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Research Article

IL-8 (also known as neutrophil-activating peptide 1) is recognized as a potent effector of neutrophil functions. Several different cell types that contact blood, namely T lymphocytes, monocytes, and endothelial cells, secrete this polypeptide following stimulation by cytokines, or lipopolysaccharide. Here we show that when IL-8 is added to blood it rapidly partitions from the plasma fluid to the blood cells and that erythrocytes account for the vast majority of this binding. Analysis of ^{125}I -IL-8 binding [(ala-IL-8] $_{77}$ form] to human red cells indicates a single, 5 nM K_d affinity class of binding sites, present at approximately 2,000 per red cell representing approximately 15 nmol of red cell IL-8 binding sites per liter of blood. These sites are protease sensitive. Their binding of IL-8 is rapidly reversible and does not result in receptor internalization, although bound IL-8 is resistant to extraction by pH 3 buffer at 5 degrees C. ^{125}I -IL-8 binding to red cells was not inhibited by epidermal growth factor or interleukin 1, but was inhibited by monocyte chemotactic peptide-1, which is not a neutrophil chemotaxin, but is a member of the same family of polypeptides as IL-8. FACS analysis of IL-8-mediated mobilization of Ca^{2+} in neutrophils indicates that the IL-8 bound to red cells is incapable of stimulating neutrophils. Thus, red cell absorption of IL-8 may function to limit [...]

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Red Blood Cells Are a Sink for Interleukin 8, a Leukocyte Chemotaxin

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Abstract

IL-8 (also known as neutrophil-activating peptide 1) is recognized as a potent effector of neutrophil functions. Several different cell types that contact blood, namely T lymphocytes, monocytes, and endothelial cells, secrete this polypeptide following stimulation by cytokines, or lipopolysaccharide. Here we show that when IL-8 is added to blood it rapidly partitions from the plasma fluid to the blood cells and that erythrocytes account for the vast majority of this binding. Analysis of ^{125}I -IL-8 binding ([ala-IL-8]₇₇ form) to human red cells indicates a single, 5 nM K_d affinity class of binding sites, present at $\approx 2,000$ per red cell representing ~ 15 nmol of red cell IL-8 binding sites per liter of blood. These sites are protease sensitive. Their binding of IL-8 is rapidly reversible and does not result in receptor internalization, although bound IL-8 is resistant to extraction by pH 3 buffer at 5°C. ^{125}I -IL-8 binding to red cells was not inhibited by epidermal growth factor or interleukin 1, but was inhibited by monocyte chemotactic peptide-1, which is not a neutrophil chemotaxin, but is a member of the same family of polypeptides as IL-8. FACS® analysis of IL-8-mediated mobilization of Ca^{2+} in neutrophils indicates that the IL-8 bound to red cells is incapable of stimulating neutrophils. Thus, red cell absorption of IL-8 may function to limit stimulation of leukocytes by IL-8 released into blood. (*J. Clin. Invest.* 1991; 88:1362-1369.) Key words: neutrophil-activating peptide 1 • monocyte chemotactic peptide 1 • erythrocyte • neutrophil • fluorescence activated cell sorter analysis • equilibrium dialysis

Introduction

Interleukin 8 is secreted from activated monocytes (1-3), T lymphocytes (4), fibroblasts (5), and endothelial cells (6, 7) and is a potent effector of neutrophils (8) and perhaps also of T lymphocytes (9). This peptide stimulates neutrophil chemotaxis, calcium mobilization (10), morphological polarization (11), and, in the presence of cytochalasin-B, azurophil granule release (10, 11). IL-8 also modulates neutrophil:endothelial interaction, causing an up to two- to threefold increase in neutrophil adhesion to resting (and therefore relatively nonadherent) endothelium (12, 13), but a 70-90% decrease in adhesion to cytokine-activated (and therefore highly adherent) endothelium (14). The nature of the effects of IL-8 administered *in vivo*

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varies dramatically depending on its localization. Thus, whereas intradermally injected IL-8 is a neutrophil chemoattractant (15, 13), intravenously injected IL-8 causes rapid transient leukopenia followed by prolonged granulocytosis (16), and also inhibits neutrophil extravasation to intradermal inflammatory sites (17). The latter action may reflect the recently described ability of IL-8 to inhibit adhesion of neutrophils to activated endothelium (14). Repeated intravenous administration of IL-8 causes lung injury similar to that which occurs in adult respiratory distress syndrome (18).

This study demonstrates that when interleukin 8 is introduced into whole blood at concentrations within its biologically active concentration range (0.1-10 nM), this cytokine becomes rapidly and efficiently bound to the red blood cells as a result of an unusual acid-resistant interaction with protein binding sites on the red cell surface. Results presented here also indicate that IL-8 bound to red blood cells is incapable of communicating with neutrophils. Thus, one function of red cell binding sites for IL-8 could be to limit neutrophil interaction with IL-8 that enters the systemic circulation.

Methods

Materials. Recombinant human [ala-IL-8]₇₇ and [ser-IL-8]₇₂ were expressed in *Escherichia coli* and purified as previously described (19). Bovine serum albumin, goat anti-rabbit IgG, platelet factor 4, Sephadex G-25 (100-300 μ bead size), micro-crystalline cellulose (type 50), and fluorescein isothiocyanate isomer-1 were obtained from Sigma Chemical Co. (St. Louis, MO). Phycoerythrin-conjugated goat anti-rabbit Fab'2 fragments were obtained from CalTag (S. San Francisco, CA). The acetoxymethyl ester of indo-1 was obtained from Molecular Probes Inc. (Eugene, OR). Heparin was obtained from The Upjohn Company (Kalamazoo, MI). Diffusion chambers and diffusion chamber filters were obtained from Nucleopore.

Polyclonal antibody against IL-8 was prepared in rabbits as described by Hébert et al. (19). ^{125}I -[ala-IL-8]₇₇ (specific activity ranging between $2-4 \times 10^7$ cpm/ μ g) was prepared and radioimmunoassay for IL-8 (maximal sensitivity ≈ 50 pM) performed as previously described (19). Enzyme-linked immunosorbent assay for tissue-type plasminogen activator was carried out as described by Ramakrishnan et al. (20).

