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# Proinflammatory Activities of S100: Proteins S100A8, S100A9, and S100A8/A9 Induce Neutrophil Chemotaxis and Adhesion<sup>1</sup>

Carle Ryckman, Karen Vandal, Pascal Rouleau, Mariève Talbot, and Philippe A. Tessier<sup>2</sup>

**S100A8 and S100A9 are small calcium-binding proteins that are highly expressed in neutrophil and monocyte cytosol and are found at high levels in the extracellular milieu during inflammatory conditions. Although reports have proposed a proinflammatory role for these proteins, their extracellular activity remains controversial. In this study, we report that S100A8, S100A9, and S100A8/A9 caused neutrophil chemotaxis at concentrations of  $10^{-12}$ – $10^{-9}$  M. S100A8, S100A9, and S100A8/A9 stimulated shedding of L-selectin, up-regulated and activated Mac-1, and induced neutrophil adhesion to fibrinogen in vitro. Neutralization with Ab showed that this adhesion was mediated by Mac-1. Neutrophil adhesion was also associated with an increase in intracellular calcium levels. However, neutrophil activation by S100A8, S100A9, and S100A8/A9 did not induce actin polymerization. Finally, injection of S100A8, S100A9, or S100A8/A9 into a murine air pouch model led to rapid, transient accumulation of neutrophils confirming their activities in vivo. These studies 1) show that S100A8, S100A9, and S100A8/A9 are potent stimulators of neutrophils and 2) strongly suggest that these proteins are involved in neutrophil migration to inflammatory sites. *The Journal of Immunology*, 2003, 170: 3233–3242.**

**D**uring inflammatory responses, neutrophils migrate in a multistep fashion (reviewed in Ref. 1) from the blood to inflammatory sites where they are involved in immune defense. First, interactions between selectins and glycans mediate neutrophils rolling along the endothelium. Neutrophils are then activated through change in  $\beta_2$  integrins to an active conformation. This conformational change thought to be induced by lipid mediators such as platelet-activating factor or chemokines like IL-8, cause neutrophils to adhere strongly to the endothelium and extravasate. Once in the tissue, they follow concentration gradients of chemoattractants (such as C5a, leukotriene B<sub>4</sub>, and IL-8, or bacterial *N*-formylated peptides) toward the inflammatory site.

Recent studies suggest that the myeloid-related proteins (MRPs)<sup>3</sup> are involved in neutrophil migration. The MRPs S100A8 and S100A9 (10.6 and 13.5 kDa, respectively (2)) are calcium-binding proteins that belong to the S100 protein family (3, 4); they are expressed almost exclusively by cells of myeloid lineage. The MRPs are constitutively expressed in the cytosol of neutrophils where they comprise up to 30% of the cytosolic proteins (5). Monocytes and differentiated macrophages in inflamed tissues also express MRPs (4–9). S100A8 and S100A9 form noncovalent homodimers and a heterodimer (S100A8/A9) in a calcium-dependent manner (10–12).

Local secretion of MRPs has been detected in chronic periodontal infections (13, 14) as well as in the serum of patients suffering

from infections and inflammatory pathologies. High serum concentrations of MRPs have been found in advanced HIV infections, adult and juvenile rheumatoid arthritis, chronic bronchitis, cystic fibrosis, systemic lupus erythematosus, and granulomatous conditions, such as tuberculosis and sarcoidosis (10, 15–20). S100A8/A9 is the main form found in the extracellular milieu and its in vitro release by neutrophils, activated monocytes, and macrophages has also been demonstrated (20–25).

Extracellular S100A8, S100A9, and S100A8/A9 mediate regulatory and biological functions, including antiproliferative, antitumor, antimicrobial, and antinociceptive activities (26–31). Like other members of the S100 family (32–34), human MRPs and their murine homologs possess proinflammatory activities. However, there are disparities in the activities ascribed to these proteins pending a clear understanding of their activities in neutrophil migration to inflammatory sites.

Newton and Hogg (35) recently demonstrated that human S100A9 stimulates neutrophil adhesion to fibrinogen by activating the  $\beta_2$  integrin Mac-1 (CD11b/CD18). The promotion of this adhesion was negatively regulated by the formation of the heterocomplex with S100A8 implying that S100A8 as well as S100A8/A9 do not stimulate neutrophil adhesion (35). Eue et al. (36) also reported that S100A8 do not enhanced monocyte adhesion to endothelial cells. However, in contrast to the latter study, they demonstrated that not only S100A9 but also S100A8/A9 enhanced monocyte adhesion to endothelial cells via Mac-1/ICAM-1 interactions. These results suggest that S100A8 does not negatively regulate S100A9 activity by forming S100A8/A9.

Studies conducted using murine MRPs demonstrated that murine S100A8, called CP-10, is a potent chemotactic factor (activity at  $10^{-13}$ – $10^{-11}$  M) for neutrophils (37), and induces sustained leukocyte recruitment in vivo (38, 39). Moreover, like human S100A9, murine S100A8 does not stimulate calcium flux, shedding of L-selectin, or up-regulation of the  $\beta_2$  integrin Mac-1 on neutrophils (35, 37, 40). In contrast to its murine homolog, human S100A8 is supposedly not chemotactic for neutrophils, but has been reported to be chemotactic for periodontal ligament cells (35, 41).

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<sup>3</sup> Abbreviation used in this paper: MRP, myeloid-related protein.

Therefore, the exact activities of S100A8 and S100A8/A9, particularly their effects on neutrophil migration, remain unknown. Harrison et al. (42) showed that murine S100A8 chemotactic activity can be efficiently inhibited by oxidation of the protein, demonstrating a certain susceptibility of S100A8 to inactivation. S100A9 activity appeared to be differently affected by oxidation (42). Therefore, the discrepancies in MRP activity could be due to differences in protein activity arising from interlaboratory variation in purification protocols or methodologies.

In this study, a different and gentle approach was used for the production and purification of the human S100A8, S100A9, and S100A8/A9 proteins. Recombinant proteins were generated and their proinflammatory properties toward neutrophils were characterized *in vitro*. S100A8, S100A9, and S100A8/A9 proved to be chemotactic for neutrophils *in vitro* and also stimulated shedding of L-selectin and up-regulation and activation of Mac-1. They also stimulated neutrophil adhesion to fibrinogen in a Mac-1-dependent manner. Neutrophil adhesion was also associated with an increase in intracellular calcium levels, but not with actin polymerization. Finally, injection of the human S100A8, S100A9, or S100A8/A9 into a murine air pouch led to a transient accumulation of neutrophils, therefore confirming their activity *in vivo*.

## Materials and Methods

### Reagents

The fluorescent dyes fura 2-AM and calcein-AM were purchased from Calbiochem (San Diego, CA). ALEXA 488-conjugated goat anti-mouse specific Abs and SYPRO orange protein gel stain were purchased from Molecular Probes (Eugene, OR). LPS purified from *Escherichia coli* serotype O26 B6, fMLP, and fibrinogen were purchased from Sigma-Aldrich (St. Louis, MO). Anti-CD11b-, CD18-, and CD32-specific hybridomas OKM1, IB4, and IV.3, respectively, were obtained from the American Type Culture Collection (Manassas, VA) and were produced as purified ascites. Anti-CD51 ( $\alpha_v\beta_3$ ) was obtained from Chemicon International (Temecula, CA). Digitonin was purchased from Calbiochem. FITC-phalloidin was purchased from Sigma-Aldrich. Anti-CD62L was purchased from BD PharMingen (San Diego, CA) and mAb 24 was a generous gift from Dr. N. Hogg (Imperial Cancer Research Fund, London, U.K.).

