Acute-phase protein α-1-acid glycoprotein mediates neutrophil migration failure in sepsis by a nitric oxide-dependent mechanism

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The reduction of circulating neutrophil migration to infection sites is associated with a poor outcome of severe sepsis. α-1-Acid glycoprotein (AGP) was isolated from the sera of severely septic patients by HPLC and acrylamide gel electrophoresis and identified by mass spectrometry. Both the isolated protein and commercial AGP inhibited carrageenin-induced neutrophil migration into the rat peritoneal cavity when administered i.v. at a dose of 4.0 μg per rat (95 pmol per rat). Analysis by intravital microscopy demonstrated that both proteins inhibited the rolling and adhesion of leukocytes in the mesenteric microcirculation. The inhibitory activity was blocked by 50 mg/kg aminoguanidine, s.c., and was not demonstrable in inducible nitric oxide synthase (iNOS) knockout mice. Incubation of AGP with neutrophils from healthy subjects induced the production of NO and inhibited the neutrophil chemotaxis by an iNOS/NO/cyclic guanosine 3,5-monophosphate-dependent pathway. In addition, AGP induced the L-selectin shedding by neutrophils. The administration of AGP to rats with mild cecal ligation puncture sepsis inhibited neutrophil migration and reduced 7-day survival from ≈80% to 20%. These data demonstrate that AGP, an acute-phase protein, inhibits neutrophil migration by an NO-dependent process and suggest that AGP also participates in human sepsis.

Sepsis is a complex clinical syndrome resulting from an inadequate host response to infection. It is one of the major causes of death in intensive care units and has a mortality rate of 30–50%. Therapy for severe sepsis (SS) and septic shock is still largely symptomatic and supportive (1).

Neutrophils are the first cells to migrate to the site of bacterial infection. They kill microorganisms by producing reactive oxygen and nitrogen species. Together with resident cells they also release cytokines and chemokines, which increase the recruitment and activation of additional neutrophils and other immune cells such as monocytes and lymphocytes at the site of infection (2). Severe experimental sepsis induced by polymicrobial (3) infection or inoculation with *Streptococcus aureus* (4) is associated with the failure of neutrophils to migrate to the site of infection. Furthermore, neutrophils obtained from septic patients have reduced migration responses to *N*-formyl-L-leucy1-L-phenylalanine (fMLP) and leukotriene B4 compared with neutrophils from healthy subjects. The inhibition of neutrophil migration is greater for cells obtained from nonsurviving septic patients than from survivors (5).

The mechanism involved in the reduction of neutrophil migration observed in sepsis is not completely understood. However, there is evidence suggesting that circulating cytokines and/or chemokines, acting via activation of the L-arginine/inducible nitric oxide synthase (iNOS)/NO pathway, reduce neutrophil rolling/adhesion to endothelial cells, resulting in the reduction of neutrophil migration to the focus of infection (3). In fact, plasma concentrations of cytokines and chemokines and NO are increased in experimental and human sepsis (5, 6), and i.v. administration of TNF-α or IL-8 inhibits neutrophil rolling, adhesion, and migration to an inflammatory site by a mechanism sensitive to NO synthase inhibitors (7). Moreover, i.v. administration of antiserum against TNF-α partially prevents the inhibition of neutrophil migration induced by systemically administered LPS (F.Q.C. and B.M.T.-M., unpublished work). However, despite the evidence that cytokines mediate the failure of neutrophil migration, clinical trials using neutralizing antibodies against cytokines or soluble receptors did not show beneficial effects on septic patients (8).

It has been suggested that serum factors may also be responsible for the inhibition of neutrophil migration, because massive fresh plasma transfusions restore neutrophil function in newborns suffering from sepsis (9). Serum factors have also been reported to play a role in the reduced neutrophil migration in other diseases, such as diabetes (10), cirrhosis (11), AIDS (12), and cancer (13). All of these diseases are associated with high susceptibility to infection. It is also well known that acute-phase proteins are present at much higher concentrations in the blood of septic patients than in healthy subjects (14). Although there is indirect evidence for the existence of factor(s) capable of inhibiting neutrophil migration in the serum of septic patients, none has been characterized. Thus, the objective of the present study was to identify non-cytokine-soluble substances in the blood of septic patients that inhibit neutrophil migration.

