHO cells*

| Chemokines | K1-CCR1 | 745-CCR1 | K1-CCR5 | 745-CCR5 |
|---|---|---|---|---|
| | | *pM* | | |
| MIP-1$\alpha$ | 116 ± 26 | 688 ± 364 | 196 ± 82 | 5090 ± 1700 |
| RANTES | 592 ± 109 | 10362 ± 44 | 322 ± 88 | 721 ± 21 |
| MIP-1$\beta$ | ND[a] | ND | 232 ± 15 | 274 ± 55 |

[a] ND, not determined.

unresponsive to stimulation by human chemokines. However, wild-type K1-CHO cells acquired the capacity to respond to MIP-1$\alpha$ and RANTES after transfection with CCR1 or to MIP-1$\alpha$, RANTES, and MIP-1$\beta$ after transfection with CCR5. This response was measured in terms of the induction of an intracellular $Ca^{2+}$ flux and the stimulation of cellular migration; these are properties shared by human cells such as monocytes, which show a physiological response to CC chemokines.

A potential role for GAGs in the chemokine-signaling process was demonstrated clearly by inhibition of the induction of the $Ca^{2+}$ flux in CHO transfectants by mixture of MIP-1$\alpha$ with the soluble GAG molecule, heparin. Heparin is structurally and biosynthetically related to the common cell surface GAG, heparan sulfate, and is known to bind a range of growth factors and cytokines with high affinity. The exact nature of the interaction between chemokines and heparin is unknown. However, it is unlikely that this is simply a nonspecific ionic interaction between cationic protein sequences and anionic GAGs. This is supported by the observation that 50–250-pg/ml quantities of heparin and heparan sulfate could inhibit the binding of RANTES to its receptor, while similar amounts of the anionic GAG, CS-A, did not prevent binding. Hence, the charge distribution on the GAG appears to be of critical importance for interaction with protein molecules (29)

In many cases, a GAG-binding motif has been identified with

a consensus BB*X*B sequence, where B is a basic residue and *X* is any other amino acid. Site-directed mutagenesis of MIP-1$\alpha$ has shown that three noncontiguous arginine residues are critical for GAG binding, with a further lysine residue also contributing to the affinity of this interaction (24). Significantly, two of these arginine residues and the contributing lysine form a BB*X*B motif.

Koopmann and Krangel (24) have shown that a singly substituted MIP-1$\alpha$ mutant that fails to interact with heparin displays wild-type binding to CCR1 and produces wild-type $Ca^{2+}$ signaling. However, a non-heparin-binding mutant of MIP-1$\alpha$ generated by Graham *et al.* (30) failed to bind to or signal through CCR1.

To investigate further the role of cell surface proteoglycans in chemokine activity, our group has performed a series of experiments using the mutant 745-CHO cell line. These cells were produced by chemical mutagenesis with ethylmethane sulfonate followed by screening for colonies of cells showing a reduced incorporation of sulfated GAGs into cell surface proteoglycans (26). Detailed study revealed that the 745-CHO cell line is deficient in xylosyltransferase, which prevents coupling and, hence, initiation of GAG chain formation on serine residues of the proteoglycan core protein (31). By contrast, wild-type CHO cells express both heparan sulfate and CS-A on their surface. As a consequence, the transfection of 745-CHO mutants with human CCR1 or CCR5 receptors provides an excellent system for investigation of the role of GAG molecules in the response to MIP-1$\alpha$.

The normal $Ca^{2+}$-flux observed following stimulation of both CCR1- and CCR5-expressing 745-CHO transfectants with a high concentration of chemokines (MIP-1$\alpha$, RANTES, MIP-1$\beta$) demonstrated the absence of an absolute requirement for GAG expression for chemokine responsiveness. This observation is consistent with the potential of non-GAG-binding, mutant MIP-1$\alpha$ molecules to activate both monocytes and CCR1-expressing human epithelial cells (24). However, despite the selection of CHO transfectants expressing approximately equal numbers of receptors, at lower ligand concentrations it was found that both CCR1- and CCR5-expressing wild-type cells were more responsive than the corresponding 745-CHO transfectants.

Previous studies by our group have shown that soluble, heparin-like GAGs are able to antagonize the biological activity of IFN-$\gamma$ *in vitro* (32). Furthermore, mixture of heparin and MCP-1 prior to incubation with mononuclear leukocytes effectively antagonized the tyrosine phosphorylation of PI 3-kinase and cellular migration produced by MCP-1 alone (33). There is also a precedent for heparin to increase the affinity of FGF molecules for their high affinity receptors on GAG-deficient cells. However, in the current study low concentrations of heparin did not have a potentiating effect on the binding of chemokines to their receptors on GAG-deficient cells, while high concentrations of heparin-like GAGs were always inhibitory.