### Recombinant proteins

Cloning, expression, and purification of human S100A8 and S100A9 was described previously (43). Briefly, human S100A8 and S100A9 (full-length) cDNA were cloned into the pET28 expression vector (Novagen, Madison, WI). Recombinant protein expression was induced with 1 mM isopropyl  $\beta$ -D-thiogalactoside in *E. coli* HMS174 (Boehringer Mannheim, Mannheim, Germany) for 16 h at 16°C. After incubation, the bacteria were centrifuged at 5000  $\times$  g for 10 min and the pellet was resuspended in PBS/NaCl (0.5 M)/imidazole (1 mM) and lysed by sonication. The lysate was then centrifuged at 55,000  $\times$  g for 30 min at 4°C, and the supernatant was collected. Recombinant His-Tag S100A8 and S100A9 were purified using a nickel column. S100A8 or S100A9 bound to the column were freed from their His-Tag by incubation with 10 U of biotinylated thrombin (Novagen) for 20 h at room temperature. Recombinant S100A8 and S100A9 were eluted with PBS. The digestion and elution processes were repeated once to cleave the remaining undigested recombinant proteins, and streptavidin-agarose (Novagen) was added to remove the contaminating thrombin. Finally, the protein solutions were passed through a polymyxin B-agarose column (Pierce, Rockford, IL) to remove endotoxins. Control for bacterial contamination of the recombinant proteins consisted of untransformed HMS 174, stimulated, lysed, and processed for purification like the recombinant proteins. Experiments using this control were conducted using the same dilutions as for MRPs. Contamination by endotoxins were <1 pg/ $\mu$ g S100A8 or S100A9 protein as measured by the *Limulus* amebocyte assay (Sigma-Aldrich). The proteins were kept at -80°C for up to 2 mo until used.

### Production of mAbs

Female BALB/c mice (4 wk old) were immunized by i.p. injections with 30  $\mu$ g of purified recombinant S100A8 or S100A9 in 50  $\mu$ l of endotoxin-free PBS (Sigma-Aldrich) mixed in an equal volume of CFA. Ab responses were enhanced by injections 14 days later with S100A8 or S100A9 using

IFA, and final boost was given on day 28 with proteins alone. On day 31, spleen cells from the immunized mice were fused with SP2 murine myeloma cells and cultured in hypoxanthine/amethopterin/thymidine selection medium. Culture supernatants of the hybridomas were screened by ELISA using plates coated with 1  $\mu$ g/ml recombinant proteins in 0.1 M carbonate buffer (pH 9.6). Positive hybridoma cells were cloned by limiting dilution. The mAb clones KVC/3E2 and KVC/2B9 showed the most distinctive recognition of the recombinant proteins S100A8 and S100A9, respectively. Both clones were isotypized as IgG1 kappa. Specificity of the mAbs was confirmed by ELISA and Western blot analysis.

### Neutrophil purification

Peripheral blood was collected in heparinized tubes from healthy adult volunteers. Neutrophils were isolated as previously described by Boyum (44) and resuspended in HBSS-H (HBSS supplemented with 10 mM HEPES (pH 7.4)) containing 1.3 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$ . The purity and cell viability of neutrophil preparations were consistently >98% as assessed by acetic blue staining and trypan blue exclusion, respectively.

### Chemotaxis assays

Neutrophil chemotaxis assays were performed in modified Boyden chambers. Increasing concentrations of MRPs or  $10^{-8}$  M fMLP diluted in HBSS-H containing 1.3 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$  was added to the lower well of the chambers. Polycarbonate filters (8- $\mu$ m pore; Neuroprobe, Gaithersburg, MD) were put in place and the upper chambers were secured. Neutrophils ( $5 \times 10^4$  cells in 200  $\mu$ l) were added to the upper chambers and allowed to migrate through the membrane for 30 min at 37°C. The upper chambers were washed once with PBS, and the membrane was then fixed in methanol and stained with Wright-Giemsa. Cells that had migrated through the membrane were counted using a microscope in five high-power fields. The results are expressed as mean number of migrated cells per high-power field. Directional migration was confirmed by adding increasing concentrations of MRPs to the upper wells. In separate experiments, 10  $\mu$ g/ml anti-S100A8 and/or anti-S100A9 were added to the lower chambers before addition of neutrophils to the upper chamber.

### Flow cytometry analysis

Neutrophils ( $2 \times 10^6$  cells/ml) were stimulated with MRPs ( $2 \times 10^{-6}$  M) at 37°C for 30 min. For the detection of CD62L, CD11b, and CD51 expression, neutrophils ( $4 \times 10^5$ ) were incubated with 1  $\mu$ g of CD62L-, CD11b-, or CD51-specific Abs, respectively, on ice for 25 min. After two washes in PBS/0.2% BSA/0.1% azide, neutrophils were incubated with ALEXA 488-conjugated goat anti-mouse specific Ab for 25 min on ice, washed twice, and fixed in PBS containing 2% formaldehyde. CD18 activation was detected by adding 1  $\mu$ g of mAb 24 to  $5 \times 10^5$  neutrophils after 20-min stimulation with MRPs and incubating at 37°C for 10 min. Cells were washed twice with PBS/0.2% BSA/0.1% azide and incubated with ALEXA 488-conjugated goat anti-mouse specific Ab for 45 min at RT. After two washes, neutrophils were fixed in PBS containing 2% formaldehyde. Detection was done with a FACSCalibur (BD Biosciences, Mountain View, CA) flow cytometer.

### Adhesion

Neutrophils were resuspended at  $5 \times 10^6$  cells/ml in HBSS-H containing 1.3 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$  and labeled with 5  $\mu$ M intracellular fluorescent dye calcein-AM for 30 min at 37°C. After washing, labeled cells were resuspended in HBSS-H containing 1.3 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$  at  $5 \times 10^6$  cells/ml, and 50  $\mu$ l was added to Costar 96-well plates, which had been coated overnight with 2 mg/ml fibrinogen (50  $\mu$ l/well). These wells contained 50  $\mu$ l of increasing concentrations (2 $\times$ ) of human S100A8, S100A9, S100A8/A9, equal volumes of mock protein purification solution, or  $10^{-7}$  M fMLP. Neutrophils were allowed to adhere for 30 min at 37°C before being washed three times by immersion in cold PBS. The adherent cells were lysed by adding ddH<sub>2</sub>O, and the fluorescence was measured using a 96-well plate fluorescence reader. In some experiments, S100A8, S100A9, S100A8/A9, or  $10^{-7}$  M fMLP were boiled or treated at room temperature with 10 or 100  $\mu$ M NaOCl for 15 min and diluted to a final concentration of 20  $\mu$ g/ml before stimulation. In other experiments, neutrophils were preincubated for 20 min with 20  $\mu$ g/ml Abs directed against CD18, CD51, or CD32 (negative control) before being added to the wells.

### Actin polymerization

Neutrophils were resuspended at a concentration of  $2 \times 10^6$  cells/ml in HBSS-H containing 1.3 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$ . The reaction was initiated by mixing  $5 \times 10^4$  cells (250  $\mu$ l) with the MRP preparations at

final concentrations of  $1 \times 10^{-6}$ – $5 \times 10^{-13}$  M. After increasing periods of incubation at RT, the reaction was stopped by adding 50  $\mu$ l of 3.7% formaldehyde and placed immediately on ice. The cells were permeabilized, and the F-actin was stained for 60 min on ice with a mixture of 0.05% (w/v) digitonin and 2  $\mu$ M FITC-phalloidin. Cells were then pelleted and washed twice in HBSS-H and resuspended in HBSS-H. The fluorescence was measured by flow cytometry on a FACSCalibur (BD Biosciences).

