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# Serum Amyloid A Induces Chemotaxis of Human Mast Cells by Activating a Pertussis Toxin-Sensitive Signal Transduction Pathway

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**Serum amyloid A (SAA) is an acute-phase protein and its concentration increases in the blood up to 1000 times during an inflammatory response. Mast cells are known to accumulate in various inflammatory processes, some of which are associated with increased levels of acute-phase reactants such as SAA. We report here that SAA can act as a mast cell chemoattractant. Recombinant SAA at concentrations corresponding to those found during the acute phase induced directional migration of human mast cells. No chemokinetic effect was observed. Preincubation of the cells with pertussis toxin inhibited SAA chemotaxis, suggesting that the effect is mediated by G proteins of the $G_i$ class. Furthermore, chemotaxis was diminished after pretreatment with genistein, a tyrosine kinase inhibitor, or bisindolylmaleimide I, a protein kinase C inhibitor. We suggest that SAA may participate in the migration of mast cells to inflamed tissues during an acute-phase response, acting through a pertussis toxin-sensitive signaling pathway.** 

*Key Words:* **inflammation; mast cell; migration; SAA.**

Mature mast cells are found almost exclusively in tissues where they execute their biological functions (1). They live in tissues for several months and their numbers under normal conditions are relatively constant. Mast cell accumulation occurs, however, in certain inflammatory conditions that include host response to microbes, tissue repair and fibrosis, delayed hypersensitivity reactions, neoplasia, allergy, and in chronic inflammatory conditions such as inflammatory bowel disease, rheumatoid arthritis and asthma (reviewed in 2). However, little is known about the mechanisms by which mast cells are recruited to these inflammatory sites. Directed migration of mast cells within tissue may be one important mechanism for rapidly increasing mast cell numbers. The recent reports that stem cell factor (SCF), transforming growth factor-$\beta$ (TGF-$\beta$), RANTES and the anaphylatoxins C3a and C5a are mast cell chemoattractants has provided support for this hypothesis (3–6).

Some of the pathological conditions which involve mast cell hyperplasia are also associated with high serum concentrations of acute phase proteins such as serum amyloid A (SAA) and C-reactive protein. Within hours after initiation of an inflammatory response, the serum concentrations of these proteins increase up to 1000-fold (7, 8). SAA is synthesized mainly by hepatocytes and its expression is regulated by the action of proinflammatory cytokines, i.e., IL-1, IL-6 and TNF-$\alpha$. However, the function of SAA remains unclear.

Prolonged or repeated inflammatory conditions associated with high serum levels of SAA can cause a reactive form of amyloidosis. SAA fragments that results from the enzymatic degradation of the 12.5-kDa SAA protein precipitate to form the amorphous amyloid fibril deposits (9, 10). Chemotrypsin-like protease has been found in rat brain mast cells capable of generating the N-terminus of the Alzheimer amyloid $\beta$-protein (11). Since mast cells have been reported to be present at sites of amyloid deposits (12), and that rSAA recently was shown to induce mast cell adhesion (13), we decided to investigate whether SAA can act as a mast cell chemotaxin. We also investigated the signal transducing pathway used for SAA-mediated mast cell migration.

Abbreviations used: Bis, bisindolylmaleimide I; CBCMC, cord blood cultured mast cells; CHx, cycloheximide; Gen, genistein; PI3-kinase, phosphatidylinositol - 3 kinase; PKC, protein kinase C; PTx, pertussis toxin; SAA, serum amyloid A; rhSAA, recombinant human SAA; SCF, stem cell factor; WN, wortmannin; ZAS, zymosan activated serum.

## MATERIALS AND METHODS

*Materials.* Human apo-Serum Amyloid A (Pepro Tech Inc., Princeton, NY); genistein, bisindolylmaleimide I (Calbiochem–Novabiochem Co., San Diego, CA); pertussis toxin, wortmannin,



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zymosan A and cycloheximide (Sigma Chemicals Co., St. Louis, MO); human plasma fibronectin (Life technologies, Gaithersburg, MD); nitrocellulose filters (Millipore/Continental Water Systems, Bedford, MA).

*Cell cultures.* The human mast cell line HMC-1 (14, 15) was cultured in Iscoveś modified Dulbecco's medium supplemented with 10% BCS, 2 mM L-glutamine, 100 IU/ml penicillin, 50 $\mu$g/ml streptomycin, and 1.2 mM $\alpha$-thioglycerol. Every 3 to 4 days the cells were passaged.