Isolation of blood cells. Neutrophils were isolated as described by Wheeler et al. (21). Erythrocytes were isolated by a modification of the procedures of Nakao et al. (22) and Beutler et al. (23). Briefly, fresh whole blood was drawn into a heparinized syringe (10 U heparin/ml whole blood) from healthy human volunteers or animals. Different blood donors were used for replicate experiments. Whole blood was then passed over two tandem 20-ml polypropylene syringes that were packed with microcrystalline cellulose/Sephadex G25 (in 3:1 ratio) and that contained cotton filters at their outlets. The columns were washed with 5-10 vol of 0.154 M NaCl. The erythrocyte-containing eluate was then washed thrice by centrifugation at 1,000 g and resuspension in

1. Abbreviations used in this paper: [ala-IL-8]₇₇, 77 amino acid form of IL-8 with NH₂-terminal alanine; [ser-IL-8]₇₂, 72 amino acid form of IL-8 with NH₂-terminal serine; MCP, monocyte chemotactic protein.

PBS containing 1% BSA. Blood cell counts were performed on a Baker 9000 hematology analyzer (Baker Instrument Co., Allentown, PA) to check for removal of white blood cells and platelets. It was determined that it was occasionally necessary to have the second column in series in order to achieve removal of greater than 99% of the leukocytes. Erythrocyte ghosts were prepared from outdated red blood cells as described by Hanahan and Ekhholm (24).

Measurement of IL-8 binding. Freshly isolated erythrocytes were diluted with binding medium (PBS containing 1% BSA) to achieve the desired cell concentrations. 250 μ l of the washed cells were then sedimented for 1 min at 13,000 g in a microfuge. 125 μ l of the supernatant was replaced with binding medium containing 125 I-IL-8 plus or minus nonlabeled IL-8 as indicated. The cells were resuspended and incubated for 15 min at 37°C, unless otherwise indicated, and layered atop 0.5 ml of binding medium containing 20% sucrose. The cells were sedimented by centrifugation for 3 min in a microcentrifuge at 13,000 g, and the radioactivity in the cell pellet and supernatant quantified in a Packard Minaxi 5000 series gamma counter (Packard Instruments Co., Inc., Downers Grove, IL). In the case of red cell ghosts, the binding assay was modified as follows. At the end of the binding incubation the contents of the binding incubation mixture were quickly diluted with 5 vol of PBS, and the ghosts were pelleted by 25 min centrifugation at 16,000 g.

The fraction of 125 I-IL-8 molecules that had specific binding activity was determined by carrying out a 15-min incubation of red cells with \approx 10,000 cpm of the radiolabeled ligand plus or minus 1 μ M nonlabeled IL-8. The "bindable" fraction was estimated from the Y axis intercept of a plot of the inverse of specifically bound radioactivity (on the Y axis) versus the inverse of cell concentration (on the X axis), as recently described by Besemer et al. (25).

FACS[®] analysis methods. IL-8 was fluoresceinated by incubation with fluorescein isothiocyanate Isomer-I (FITC) in 0.5 M NaHCO₃ at pH 9.0 at a ratio of 1:10 FITC/IL-8 (wt/wt). The reaction proceeded for 90 min at 20°C. The solution was neutralized and BSA at 0.04% final concentration was added. Free FITC was removed by centrifugation through a Centricon 3 unit (Amicon Division of W. R. Grace and Co., Danvers, MA). The FITC/protein ratio was \approx 2. Immediately, 2×10^5 red cells were stained with FITC-IL-8 by coincubation for 1 h at 5°C in PBS containing 2% fetal calf serum. The cells were washed thrice and analyzed on a Coulter Elite flow cytometer (Coulter Electronics Inc., Hialeah, FL), using 488-nm laser excitation (15 mW) and emission detected using a 525-nm (\pm 20 nm) band pass filter. Some samples were incubated simultaneously with a 100-fold excess of unlabeled IL-8, except the 100- and 250-nM points which were incubated with 5 μ M unlabeled IL-8. For indirect measurement of IL-8 binding to red blood cells, the cells were incubated with IL-8 for 1 h, and then incubated with a 1:200 dilution of the rabbit anti-IL-8 antisera. The cells were then incubated with phycoerythrin-conjugated goat anti-rabbit Fab'2 and analyzed by FACS using a 575-nm (\pm 25 nm) band pass filter. All incubations were performed on ice and the cells were washed three times between staining steps with PBS containing 2% fetal calf serum. Samples were analyzed by calculation of the peak mean fluorescence intensity normalized to the control (autofluorescence sample for FITC-IL-8 staining; polyclonal and anti-rabbit phycoerythrin antibody stained sample for indirect staining).

Calcium mobilization was measured by loading neutrophils (1×10^6 /ml) with 10 μ M indo-1 acetoxymethyl ester (26) for 15 min at 37°C in RPMI medium. The cells were analyzed by FACS using 200 mW UV excitation (351.1–363.8 nm) with fluorescence emission collected as a ratio of 405:525 nm as described by June and Rabino-vitch (27).

Results

Absorption of IL-8 by red blood cells. IL-8 is found in several different forms. The most common, [ala-IL-8]₇₇, and [ser-IL-8]₇₂, are distinguished solely by the 5 amino acid extension at

the amino-terminus of the former. Qualitatively, their activities are similar, with [ser-IL-8]₇₂ exhibiting somewhat greater activity (19). Unless noted otherwise, herein, the term IL-8 will refer to [ala-IL-8]₇₇, which is the form of IL-8 secreted by endothelial cells (14) and the IL-8 form used for most of the following experiments. Because a number of signal-transmitting polypeptides have humoral binding proteins (28, 29), we sought to determine whether blood contained an IL-8-binding protein by adding 125 I-IL-8 to plasma and monitoring its size by gel filtration. This experiment did not suggest that IL-8 became absorbed to a soluble binding protein (M. Mohler, unpublished data). However, as shown by the radioimmunoassay data in Table I, when IL-8 in the nanomolar concentration range was added to human whole blood it became depleted from the plasma. Thus, whereas 73% was lost when IL-8 was added at 2 nM, little or none was lost when IL-8 was added at \approx 100 nM. In contrast, IL-8 added to plasma was detectable at roughly undiminished concentration at all concentrations examined. Direct evidence that the IL-8 was being absorbed by the blood cells was obtained by incubating 10 nM 125 I-IL-8 in human whole blood for 10 min and then counting the radioactivity associated with cells and plasma supernatant. About 55% of the radioactivity rapidly redistributed from the plasma to the cells (Fig. 1).