#### Intracellular calcium measurements

Neutrophils ( $10^7$  cells/ml) were loaded with fura 2-AM (1  $\mu$ M; 30 min at 37°C), washed, and resuspended at  $2 \times 10^6$  cells/ml in HBSS-H supplemented with 1.3 mM  $\text{CaCl}_2$ . The fluorescence was measured in a spectrofluorometer (SLM 8000; SLM-Aminco, Urbana, IL) in presence of fMLP ( $10^{-7}$  M) or increasing concentrations of human S100A8, S100A9, or S100A8/A9 at excitation and emission wavelengths of 340 and 510 nm, respectively. The internal calcium concentrations were calculated as described by Tsien et al. (45).

#### Air pouch experiments

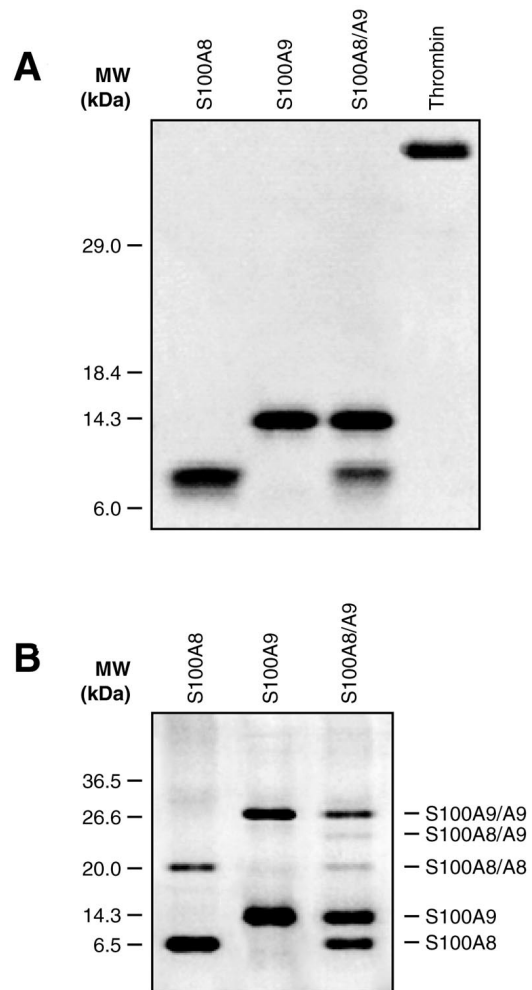
Ten- to 12-wk-old CD-1 and C3H/HeJ mice were obtained from Charles River (St. Columban, Quebec, Canada) and The Jackson Laboratory (Bar Harbor, ME), respectively. Air pouches were raised on the dorsum by s.c. injection of 3 ml of sterile air on days 0 and 3. On day 7, 1 ml of S100A8, S100A9, or an equimolar ratio of S100A8 and S100A9 dissolved in endotoxin-free PBS at concentrations ranging from 0.1 to 10  $\mu$ g/ml or LPS (1  $\mu$ g/ml) was injected into the air pouches. At specific time points, the mice were killed by  $\text{CO}_2$  asphyxiation, the air pouches were washed once with 1 ml PBS/5 mM EDTA, and then twice with 2 ml PBS/5 mM EDTA, and the exudates were centrifuged at  $500 \times g$  for 5 min at room temperature. Cells were counted with a hemacytometer following acetic blue staining. Characterization of leukocyte subpopulations migrating into the pouch space was performed by Wright-Giemsa staining of cytopins.

## Results

#### Production of recombinant S100A8, S100A9, and S100A8/A9

To date, discrepancies exist between the activities of S100A8, S100A9, and S100A8/A9. These differences could be consecutive to improper folding or inactivation of the proteins during purification. To alleviate these problems, His-tagged recombinant proteins were generated and produced in *E. coli* using a pEt28 expression vector. Production yields were kept low by inducing protein synthesis at 16°C to maximize proper folding, and the purification process was performed at physiological pH, in absence of potentially denaturing reagents such as DTT and imidazole. In addition, the purification protocol did not include any unfolding-refolding steps. Proteins with only three extra N-terminal amino acids (Gly-Ser-His-Met- . . .) were generated after thrombin cleavage of the polyhistidine tail.

Recombinant MRPs were first analyzed on SDS-PAGE gels and stained with SYPRO orange (sensitivity limit of 4–8 ng/band). As shown in Fig. 1A, S100A8 and S100A9 produced using this protocol migrated to their expected molecular masses (8 and 14 kDa, respectively) and were free of contamination from bacteria-derived proteins and thrombin. These results also confirmed the absence of thrombin-cleaved products of S100A8 or S100A9. Analysis of S100A8 and S100A9 on nonreducing gels revealed the presence of monomers and homodimers (Fig. 1B). As expected, S100A8 and S100A9 also formed the heterodimer S100A8/A9 when mixed together (Fig. 1B). Although we observed variability in the relative concentrations of monomers, homodimers, and heterodimers, these concentrations were similar to the ones observed by other laboratories for native and recombinant human or murine proteins (11, 42, 46). The presence of monomers might result from the dissociation of homodimers and heterodimers caused by SDS in the nonreducing gels.

