# Serum Amyloid A Is a Chemoattractant: Induction of Migration, Adhesion, and Tissue Infiltration of Monocytes and Polymorphonuclear Leukocytes

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## Summary

Serum amyloid A (SAA) is an acute phase protein that in the blood is bound to high density lipoproteins; SAA is secreted mainly by hepatocytes, and its concentration increases in the blood up to 1000 times during an inflammatory response. At present, its biological function is unclear. Since some forms of secondary amyloidosis are caused by deposition in tissues of peptides derived from the SAA and leukocytes seem to be involved in this process, we investigated the effect of human SAA on human monocytes and polymorphonuclear cells (PMN). When recombinant human SAA (rSAA) was used at concentrations corresponding to those found during the acute phase (>0.8 $\mu$M), it induced directional migration of monocytes and polymorphonuclear leukocytes. Preincubation of rSAA with high density lipoproteins blocked this chemoattractant activity for both monocytes and PMN. rSAA also regulated the expression of the adhesion proteins CD11b and leukocyte cell adhesion molecule 1 and induced the adhesion of PMN and monocytes to umbilical cord vein endothelial cell monolayers. When subcutaneously injected into mice, rSAA recruited PMN and monocytes at the injection site. On the basis of these data, we suggest that SAA may participate in enhancing the migration of monocytes and PMN to inflamed tissues during an acute phase response.

Within hours after initiation of an inflammatory response, the serum concentrations of acute phase proteins such as serum amyloid A (SAA)[1] and C-reactive protein increase up to 1,000-fold (1–3). Each of these proteins has unique functions during the acute phase response. The complement proteins enhance the immune reaction of the host, proteinase inhibitors regulate enzyme activities, and C-reactive protein is an opsonizing factor for cellular breakdown products and many bacterial antigens and regulates some immune functions (4, 5). 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, a condition characterized by deposition of Congo-red birefringent nonbranching fibrils in peripheral tissues with progressive loss of organ function. SAA fragments (such as the 8-kD AA fragment), that result from the enzymatic degradation of the 12.5-kD SAA protein precipitate to form the amorphous amyloid fibril deposits (6, 7). Since monocytes and polymorphonuclear cells are a source of enzymes that can convert SAA into the AA fragment (8, 9) and since these inflammatory cells have been reported to be present at sites of amyloid deposits (10), we decided to investigate whether SAA could modulate the migration of monocytes and polymorphonuclear cells (PMN) to tissues.

## Materials and Methods

*Cells.* Peripheral blood leukocytes enriched for mononuclear cells or for PMN were obtained from normal healthy volunteers at the National Institutes of Health Clinical Center Transfusion Medicine Department (Bethesda, MD) by leukapheresis. PBMCs were purified by Ficoll-Hypaque (Lymphoprep; Sigma Chemical Co., St. Louis, MO) gradient centrifugation (11). Monocytes con-

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[1] *Abbreviations used in this paper:* HDL, high density lipoprotein; HUVEC, human umbilical cord vein endothelial cells; LECAM-1, leukocyte cell adhesion molecule 1; PMN, polymorphonuclear cells; SAA, serum amyloid A; rSAA; recombinant SAA.

stituted 30–40% of PBMCs as determined by a direct immuno-fluorescence assay using the mAb CD14-PE (Amac, Westbrook, ME). PMN were purified essentially as described (11). The preparations contained at least 95% PMN as judged by morphological criteria; the remaining cells were typically lymphocytes.

*Mice.* BALB/c mice were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD).

*Recombinant (r)SAA.* Human rSAA was a kind gift of Dr. Robert Goldman of Peprotech (Princeton, NJ). Its primary structure corresponds to that of SAA-1α except the addition of a methionine at the $NH_2$ terminus, the substitution of aspartic acid for asparagine at position 60, and histidine for arginine at position 71. These substitutions have been previously reported to occur in natural SAA variants (12). The gene was expressed in *Escherichia coli*