Human mast cells were developed *in vitro* as previously described (16). Briefly, mononuclear cells were separated from human heparinized umbilical cord blood by Ficoll–Plaque gradient centrifugation and cultured in complete RPMI 1640 medium, and recombinant human stem cell factor at 50 ng/ml (Immunex Inc., Seattle, WA). The medium was changed weekly.

*Migration assay.* The migration of HMC-1 and SCF-dependent human cord blood cultured mast cells (CBCMC) was assayed in a modified Boyden chamber by means of the leading front technique (17). Micropore filters (150 $\mu$m thick nitrocellulose) with a pore size of 8 $\mu$m, were coated with human plasma fibronectin at a concentration of 10 $\mu$g/ml in room temperature overnight. The filters were air dried for at least 60 min before use. The assay was performed in a 48-well micro-Boyden chemotaxis chamber (Neuroprobe Inc., Cabin John, MD) as previously described (3). SAA concentrations to be tested were diluted in medium. Thirty microliters of the attractant were added to each well below the filter, and 50 $\mu$l of the cell suspension ($1.5 \times 10^6$ cells/ml) were added above the filter. After 150 min at 37°C and 5% $CO_2$, the filters were fixed, stained with Mayer's–Hemalum solution, and mounted. The migration of mast cells suspended in medium with 10% BCS against medium with 10% BCS, and with the same filter in the Boyden chamber, served as control and was referred to as 100% migration. Cell chemotaxis was assayed as the migration of the two furthest migrating cells visible in focus of one high power field (10x20). The migration distance on each filter was calculated as the mean of the readings of three different areas of the filter. The assay was always done in at least triplicate. A checkerboard analysis of mast cell motility was conducted according the method of Zigmond and Hirsch (17).

*Zymosan-activated serum.* Pooled fresh serum from healthy donors anti-coagulated with 0.5 M amino-*n*-caproic acid was treated with 1.5 mg zymosan A/ml serum for 30 min at 37°C. The activated serum was then centrifuged, aliquoted, and stored at −70°C. Zymosan activated serum (ZAS) serves as positive control for mast cell migration. The zymosan activates the complement where the factors C3a and C5a are known chemoattractants for human mast cells (4).

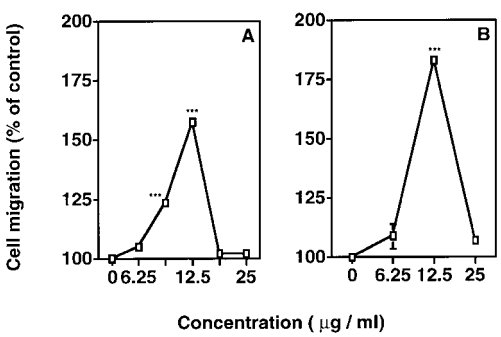


**FIG. 1.** Chemotactic response of HMC-1 cells (A) and SCF-dependent cord blood cultured human mast cells (B) to rhSAA. Cell migration toward the chemoattractant was calculated as percentage migration toward medium + 10% FCS. Results are mean ± SEM of four independent experiments performed in triplicate. A significant response was obtained as indicated ($p < 0.001$, ***).

**TABLE 1**

Comparison of Mast Cell Migration in Response to Different Chemotaxins

| Chemotaxin | Migration[a] | $ED_{50}$ (nM) |
|---|---|---|
| SAA | 157 | 800 |
| C3a | 256 | 0.5 |
| SCF | 143 | 0.6 |

[a] Migration of HMC-1 cells in response to optimal concentration of SAA, C3a, or SCF is given as percentage migration compared to medium control. $ED_{50}$ values give the half-maximal effective concentration in nM.

*Treatment with inhibitors.* HMC-1 cells were treated with pertussis toxin (0.5 $\mu$g/ml), genistein (0.5 $\mu$g/ml), wortmannin (100 nM), bisindolylmaleimide I (100 nM), or cycloheximide (20 $\mu$g/ml). Inhibitor treatment was performed by incubating $2.5 \times 10^6$ cells/ml for 90–120 min at 37°C in 5% $CO_2$ with each inhibitor in complete medium, and were then washed and resuspended at $1.5 \times 10^6$ cells/ml in complete medium before chemotaxis assay.

*Statistical analysis.* For statistical analysis we used an analysis of variance (ANOVA), followed by multiple comparison by Fisher's method. * denotes $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Values presented are the means ± SEM.