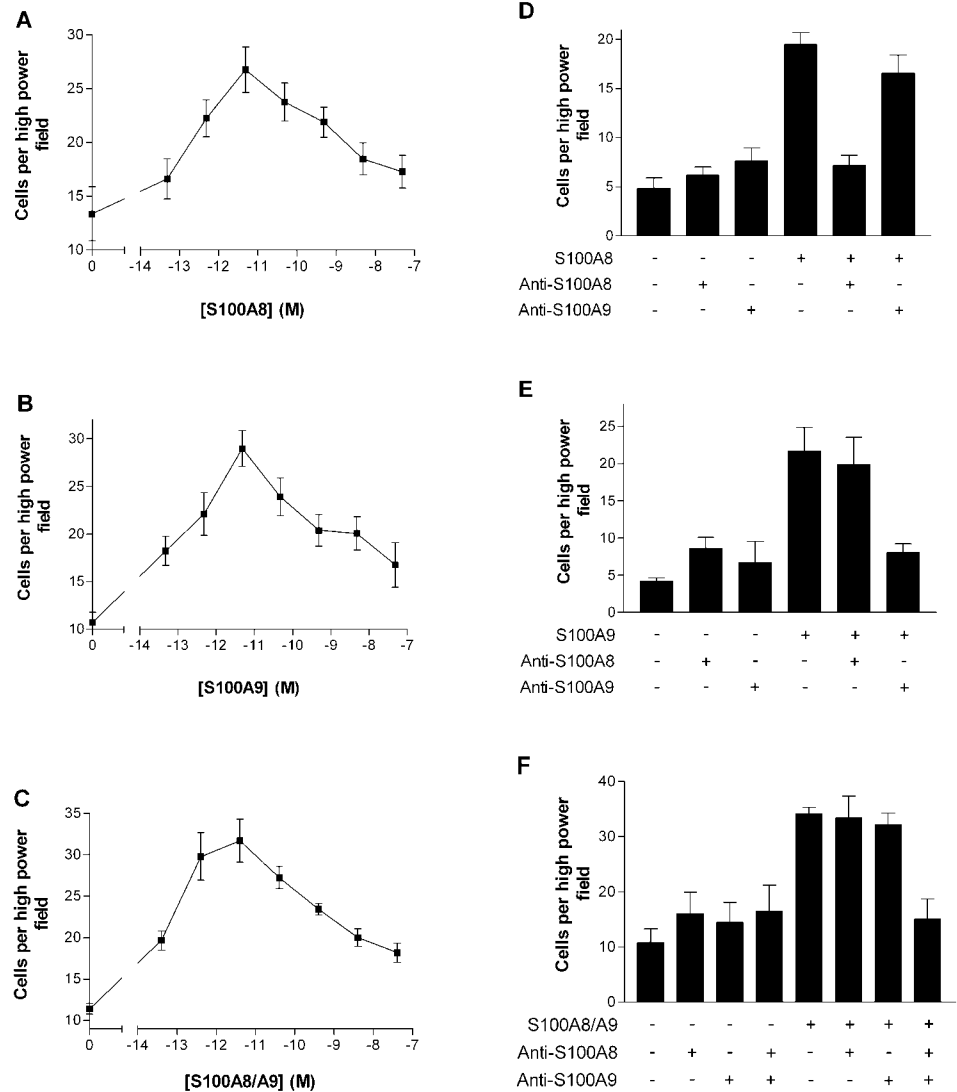


**FIGURE 1.** SDS-PAGE analysis of recombinant S100A8 and S100A9. Thrombin (0.4 U/lane) or recombinant S100A8 and S100A9 (3  $\mu$ g/lane) were mixed together (in presence of HBSS-H containing 1.3 mM  $\text{Ca}^{2+}$ ) or not before being separated on SDS-PAGE under reducing (A) or nonreducing (B) conditions and stained with Sypro orange. The presence of the monomeric, homodimeric, and heterodimeric forms of the proteins is shown.

#### S100A8, S100A9, and S100A8/A9 are potent chemotactic factors for neutrophils in vitro

Recombinant MRP activities were first evaluated in vitro in chemotaxis assays using modified Boyden chambers. S100A8, S100A9, and S100A8/A9 proved to be chemotactic for neutrophils at concentrations varying from  $10^{-12}$ – $10^{-10}$  M (Fig. 2, A–C). Neutrophil migration was inhibited by adding mAbs against S100A8 and/or S100A9 to the bottom chambers (Fig. 2, D–F), demonstrating that the migration detected was consecutive to activation of neutrophils by the recombinant S100 proteins. To confirm that cell migration was the result of induced directional migration, increasing concentrations of MRPs were added to the upper chambers. As shown in Tables I, II and III, addition of the same proteins to the upper chambers significantly reduced neutrophil migration through the membranes, confirming that S100A8, S100A9, and S100A8/A9 induced neutrophil chemotaxis. A small increased migration of neutrophils has also been observed when  $1 \times 10^{-11}$  M MRPs were added to the upper chambers, suggesting that they also stimulated random migration of neutrophils.

**FIGURE 2.** S100A8, S100A9, and S100A8/A9 are potent chemotactic factors for neutrophils. Chemotaxis assays were performed in modified Boyden chambers. HBSS-H (control) or increasing concentrations of S100A8 (A), S100A9 (B), or S100A8/A9 (C) were added to the lower chamber. Neutrophils were added to the upper chamber and allowed to migrate through the membrane for 30 min. Migrated cells were stained with Wright-Giemsa and counted. Neutralization of the activity was performed by adding 10  $\mu\text{g/ml}$  anti-S100A8 and/or anti-S100A9 to the lower wells containing  $10^{-11}$  M S100A8 (D), S100A9, (E) or S100A8/A9 (F). Results are the mean number of migrated cells per high power field. Data shown represent mean  $\pm$  SEM of at least three separate experiments performed on neutrophils from different donors.



S100A8, S100A9, and S100A8/A9 induce shedding of L-selectin, and up-regulation and activation of Mac-1

S100A9 and S100A8/A9 had previously been shown to stimulate neutrophil adhesion (35, 36). To verify whether our recombinant MRPs also stimulated adhesion, neutrophils were incubated with S100A8, S100A9, and S100A8/A9, and the level of expression of

the adhesion molecules CD62L (L-selectin), CD11b (Mac-1), and CD51 ( $\alpha_v\beta_3$ ) on the cell surface was examined. In addition, the state of activation of Mac-1 was evaluated using the activation reporter mAb 24. As shown in Fig. 3, MRPs induced shedding of L-selectin (CD62L) from the neutrophil surface. In contrast, expression of CD11b, but not CD51 was up-regulated following stimulation with MRPs. As Mac-1 is stored in specific and gelatinase granules, this suggests that MRPs induced neutrophil degranulation. Stimulation with MRPs also led to the change of  $\beta_2$

Table I. Checkerboard analysis of neutrophil chemotaxis induced by S100A8

S100A8 Concentration in Lower Chamber (M)	S100A8 Concentration in Upper Chamber (M)		
	0	$10^{-12}$	$10^{-11}$
0	$10.4 \pm 0.6^a$	$15.8 \pm 1.8$	$18.0 \pm 0.4$
$10^{-12}$	$25.3 \pm 0.6$	$17.0 \pm 0.5^*$	$19.4 \pm 1.7^{**}$
$10^{-11}$	$25.8 \pm 1$	$19.4 \pm 1.7^{**}$	$15.8 \pm 0.9^*$

<sup>a</sup> Data are expressed as the number of migrated cells per high-power field in response to different concentrations of S100A8 in the lower and upper chambers. Values represent the mean  $\pm$  SEM of three experiments carried out on neutrophils from different donors.

<sup>\*</sup>,  $p < 0.001$ , Student's *t* test (S100A8 in both chambers vs S100A8 in lower chamber only).

<sup>\*\*</sup>,  $p < 0.01$ , Student's *t* test (S100A8 in both chambers vs S100A8 in lower chamber only).

Table II. Checkerboard analysis of neutrophil chemotaxis induced by S100A9

S100A9 Concentration in Lower Chamber (M)	S100A9 Concentration in Upper Chamber (M)		
	0	$10^{-12}$	$10^{-11}$
0	$12.7 \pm 2.2^a$	$20.7 \pm 1.7$	$18.2 \pm 1.8$
$10^{-12}$	$26.0 \pm 2.1$	$18.2 \pm 0.7^*$	$21.6 \pm 0.7$
$10^{-11}$	$25.5 \pm 0.8$	$25.5 \pm 0.6$	$17.8 \pm 0.3^*$

<sup>a</sup> Data are expressed as the number of migrated cells per high-power field in response to different concentrations of S100A9 in the lower and upper chambers. Values represent the mean  $\pm$  SEM of three experiments carried out on neutrophils from different donors.

<sup>\*</sup>,  $p < 0.05$ , Student's *t* test (S100A9 in both chambers vs S100A9 in lower chamber only).

Table III. Checkerboard analysis of neutrophil chemotaxis induced by S100A8/A9

S100A8/A9 Concentration in Lower Chamber (M)	S100A8/A9 Concentration in Upper Chamber (M)		
	0	10 <sup>-12</sup>	10 <sup>-11</sup>
0	12.3 ± 2.6 <sup>a</sup>	19.0 ± 0.8	18.6 ± 0.9
10 <sup>-12</sup>	29.8 ± 0.8	19.0 ± 1.9*	22.3 ± 1.2**
10 <sup>-11</sup>	30.3 ± 1.7	27.7 ± 1.0	18.6 ± 1.3*

<sup>a</sup> Data are expressed as the number of migrated cells per high-power field in response to different concentrations of S100A8/A9 in the lower and upper chambers. Values represent the mean ± SEM of three experiments carried out on neutrophils from different donors.

\* , *p* < 0.01, Student's *t* test (S100A8/A9 in both chambers vs S100A8/A9 in lower chamber only).