Results

Isolation of Neutrophil Migration-Inhibitory Proteins from the Serum of Septic Patients. The data in Fig. 1 show that i.p. administration of carrageenin (Cg) (100 μg per cavity) to rats pretreated with saline induced a significant amount of neutrophil migration compared with the control (C) group injected i.p. with saline ($P < 0.05$). Pretreatment of the rats with serum from septic patients (SHS) (40 mg of protein per rat) significantly reduced Cg-induced neutrophil migration ($P < 0.05$). The small and statistically nonsignificant reduction in neutrophil migration caused by serum obtained from normal subjects [compare normal human serum (NHS), 40 mg of protein per rat, with SHS] was probably due to α-1-acid glycoprotein (AGP) present in

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The authors declare no conflict of interest.

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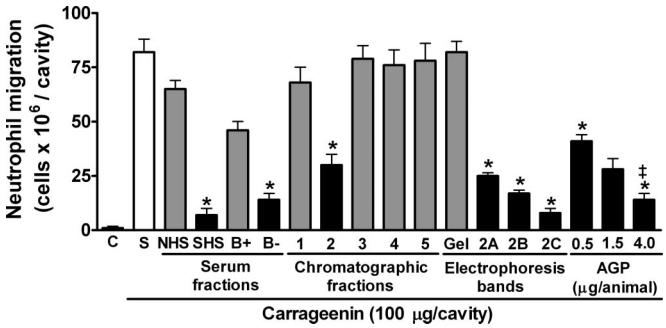

Fig. 1. Neutrophil migration to the rat peritoneal cavity. Four hours after the injection of carrageenin (100 μg per cavity, i.p.) or saline (0.2 ml per cavity) the cells in the peritoneal cavity were collected, and neutrophils were counted (see *Methods*). The bars in the graph reflect the following (samples were administered i.v. 30 min before Cg): (*i*) Saline (0.2 ml) or NHS and septic human serum (SHS) were injected i.v. at 40 mg of protein per rat. (*ii*) The materials from the serum of septic patients that adsorbed to the Sepharose blue column (B+), and those that did not adsorb (B−) were injected i.v. at 15 mg of protein per rat. (*iii*) The numbers, 1–5, indicate the effluent pools from HPLC identified in SI Fig. 5. Those samples were injected i.v. at 20 μg of protein per rat. (*iv*) Diffusates of bands 2A, 2B, and 2C (shown in Fig. 2*A*) were injected i.v. at 4.0 μg of protein per rat. The GEL was the diffusate from a portion of the gel that was free of protein. (*v*) Bars labeled AGP indicate the effects of commerical samples of AGP upon neutrophil migration induced by Cg. *, $P < 0.05$, compared with saline; ‡, $P < 0.05$ compared with AGP (0.5 μg per animal). Data are reported as means ± SEM × 10^6 neutrophils per cavity for five animals in each group. Data were analyzed by ANOVA followed by the Bonferroni *t* test. C, control; S, saline.

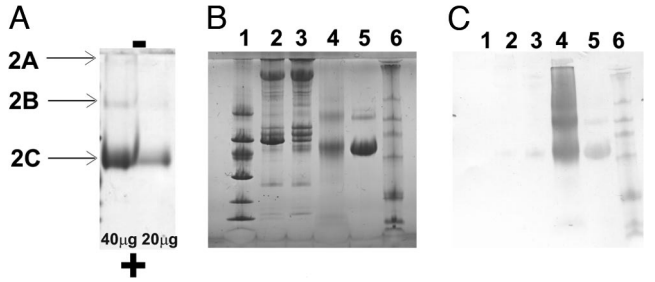

Fig. 2. Native PAGE, SDS/PAGE, and Western blot analysis of fraction B−. (*A*) Native PAGE of 20 and 40 μg of HPLC pool 2. The positions of bands 2A, 2B, and 2C are indicated by the arrows. This result was reproducible in triplicate experiments. (*B*) SDS/PAGE: lane 1, 1 μg of molecular mass marker (94, 67, 43, 30, 21.1, and 14.4 kDa, from top to bottom); lane 2, 1 μg of SHS; lane 3, 1 μg of B−; lane 4, 1 μg of HPLC pool 2; lane 5, 1 μg of commercial human AGP; lane 6, 1 μg of the prestained molecular mass marker (250, 98, 64, 50, 36, 30, 16, 6, and 4 kDa, from top to bottom). (*C*) Western blot analysis of the same sample as in *B*. A mouse monoclonal antibody against human AGP was used for detection.

normal human serum. Indeed, the level of AGP in normal human plasma was 0.55 ± 0.04 mg/ml, $n = 5$, compared with 1.58 ± 0.2 mg/ml, $n = 9$, in plasma from septic patients ($P < 0.002$, Student's t test). Plasma and serum levels of AGP from septic patients were the same.