and the protein was purifed from bacterial extracts by precipitation with 1 M acetic acid at pH 5 followed by several steps of extraction with 40% acetonitrile in 0.1% trifluoroacetic acid/water. The rSAA in the supernatant was isolated by preparative reverse phase HPLC and lyophilized. Before being used, lyophilized rSAA was reconstituted in water. The rSAA was >99% pure as shown by reverse phase HPLC and SDS-PAGE. Western blotting analysis with anti–human SAA antibodies (Calbiochem-Novabiochem Corp., La Jolla, CA) specifically revealed a band of 12 kD in reducing conditions that is the size predicted by the nucleotide sequence. When nonreducing conditions were used, we could also detect polymeric forms of the protein; however, for the calculation of molar concentration we used the molecular weight of the monomeric form (12.5 kD). Phospholipids were not detectable (<10 nmoles) in 4 μM rSAA as assessed according to the molybdate assay (13). Aliquots of 4
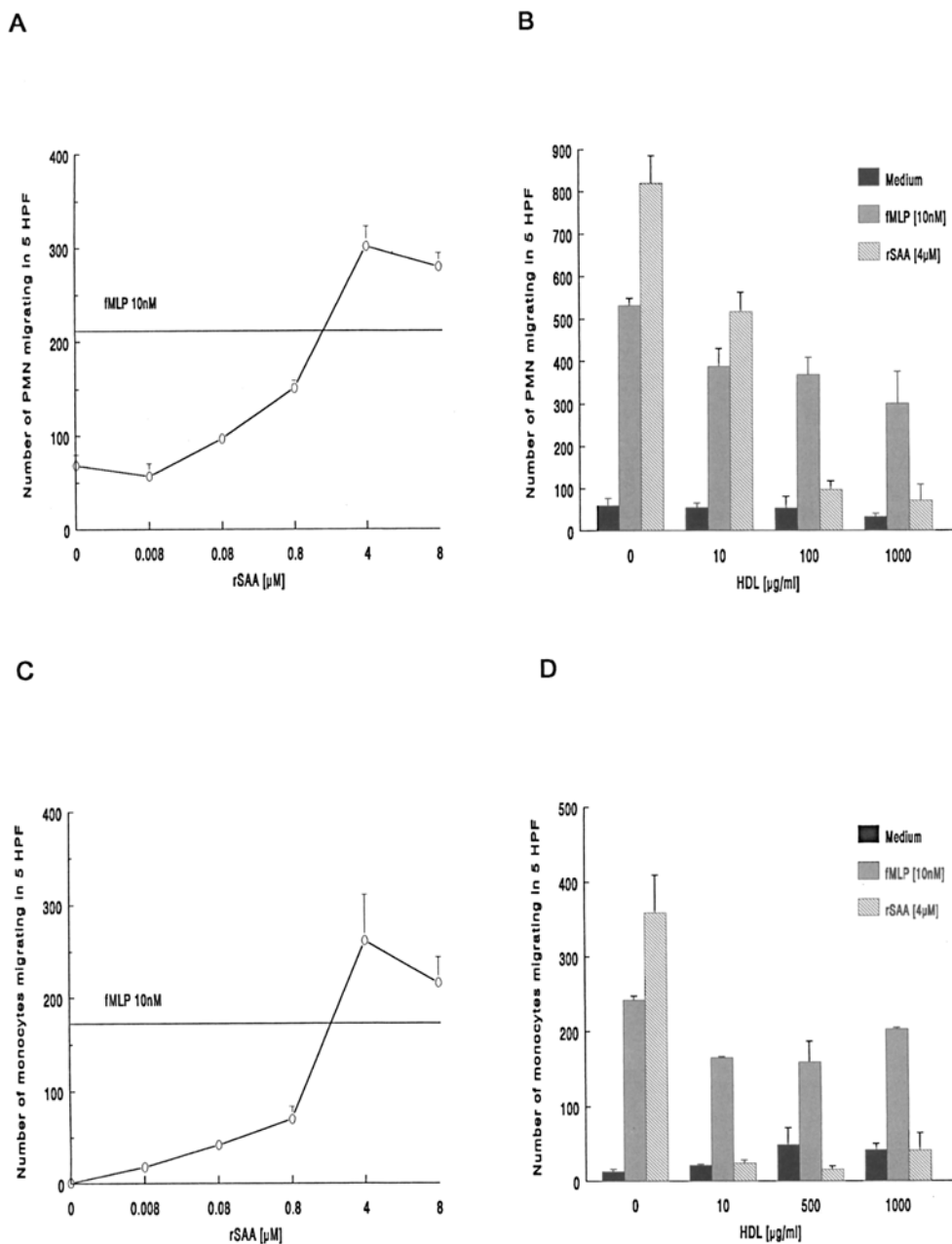