Repeated low speed (600 g) centrifugation and removal of the buffy coat did not diminish the 125 I-IL-8 binding capacity of the blood cells (data not shown), indicating that the binding activity was a property of the red cells. Therefore, red cells were

Table I. Disappearance of Soluble IL-8 from Whole Blood*

Condition	IL-8 added	IL-8 recovered	Percentage recovered
			nM
Addition to whole blood	2	0.54	27
	8	2.98	37
	32	12.47	39
	128	136.64	107
	512	507.65	99
	2,050	2,978.43	145
Addition to plasma	2	1.88	94
	8	5.79	72
	32	26.35	82
	128	144.07	113
	512	515.10	101
	2,050	2,327.90	114

* Plasma was derived from freshly drawn heparinized (10 U/ml) whole blood by twice sedimenting the blood cells in a centrifuge at 1000g for 4 min. The cells from 250- μ l aliquots of whole blood were sedimented by centrifugation for 1 min in a microfuge, and 125 μ l of the plasma supernatant was removed. In duplicate, 125 μ l of plasma spiked with IL-8, to generate the indicated IL-8 concentrations, was then added back to the cell pellets and to 125- μ l plasma aliquots. The cells were resuspended and then incubated with the plasma dilutions of IL-8 for 15 min at 37°C. The cells were then sedimented and soluble IL-8 was quantified in the resulting cell plasma supernatants and plasma dilutions by radioimmunoassay (19) (maximum sensitivity = 50 pM) using standards diluted in plasma derived from the same blood. The measured concentrations of IL-8 added to plasma generally conform to the theoretical concentrations based on the known amounts of IL-8 added.

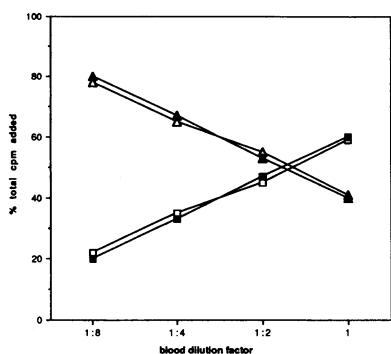


Figure 1. Binding of ^{125}I -IL-8 to unfractionated blood cells and isolated erythrocytes. Purified red blood cells and unfractionated whole blood cells, both in 1% BSA/PBS, were diluted to 3.7×10^9 cells/ml, as determined with a Coulter Counter (Coulter Electronics Inc.). These ("1x") preparations, and the

indicated dilutions thereof, were incubated with 10 nM ^{125}I -IL-8 for 20 min at 37°C and the cell-associated radioactivity measured. Closed and open symbols represent experiments with isolated red blood cells, or unfractionated blood cells, respectively. Squares represent fraction of radiolabeled ligand bound to cells. Triangles represent fraction of radiolabeled ligand not bound to cells.

highly purified and their ^{125}I -IL-8 binding activity was compared with that of unfractionated blood cells. As shown in Fig. 1, purified human red cells at their concentration in whole blood ($\approx 4 \times 10^9/\text{ml}$) bound $\sim 55\%$ of ^{125}I -IL-8 added at 10 nM. This percentage decreased linearly with serial dilution of the cells, down to $\sim 20\%$ at a 1:8 cell dilution. The ^{125}I -IL-8 binding capacities of unfractionated blood cells and of isolated red cells were equivalent. Thus, the red blood cells were responsible for the extensive binding of ^{125}I -IL-8 by blood cells.

As predicted by radioimmunoassay of IL-8 added to whole blood (Table I), ^{125}I -IL-8 binding to red cells was saturable (Fig. 2). At ^{125}I -IL-8 concentrations up to ~ 1 nM, $\sim 70\%$ of the input ligand became absorbed to the cells, whereas at 100 nM ^{125}I -IL-8 this fraction had decreased to $\sim 10\%$ (Fig. 2, closed bars). Furthermore, excess nonlabeled IL-8 inhibited the binding of ^{125}I -IL-8 (see below). These results indicated that red cells contained specific binding sites for IL-8. About 50–70% of the radioiodinated IL-8 molecules (depending on the preparation) had the capacity to bind to red cell specific binding sites (see Methods). Besemer et al. (25) made a similar observation with respect to binding of their radioiodinated IL-8 preparations to neutrophil receptors. The cross-hatched bars in Fig. 2 show

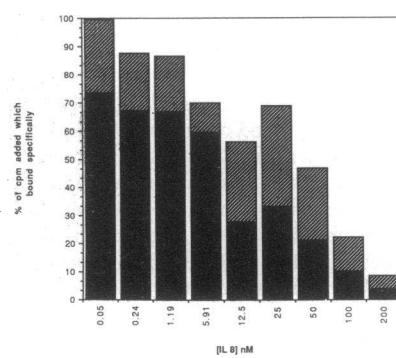


Figure 2. Absorption of ^{125}I -IL-8 by erythrocytes. Red cells at $4.0 \times 10^9/\text{ml}$ were incubated with ^{125}I -IL-8 at the indicated concentrations with or without 1 μM nonlabeled IL-8, for 15 min at 37°C. The percentage of radioactivity that was specifically bound is shown by the closed bars. The levels indicated by the

cross-hatched bars show the percentage of "bindable" ^{125}I -IL-8 that was specifically bound, derived by subtracting the fraction of ligand that was found (see protocol in Methods) to be incapable of binding. This graph represents data from two experiments.

that the fraction of "bindable" ^{125}I -IL-8 absorbed by red cells exceeded 80% at ^{125}I -IL-8 concentrations below ~ 1 nM. It is noteworthy that red cell absorption of IL-8 had its maximal impact on soluble IL-8 levels in the IL-8 concentration range over which neutrophil responses have been shown to occur and be dose dependent (reference 8, and Fig. 8 below).