## RESULTS

*Chemotactic response of human mast cells to rhSAA.* The chemotactic activity of rhSAA for human mast cells was determined in a modified Boyden chamber. Figure 1 shows a typical bell-shaped dose–response curve of mast cell migration with higher concentrations resulting in a loss of directed migration. However, in contrast to other mast cell chemoattractants studied, such as SCF and C3a (3, 4), rhSAA induced mast cell migration in a more narrow concentration interval. The optimal concentration of rhSAA for the induction of mast cell migration was 12.5 $\mu$g/ml (1 $\mu$M), which is higher than normal serum level (<0.1 $\mu$M), but well below the levels seen in inflammatory conditions. HMC-1 cells and CBCMC showed a similar response to optimal concentration of rhSAA, but the efficacy were different, 157 and 183% respectively. This is comparable with SCF, but lower than C3a (Table 1). The $ED_{50}$ value was estimated to approximately 800 nM, that is 1000 times higher compared to SCF and C3a (Table 1).

The ability of rhSAA to stimulate directional migration (chemotaxis) vs random migration (chemokinesis) was analyzed employing a checkerboard analysis. As shown in Table 2, rhSAA was found to be chemotactic but not chemokinetic for HMC-1 cells.

*Chemotactic response of mast cells to rhSAA is pertussis toxin sensitive.* To evaluate whether SAA uses a G-protein-dependent signaling pathway, HMC-1 cells were treated with PTx prior chemotaxis assay. Pre-incubation with PTx inhibits the chemotactic re-

**TABLE 2**

Checkerboard Analysis of rhSAA-Induced HMC-1 Cell Migration[a]

| | Upper well | | | |
|---|---|---|---|---|
| Lower well | 0 | 9.4 | 12.5 | 19 |
| 0 | 100 ± 0 | 95.5 ± 9.5 | 88.5 ± 7.5 | 100.5 ± 2.5 |
| 9.4 | 113.5 ± 1.5 | 104 ± 11 | 104.5 ± 8.5 | 105.5 ± 7.5 |
| 12.5 | 169 ± 12 | 167.5 ± 12.5 | 168.5 ± 17.5 | 143 ± 13 |
| 19 | 116 ± 6 | 112.5 ± 1.5 | 110 ± 7 | 114 ± 3 |

[a] Different concentrations of rhSAA were placed in the upper and/or lower wells of the chemotaxis chamber. The chemotaxis assay was performed as described under Materials and Methods. Results are expressed as mean cell migration compared to control ± SEM ($n = 6$).

sponse of mast cells to rhSAA (Fig. 2A). G proteins are thought to trigger second messengers that activate protein kinase C (PKC). To examine whether PKC activation might be of importance for rhSAA-induced chemotaxis, mast cells were pre-treated with bisindolylmaleimide I. As shown in Fig. 2B, the migratory response was significantly attenuated, indicating that rhSAA-mediated mast cell chemotaxis is mediated through a partial PKC dependent pathway. We next evaluated whether other kinases are required for SAA induced migration. Cells were treated with genistein to inhibit tyrosine kinases, and wortmannin to inhibit PI3-kinase. Genistein partly inhibited the migratory response, while the response where attenuated, although not significantly, by wortmannin (Figs. 2C and 2D). These results suggest that SAA-induced mast cell chemotaxis is mediated via a SAA-receptor coupled to Gi-protein that transduce the signals via tyrosine kinases.

*Mast cell migration is dependent on protein synthesis.* We next evaluated if protein synthesis is required for SAA-induced migration. Mast cells were treated with cycloheximide before chemotaxis assay towards rhSAA, SCF or ZAS. SAA-induced migration as well as migration to SCF and ZAS was found to be dependent on protein synthesis (Fig. 3).