When SHS was filtered through blue Sepharose resin to remove albumin, most of the neutrophil migration-inhibitory activity was recovered in the fraction that was not adsorbed to the resin (B−). Compare B− (15 mg of protein per rat) with the fraction adsorbed to the resin B+ (15 mg of protein per rat) in Fig. 1.

A typical HPLC-UV absorbance profile of 5.2 mg of fraction B− is shown in supporting information (SI) Fig. 5. The biological activity of 20 μg of protein from each of the five effluent pools identified on the horizontal line of SI Fig. 5 is reported in Fig. 1. Neutrophil migration-inhibitory activity was detected only in pool 2.

When a portion of pool 2 (40 μg) was submitted to PAGE under nondenaturing conditions, three diffuse bands were detected with colloidal Coomassie (Fig. 2*A*). Neutrophil migration inhibitory activity was demonstrable in the diffusate from all three electrophoretic bands (2A, 2B, 2C) when 4.0 μg of protein per rat was assayed as shown in Fig. 1. In contrast, the diffusate from that part of the gel not stained for protein (GEL) did not inhibit neutrophil migration (Fig. 1).

Mass Spectrometric Identification of AGP. Mass spectrometric analysis of the tryptic peptides obtained from fraction 2C identified the protein as AGP (Swiss-Prot accession no. P02763). Proteins 2A and 2B were not recovered in sufficient amounts to be identified unambiguously. The peptide mass fingerprint of fraction 2C is presented in SI Fig. 6*A* and shows the deconvolution of double-charged ions to single-charged ions obtained with the MaxEnt 3 algorithm. The six peptides listed in SI Table 1 were detected in the tryptic hydrolysate, and their m/z values were <0.3 μ different from the value expected on the basis of the amino acid sequence of AGP. Four of the six peptides, identified with asterisks in SI Table 1, were submitted to collision-induced dissociation-tandem mass spectrometry (CID-MS/MS) and their amino acid sequences were deduced from the ion fragment pattern. SI Fig. 6*B* shows a typical sequencing result, i.e., the spectrum of the peptide ion m/z 581 [M + $2H^+$] (M_r 1159.5). The deduced amino acid sequence was WFYLxASAFR, which corresponds to residues 43–51 of AGP. The sequences of the four peptides identified in SI Table 1 correspond to 23% of the amino acid sequence of AGP, and all six peptides correspond to 35%.

The tryptic mass fingerprint obtained from a commercial sample of AGP did not differ significantly from that obtained from fraction C isolated from the serum of patients with sepsis. Five ions corresponding to expected masses of tryptic peptides were common to both samples of AGP (data not shown) and are indicated by the arrows in SI Fig. 6*A*. Moreover, the results of mass spectrometric analysis were confirmed by Western blotting using an anti-human AGP monoclonal antibody (Fig. 2*C*).

A Commercial Sample of AGP Inhibits Neutrophil Migration and Leukocyte Rolling/Adhesion by a NO-Dependent Process. Although the mass spectrometric demonstration of the presence of AGP in a protein fraction, which inhibited neutrophil migration was convincing, it was necessary to demonstrate that a characterized commercial sample of AGP inhibited neutrophil migration. Fig. 1 shows that pretreatment of the rats with different doses of commercial AGP inhibited the neutrophil migration stimulated by Cg. There was a statistical difference between the responses to 0.5 and 4.0 μg per rat, indicating that the response was dose-dependent. The data in Fig. 3*A* also show that pretreatment of the rats with an inhibitor of iNOS, aminoguanidine (AG) (50 mg/kg, s.c.), 30 min before administration of AGP or fraction 2C completely prevented the inhibition of neutrophil migration obtained with 4.0 μg per rat of these proteins. Moreover, administration of AGP (4.0 μg per mouse, i.v.) significantly inhibited ($P < 0.05$) the neutrophil migration induced by Cg (500 μg per cavity) in C57BL/6 (WT) mice (9.14 ± 0.79 × 10^6 neutrophils per cavity for control group vs. 4.78 ± 0.79 × 10^6 neutrophils per cavity for AGP group), but not in iNOS$^{-/-}$ mice (9.57 ± 1.15 × 10^6 neutrophils per cavity for control group vs. 9.14 ± 0.79 × 10^6 neutrophils per cavity for AGP group). We have observed that AGP does not inhibit the migration of mononuclear cells 6 h after Cg injection (data not show).