\*\* , *p* < 0.001, Student's *t* test (S100A8/A9 in both chambers vs S100A8/A9 in lower chamber only).

integrin conformation to an active form, as reported by the activation reporter mAb 24.

*S100A8, S100A9, and S100A8/A9 stimulate neutrophil adhesion to fibrinogen*

The effect of MRPs on the expression of adhesion molecule on neutrophils suggested that they modify neutrophil adhesion. Thus, we examined neutrophil adhesion to fibrinogen following stimulation with MRPs. Stimulation of neutrophil adhesion by S100A8/A9 was comparable to that of fMLP while the same concentrations of S100A8 and S100A9 stimulated a weaker adhesion (Fig. 4). Maximal adhesion was observed between 10<sup>-7</sup> and 10<sup>-6</sup> M S100A8/A9 and at 10<sup>-6</sup> M S100A8 or S100A9. This effect was inhibited by >80% when the proteins were heat inactivated (Fig. 5A). Boiling fMLP did not reduce its activity; thus our results were not confounded by the proteins being contaminated with formyl peptides. Moreover, stimulation of neutrophils with a mock protein preparation did not induce their adhesion (Fig. 5B), ruling out the possibility that the activities attributed to the MRPs were a consequence of bacterial or thrombin contamination.

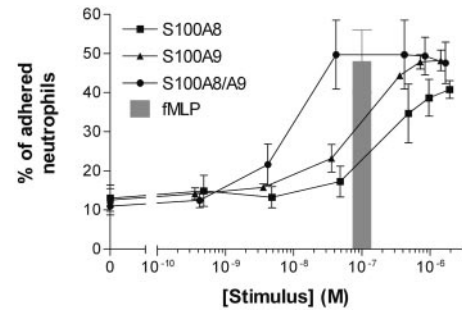


FIGURE 4. MRPs stimulate neutrophil adhesion to fibrinogen. Neutrophils were stimulated with increasing concentrations of S100A8, S100A9, or S100A8/A9, 10<sup>-7</sup> M fMLP, or PBS (control) for 30 min at 37°C, and the number of adhered neutrophils was determined as described. Results are percentage of adhered neutrophils to fibrinogen. Data shown represent mean ± SEM of three experiments performed on neutrophils from different donors.

It has been demonstrated that murine S100A8 can be efficiently inactivated by oxidation (42, 47). Therefore, we investigated whether the human MRPs could be inactivated by oxidation as well. As shown in Fig. 5C, neutrophil adhesion to fibrinogen was inhibited by >95% when S100A8 was treated with 100 μM NaOCl before stimulation, suggesting that human S100A8 can be inactivated by oxidation like its murine counterpart. In contrast, NaOCl (100 μM) had no effect on S100A9 and S100A8/A9 activity. Because hypochlorite is an oxidant generated at inflammatory sites, it could also be involved in the regulation of human S100A8 activity (42, 47). This result suggests that the lack of activity of S100A8 reported by others could be due to oxidation of the protein during purification and/or storage.

*MRP-stimulated neutrophil adhesion to fibrinogen is mediated by Mac-1 but not α<sub>v</sub>β<sub>3</sub>*

Mac-1 (CD11b/CD18) and α<sub>v</sub>β<sub>3</sub> (CD51/CD61) are two integrins expressed by neutrophils known to bind fibrinogen (48, 49). To

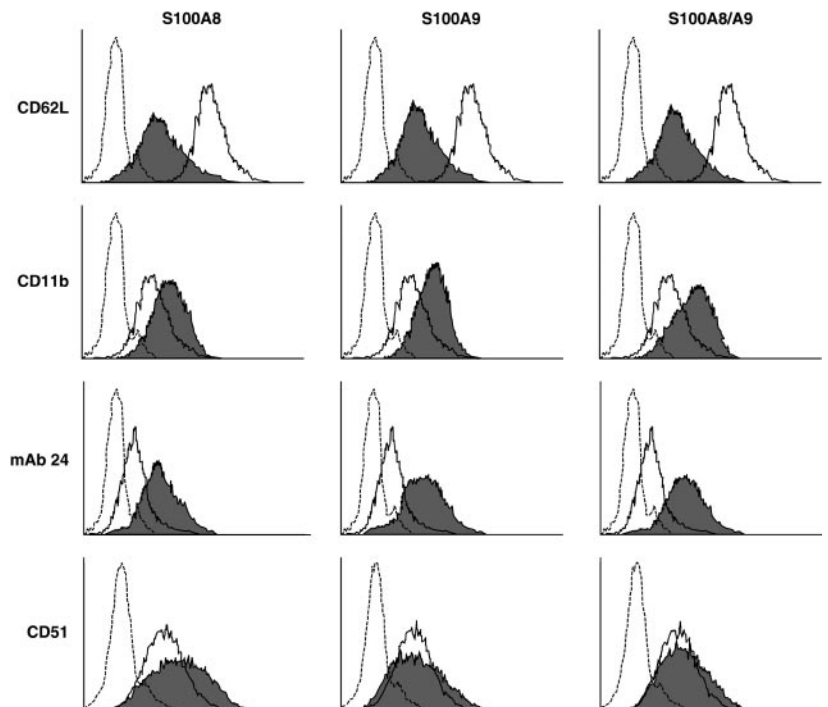
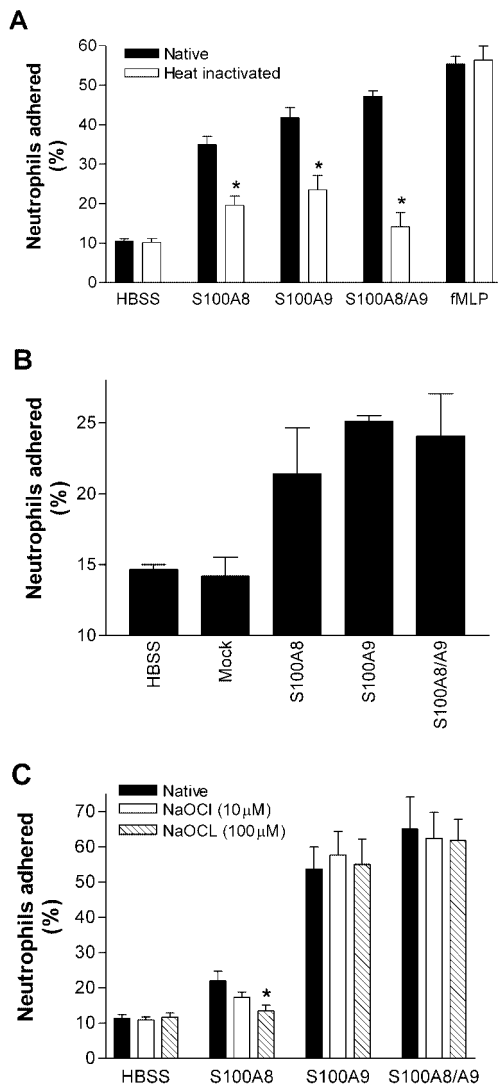


FIGURE 3. MRPs induce shedding of CD62L, up-regulation of CD11b, and activation of CD18. Expression of CD62L, CD11b, CD51, and activation of CD18 (mAb 24) was analyzed on MRP-stimulated neutrophils by flow cytometry analysis. Neutrophils were stimulated with MRPs (2 × 10<sup>-6</sup> M) for 30 min at 37°C and incubated with CD62L, CD11b, CD51, or CD18 activation-specific Ab. Histograms of MRP-stimulated neutrophils are illustrated in gray, while unstimulated neutrophils (HBSS) appear in open histograms. Non-specific stainings are illustrated by a dotted line. Data shown are from an experiment representative of two others conducted on cells from different donors.