**Figure 1.** rSAA exerts chemoattractant activity for PMN (*A*) and monocytes (*C*). Different concentrations of rSAA were placed in the lower wells of microchemotaxis chamber. PMN (*A*) or monocytes (*C*) (1.5 × $10^6$ cells/ml in RPMI 1640 containing 1% BSA) were added in the upper wells. The two wells were separated by a 5-μm pore-size polycarbonate filter. After incubation at 37°C in air with 5% $CO_2$ (60 min for PMN and 90 min for monocytes), filters were removed, fixed, and stained as described (11). The results are expressed as the mean (± SD) number of cells that migrated across the filter in five high power fields (HPF) counted in triplicate. In each experiment FMLP (10 nM) was used as positive control. In *B* and *D*, rSAA (4 μM), FMLP (10 nM), or medium were incubated for 5 h at 37°C with freshly prepared HDL and then tested for chemoattractant activity on PMN (*B*) and monocytes (*D*) (1.5 × $10^6$ cells/ml).
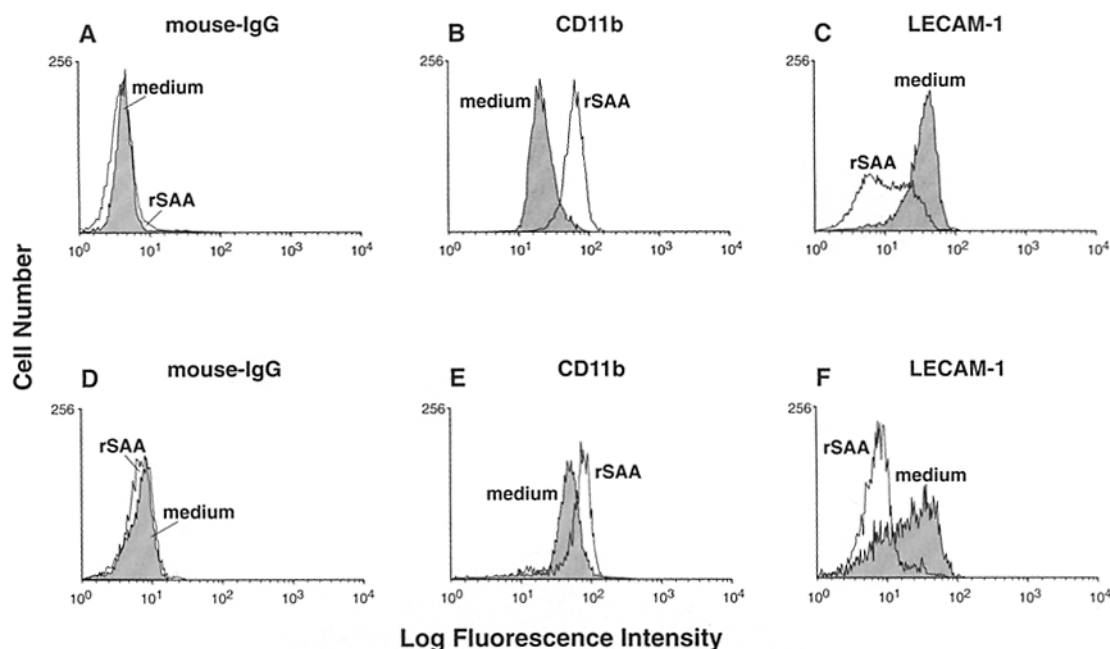
**Figure 2.** Modulation of expression of adhesion molecules by rSAA in PMN (*A–C*) and monocytes (*D–F*). Whole blood was incubated with medium alone or rSAA (4 $\mu$M) for 15 min at 37°C. After washing, cells were stained with control mouse-IgG (*A* and *D*), or CD11b (*B* and *E*), or LECAM-1 (*C* and *F*) monoclonal antibodies. Neutrophils (*A–C*) or monocytes (*D–F*) were gated on the basis of physical parameters (forward and side scatter). The x-axis represents the intensity of fluorescence expressed in a log scale as mean channel and the y-axis the number of cells/channel.

$\mu$M rSAA were negative for endotoxin in Limulus Amebocyte lysate assay (sensitivity 0.06 IU/ml) (BioWhittaker, Walkersville, MD).

*High Density Lipoprotein (HDL) Purification.* HDL was isolated from human plasma by floatation on discontinuous NaBr gradients by the method of Havel et al. (14). Before being used, HDL preparations were dialysed against two changes of PBS and then against RPMI. The protein concentration was determined using a protein assay (Bio-Rad Laboratories, Inc., Richmond, CA). The purity of HDL was verified by SDS-PAGE.

*Migration Assays.* Migration of monocytes and PMN, was evalu-

ated by a microchamber technique as described (15). For PBMC, 5-$\mu$m pore-size polycarbonate filters were employed. Under the assay condition employed, only monocytes, in PBMC preparation, migrated across the filter. Polyvinylpyrrolidone (PVP)-free polycarbonate filters were used for PMN. At the end of the incubation, filters were removed, fixed, and stained by Diff-Quik (Harleco, Gibbstown, NJ), and five oil immersion fields were counted after coding samples. In each assay *N*-formylmethionyl-leucyl-phenylalanine (FMLP; Sigma Chemical Co.) at a concentration of 10 nM was used as a standard chemoattractant for monocytes and PMN.