Because neutrophil migration depends on leukocyte–endothelium interactions, we examined the effect of fraction 2C and AGP on neutrophil rolling and adhesion to mesenteric postcapillary venules. The Cg-induced increase in leukocyte

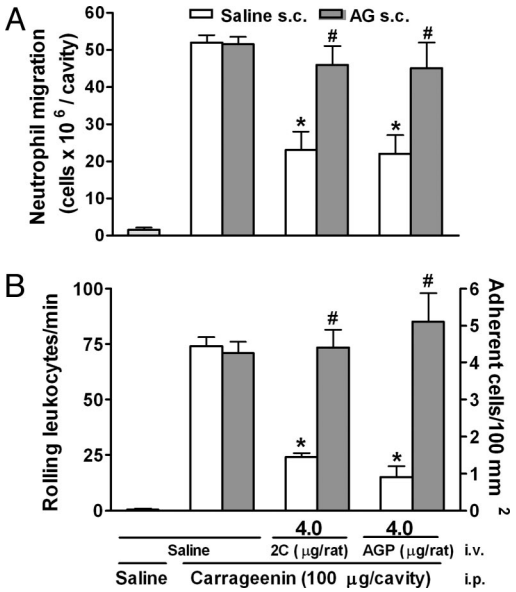

Fig. 3. AGP inhibited neutrophil migration and leukocyte rolling/adhesion by a NO-dependent process. (*A*) Neutrophil migration into the peritoneal cavity of rats was determined 4 h after i.p. Cg administration. The results of neutrophil migration are reported as means ± SEM of the number of neutrophils × 10^6 in the peritoneal cavity ($n = 5$). *, $P < 0.01$, compared with saline i.v. plus Cg i.p.; #, $P < 0.01$, compared with AGP or fraction 2C i.v. plus Cg i.p. (*B*) The *ex vivo* measurement of rolling and adhesion was carried out 2 and 4 h after Cg administration, respectively. AG (50 mg/kg) was injected s.c. 30 min before i.v. administration of fraction 2C (4.0 μg per animal) or AGP (4.0 μg per animal) and 1 h after Cg was injected i.p. The results of rolling are reported as the mean ± SEM number of rolling leucocytes per minute ($n = 5$) and, for adhesion, as the mean ± SEM number of adherent cells per 100 mm^2 ($n = 5$). *, $P < 0.01$, compared with saline i.v. plus Cg i.p.; #, $P < 0.01$, compared with AGP or fraction 2C i.v. plus Cg i.p.

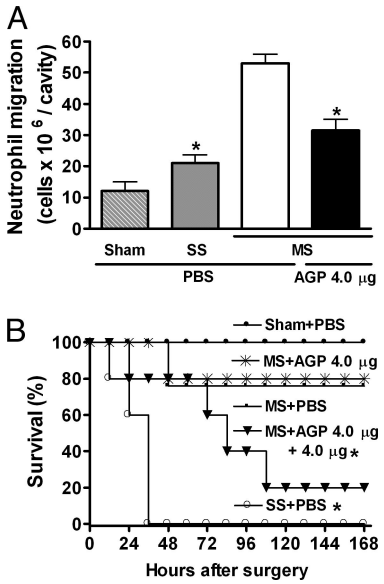

Fig. 4. AGP inhibited neutrophil migration and increased mortality in rats with sepsis induced by cecum ligation and puncture. (*A*) Neutrophil migration to the site of infection in the peritoneal cavity. AGP was administered to rats with MS 30 min before surgery, and migration was measured 4 h after surgery. Rats with SS and Sham-operated rats received only PBS. Data are reported as the means ± SEM (number of neutrophils × 10^6 per cavity, $n = 5$). *, $P < 0.05$, compared with the MS-plus-PBS and Sham-plus-PBS groups. (*B*) Mortality data are reported for 10–12 animals in each group. *, $P < 0.01$, compared with Sham, MS plus PBS, and MS plus 4.0 μg of AGP (Mantel–Cox log–rank test).

rolling and adhesion (Fig. 3*B*) was significantly reduced by pretreatment with 4.0 μg per rat of AGP or fraction 2C.

