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Proinflammatory Activities of S100: Proteins S100A8, S100A9, and S100A8/A9 Induce Neutrophil Chemotaxis and Adhesion

Carle Ryckman, Karen Vandal, Pascal Rouleau, Mariève Talbot, and Philippe A. Tessier

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.


During 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 β₂ 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 B₄, and IL-8, or bacterial N-formylated peptides) toward the inflammatory site.

Recent studies suggest that the myeloid-related proteins (MRPs) 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 β₂ 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 monocye 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 has been shown to calculate calcium flux, shedding of L-selectin, or up-regulation of the β₂ 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).
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 calcine-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). LFS purified from Escherichia coli serotyp 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 (α,β) 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 β-D-thiogalactoside in E. coli HMS174 (Boehringer Mannheim, Mannheim, Germany) for 16 h at 16°C. After incubation, the bacteria were centrifuged at 5000 × 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 × 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 I.U. of biotinylated thrombin (Novagen) for 16°C before being washed three times by immersion in cold PBS. The purity and cell viability of the recombinant proteins 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 Ca2++ and 0.8 mM Mg2+ was added to the lower well of the chambers. Poly carbonate filters (8-μm pore; Neuroprobe, Gaithersburg, MD) were put in place and the upper chambers were secured. Neutrophils (5 × 104 cells in 200 μl) 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 μl/well 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 × 106 cells/ml) were stimulated with MRPs (2 × 10−6 M) at 37°C for 30 min. For the detection of CD62L, CD11b, and CD51 expression, neutrophils (4 × 105) were incubated with 1 μg 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 μg of mAb 24 to 5 × 105 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 × 106 cells/ml in HBSS-H containing 1.3 mM Ca2++ and 0.8 mM Mg2+ and labeled with 5 μM intracellular fluorescent dye calcine-AM for 30 min at 37°C. After washing, labeled cells were resuspended in HBSS-H containing 1.3 mM Ca2++ and 0.8 mM Mg2+ at 5 × 106 cells/ml, and 50 μl was added to Costar 96-well plates, which had been coated overnight with 2 mg/ml fibrinogen (50 μl/well). These wells contained 30 μl of increasing concentrations (2×) of human S100A8/A9, S100A8, or S100A9, or 0.8 mM FMLP. The purity of each sample was tested by acetic blue staining and trypan blue exclusion, respectively. 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 ddH2O, and the fluorescence was measured using a 96-well plate fluorescence reader. In some experiments, S100A8, S100A9, S100A8/A9, or 10 μl/ml FMLP were boiled or treated at room temperature with 10 or 100 μM NaOCl for 15 min and diluted to a final concentration of 20 μg/ml before stimulation. In other experiments, neutrophils were preincubated for 20 min with 20 μg/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 × 106 cells/ml in HBSS-H containing 1.3 mM Ca2++ and 0.8 mM Mg2+. The reaction was initiated by mixing 5 × 104 cells (250 μl) with the MRP preparations at 37°C. After 10 min, coverslips were collected and stained with either ALEXA 488 or 568 conjugated phallidin (Molecular Probes) and resuspended in HBSS-H containing 1.3 mM Ca2++ and 0.8 mM Mg2+. The purity and cell viability of the neutrophil preparations were consistently >98% as assessed by acetic blue staining and trypan blue exclusion, respectively. Both mAbs were isotype-matched 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 Boyun and Tagoh (44) and resuspended in HBSS-H (HBSS supplemented with 10 mM HEPES [pH 7.4]) containing 1.3 mM Ca2++ and 0.8 mM Mg2++. The purity and cell viability of the neutrophil preparations were consistently >98% as assessed by acetic blue staining and trypan blue exclusion, respectively.

**Production of mAbs**

Female BALB/c mice (4 wk old) were immunized by i.p. injections with 30 μg of purified recombinant S100A8 or S100A9 in 50 μl 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/aminopterin/thymidine selection medium. Culture supernatants of the hybridomas were screened by ELISA using plates coated with 1 μg/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 isotype-matched as IgG1 kappa. Specificity of the mAbs was confirmed by ELISA and Western blot analysis.
final concentrations of $1 \times 10^{-6}$ to $5 \times 10^{-13}$ M. After increasing periods of incubation at RT, the reaction was stopped by adding 50 µl 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 µM 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 (10⁷ cells/ml) were loaded with fura 2-AM (1 µM; 30 min at 37°C), washed, and resuspended at 2 × 10⁶ cells/ml in HBSS-H supplemented with 1.3 mM CaCl₂. 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 µg/ml or LPS (1 µg/ml) was injected into the air pouches. At specific time points, the mice were killed by CO₂ 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 × g for 5 min at room temperature. Cells were counted with a hematocytometer following acetic blue staining. Characterization of leukocyte subpopulations migrating into the pouch space was performed by Wright-Giemsa staining of cytospins.

**Results**

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

To date, discrepancies exist between the activities of S100A8, S100A9, or an equimolar ratio of S100A8 and S100A9 dissolved in endotoxin-free PBS at concentrations ranging from 0.1 to 10 µg/ml or LPS (1 µg/ml). The presence of monomers, homodimers, and heterodimers of the proteins is shown.

FIGURE 1. SDS-PAGE analysis of recombinant S100A8 and S100A9. Thrombin (0.4 U/lane) or recombinant S100A8 and S100A9 (3 µg/lane) were mixed together (in presence of HBSS-H containing 1.3 mM Ca²⁺) 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.