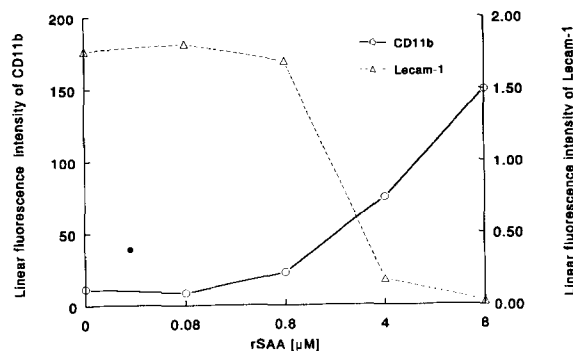*FACS® Analysis.* Whole blood treated with rSAA was pre-



**Figure 3.** Expression of CD11b and LECAM-1 on PMN: dose-response relationship. PMN were incubated for 15 min at 37°C with medium or rSAA. Cells were washed and stained with control mouse-IgG, CD11b, or LECAM-1 monoclonal antibodies as described in Materials and Methods section. The expression of adhesion molecules on PMN was evaluated by FACS® anlaysis using linear units converted from log fluorescence intensity of the peak.
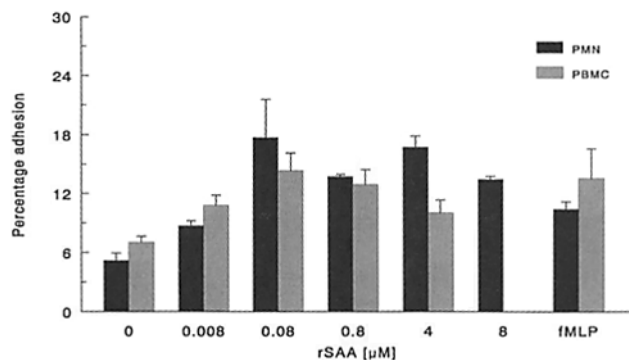