In agreement with the data in Fig. 3, 6 h after *in vitro* stimulation of human neutrophils with LPS (10 ng/ml) or AGP (50 or 500 μg/ml) there was a significant increase ($P < 0.05$) in supernatant nitrite content (2.10 ± 0.33, 2.35 ± 0.31, 2.8 ± 0.35 μM nitrite, respectively) compared with the cells without stimulation (0.98 ± 0.24 μM nitrite). In addition, pretreatment with AG (15 μM, 30 min) abolished the increase in nitrite content induced by 500 μg/ml AGP (0.92 ± 0.22 μM nitrite). AGP-induced NO production by human neutrophils was confirmed by visualization under a confocal microscope with 4,5-diaminofluorescein diacetate (DAF-2DA) fluorescence (data not shown). Furthermore, *in vitro*, AGP inhibited the neutrophil–chemotaxis response stimulated by IL-8, which was prevented by aminoguanidina, *N*-(3-(aminomethyl)benzyl)acetamidine (1400W), or 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (SI Fig. 7*A*). Similarly, the mouse neutrophil–chemotaxis response to MIP-2 was also inhibited by AGP (data not shown). AGP also induced neutrophil L-selectin shedding, similarl to that induced by LPS (SI Fig. 7*B*). Moreover, AGP labeled with FITC was able to bind human neutrophils (SI Fig. 7*C*), which was inhibited by preincubation with AGP without FITC, fetuin, or fucoidan. Fucoidan is a sulfated polysaccharide that binds P- and L-selectin and has been extensively used as a selectin blocker (15). Fetuin is a glycoprotein that consists of a single polypeptide chain that has three heteropolysaccharide units made up of sialic acid, galactose, *N*-acetylglucosamine, and mannose (16). These experiments suggest that AGP interacts with structures of the neutrophils, such as recently shown sialic acid binding Ig-like lectins (Siglecs) (17).

AGP Inhibits Neutrophil Migration and Decreases Survival in the Perforated Cecum Model of Sepsis. Compared with the Sham-operated group, significant neutrophil migration into the peritoneal cavity was observed in the mild sepsis (MS) group (Fig. 4*A*), whereas the levels of plasma AGP were similar in the two groups (4.57 ± 0.46 mg/dl and 4.20 ± 0.79 mg/dl, respectively). However, the SS group showed reduced neutrophil migration into the peritoneal cavity and increased levels of plasma AGP (26.92 ± 1.56 mg/dl, $P < 0.05$ vs. Sham). In addition, the i.v. administration of AGP (4.0 μg per rat) to MS rats 30 min before surgery significantly reduced neutrophil migration compared with MS rats that received only PBS (Fig. 4*A*).

The effect of AGP on the survival of septic rats is shown in Fig. 4*B*. MS rats treated or not with 4.0 μg per rat of AGP i.v. after surgery had a survival rate of ≈80% at 168 h. In contrast, the survival of MS rats treated i.v. with 4.0 μg per rat of AGP 30 min before surgery and 12 h after surgery was reduced to 20% at 108 h. All SS rats were dead 36 h after surgery. Thus, the inhibition of neutrophil migration to the site of infection by AGP increased mortality due to sepsis.

Discussion

The acute phase protein AGP was isolated from serum of patients with SS. The purified protein, as well as the commercial sample of AGP, inhibited Cg-induced neutrophil migration to the peritoneal cavity by an NO-dependent mechanism. The present data suggest that the reduction of migration is probably the result of the inhibition of rolling and adhesion of neutrophils to the endothelium. Furthermore, in a well characterized experimental model of sepsis based on perforation of the cecum, which is similar to clinical sepsis, microgram quantities of AGP

administered i.v. inhibited neutrophil migration to the infection site and significantly increased mortality.

Our search for a soluble factor in serum that could inhibit neutrophil migration in the course of sepsis was based on several lines of evidence (18, 19) that suggested that serum of septic patients contains substances of this nature, but none have been isolated or identified by others. One of the most remarkable indirect indications of the existence of an important factor in the serum of septic patients is that massive fresh plasma transfusions restore neutrophil function to newborns suffering from sepsis (9). The reduction of neutrophil migration has been described in experimental polymicrobial (3) and Gram-positive (4) sepsis, and the extent of the reduction was related to the intensity of sepsis. Moreover, neutrophils obtained from septic patients, mainly from nonsurvivors, also present reduced chemotactic activity (5). As is the case for many acute phase proteins, the concentration of AGP is increased in the serum of septic patients (14), suggesting that AGP may reduce neutrophil migration during the inflammatory process because of the large number of negatively charged sialyl Lewis X residues on its surface, which could inhibit interactions between the membranes of neutrophils and endothelial cells (20). In agreement with this, we showed that binding of AGP by neutrophils is inhibited by fetuin, a glycoprotein with a high content of sialyl Lewis X residues. Indeed the level of AGP was three times higher in plasma from septic patients compared with normal controls, as well as in rats submitted to SS when compared with MS.