As an independent test for red blood cell absorption of IL-8, we examined the influence of red cells on the equilibration of IL-8 across the compartments of a diffusion chamber. Red blood cells at $4 \times 10^9/\text{ml}$ were placed in the lower compartment, which was separated from the upper compartment by a filter containing pores with a 5 μm diameter. IL-8 was added to either compartment, the chambers were incubated overnight on a rotary shaker at 23°C, and the amount of IL-8 in the buffer fluid of the two compartments was determined by radioimmunoassay. Control experiments with ^3H -inulin indicated that the red cells did not pose a significant barrier to diffusion, and that the concentration of this inert molecule approached equilibrium (Table II). As shown in Table II, when IL-8 was added to the red cell side no detectable IL-8 antigen escaped to the opposite compartment or was found in the buffer in the red cell compartment. Red cells also caused depletion of IL-8 when the IL-8 was added to the compartment opposite to the red cells. The amount of depletion of IL-8 antigen in this case, although not complete, was also large. Data similar to those given in Table II were obtained in all (three) replicate experiments. Red cells did not act as a sink for proteins in general in these experiments because their presence did not influence the diffusion of tissue type plasminogen activator across the diffusion chambers (data not shown).

Properties of the red cell IL-8 interaction. Specific binding of ^{125}I -IL-8 reached steady-state at the earliest time points that could be taken (see Fig. 6 below). Fig. 3 illustrates that this interaction was also rapidly reversible. ^{125}I -IL-8 was incubated with red cells for 15 min at 37°C, and then the incubation mixture was diluted 100-fold. At various times the cells were sedimented and their specifically-bound ^{125}I -IL-8 was measured. As shown, virtually all of the specifically-bound ligand had dissociated at the earliest (" t_0 ") time point after the dilution. Because the centrifugation and manipulations at each time point consumed about 5 min, the experimental protocol did not allow measurement of the $t_{1/2}$ for dissociation of ^{125}I -IL-8. Nevertheless, the data indicate that this is considerably less than 5 min.

About 70% of the total binding of 0.1 nM ^{125}I -IL-8 was eliminated in the presence of 1 μM nonlabeled IL-8 (Fig. 4). In contrast, unrelated polypeptides, epidermal growth factor and interleukin 1, at 1 μM concentrations did not detectably influence ^{125}I -IL-8 binding (data not shown). Analysis of the ligand dose dependence of steady-state binding using the LIGAND program (30) indicated that red cells display a single affinity class of ^{125}I -IL-8 binding sites, at 1,500–3,000 per cell with a ≈ 5 nM K_d (Fig. 4). This translates to about 15 nmol of red cell binding sites per liter of whole blood.

Independent analysis of IL-8 binding sites on red cells was carried out using a FACS to examine the interaction of FITC-labeled IL-8 with red cells. As shown in Fig. 5 A, this IL-8 derivative bound to red cells with a ≈ 10 nM K_d , and the binding was inhibited in the presence of excess native IL-8. In addition, native IL-8 was incubated with red cells, and binding of the ligand was detected indirectly (Fig. 5 E). Although this

Table II. Diffusion of IL-8 in the Presence and Absence of Erythrocytes

Condition	Initial condition	Chamber side	Final soluble [IL-8]	Range	Total soluble IL-8 recovered	[³ H] Inulin ratio (top/bottom)
			<i>nM</i>		<i>%</i>	
1	buffer	Top	LTS	(---)	0	1.04
	RBCs +0.5 nM IL-8	Bottom	LTS	(---)		
2	buffer	Top	0.17	0	68	0.77
	0.5 nM IL-8	Bottom	0.17	0.02		
3	0.5 nM IL-8	Top	0.06	0.04	12	0.99
	RBCs	Bottom	LTS	(---)		
4	0.5 nM IL-8	Top	0.185	0.03	66	1.02
	buffer	Bottom	0.145	0.09		

* Red blood cells were isolated from freshly drawn heparinized (10 U/ml) human whole blood, and resuspended to 5×10^9 cells/ml in PBS buffer containing 1% BSA. 200 μ l of the various mixtures indicated were added to the bottom of a chemotactic chamber (Nucleopore Corp., Pleasanton, CA). After securing a 5.0 μ m pore diameter filter over the bottom chamber, 200 μ l of the indicated mixtures were added to the top chamber (on the filter). Duplicate chemotactic chambers were set up for each condition. In the first condition indicated, the red blood cells were preincubated with 0.5 nM IL-8 at 37°C for 15 min before the direct addition of the mixture to the bottom chamber. The top of the chemotactic chamber was then covered with parafilm, and incubated on a rotary shaker at room temperature for 24 h. The contents of each chamber side were then collected. The red blood cells in the samples from conditions 1 and 3 were sedimented by a 3-min centrifugation in a microfuge. Radioimmunoassay was used to determine the concentration of IL-8 in the resulting supernatants and chamber solutions. LTS, less than standard; <50 pM.

indirect method does not allow for subtraction of nonspecific binding, it is noteworthy that the slope of the binding versus concentration curve was substantially steeper at ligand concentrations below ≈ 15 nM than at higher ligand concentrations, consistent with IL-8 binding to a saturable site on the cells that had approximately the K_d predicted by our other experiments.

Resistance of cell-bound polypeptide ligands to extraction by pH 2.5–3.0 buffer on ice is a commonly used criterion for internalized ligand because, in general, noncovalent protein: protein interactions are disrupted at this pH (31, 32). Red cells were incubated with ¹²⁵I-IL-8 for various times at 37°C and then incubated in 0.1 M glycine buffer (pH 3) for 3 min on ice. As shown in Fig. 6, regardless of the length of time of incubation of red cells with ¹²⁵I-IL-8 (up to 90 min), little ($\approx 20\%$) of the bound ¹²⁵I-IL-8 dissociated from the cells upon pH 3 treatment at 5°C. This at first suggested that the ligand had been internalized. However, it was also determined that $> 90\%$ of