**Figure 4.** Induction of cells adhesion to endothelial cells. $^{51}$Cr-labeleed PBMC or PMN ($10^6$ cells/ml) were incubated with rSAA, FMLP (10 nM), or medium alone for 15 min at 37°C. Cells were washed and left for 30 min at 37°C to adhere to endothelial monolayers cultured in 24-flat-bottomed well plates. After three careful washings, the cells adherent to endothelial monolayer were determined by measuring the radioactivity in the cell lysates.
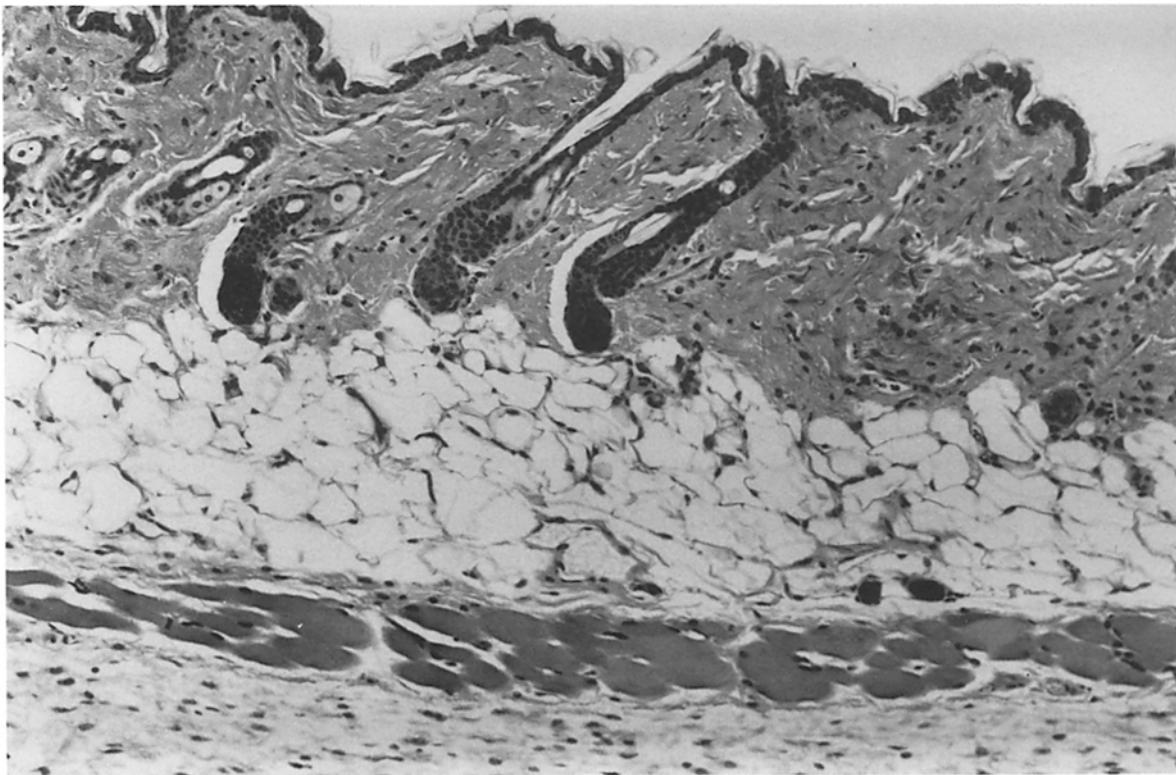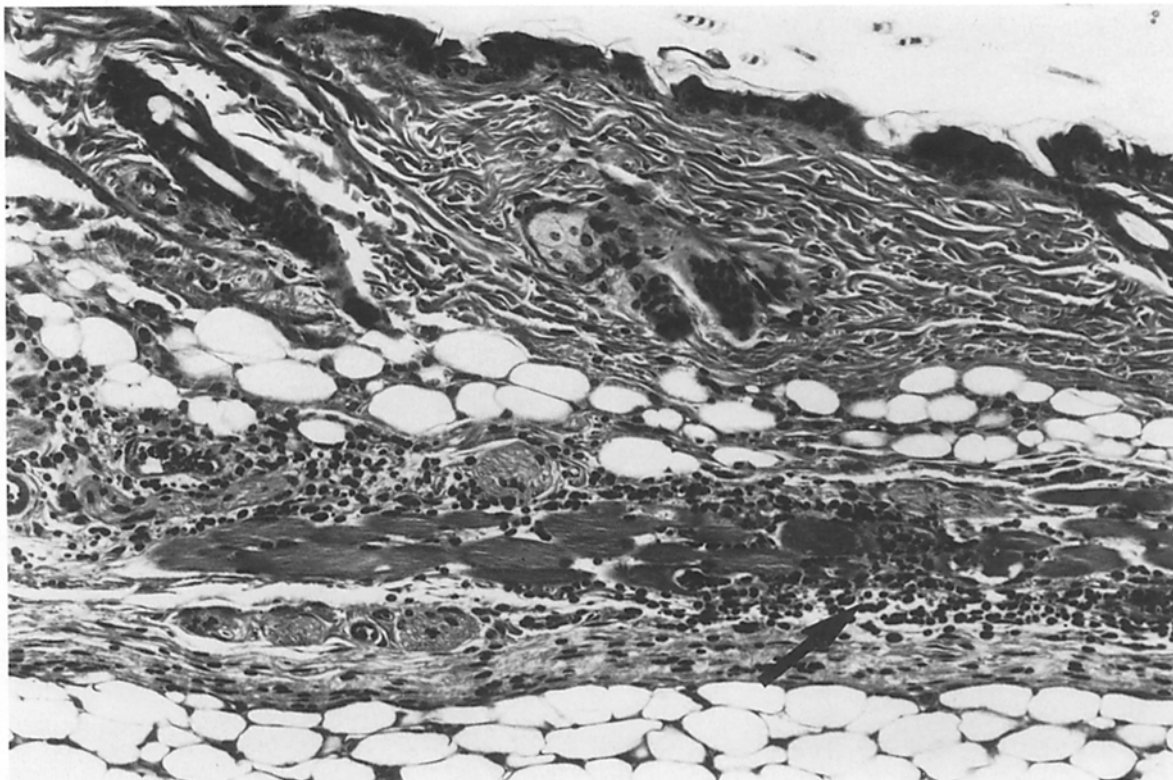
**Figure 5.** Cellular infiltration in subcutaneous tissues after injections of rSAA in BALB/c mice. BALB/c mice were injected subcutaneously daily with either 500 μg of HDL (in 0.2 ml of buffer) or 0.8 nmol of rSAA (in 0.2 ml of buffer) for a total of three injections. The injection site was biopsied at 72 h and fixed in formalin. The coded slides were then examined for the extent of cellular infiltrate and evaluated by a histopathologist. (*A*) Subcutaneous tissue of a mouse injected with HDL for 3 d (33×). No evidence of significant infiltration is present. (*B*) Site of subcutaneous injections with SAA for 3 d. (50×). Note the infiltration by mononuclear cells and PMN (*arrow*). (*C*) Higher magnification of the infiltrate noted in Fig. 5 *B* (132×). Representative of three experiments with three to four mice/group.
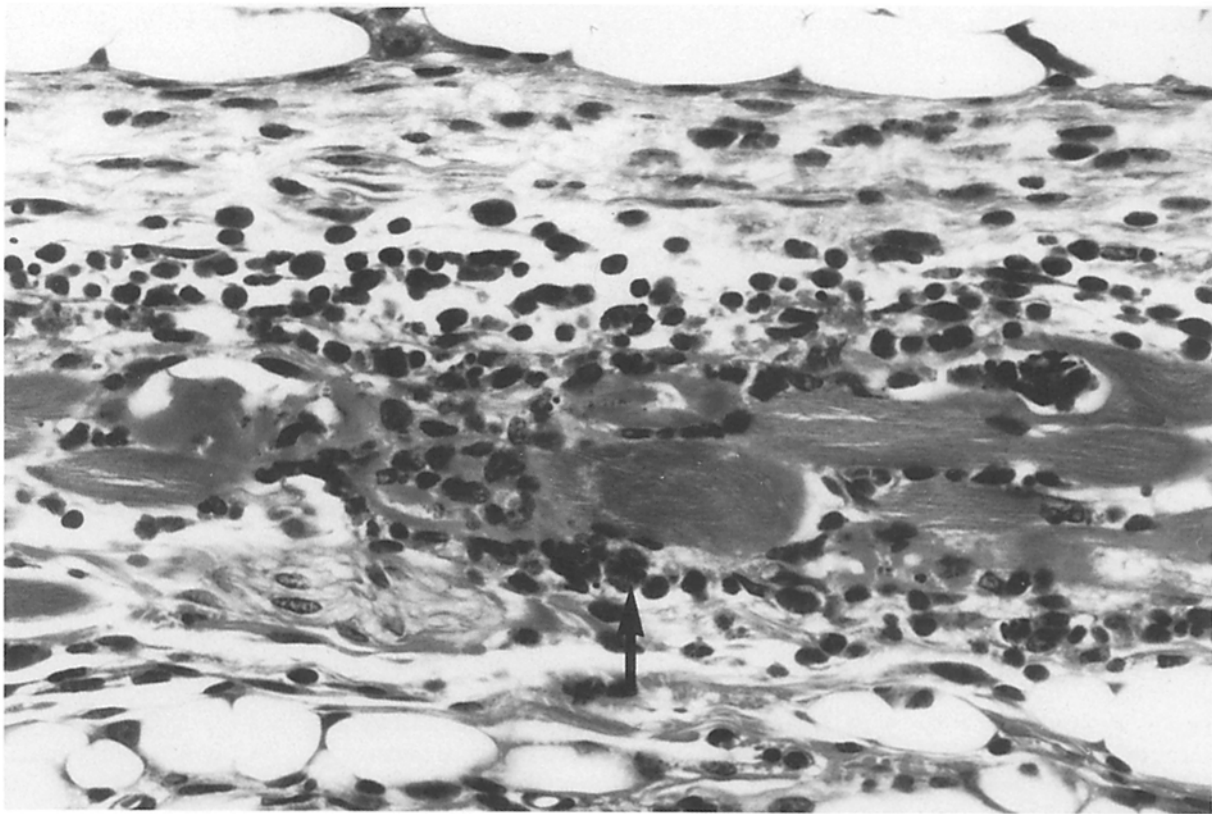
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incubated with buffer (RPMI and 1% BSA) containing 1 mg/ml of rabbit γ-globulins. Each sample of 100 μl of blood was washed and incubated with saturating concentrations of CD11a, CD11b, CD11c (Amac Inc., Westbrook, ME), leukocyte cell adhesion molecule 1 (LECAM-1; Endogen Inc., Boston, MA), or control mouse-IgG (Coulter Corp., Hialeah, FL) for 30 min at 4°C. Cells were washed two times with PBS, resuspended in 100 μl of PBS, and incubated at 4°C for 30 min with 4 μl of FITC-conjugated goat anti–mouse IgG (Tago Inc., Camarillo, CA). Red blood cells were then lysed by incubating the blood with 4 ml ACK buffer (Quality Biological Inc., Gaithersburg, MD) for 5 min at room temperature. Cells were washed three times with PBS, resuspended in PBS plus 1% paraformaldehyde, and analysed by a FACS® (Epics Profile; Coulter Corp.). At least 10,000 events were acquired, and on the basis of forward and side scatter the window for monocyte or neutrophil gated cells was set. The following formula was used to convert log units: linear units $= 10^{([4/255] \times \text{mean channel})}$.