The methods used to isolate AGP, i.e., blue Sepharose, C_{18}-HPLC, and PAGE, were standard. However, the inability to quantify the biological response to AGP or to measure AGP made it impossible to either monitor the recovery of activity or the active substance at each purification step or to refine any step. We recovered $\approx$100 μg of AGP from 68 ml of serum, which is very low considering that the blood of septic patients contains $\approx$1.58 mg/ml AGP. The protein fractions 2A and 2B had biological activity but were not obtained in sufficient quantity to be identified unambiguously. Their electrophoretic mobility in native PAGE indicated that they were less negatively charged than AGP. If these species were in fact AGP, then these differences in mobility might be the consequence of differences in sialic acid content, because human AGP contains 10–12% of this acidic sugar, and the protein has a pI of 2.8–3.8. Normal human serum contains 12–20 glycoforms of AGP, and specific changes in the number and charge of glycoforms are observed during the initiation of the acute-phase reactions (20, 21).

The reduction of neutrophil migration in sepsis appears to be mediated by NO and seems to be due to the attenuation of rolling and adhesion of neutrophils to endothelial cells of the microcirculation. In fact, reduction of these parameters is observed in mice submitted to SS induced by perforation of the ligated cecum (6). The process is prevented by pretreatment of the animals with iNOS inhibitors or by the use of iNOS$^{-/-}$ mice (22), and increases in neutrophil rolling/adhesion and in the expression of the adhesion molecules can be observed in both situations, whereas NO donors decrease these effects (23, 24). Both authentic AGP and natural purified AGP reduced neutrophil migration, rolling, and adhesion of leukocytes to the endothelium of the mesenteric microcirculation of rats also by an NO-dependent mechanism. Pretreatment of animals with AG or the use of iNOS$^{-/-}$ mice abolished the inhibitory effect of AGP. Moreover, *in vitro*, AGP induces the NO production and inhibited the neutrophil chemotaxis by an iNOS/NO/cyclic guanosine 3,5-monophosphate-dependent pathway.

To show that circulating AGP mediates the failure of neutrophils to migrate to the focus of infection, consequently aggravating the sepsis condition, we administered AGP i.v. to animals with mild nonsevere sepsis (MS animals) and observed the reduction of neutrophil migration to the infection focus. MS rats receiving AGP had a mortality rate similar to that observed in SS animals, showing that neutrophil migration to the focus of infection is a pivotal event for the resolution of infection. Additionally, *in vitro*, AGP induced shedding of L-selectin by neutrophils. Shedding of L-selectin has been shown in sepsis-activated neutrophils. Thus, high serum levels of L-selectin as a consequence of neutrophil shedding has been used as a predictor of severity of septic patients (25).

Circulating cytokines and chemokines, such as TNF-α, IL-1β, and IL-8, may mediate the failure of neutrophil migration observed in sepsis (7) by a mechanism dependent on NO release (3). The reduction of neutrophil rolling, adhesion, and migration observed in sepsis or after i.v. administration of TNF-α or IL-8 is inhibited by pretreatment of the animals with iNOS inhibitors or by the use of iNOS$^{-/-}$ mice (22). An increase in neutrophil rolling/adhesion and in the expression of the adhesion molecules is observed after the administration of both cytokines, whereas NO donors decrease these effects (23, 24). The fact that AGP was increased in the serum of septic patients and that the cytokines are major modulators of AGP gene expression (20) indicates the importance of determining whether the inhibitory effect of cytokines on neutrophil migration is also an AGP-dependent process. Moreover, it is also important to determine whether AGP is the serum factor that mediates the reduction of neutrophil migration reported for other diseases, such as diabetes (10), cirrhosis (11), AIDS (12), and cancer (13).