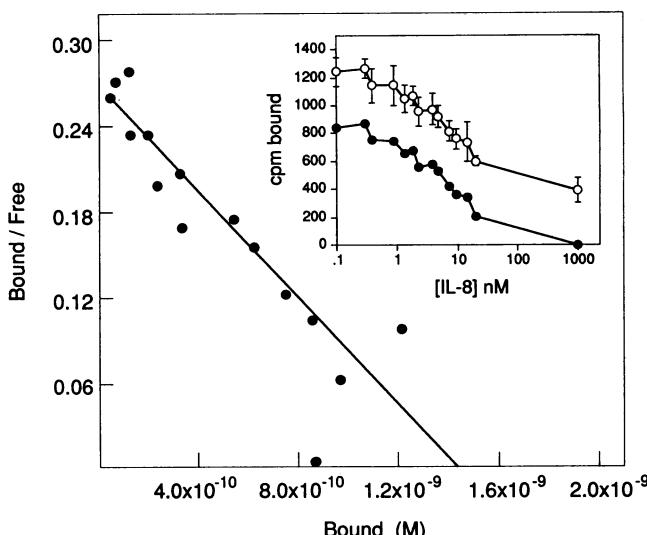


Figure 4. Analysis of ¹²⁵I-IL-8 binding to human red blood cells. Erythrocytes (1.22×10^8 cells/250- μ l aliquot) were sedimented by a 1-min centrifugation in a microfuge. 125 μ l of the resulting supernatants were replaced with 125 μ l mixtures of 0.1 nM ¹²⁵I-IL-8 and increasing concentrations of nonlabeled IL-8 (two-fold dilutions from 0.2–40 nM, and 1,000 nM) in 1% BSA/PBS, each point in quintuplicate. The cells were resuspended and incubated at 37°C for 20 min. The cell suspensions were each layered onto a 0.5 ml 20% sucrose/0.1% BSA/PBS cushion, the cells sedimented and the radioactivity in the cell pellets and supernatants quantified. The LIGAND program of Munson and Rodbard (30) was then used to estimate binding site number and affinity and develop a Scatchard plot of the data. The inset shows the binding data plotted as a function of the logarithm of IL-8 concentration (nM), with open and closed symbols showing total and specific binding, respectively. This analysis is representative of four separate competition binding experiments. The error bars represent SD of ¹²⁵I-IL-8 total binding to red cells in the presence of the respective concentration of cold ligand.

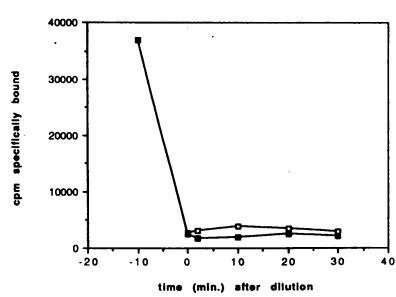


Figure 3. Time course of dissociation of specifically bound IL-8. Red blood cells at 5.1×10^9 cells/ml were incubated with 5 nM ¹²⁵I-IL-8 with or without 1 μ M nonlabeled IL-8 for 15 min at 37°C. An aliquot of the suspension was removed to measure cell-associated radioactivity and the rest was diluted 100-fold in 1% BSA/PBS. The mixtures were then incubated at 4° or 37°C, and at the indicated time points aliquots were taken for measurement of cell-associated radioactivity. Shown is the radioactivity specifically bound to 6×10^8 cells. Open square, 37°C; closed square, 4°C. This figure is representative of four experiments.

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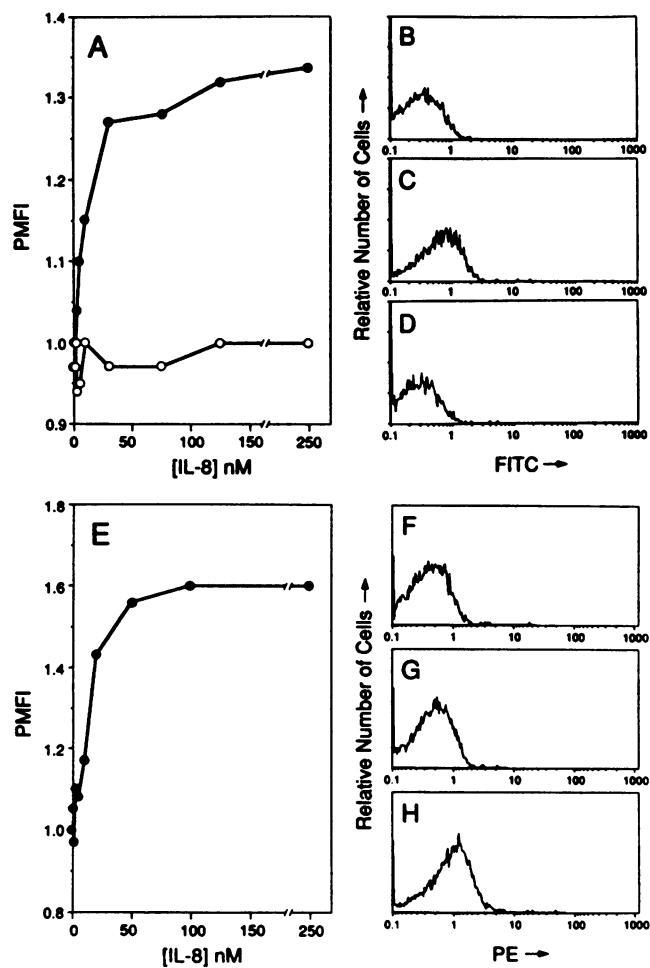


Figure 5. Flow cytometric analysis of binding of IL-8 to human red blood cells. (A) Binding of FITC-IL-8 to cells with (open symbols) or without (closed symbols) addition of 100-fold excess of unconjugated IL-8. Plotted is the peak mean fluorescence intensity (PMFI) normalized to the control. Representative fluorescence histograms of FITC-IL-8 binding are shown to the right of the graph, illustrating autofluorescence (B); 75 nM FITC-IL-8 (C); and 75 nM FITC-IL-8 plus 7.5 μ M unconjugated IL-8. E-H display measurement of IL-8 binding to red cells using an indirect staining approach. Cells were stained with various concentrations of IL-8, followed by polyclonal sera against IL-8 (D), followed by goat anti-rabbit phycoerythrin-conjugated Fab₂ and analyzed by FACS (E). Representative histograms are shown to the right: F, anti-rabbit phycoerythrin-conjugated antibody only; G, polyclonal sera plus anti-rabbit phycoerythrin-conjugated antibody; H, IL-8 plus polyclonal sera plus anti-rabbit phycoerythrin-conjugated antibody. Data from direct and indirect FACS analysis is representative of three experiments for each method.

the bound ligand rapidly dissociated from the cells upon the addition of excess IL-8 (0.5 μ M; Fig. 6). Thus, 125 I-IL-8 became associated with the red cell surface in an unusual pH 3-resistant interaction, and did not become internalized. Other experiments demonstrated that 125 I-IL-8 did not become internalized by red cells even after incubation overnight at 37°C, as virtually all of the specifically bound 125 I-IL-8 was displaced by addition of excess nonlabeled IL-8.