**FIGURE 5.** Inactivation of the MRPs by heat denaturation and oxidation. Neutrophils adhesion was stimulated with  $4 \times 10^{-7}$  M native or heat-inactivated S100A8, S100A9, S100A8/A9,  $10^{-7}$  M fMLP, or HBSS-H (control) (A);  $4 \times 10^{-7}$  M native S100A8, S100A9, or S100A8/A9, or the same volume of mock protein purification solution (B); or  $4 \times 10^{-7}$  M native S100A8, S100A9, or S100A8/A9, or oxidized with 10 or 100  $\mu$ M NaOCl, for 30 min at 37°C (C). Results are percentage of adhered neutrophils to fibrinogen. Data shown represent mean  $\pm$  SEM of four experiments performed on neutrophils from different donors. \*,  $p < 0.01$  compared with native S100 proteins (Dunnett multiple comparison test).

study the mechanism of MRP-induced neutrophil adhesion, their role in neutrophil adhesion to fibrinogen was evaluated. Specific Abs directed against CD18 blocked MRP-stimulated and unstimulated neutrophil adhesion to fibrinogen. In contrast, the anti-CD51 Ab did not diminish neutrophil adhesion. CD32 Ab, used as negative control, did not influence neutrophil adhesion either (Fig. 6). These results demonstrate that MRP-stimulated neutrophil adhesion to fibrinogen is mediated by the  $\beta_2$  integrin Mac-1.

#### Inability of S100A8, S100A9, and S100A8/A9 to stimulate neutrophil actin polymerization

To evaluate the level of activation of neutrophils, the effect of MRPs on actin polymerization was next investigated. fMLP induced a rapid and transient neutrophil actin polymerization at a concentration of  $10^{-7}$  M. However, S100A8, S100A9, and

S100A8/A9 did not induce significant neutrophil actin polymerization (Fig. 7). This contrasts with previously reported observations indicating that murine neutrophils stimulated with murine S100A8 had increased actin polymerization (37). These results indicate that, unlike classical chemotactic factors, the human MRPs possess restricted activating activities toward neutrophils.

#### MRPs induce calcium mobilization in neutrophils

Calcium mobilization is also frequently associated with neutrophil adhesion and chemotaxis. Because murine S100A8 does not induce calcium mobilization in neutrophils (37) and S100A12 induces calcium mobilization in monocytoic cells, but not in neutrophils (Ref. 50; M. Talbot and P. A. Tessier, unpublished observations), we investigated the effect of MRPs on calcium mobilization in human neutrophils was next investigated. Human neutrophils had resting  $\text{Ca}^{2+}$  levels of 100 nM. Neutrophil stimulation with fMLP ( $10^{-8}$  M) led to a transient increase in  $\text{Ca}^{2+}$  (Fig. 8A). Stimulation of neutrophils with S100A8, S100A9, and S100A8/A9 also increased calcium levels, although not as high as fMLP (Fig. 8, B, C, and D). This mobilization was observed at concentrations of  $10^{-6}$ – $10^{-7}$  M but not at lower concentrations of MRPs.

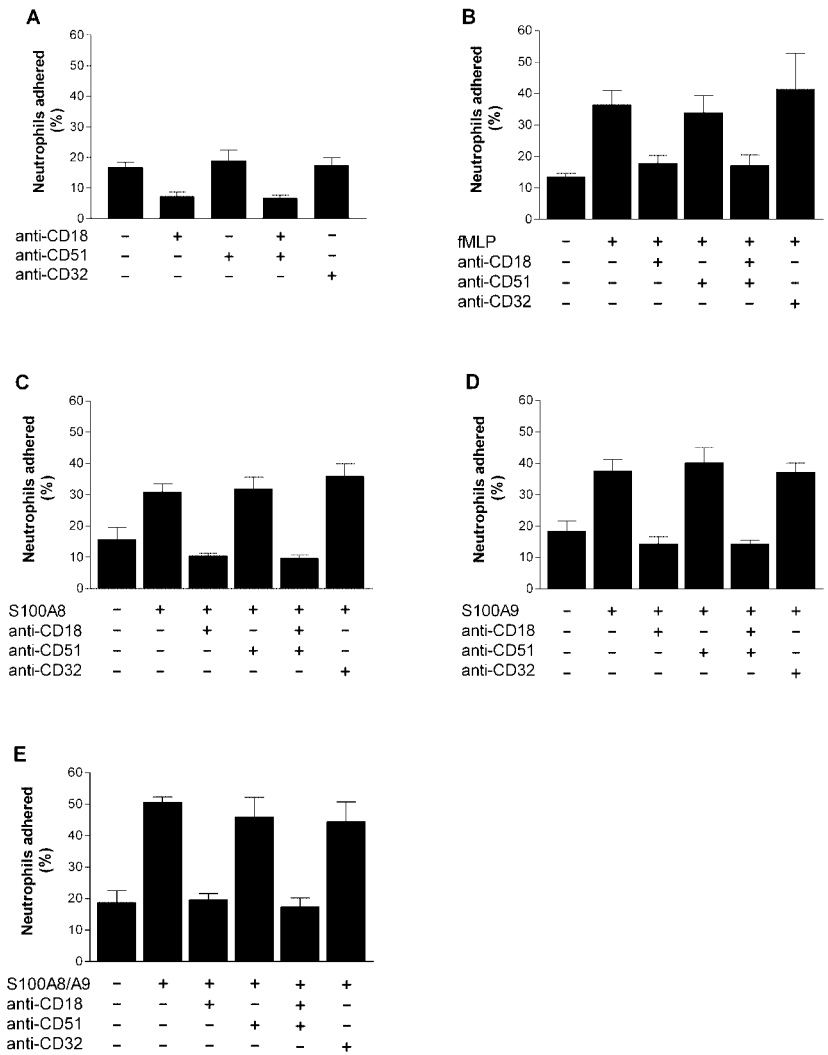
#### MRPs induce neutrophil accumulation in vivo

To determine the biological activities of the MRPs, S100A8 and S100A9 were injected into the murine air pouch and cells migrating to the air pouch environment were quantified. Injection of both S100A8 and S100A9 led to the accumulation of leukocytes. From 90 to 95% of the migrating leukocytes were neutrophils (Fig. 9A). Neutrophils were recruited to the air pouch within 3 h postinjection. The accumulation was maximal between 6 and 12 h postinjection, dropping to control levels by 24 h (Fig. 9A). Monocytes were also recruited to the air pouch, with maximum migration observed at 12 h postinjection (Fig. 9B). No significant migration was detected in PBS- or mock protein preparation-injected mice (data not shown), suggesting that these activities are not caused by contamination of the proteins with bacteria-derived Ni-binding proteins or thrombin.

In a second series of experiments, increasing concentrations of MRPs were injected into the air pouch and leukocyte accumulation was measured. Injection of 0.1  $\mu$ g of MRPs was sufficient to induce an inflammatory response in mice (Fig. 10A). Leukocyte accumulation was maximal at a dose of 10  $\mu$ g. No migration of leukocytes was detected at quantities  $<0.1$   $\mu$ g (data not shown). MRPs were also injected in the air pouch of C3H/HeJ mice. Those mice do not respond to LPS due to a deficiency in Toll-like receptor 4 (51). Injection of 10  $\mu$ g of S100A8, S100A9, or S100A8/A9 did induce an inflammatory reaction (Fig. 10B), thus confirming that the activities of the proteins were not due to LPS contamination. As expected, even high concentrations of LPS (up to 1  $\mu$ g/ml) did not induce significant leukocyte migration.