## DISCUSSION

Mast cells are resident cells found in most tissues. They have for long time been considered as stationary cells with no or low desire to migrate. The use of the chemotaxis assay has allowed the identification of several mast cell chemoattractants, such as SCF, C3a and C5a, RANTES, TGF-$\beta$, and angiogenic factors (3, 4, 6, 18–20). The finding that SAA is a chemotactic factor for mast cells discloses a new mast cell chemoattractant that might be involved in the accumulation of

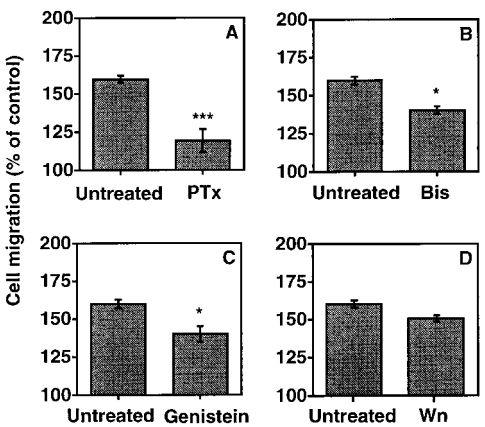


**FIG. 2.** Analysis of signal transduction involved in SAA-mediated mast cell migration. Cells were pre-treated with (A) pertussis toxin to inhibit $G_i$-proteins (B) genistein to block tyrosine kinases, (C) bisindolylmaleimide I to inhibit protein kinase C, or (D) wortmannin to inhibit PI3-kinase. Results are mean ± SEM of three independent experiments performed in triplicate. A significant response was obtained as indicated ($p < 0.05$, *; $p < 0.001$, ***). PTx, pertussis toxin; Gen, genistein; Bis, bisindolylmaleimide I, Wn, wortmannin.

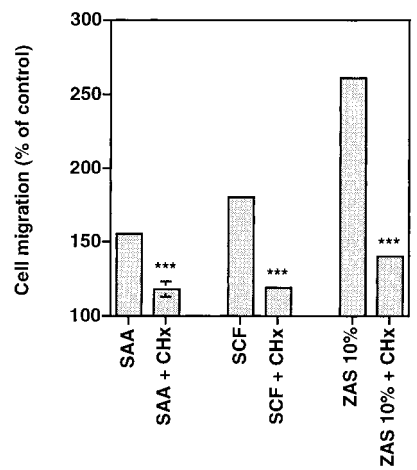


**FIG. 3.** Mast cell migration is dependent on protein synthesis. HMC-1 cells were pre-treated with cycloheximide. The cells were assayed for migratory response to SAA at 12.5 $\mu$g/ml, stem cell factor at 50 ng/ml or zymosan activated serum at a 10% solution. Results are mean ± SEM of three independent experiments performed in triplicate. A significant response was obtained as indicated ($p < 0.05$, *; $p < 0.001$, ***).

mast cells in acute inflammation. The optimal concentration at which rhSAA induces mast cell migration is 1 $\mu$M (Fig. 1). This concentration is higher than normal serum level of 0.08 $\mu$M, but fall in the range of SAA concentrations reached during acute phase when the serum concentration of SAA can reach 80 $\mu$M (7, 8, 21).

Although most serum SAA is associated with HDL, which acts as a natural inhibitor of the chemotactic activity of SAA, it has been reported that leukocyte-derived enzymes can degrade SAA to smaller molecules and thereby may release a biologically active SAA (22–24). Because SAA binds to HDL at equimolar ratios (25), it is possible that higher concentrations of locally produced SAA could result in a gradient of free active SAA with consequent recruitment of mast cells into local inflammatory sites. In a previous report it was shown that SAA induced adhesion of mast cells to extracellular matrix components, without activating the cells to release mediators (13). Thus, SAA might provide two signals necessary for leukocyte migration, one for adhesion and the second stimulating migration.

We have found that SAA-mediated mast cell migration is mediated through a PTx sensitive signal transducing pathway indicating the involvement of G proteins of the $G_i$ class. Our data also suggest that PKC may be involved in SAA-induced migration of mast cells. This is based on the inhibition observed by bisindolylmaleimide I, a PKC inhibitor. Similar results have been shown for SAA-mediated migration of monocytes (26). Thus, these results indicate that SAA-mediated chemotaxis are mediated by similar signal transducing pathways in different cell types. Furthermore, our data also indicate the involvement of tyrosine kinase activation as the tyrosine kinase inhibitor, genistein, significantly inhibited the migratory response (Fig. 2). Tyrosine kinase activation is reportedly also involved in SAA-mediated adhesion (13), suggesting that these two activities are possibly mediated by similar signaling pathways in mast cells.

Some of the pathological conditions which involve mast cell hyperplasia are also associated with high serum concentrations of acute phase proteins such as SAA (1). The present study indicates that SAA can induce mast cell migration by specific receptors coupled to G proteins. Thus, SAA appears to be one of the chemoattractants which governs mast cell distribution and promotes their accumulation in inflammation.

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