In contrast to the resistance of red cell-bound 125 I-IL-8 to extraction by pH 3 buffer in the cold, this bound ligand was rapidly extracted by the same pH 3 buffer at 37°C (data not

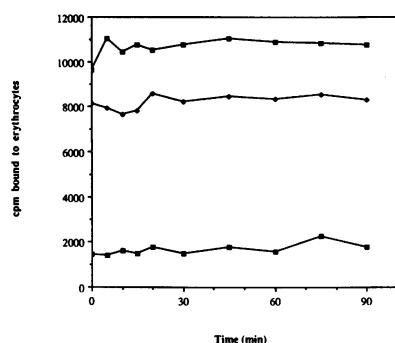


Figure 6. Extraction of IL-8 bound to red blood cells. Red blood cells at 4.85×10^9 cells/ml were incubated with 10 nM 125 I-IL-8 at 37°C. At the time points indicated, three 250- μ l aliquots were removed and treated as follows: the cells were sedimented through a sucrose cushion and the radioactivity in the cell pellets

counted (open square). The cells were sedimented by centrifugation in a microfuge for 1 min. 125 μ l of the supernatant was replaced with 0.1 M glycine/0.154 M NaCl buffer at pH 3.0. The cells were resuspended and incubated at 4°C for 3 min. The cell suspension was then sedimented through a sucrose cushion, and the radioactivity in the resulting supernatant and cell pellet was quantified (closed diamond). The cells were sedimented by centrifugation in a microfuge for 1 min. 125 μ l of the supernatant was replaced with buffer containing nonlabeled IL-8 at a final concentration of 5 μ M. The cells were resuspended and incubated at 4°C for 3 min. The cell suspension was then sedimented through a sucrose cushion and the radioactivity in the cell pellet was quantified (closed square). This figure is representative of five experiments.

presented). At this temperature the acidic buffer extracted the bound 125 I-IL-8 as efficiently as did excess nonlabeled IL-8. It is noteworthy that the opposite effect of temperature on pH 3 extraction of neutrophil-bound 125 I-IL-8 (sensitivity at 4°C and resistance at 37°C) has been reported (25). The possibility that the red cell binding sites for IL-8 were located on the plasma membrane was supported by the finding that red cell ghosts specifically bound 125 I-IL-8 (data not shown; see Methods for experimental details).

Red cell binding of 125 I-IL-8 was not significantly altered by preincubation of the cells with high levels of trypsin (100 μ M). However, preincubation of the cells with similar amounts of chymotrypsin or pronase reduced their specific binding of 125 I-IL-8 by 50 and 100%, respectively (Fig. 7 A). These treatments did not cause detectable hemolysis. The loss of binding was not caused by digestion and inactivation of the 125 I-IL-8 ligand by residual protease that did not wash off of the red cells after their protease treatment. SDS polyacrylamide gel electrophoresis (carried out in the presence of dithiothreitol) and autoradiography indicated that the 125 I-IL-8 ligand was intact after the binding incubations (data not presented). Moreover, the recovered ligand bound to red cells that had not been preincubated with protease (Fig. 7 B). These results thus suggest that the red cell binding site for IL-8 is a protein.

IL-8 bound to red cells is not accessible to neutrophils. Because neutrophils are recognized as a primary target of IL-8 action (8), we designed an experiment to determine whether IL-8 that was bound to red cells could stimulate neutrophils. The previously described rise in cytosolic free calcium that occurs as an early response of neutrophils to IL-8 (11) was chosen as an indicator of IL-8 signal transmission. Neutrophils were loaded with the calcium-sensitive dye, indo-1, incubated with IL-8, and calcium ion levels measured in a fluorescence-activated cell sorter. Fig. 8 shows the recording of calcium mobilization in neutrophils induced by IL-8. Dose-response curves

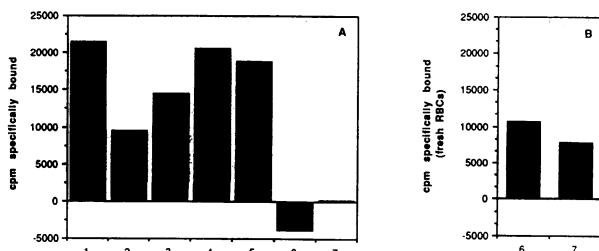
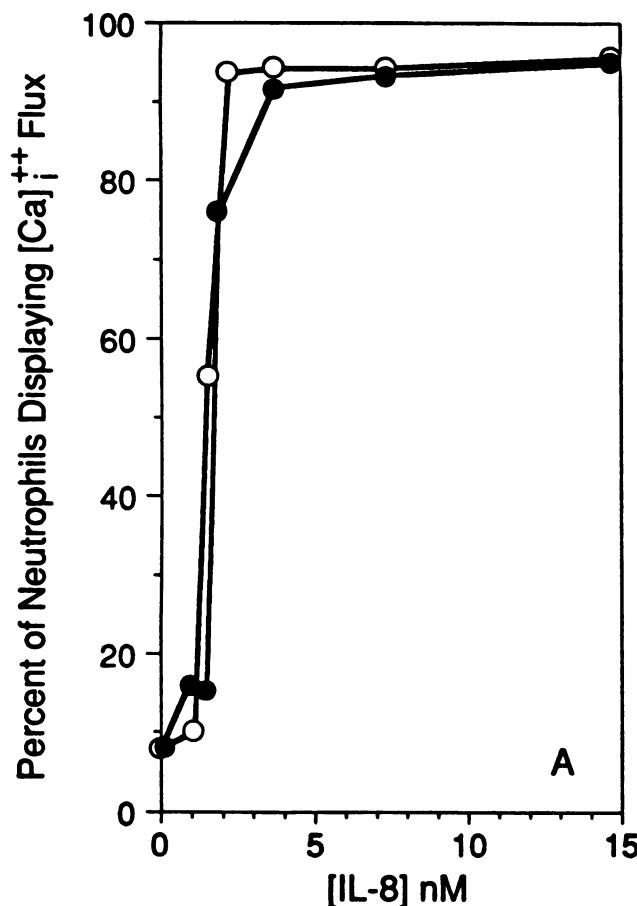