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.

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 $10^{-12}$ to $10^{-10}$ 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 μg/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 ± 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 (α,β3) 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 β2.

Table I. **Checkerboard analysis of neutrophil chemotaxis induced by S100A8**

<table>
<thead>
<tr>
<th>S100A8 Concentration in Lower Chamber (M)</th>
<th>S100A8 Concentration in Upper Chamber (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10^{-12}</td>
</tr>
<tr>
<td>10^{-12}</td>
<td>10^{-11}</td>
</tr>
<tr>
<td>10^{-11}</td>
<td>10^{-10}</td>
</tr>
<tr>
<td>10^{-10}</td>
<td>10^{-9}</td>
</tr>
</tbody>
</table>

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 ± SEM of three experiments carried out on neutrophils from different donors.

* 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 ± SEM of three experiments carried out on neutrophils from different donors.

**Table II. Checkerboard analysis of neutrophil chemotaxis induced by S100A9**

<table>
<thead>
<tr>
<th>S100A9 Concentration in Lower Chamber (M)</th>
<th>S100A9 Concentration in Upper Chamber (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10^{-12}</td>
</tr>
<tr>
<td>10^{-12}</td>
<td>10^{-11}</td>
</tr>
<tr>
<td>10^{-11}</td>
<td>10^{-10}</td>
</tr>
<tr>
<td>10^{-10}</td>
<td>10^{-9}</td>
</tr>
</tbody>
</table>

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 ± SEM of three experiments carried out on neutrophils from different donors.

* 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 ± SEM of three experiments carried out on neutrophils from different donors.

* p < 0.01, Student’s t test (S100A9 in both chambers vs S100A8 in lower chamber only).

**p** < 0.05, Student’s t test (S100A9 in both chambers vs S100A8 in lower chamber only).
Table III.  Checkerboard analysis of neutrophil chemotaxis induced by S100A8/A9

<table>
<thead>
<tr>
<th>S100A8/A9 Concentration in Lower Chamber (M)</th>
<th>S100A8/A9 Concentration in Upper Chamber (M)</th>
<th>% of adhered neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>12.3 ± 2.6*</td>
</tr>
<tr>
<td>10^{-12}</td>
<td>0</td>
<td>19.0 ± 0.8</td>
</tr>
<tr>
<td>10^{-11}</td>
<td>0</td>
<td>18.6 ± 0.9</td>
</tr>
<tr>
<td>10^{-12}</td>
<td>10^{-12}</td>
<td>29.8 ± 0.8</td>
</tr>
<tr>
<td>10^{-11}</td>
<td>10^{-11}</td>
<td>20.0 ± 1.9</td>
</tr>
<tr>
<td>10^{-11}</td>
<td>10^{-11}</td>
<td>22.3 ± 1.2**</td>
</tr>
<tr>
<td>10^{-11}</td>
<td>10^{-11}</td>
<td>18.6 ± 1.3*</td>
</tr>
</tbody>
</table>

* 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^{-7} and 10^{-6} M S100A8/A9 and at 10^{-7} 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.

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, neutral 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 αvβ3.

Mac-1 (CD11b/CD18) and αvβ3 (CD51/CD61) are two integrins expressed by neutrophils known to bind fibrinogen (48, 49). To
To evaluate the level of activation of neutrophils, the effect of neutrophil actin polymerization was next investigated. IMLP 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 monocytes, but not in neutrophils (Ref. 50; M. Talbot and P. A. Tessier, unpublished observations), we investigated the effect of MRPs on calcium mobilization. The effect of S100A8, S100A9, and S100A8/A9 on calcium mobilization in human neutrophils was next investigated. Human neutrophils had resting Ca^{2+} levels of 100 nM. Neutrophil stimulation with IMLP ($10^{-6}$ M) led to a transient increase in Ca^{2+} (Fig. 8A). Stimulation of neutrophils with S100A8, S100A9, and S100A8/A9 also increased calcium levels, although not as high as IMLP (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 µg of MRPs was sufficient to induce an inflammatory response in mice (Fig. 10A). Leukocyte accumulation was maximal at a dose of 10 µg. No migration of leukocytes was detected at quantities $<0.1$ µg (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 µg 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 µg/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 iso-electric 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 pro-inflammatory properties. Injection of human S100A8, S100A9, or S100A8/A9 in the murine air pouch induced...
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 suggest 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-κB 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.
Murine S100A8 and, to a lower level, S100A9 can be efficiently oxidized by OCl⁻/H₂O₂ 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

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**FIGURE 8.** S100A8, S100A9, and S100A8/A9 stimulate neutrophil calcium mobilization. Neutrophils were incubated with 10⁻⁷ M fMLP (A), or 1 × 10⁻⁶ M S100A8 (B), S100A9 (C), or S100A8/A9 (D). Intracellular calcium flux was measured by quantification of the fluorescence with a spectrophuorometer. 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.

**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 ± 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 μg 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 ± 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 $10^{-10}$ M (50). Murine S100A8 is also chemotactic for neutrophils but induces chemotaxis at lower concentrations ($10^{-11}$–$10^{-13}$ 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$^+$ T lymphocytes (34). Finally, S100L (S100A2a) 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 content could therefore result in their inactivation.

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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