## Discussion

In former studies, the activity of MRPs has been investigated using either native or sometimes recombinant proteins, purified by isoelectric focusing or in presence of reducing agents such as DTT (35, 36). These purification protocols could potentially result in partial or total inactivation of the proteins. In this study, a different approach was used for the production and purification of the human S100A8, S100A9, and S100A8/A9 proteins to enable the study of their proinflammatory properties. Injection of human S100A8, S100A9, or S100A8/A9 in the murine air pouch induced

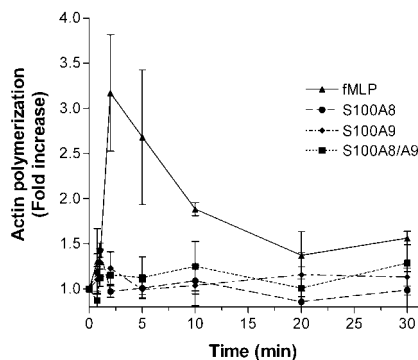


**FIGURE 6.** MRPs stimulate neutrophil adhesion via CD18 but not CD51. Neutrophils were incubated with CD18-, CD51-, and CD32-specific Ab, and neutrophil adhesion was stimulated with HBSS-H (A),  $3\text{--}4 \times 10^{-7}$  M S100A8 (B), S100A9 (C), or S100A8/A9 (D), or  $10^{-7}$  M fMLP (E) for 30 min at  $37^\circ\text{C}$ . Results are percentage of adhered neutrophils to fibrinogen. Data represent mean  $\pm$  SEM of the experiments performed on neutrophils from different donors.

an inflammatory reaction resulting in a rapid and transient accumulation of neutrophils. This migration could be explained by the chemotactic and proadhesive activities of the MRPs toward neutrophils *in vitro*. Neutrophil adhesion was associated with an increase in intracellular calcium levels but not with actin polymerization. These studies demonstrate that S100A8, S100A9, and S100A8/A9 are potent stimulators of neutrophils and strongly sug-

gest that they are involved in neutrophil migration to inflammatory sites.

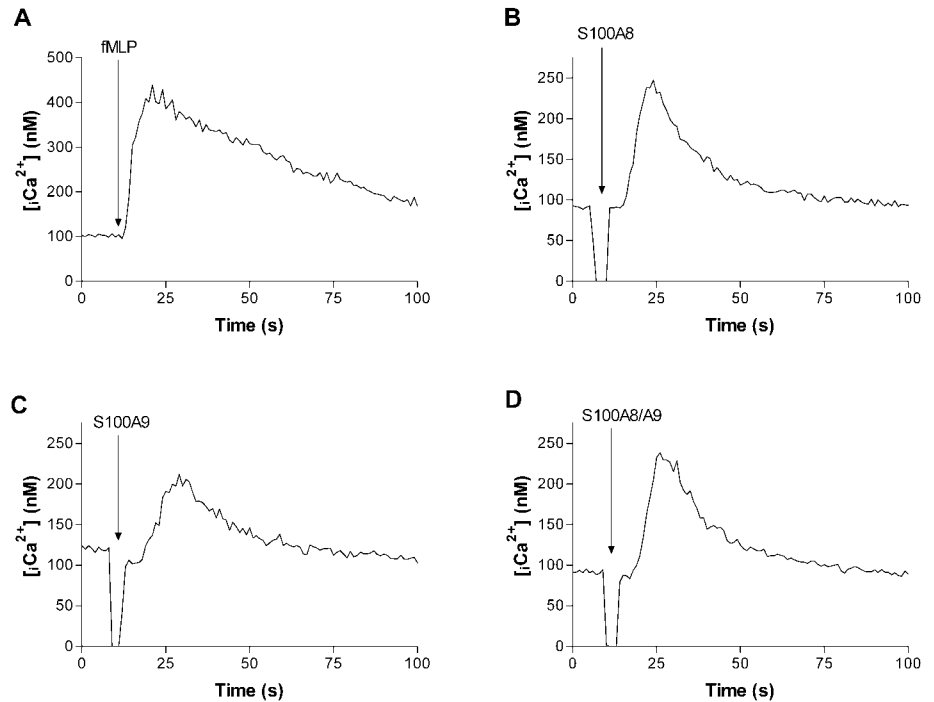
To date, the proinflammatory activities of the MRPs remained elusive. Human S100A9 has been reported to stimulate neutrophil adhesion to fibrinogen, but its activity was negatively regulated by forming the heterocomplex S100A8/A9 with S100A8 (35). Another study recently demonstrated that S100A9, S100A8/A9, but not S100A8, enhanced monocyte adhesion to endothelial cells, indicating that S100A8 does not negatively regulate S100A9 activity toward monocytes by forming S100A8/A9 (36). In addition, S100A8/A9 was also associated with monocyte recruitment by facilitating their migration through the endothelium (36). In these reports, S100A8 had no activity toward neutrophils and monocytes. The only activities attributed to human S100A8 were its capacity to activate HIV replication in human monocytic infected cells (52) and its chemotactic activity toward periodontal ligament cells (41). In addition, we recently demonstrated that S100A8 stimulates HIV-1 transcription and production in infected T lymphocytes through NF- $\kappa$ B activation (43). Therefore, it was unclear whether S100A8 exerts proinflammatory activities by itself. In this study, we demonstrate that human S100A8 possess proinflammatory activities toward neutrophils. We show that recombinant S100A9, S100A8/A9, and also S100A8 stimulate human neutrophil chemotaxis, adhesion, and other proinflammatory activities, proving that they all exert activities toward neutrophils.



**FIGURE 7.** MRPs do not stimulate actin polymerization. Neutrophils were stimulated with  $10^{-7}$  M fMLP or  $4 \times 10^{-7}$  M MRPs for increasing periods of time at  $37^\circ\text{C}$ . The reaction was stopped, and F-actin was stained. Results represent mean  $\pm$  SEM of three experiments performed on neutrophils from different healthy donors.

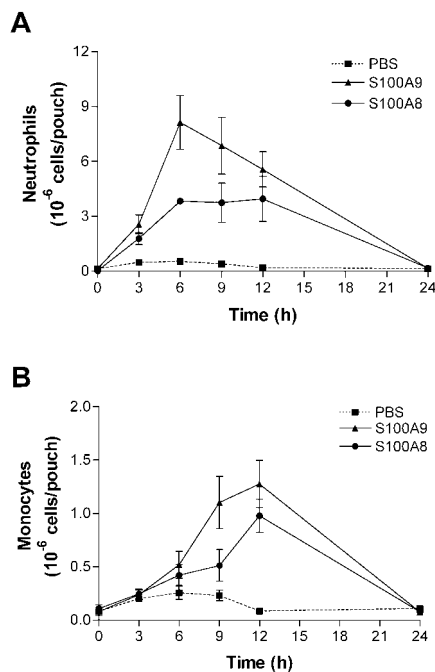


**FIGURE 8.** S100A8, S100A9, and S100A8/A9 stimulate neutrophil calcium mobilization. Neutrophils were incubated with  $10^{-7}$  M fMLP (A), or  $1 \times 10^{-6}$  M S100A8 (B), S100A9 (C), or S100A8/A9 (D). Intracellular calcium flux was measured by quantification of the fluorescence with a spectrofluorometer. Internal concentrations were calculated as described by Tsien et al. (45). Data shown are the result of one experiments representative of two other experiments performed on neutrophils from different healthy donors.