For each sample we evaluated the total mean channel value after subtracting the mean channel value of the control Ig sample which had less than 1% background staining.

*Adhesion Assay.* Human umbilical cord (courtesy of the Obstetric Department, Frederick Memorial Hospital, Frederick, MD) vein endothelial cells (HUVEC) were prepared as described previously (16) and were used from the first to the third passage. PBMCs or PMN were resuspended in 250 μl of RPMI 1640 with 5% FCS and incubated for 60 min with 50–100 μCi $^{51}$Cr (Amersham Corp., Arlington Heights, IL). Cells were washed twice with RPMI containing 5% FCS and resuspended in this medium at $10^6$ cells/ml. Confluent endothelial cell monolayers in 24-well flat bottom plates were washed twice with RPMI 1640 before the ad-

dition of leukocytes in the adhesion assay. $0.5 \times 10^6$ leukocytes were added in a volume of 500 μl/well and incubated at 37°C for 30 min. On completion of the incubation period, the endothelial cell monolayers were washed three times with prewarmed RPMI containing 5% FCS and cells remaining in the wells were lysed by the addition of 1 N NaOH. Lysates were transferred to tubes and counted in a gamma counter. The radioactivity incorporated by $0.5 \times 10^6$ leukocytes was counted as total cpm. An aliquot of $0.5 \times 10^6$ leukocytes after incubation for 30 min at 37°C in the absence of endothelial monolayer, was centrifuged and the radioactivity measured in the supernatant was taken as spontaneous release. Percentage adhesion was calculated as following: Percent adhesion = (cpm obtained from leukocytes adherent to HUVEC/total cpm − cpm of spontaneous release) × 100.

*Histology.* Injection sites from three adult BALB/c mice/experimental group were biopsied and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Coded slides were read by a histopathologist.

## Results and Discussion

Various doses of rSAA were placed in the lower wells of a microchemotaxis chamber and PMN or PBMC were placed in the upper wells. rSAA induced cell migration in a dose-dependent manner (0.08–8 μM) of PMN and monocytes (Fig. 1, *A* and *C*). To distinguish between chemokinetic and chemotactic effects of rSAA on monocytes or PMN, we performed checkerboard assays by placing different concentrations of rSAA in the upper and/or lower wells. Under nega-

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tive gradient conditions (higher SAA concentration in the upper wells) no significant migration was observed. With equal SAA concentration in the upper and lower wells, only a small increase in migration by PMN occurred, indicating a minor but significant chemokinetic effect on PMN (data not shown).

Since in the blood, SAA is almost exclusively associated with HDL (17), we investigated whether the binding of SAA to HDL could affect the chemoattractant activity of SAA. HDLs rich in natural human SAA (Calbiochem-Novabiochem) at concentrations containing 0.008–8 $\mu$M of SAA had no chemoattractant activity on monocytes or PMN cells (data not shown). To address the effect of HDLs on the chemotactic activity of rSAA, we preincubated rSAA (4 $\mu$M), FMLP (10 nM), or medium with purified HDL (at concentrations from 10 to 1000 $\mu$g/ml) for 5 h at 37°C. Whereas the FMLP chemoattractant activity was only minimally affected by preincubation with HDLs, the chemotactic effect of rSAA on monocytes and PMN was inhibited in a dose-dependent manner by HDLs (Fig. 1, B and D). At concentrations above 100 $\mu$g/ml, HDLs almost completely blocked the activity of rSAA (4 $\mu$M), suggesting that HDLs may function as a natural inhibitor of SAA. A time course showed that SAA chemoattractant activity is inhibited by 70% after at least 1 h of preincubation of rSAA with HDLs at 37°C (data not shown). Conversely, a natural SAA preparation biochemically separated from HDLs (18) also exerted chemotactic activity with a dose response curve similar to rSAA (data not shown).

Cell surface adhesion proteins such as integrins and leukocyte selectin (LECAM-1) are crucial in mediating the adhesion of leukocytes to blood vessel walls and extravasation into tissues. Chemotactic factors like IL-8, FMLP and C5a are able to modulate the expression of CD18/CD11b, CD18/CD11c, LECAM-1 and induce leukocyte adhesion (19). We therefore examined the effect of rSAA on the expression of adhesion molecules (CD11a, CD11b, CD11c, LECAM-1) on leukocytes. Since in many cases, monocytes, after purification from blood have already lost LECAM-1 from the cell surface and express considerable CD18/CD11b, whole blood was used in these assays. PMN incubated with rSAA showed a remarkable enhancement of expression of CD18/CD11b (Fig. 2, A and B), a minor increase of CD18 /CD11c (data not shown), and the loss of LECAM-1 from the cell surface (Fig. 2, A and C), while CD18/CD11a was not affected (data not shown). The effects of rSAA on expression of CD18/CD11b and LECAM-1 were dose dependent as shown in Fig. 3 A. rSAA induced similar changes in the expression of adhesion molecules on monocytes (Fig. 2, D–F).