AGP belongs to the lipocalin family, which also includes several other proteins such as α-1-microglobulin, glycodelin, neutrophil lipocalin, and complement C8-γ. Most of these present several similar inflammatory and immunomodulatory activities, such as inhibition of neutrophil and monocyte adherence to the endothelium and inhibition of antigenic or mitogenic stimulation of lymphocytes (26). It is important to determine whether other lipocalins are also involved in the mediation of neutrophil migration failure observed in sepsis.

The data presented here provide insights into the physiopathology of sepsis, in that the ability of the circulating acute phase protein AGP to inhibit neutrophil migration was shown to be dependent on NO. To our knowledge, it is the first demonstration of the deleterious effect of an acute phase protein in sepsis. Extrapolating our results to patients with sepsis we suggest that the acute phase protein AGP mediates the failure of neutrophil migration in human sepsis. Blockade of the release or of the biological activity of AGP may constitute a new therapeutic target in the control of human sepsis.

Materials and Methods

Chemicals. Cg, human AGP (99% purity SDS/PAGE), AG, *Escherichia coli* LPS, DMEM, May–Grunwald–Giemsa stain, and the mouse monoclonal anti-AGP antibody were purchased from Sigma. TFA and tribromoethanol were products of Aldrich, and sequencing-grade bovine trypsin was purchased from Promega. Acetonitrile and methanol were HPLC grade from Merck. Blue Sepharose 6 Fast Flow resin and electrophoresis reagents were products of Amersham Pharmacia Biotech. Serva Blue G was purchased from Serva. Amino acid standards and phenylisothiocyanate (PITC) were obtained from Pierce. All other chemicals were reagent-grade or equivalent.

Septic Patients. This protocol was approved by the Ethics Committee of the Faculty of Medicine of Ribeirão Preto (FMRP), University of São Paulo, and written informed consent was obtained from all patients (or persons responsible for them) and normal volunteers. Sixty-eight patients (53% female) in the intensive therapy unit of the University Hospital, FMRP, were enrolled in the study. Patients were prospectively identified as having SS according to APACHE II criteria (27). For APACHE scores, see *SI Methods*.

Blood Collection. Human blood was collected into two vials, one of which contained anticoagulant (heparin, 5,000 units/liter). The serum was separated by centrifugation and pooled for the isolation of neutrophil migration-inhibitory proteins. Plasma was separated by centrifugation and used for the measurement of AGP. A similar procedure was used to collect plasma from rats (septic and controls). Both plasma and serum were stored at −70°C.

Measurement of Plasma AGP. AGP was measured in human and rat plasma by an immunoturbidimetric method using a kit manufactured by Quibasa Química Básica. The measurements were performed in duplicate, varied ≤10%, and were carried out at the same time.

Animals. The care and treatment of the animals was based on the *Guide for the Care and Use of Laboratory Animals* (28), and the research was approved by the Animal Research Ethics Committee of the FMRP. Male Wistar rats (200–250 g) and C57BL/6 (WT) or iNOS-deficient ($iNOS^{-/-}$) mice (18–22 g) were used. The mice were obtained from the Animal Facility of the FMRP and housed in the Animal Room of the Department of Pharmacology at 23–25°C with free access to water and food. Breeding pairs of mice with targeted disruption of the iNOS gene were obtained from The Jackson Laboratories.

Neutrophil Migration to the Rat Peritoneal Cavity. AG (50 mg/kg) or saline were injected s.c. 30 min before i.v. AGP administration. One hour after i.v. administration, neutrophil migration was induced by i.p. injection of Cg (100 μg per cavity) or sepsis. The animals were killed 4 h after Cg administration or surgery, and neutrophil migration was assessed (3).

Leukocyte Rolling and Adhesion to the Rat Mesenteric Microcirculation Analyzed by Intravital Microscopy. Briefly, rats were anesthetized with tribromoethanol (250 mg/kg, i.v.) and mesenteric tissue was withdrawn for microscopic examination (6). Rolling leukocytes were defined as those white blood cells that moved at a lower velocity than erythrocytes in the same stream and were measured at 10-min intervals, 2 h after the Cg stimulus. In contrast, adherent leukocytes were measured 4 h after stimulation with Cg. White blood cells that did not move with respect to the venular endothelium for >30 s during a 5-min period of observation were reported as the number of adherent cells per 100 μm^2 of venule. Rats used for the neutrophil rolling and adhesion assay received AG, AGP, fraction 2C, and Cg as described above for the neutrophil migration assay.