A series of radioligand binding experiments demonstrated that both CCR1 and, to a greater degree, CCR5 showed a higher affinity for MIP-1$\alpha$ when expressed in wild-type CHO cells than when in the GAG-deficient mutant cells. These results are consistent with the $Ca^{2+}$ flux data and support the notion that expression of cell surface GAGs is not an absolute requirement for CCR1 or CCR5 ligation or signaling but does improve the sensitivity of cells to chemokine stimulation. However, when these experiments were repeated with RANTES and MIP-1$\beta$, the absence of GAGs had no significant effect on the binding affinity to their receptors CCR1 and CCR5. The data showing a decreased affinity for MIP-1$\alpha$ to seven-transmembrane receptors CCR1 and CCR5 in GAG-deficient cells is consistent with the results recently published by Hoogewerf *et al.* (34) showing a lower affinity for MIP-1$\alpha$ and RANTES binding to CCR1 on cells pretreated with glycosidase enzymes. In contrast, results published by Graham *et al.* (30) using a dimeric sequence variant of MIP-1$\alpha$ show for both wild-type and GAG-deficient CHO cells transfected with CCR1 that proteoglycan presentation is not important for interaction of MIP-1$\alpha$. Furthermore, this group demonstrated that neither heparin nor heparan sulfate inhibited binding at concentrations up to 2 mg/ml. The binding affinity reported by Graham *et al.* was 185-fold greater than that in the current report, suggesting that wild-type MIP-1$\alpha$ may behave differently from the modified dimeric species.

In both $Ca^{2+}$ flux and chemotaxis assays, CCR1- and CCR5-expressing wild-type cells were more responsive than corresponding 745-CHO transfectants when stimulated with RANTES and MIP-1$\beta$. Paradoxically, however, there was no significant difference between the binding affinities of RANTES or MIP-1$\beta$ to wild-type or 745-CHO transfectants.

The mechanism by which cell surface GAGs can potentiate the sensitivity of cells expressing CCR1 or CCR5 to stimulation by MIP-1$\alpha$, while soluble heparin can antagonize this chemokine, remains unclear. However, the binding of MIP-1$\alpha$ to the surface of untransfected GAG-expressing CHO cells was greater than to the GAG-deficient mutant cells. This finding is consistent with the decreased binding of MIP-1$\alpha$ to cells expressing neither CCR1 nor CCR5 following treatment with glycosidase enzymes, which remove heparan sulfate and CS-A (34). These results suggest that binding to noncognate GAG sites provide a mechanism for the sequestration of chemokines onto the cell surface.

Further studies have shown that cell surface GAGs potentiate the multimerization of chemokines such as MIP-1$\alpha$, although the biological relevance of this remains unclear (34). Multimerization of MIP-1$\alpha$ can also occur in solution (24), where it is enhanced by the presence of heparin. It is possible that inappropriate GAG binding in solution will change a chemokine's conformation, resulting in failure of the normal binding to cell surface receptors.

The chemokine binding to GAGs may be important for stabilization of chemokines in the form of solid phase gradients for presentation to passing leukocytes. However, recent experiments suggest that there is even greater and more fundamental involvement of GAGs in chemokine function. In support of this idea, enzymatic removal of cell surface GAGs results in a significant reduction in apparent affinity of RANTES and MIP-1$\alpha$ for cell surface receptors (34). Heparan sulfate synergizes with RANTES to inhibit HIV-1 replication in monocyte (35). Furthermore, the removal of cell surface GAGs has been shown to block the inhibitory effect of RANTES on human immunodeficiency virus-1 replication in PM1 T cells (36) and to block intracellular signaling in PBL (37). In contrast, an IL-8 mutant that binds poorly to GAGs displays reduced activity in a neutrophil chemotaxis assay that is thought to depend on both receptor binding and solid phase immobilization, but the mutant displays normal activity in a neutrophil elastase release assay that is thought to depend only on receptor binding (38). Nonheparin binding mutants generated for MIP-1$\alpha$ (24) and MIP-1$\beta$ (25) demonstrate that the ability of these chemokines to bind to GAGs is not essential for functional interaction with its receptor.

In summary, it has been shown that high concentrations of MIP-1$\alpha$ can stimulate CHO cells through human CCR1 and CCR5 and that this process is independent of the presence or absence of cell surface GAGs. However, the sensitivity of these cells to low concentrations of MIP-1$\alpha$ was enhanced by the

presence of cell surface GAGs, which increased the effective affinity of the specific CC chemokine receptors. The sensitivity of these cells was also enhanced by the presence of cell surface GAGs when stimulated with RANTES and MIP-1$\beta$ in functional biological assays, but the presence or absence of GAGs did not alter the apparent affinity for these ligands. It is possible that cell surface GAGs play an important role *in vivo* by sequestering chemokines onto a multiplicity of relatively low affinity sites, thereby raising the effective ligand concentration or altering the conformation of chemokines within the microenvironment of their specific receptors. Hence, it is possible that the parameters for receptor binding and cell activation by chemokines may vary according to biological assay, the receptor type, and the local type and distribution of GAGs.