We next investigated whether the modulation of the expression of the adhesion antigens CD11b and LECAM-1 by rSAA could affect the ability of PMN and PBMCs to adhere to endothelial cell monolayer. $^{51}$Cr labeled PMN or PBMCs were preincubated for 15 min with medium, FMLP (10 nM) or rSAA at various concentrations. The cells were washed and incubated with endothelial monolayers for 30 min as described in Fig. 4. rSAA at concentrations above 0.08 $\mu$M was

as active as an optimal concentration of FMLP (10 nM) in inducing the adhesion of PBMC or PMN to endothelial cell monolayer (Fig. 4). The bell shaped dose-response curves were similar to that observed in chemotaxis assays. However, the concentration required to induce optimal adhesion is slightly lower than that required in chemotaxis. Although in adhesion molecule expression and HUVEC monolayer adhesion experiments whole blood or FCS was used, the HDL present in the blood or in the FCS failed to show inhibitory effect. This could be due to the relatively short interaction time of rSAA with HDLs, since, in chemotaxis experiments, a minimum of 60 min preincubation time was required for HDLs to inhibit the activity of rSAA.

Human rSAA, 0.8 nmol in 0.2 ml of buffer, HDL, 500 $\mu$g in 0.2 ml of buffer, or buffer alone, were injected subcutaneously daily into normal adult BALB/C mice. After three doses, the animals were sacrificed at 72 h and histological examination of injected areas was performed. rSAA induced infiltration of monocytes and PMN in the dermis and adipose tissues at 72 h (Fig. 5, B and C) that could not be detected in the animals injected with HDL (Fig. 5 A) or buffer alone (data not shown). This was consistently observed in three separate experiments. With 8 nmol of rSAA injected, a multifocal accumulation of monocytes and PMN was also observed in the panniculus (data not shown). No lymphocytic infiltration was detected at these time points and this dose range as determined by immunohistology (data not shown). The inhibitory effect of HDL on in vitro chemotactic activity of rSAA is currently being examined in vivo. Preliminary evidence suggests that preincubation of rSAA with HDL for 6–12 h reduces the ability of rSAA to induce leukocyte infiltration in subcutaneous tissue.

The optimal concentrations at which rSAA induces leukocyte migration, adhesion and tissue infiltration range between 0.8–4 $\mu$M. These concentrations are higher than the normal serum level of 0.08 $\mu$M, but are characteristic of levels of rSAA reached during the acute phase when the serum concentration of SAA exceeds 40 $\mu$M (2). However, most of the plasma SAA is bound to HDL, which acts as an inhibitor suggesting that SAA, to be active, needs to be released from the HDL complexes, spontaneously or perhaps by enzymatic cleavage. It has been reported that leukocyte-derived enzymes can degrade SAA to an 8-kD fragment and other small peptides (8, 9, 20). At sites of inflammation where proteolytic enzymes are released by leukocytes and proteinase inhibitors are inactivated, SAA may be released from HDL in a free form and create a concentration gradient to allow recruitment of inflammatory cells.

Since SAA is an amphipatic protein and interacts with phospholipids (13, 21) we wondered if lipids, bound to recombinant SAA, could account for its chemoattractant activity. However, we could not detect phospholipids associated with the rSAA. Treatment with trypsin at increasing ratios of trypsin/SAA caused degradation of the protein (as assessed by SDS-PAGE) together with loss of the corresponding chemotactic activity (data not shown). Furthermore, preparations of HDL which are rich in lipids, were not chemoattractants, but inhibited rSAA chemotactic activity (Fig. 2,

*B* and *D*). On the basis of these data we believe that the chemotactic activities of rSAA are attributable to the protein itself.

In Familial Mediterranean Fever, intermittent attacks of fever are associated with aseptic arthritis and serositis with accumulation of leukocytes in joints and in serosal surfaces. During these attacks, the levels of SAA in the blood increases and after many years, some patients develop systemic amyloidosis with deposits of amyloid fibrils in several organs (22). The fact that leukocytes accumulate in the synovial fluid and colchicine, an inhibitor of leukocyte chemotaxis and degranulation (23), decreases the frequency and the severity of these attacks implies a potential role of SAA in this inflammatory disease.

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