Figure 7. IL-8 binding to erythrocytes pretreated with proteases. (A) Erythrocytes at 3.5×10^9 cells/ml were incubated with the proteases indicated below for 30 min at 37°C. A subset of these cells underwent a second protease incubation for a further 30 min by replacing the protease-containing supernatant with fresh solution of 200 μ g/ml protease. The protease-treated cell suspensions were then washed by repeated sedimentation and resuspension, diluted tenfold in PBS containing 1 mM PMSF, 3 mM EDTA, 100 μ g/ml aprotinin, and 10% fetal calf serum. The treated red blood cells were then diluted tenfold and washed twice in 1% BSA/PBS before resuspension in 5 nM 125 I-IL-8 at the original (250 μ l) volume. The cells were incubated at 37°C for 15 min, sedimented, and the radioactivity in the cell pellet quantified. (B) 100 μ l of the 125 I-IL-8-containing supernatants (80% of volume) from the cells that had been incubated with pronase were then used to resuspend 100 μ l of fresh red blood cells that had been sedimented by centrifugation. The cells were then incubated for 15 min at 37°C, sedimented, and the radioactivity in the cell pellet quantified. The data represents reproducible data attained in four experiments. Key to protease treatment conditions: 1, buffer control; 2, chymotrypsin (two incubations); 3, chymotrypsin (one incubation); 4, trypsin (two incubations); 5, trypsin (one incubation); 6, pronase (two incubations); 7, pronase (one incubation).



generated with both $[ala-IL-8]_{77}$, and $[ser-IL-8]_{72}$ forms of IL-8 were very steep, indicating little response below ≈ 1 nM IL-8 and maximal response above ≈ 2 nM IL-8 (Fig. 8, A). IL-8 at 10 pM elicited little if any release of calcium ion (Fig. 8, B). Approximately half of the cell population mobilized calcium in response to 1.5 nM IL-8 (Fig. 8, C), and this fraction approached 100% at 10 nM IL-8 (Fig. 8, D).

To determine whether IL-8 bound to red cells was accessible to neutrophils, red cells were loaded with IL-8 by incubation with the polypeptide at concentrations from 0.1 to 100 nM. Unbound IL-8 was removed by sedimentation and resuspension of the cells in IL-8-free buffer. The cells were not diluted during the washing step, to prevent the loss of bound IL-8 that occurs on dilution (Fig. 3). (This wash procedure resulted in efficient retention of the bound IL-8, as indicated by a control experiment carried out with 10 nM 125 I-IL-8. In this case 7.7 nM 125 I-IL-8 became bound and 7.0 nM 125 I-IL-8 was retained by the red cells after the wash [data not shown]). The red cells were then mixed with indo-1-loaded neutrophils at a neutrophil/red cell ratio of 1:100. The cell mixtures were then immediately injected into the FACS, gating on the neutrophil population. As shown in Fig. 9, no detectable rise in neutrophil cytoplasmic free calcium occurred in these mixtures. This failure to respond to IL-8 was not caused by damage to the neutrophils or loss of sensitivity of the cells to IL-8, because excess (100 nM) IL-8 added to these mixtures triggered a rapid large scale increase in intracellular free calcium.

Monocyte chemotactic peptide-1 binding to red cells. Reasoning that red cells might serve as a sink for other chemotactic

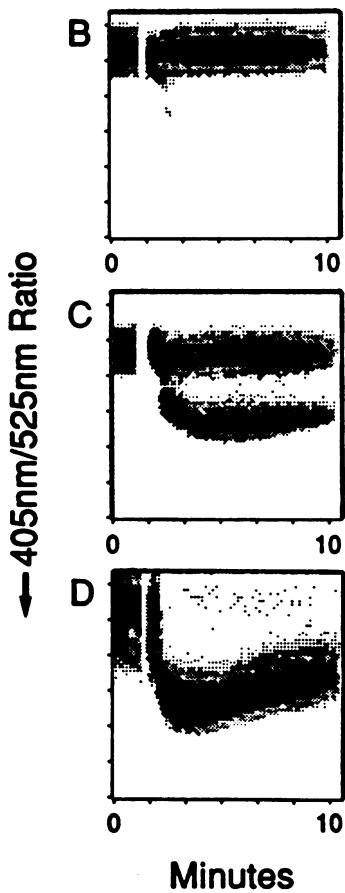


Figure 8. Flow cytometric analysis of Ca^{2+} mobilization in neutrophils stimulated with IL-8. A shows the percentage of neutrophils responding (integrated at 2–4 min after addition of IL-8) as a function of IL-8 concentration. The dose-response curves for both $[ala-IL-8]_{77}$ and $[ser-IL-8]_{72}$ are shown (closed and open circles respectively). Representative bivariate histograms plotting the 405/525 nm fluorescence ratio against time are shown in B, C, and D. The cells were equilibrated and a baseline ratio established before the addition of either 10 pM (B), 1.5 nM (C), or 10 nM (D) IL-8. Baseline ratio shown in each histogram before the break representing the time of IL-8 addition. This data is representative of several experiments where Ca^{2+} mobilization has been monitored to determine neutrophil activation.