## REFERENCES


1. Springer, T. A. (1994) *Cell* **76,** 301–314
2. Butcher, E. C., and Picker, L. J. (1996) *Science* **272,** 60–66
3. Kirby, J. (1996) in *Transplantation Biology: Cellular and Molecular Aspects* (Tilney, N. L., Strom, T. B., and Paul, L. C. eds) pp. 137–149, Raven Publishers, Philadelphia
4. Baggiolini, M. (1998) *Nature* **392,** 565–568
5. Rollins, B. J. (1997) *Blood* **90,** 909–928
6. Baggiolini, M., Dewald, B., and Moser, B. (1994) *Advances Immunol.* **55,** 97–179
7. Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A., and Schall, T. J. (1997) *Nature* **385,** 640–644
8. Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and Zlotnik, A. (1994) *Science* **266,** 1395–1399
9. Witt, D. P., and Lander, A. D. (1994) *Curr. Biol.* **4,** 394–400
10. Tanaka, Y., and Aso, M. (1998) *Trends Glycosci. Glycotechnol.* **10,** 153–160
11. Husman, A. M. D., and Schuitemaker, H. (1998) *Trends Microbiol.* **6,** 244–249
12. Bonini, J. A., Martin, S. K., Dralyuk, F., Roe, M. W., Philipson, L. H., and Steiner, D. F. (1997) *DNA Cell Biol.* **16,** 1249–1256
13. Premack, B. A., and Schall, T. J. (1996) *Nat. Med.* **2,** 1174–1178
14. Rot, A. (1993) *Eur. J. Immunol.* **23,** 303–306
15. Gilat, D., Hershkoviz, R., Mekori, Y. A., Vlodavsky, I., and Lider, O. (1994) *J. Immunol.* **153,** 4899–4906
16. Tanaka, Y., Kimata, K., Adams, D. H., and Eto, S. (1998) *Proc. Assoc. Am. Phys.* **110,** 118–125
17. Ruoslahti, E., and Yamaguchi, Y. (1991) *Cell* **64,** 867–869
18. Luster, A. D., Greenberg, S. M., and Leder, P. (1995) *J. Exp. Med.* **182,** 219–231
19. Lortatjacob, H., and Grimaud, J. A. (1992) *Biochim. Biophys. Acta* **1117,** 126–130
20. Maione, T. E., Gray, G. S., Hunt, A. J., and Sharpe, R. J. (1991) *Cancer Res.* **51,** 2077–2083
21. Webb, L. M. C., Ehrengruber, M. U., Clark-Lewis, I., Baggiolini, M., and Rot, A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7158–7162
22. Kuschert, G. S. V., Hoogewerf, A. J., Proudfoot, A. E. I., Chung, C-W., Cooke, R. M., Hubbard, R. E., Wells, T. N. C., Sanderson, P. N. (1998) *Biochemistry* **37,** 11193–11201
23. Moy, F. J., Safran, M., Seddon, A. P., Kitchen, D., Bohlen, P., Aviezer, D., Yayon, A., and Powers, R. (1997) *Biochemistry* **36,** 4782–4791
24. Koopmann, W., and Krangel, M. S. (1997) *J. Biol. Chem.* **272,** 10103–10109
25. Koopmann, W., Ediriwickrema, C., and Krangel, M. S. (1999) *J. Immunol.* **163,** 2120–2127
26. Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3197–3201
27. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell* **64,** 841–848
28. Samson, M., Labbe, O., Mollereau, C., Vassart, G., and Parmentier, M. (1996) *Biochemistry* **35,** 3362–3367
29. Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P., and Gallagher, J. T. (1998) *J. Biol. Chem.* **273,** 22936–22942
30. Graham, G. J., Wilkinson, P. C., Nibbs, R. J., Lowe, S., Kolset, S. O., Parker, A., Freshney, M. G., Tsang, M. L., and Pragnell, I. B. (1996) *EMBO J.* **15,** 6506–6515
31. Esko, J. D., Elgavish, A., Prasthofer, T., Taylor, W. H., and Weinke, J. L. (1986) *J. Biol. Chem.* **261,** 15725–15733
32. Douglas, M. S., Rix, D. A., Dark, J. H., Talbot, D., and Kirby, J. A. (1997) *Clin. Exp. Immunol.* **107,** 578–584
33. Douglas, M. S., Ali, S., Rix, D. A., Zhang, J. G., and Kirby, J. A. (1997) *Immunology* **92,** 512–518
34. Hoogewerf, A. J., Kuschert, G. S. V., Proudfoot, A. E. I., Borlat, F., Clark-Lewis, I., Power, C. A., and Wells, T. N. C. (1997) *Biochemistry* **36,** 13570–13578
35. Wagner, L., Yang, O. O., Garcia-Zepeda, E. A., Ge, Y., Kalams, S. A., Walker, B. D., Pasternack, M. S., and Luster, A. D. (1998) *Nature* **391,** 908–911
36. Oravecz, T., Pall, M., Wang, J., Roderiquez, G., Ditto, M., and Norcross, M. A. (1997) *J. Immunol.* **159,** 4587–4592
37. Burns, J. M., Gallo, R. C., DeVico, A. L., and Lewis, G. K. (1998) *J. Exp. Med.* **188,** 1917–1927
38. Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E., and Rot, A. (1997) *Cell* **91,** 385–395