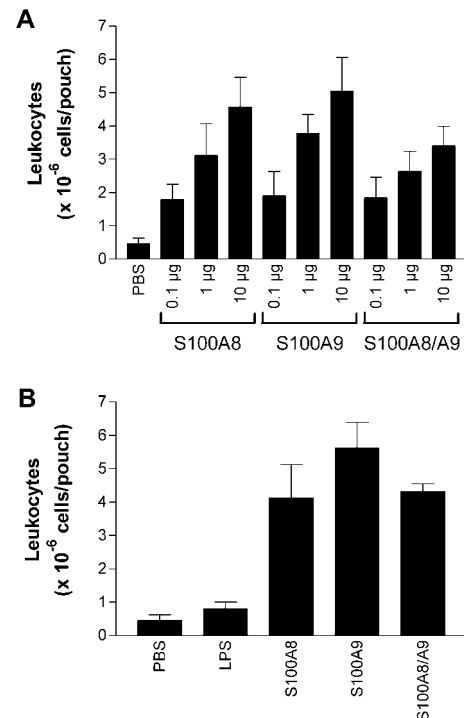


Murine S100A8 and, to a lower level, S100A9 can be efficiently oxidized by  $\text{OCl}^-$  anions and copper, sometimes resulting in the inactivation of S100A8 chemotactic activity (42, 48). In this study, we present similar results with human S100A8 and demonstrate that S100A8 but not S100A9 or S100A8/A9 can be inactivated by oxidation with NaOCl. These results demonstrate that human

S100A8 possesses a similar susceptibility toward inactivation by oxidation as does its murine counterpart. This result suggests that the absence of S100A8 activity described by others might be due



**FIGURE 9.** S100A8 and S100A9 induce an inflammatory reaction when injected in the air pouch model. Dorsal air pouches were raised on 10- to 12-wk-old CD-1 mice. Ten micrograms of S100A8, S100A9, or PBS (controls) was injected into the air pouches. At increasing time points, the exudates were harvested and the number of emigrated leukocytes was quantified. Neutrophils (A) and monocytes (B) were quantified after cyto-spin and Wright-Giemsa staining of the exudates. Data represent the mean  $\pm$  SEM of seven mice.



**FIGURE 10.** S100A8, S100A9, and S100A8/A9 induce an inflammatory reaction in a dose-dependent manner when injected in the air pouch model. A, Dorsal air pouches were raised on 10- to 12-wk-old CD-1 mice. Increasing concentrations of S100A8, S100A9, or S100A8/A9, or PBS (controls) were injected into the air pouches. B, Dorsal air pouches were raised on 10- to 12-wk-old C3H/HeJ mice. One microgram of LPS, or 10  $\mu\text{g}$  of S100A8, S100A9, or S100A8/A9, or PBS (controls) was injected into the air pouches. Six hours later, the exudates were harvested, and the number of emigrated leukocytes was quantified. Data represent the mean  $\pm$  SEM of seven mice.

to oxidation and consequent deactivation of the protein during purification. In addition, the activities for S100A9 and S100A8/A9 reported by others could be explained by their relative resistance to inactivation by oxidation. Heat denaturation, which consists of unfolding and refolding of the proteins, also inactivates the proteins. The presence of denaturing agents such as DTT or imidazole during the purification process could therefore result in their inactivation. Thus, we propose that the discrepancies in MRPs activities could have resulted from interlaboratory variance in methodology, in particular protein oxidation or denaturation during the purification process or during the manipulation of the proteins, which could be responsible for the inactivation of the MRPs.

In the present study, S100A8, S100A9, and S100A8/A9 proved to be potent chemoattractants for neutrophils at concentrations of  $10^{-12}$ – $10^{-10}$  M. Their chemotactic activities are similar to those of another MRP, S100A12, which is chemotactic for neutrophils at a concentration of  $10^{-9}$  M (50). Murine S100A8 is also chemotactic for neutrophils but induces chemotaxis at lower concentrations ( $10^{-13}$ – $10^{-11}$  M) (37). The chemotactic activity of the S100 proteins is not restricted to neutrophils. Murine S100A8 is also chemotactic for mononuclear cells but not lymphocytes (38, 39, 53). In addition, S100A12 is chemotactic for monocytes and THP-1 cells (50), while another S100 protein, S100A7, is chemotactic for neutrophils and CD4<sup>+</sup> T lymphocytes (34). Finally, S100L (S100A2) is chemotactic toward eosinophils (33). The chemotactic activity shared by several S100 proteins suggests that this family of proteins might represent a new group of chemotactic factors.

MRPs proved not only to stimulate chemotaxis of neutrophils but also to induce neutrophil adhesion and calcium mobilization. However, not all MRPs have the same activating potential toward neutrophils. Although all MRPs induced neutrophil chemotaxis at relatively similar concentrations, S100A8/A9 stimulated neutrophil adhesion at lower concentration than did S100A9 or S100A8, the latter being a less potent inducer. In addition, the activities of the MRPs varied according to their concentration. At lower concentrations, MRPs were chemotactic for neutrophils, while they stimulated neutrophil adhesion at higher concentrations. This suggests that MRPs could attract neutrophils at low concentrations, while higher concentrations could help retain them at the inflammatory site. Surprisingly, neutrophil migration stimulated by MRPs occurred without increases in polymerized actin content. However, actin cytoskeleton reorganization might occur following stimulation with S100 proteins while the total amount of polymerized actin remained unchanged.

MRPs have been described in several infections and immune diseases including tuberculosis, bronchitis, and arthritis (10, 15–20), suggesting a role for these proteins in the inflammatory process. Unpublished observations from our laboratory demonstrate that neutrophil recruitment induced by monosodium urate crystals, the etiologic agent of gout, is inhibited when both S100A8 and S100A9 are inactivated by passive immunization of mice with specific anti-S100A8 and anti-S100A9 Abs (C. Ryckman, K. Vandal, and P.A. Tessier, manuscript in preparation). These results confirm the importance of S100A8, S100A9, and S100A8/A9 in neutrophil migration to inflammatory sites *in vivo*. Stimulation of neutrophil chemotaxis and adhesion by S100A8, S100A9, and S100A8/A9, corroborates MRP's implication in neutrophil localization, adhesion, and transendothelial migration to inflammatory sites, shedding new light on the role of MRPs in neutrophil migration during inflammation.

The current paradigm of leukocyte migration suggests that, following selectin-induced rolling, neutrophils are activated by chemokines, resulting in a change of conformation of  $\beta_2$  integrins to

their active conformation. This results in neutrophil strong adhesion to the endothelium and allows the transendothelial migration to occur. Leukocytes are then guided to the inflammatory site by a gradient of chemotactic factors. Chemokines have been suggested to act as activators of both neutrophil adhesion to endothelium and chemotaxis in the tissue. The results presented in this study suggest that MRPs could play the same role. S100A8 and S100A9 have been detected on the endothelium in inflamed tissues (54), and S100A9 has been shown to bind specifically to  $\alpha_2$ -macroglobulin on human endothelial cells (55), suggesting that they could induce neutrophil strong adhesion to the endothelium. In addition, the presence of S100A8/A9 has been described at sites of inflammation, suggesting that it could direct neutrophil migration in the tissue as well. Therefore, the MRPs could exert relatively the same activities as chemokines. Further studies will be necessary to confirm these hypotheses.

In conclusion, the present study demonstrates that S100A8, S100A9, and S100A8/A9 are potent inducers of neutrophil chemotaxis and adhesion. Together with other S100 proteins such as S100A12, S100A7 and S100A2, they could represent a new class of chemotactic factors contributing to neutrophil migration to inflammatory sites.

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