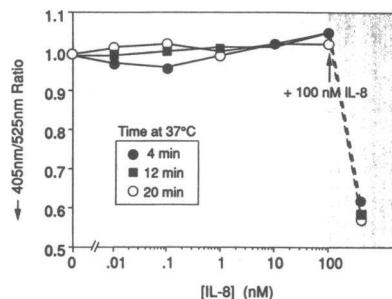


Figure 9. Measurement of free Ca^{2+} in neutrophils incubated with red cells that were pre-loaded with IL-8. Red cells at 4×10^9 cells/ml in 0.5 ml were preincubated with the indicated amounts of IL-8 for 4, 12, or 20 min at 37°C. Unbound IL-8 was removed by two 250 μl

washes with 1% BSA/PBS. Neutrophils loaded with Indo-1 as described in Methods, were then mixed with the red cells at a neutrophil to red cell ratio of 1:100. The red cells were electronically gated out using a combination of forward angle light scatter, 90° light scatter and Indo-1 fluorescence. Therefore, displayed is the Indo-1 response (405/525 nm ratio) of the neutrophils only. At the arrow 100 nM IL-8 was added to a subset of the neutrophil/IL-8-loaded red cell mixtures. This data is representative of two experiments.

cytokines, we have examined the interaction of red cells with monocyte chemotactic peptide-1 (MCP-1), a recently defined chemotaxin produced by vascular smooth muscle cells and cytokine-stimulated peripheral blood mononuclear leukocytes (33, 34). MCP-1 is a member of the same relatively large family of polypeptides that includes IL-8 (34, 35). Unlike IL-8, MCP-1 does not have chemotactic activity for neutrophils (33).

Similar to the results with ^{125}I -IL-8, addition of 1 nM ^{125}I -MCP-1 (details of the purification and iodination of the native human polypeptide is described in reference 36) to purified red cells at 4×10^9 per ml resulted in 63% of the radiolabeled ligand becoming bound to the cells, and 70% of the binding was inhibited by a 100-fold excess of nonlabeled MCP-1 (data not shown). The total binding ($\pm \text{SD}$) of 0.1, 1, and 5 nM ^{125}I -MCP-1 ($61,687 \pm 568$, $569,318 \pm 13,830$, and $2,161,235 \pm 45,232$ CPM respectively) to 7.4×10^8 red cells was inhibited to a similar extent by both 500 nM nonlabeled MCP-1 ($18,140 \pm 1,897$, $194,351 \pm 8,989$, and $800,939 \pm 94,130$ CPM bound) and 500 nM nonlabeled IL-8 ($22,910 \pm 2178$, $194,656 \pm 10,259$, and $920,587 \pm 69,988$ CPM bound). These results suggest that both MCP-1 and IL-8 bind to the same receptor on the red cell surface.

Discussion

The major point of this paper is that red blood cells carry on their surfaces IL-8-binding proteins that significantly diminish the concentration of soluble IL-8 in blood plasma. The evidence for IL-8 extraction by red cells comes from examination of ^{125}I -IL-8 binding, from FACS detections of FITC-IL-8 binding and native IL-8 binding (determined using anti-IL-8 antibody), and from examination of the influence of red cells on the diffusion of IL-8 across the compartments of diffusion chambers. The ^{125}I -IL-8 binding data indicate that this redistribution of IL-8 from plasma onto red cells is mediated by specific high affinity (≈ 5 nM K_d) binding sites for IL-8 that are present on the red cell surface at $\approx 2,000$ sites per cell.

Although there are numerous examples of cytokine and hormone binding proteins in blood (28, 29), in general these are soluble plasma proteins. We know of no other case in which the red blood cell acts as a major sink for a signal-transmitting polypeptide. Red cells do bind insulin and insulin-like growth

factors, but not so extensively as to substantially reduce the soluble levels of these hormones (37, 38, 39). The red cell surface is also a key site for control of other proteins that play a role in inflammatory processes. The transmembrane protein CR1 is an essential cofactor for the cleavage of C3bi, and is also a carrier of immune complexes to the liver for clearance (40, 41). Decay accelerating factor, which is associated with the red cell membrane by a fatty acyl linkage, inhibits the assembly and accelerates the decay of C3 convertase (41, 42).

These results, although novel, are generally consistent with the literature. In a recently reported screening study Besemer et al. (25) detected very little specific binding of ^{125}I -IL-8 to red cells, but in these experiments the number of red cells used in the binding incubations was less than 1% of the number used here. These investigators also noted that their study did not address the possibility that red cells might have IL-8 receptors with a $K_d > 3$ nM. Our results are also not inconsistent with the view that the neutrophil is a principal responsive target cell for IL-8. Previous studies show that neutrophils carry at least 30,000 IL-8 receptors per cell (43, 25). The 1,000-fold greater abundance of red cells over neutrophils in blood accounts for our finding that red cells are responsible for the bulk of the binding of ^{125}I -IL-8 to cells in whole blood.

Our finding that IL-8 absorbed to red cells did not stimulate an increase in neutrophil free calcium suggests that red cells limit neutrophil interaction with IL-8 in the bloodstream. This function could be physiologically important, as endotoxin or inflammatory cytokines stimulate the accumulation of large amounts of IL-8 by monocytes, T lymphocytes, and endothelial cells in whole blood (references 1, 4, and 44, W. C. Darbonne, unpublished data). Studies in which IL-8 has been injected intravenously indicate that even transiently existing, modest, intravascular levels of this chemotaxin/agonist inhibit targeting of neutrophils to sites of inflammation (17). Moreover, the activity of extravascularly administered or secreted IL-8 to induce leukocyte chemotaxis depends on the nature of IL-8 concentration gradients that develop across capillaries. Neutrophil responses to IL-8 occur within seconds and exhibit dose dependence over the ~ 0.1 – 5 nM range (8). Significantly, these results show that red cells bind IL-8 extraordinarily rapidly, and extract $> 80\%$ of soluble IL-8 when the cytokine is added to blood in this concentration range. Binding and neutralization of IL-8 by red cells may provide a line of defense against systemic activation and chemotactic disorientation of neutrophils.

Our results showing the competitive binding of IL-8 and MCP-1 to red cells suggests that red cells may act as a general intravascular sink for soluble chemotaxins. This effect could localize the activation of the respective target cell(s) at the site of the intravascular secretion, and/or properly orient the chemotaxin concentration gradient across the vascular wall. These findings should prompt an examination of the interactions of red cells with other polypeptides on the rapidly growing list of chemotactic cytokines.

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