Sepsis Induced by Perforation of the Cecum. Sepsis was induced by cecum ligation and puncture (29). We have standardized the number of punctures in cecum ligation and determined the number of punctures that caused MS or SS in control rats. Survival rate was determined daily for 7 days after surgery. The animals in the MS group received PBS or AGP i.v., in the quantities indicated in Fig. 4, 30 min before or 30 min before and 12 h after surgery. Blood was collected by cardiac puncture from rats 6 h after surgery, and the plasma was separated for the determination of AGP content.

Nitric Oxide Production by Human Neutrophils. Human neutrophils were obtained from heparinized venous blood (10 ml) of healthy subjects and isolated by Percoll gradient (30). The viable isolated neutrophils (>95%, as determined by Trypan blue exclusion) were resuspended in DMEM. Neutrophils (1×10^6/500 μl) were incubated for 6 h at 37°C in an atmosphere of 5% CO_2/95% O_2 in DMEM without (control) or with AGP (50 or 500 μg/ml), LPS (10 ng/ml), or AGP (500 μg/ml) plus AG (15 μM, 30 min before AGP). Six hours after incubation, cells were centrifuged, and the supernatants were collected for nitrite determination. The nitrite concentration was determined using an NO analyzer (NOA 280; Sievers Instruments). Sodium nitrite (Sigma) was used as a standard (31).

Protein Determination. Amino acid composition and total protein were determined by amino acid analysis using the phenyl thiocyanate method (PTC) method (32). Briefly, samples were hydrolyzed with 6 M HCl in the vapor phase (110°C) for 22 h (33), derivatized with PITC, and applied to a Picotag C_{18} column (Waters). Amino acid content was calculated using an amino acid standard subjected to the same derivatization procedure.

Chromatography on Blue-Sepharose. A pool of 1 ml of serum from each septic patient (68 ml) was dried in a Speed Vac rotary centrifuge (Savant Instruments) and rehydrated with 34 ml of 0.1 M sodium phosphate buffer, pH 6.0. This 2× concentrated serum was filtered three times through a Blue-Sepharose 6 Fast Flow column (25 × 250 mm, 122-ml bed volume) to remove most of the albumin (34). The column was washed to elute proteins that were not absorbed. The eluate was combined and denoted fraction B−. The column was then washed to strip albumin and other proteins from the resin, and this effluent was denoted fraction B+. Fractions B− and B+ were dialyzed against Milli-Q water.

HPLC. Fraction B−, which contained most of the neutrophil migration inhibitory activity, was submitted to HPLC on a Vydac C_{18} column (Grace Vydac) at room temperature by using a Shimadzu Li Chromatography System (see *SI Methods*).

Native Electrophoresis. The HPLC effluent containing neutrophil migration inhibitory activity, pool 2 (Fig. 1), was submitted to 12.5% homogeneous PAGE in a Hoeffer Mini VE system (GE–Amersham Pharmacia). One lane was stained with 0.1% colloidal Coomassie blue (35) to locate protein, and the gel of four unstained lanes was cut into bands on the basis of alignment with the protein-stained lane. A control for catalysts and other substances in the gel was prepared from an equivalent amount of gel. Protein was eluted from the gel fragments by passive diffusion into 1.0 ml of PBS overnight at 4°C, and the gels were reextracted four times with 500 μl of PBS for 2 h (36). The eluted proteins were dialyzed against Milli-Q water and concentrated to 2.0 ml.

SDS/PAGE and Western Blot Analysis. One microgram each of SHS, B− fraction, HPLC pool 2, and commercial human AGP and 1 μg of molecular weight marker (Amersham Pharmacia) were separated on a precast 8–25% PhastGel by using PhastGel SDS buffer strips (Fig. 2*B*), and the gels were stained with 0.1% colloidal Coomassie blue. After SDS/PAGE, the same proteins as in Fig. 2*B* were transferred to a nitrocellulose filter (Amersham Pharmacia Biotech). The blot was then probed with a mouse antiserum against human AGP, incubated sequentially with a biotinylated rabbit anti-mouse serum and streptavidin–alkaline phosphatase, and developed colorimetrically with 3,3-diaminobenzidine (DAB).

Mass Spectrometry. Proteins purified by native PAGE and a commercial AGP sample were heat denatured and hydrolyzed with 0.5 μg of Promega modified trypsin. The mass fingerprint of the tryptic peptides was obtained with an electrospray triple-quadrupole mass spectrometer, Quattro II (Micromass). Peptide sequences were obtained by CID-MS/MS with argon as the collision gas. The amino acid sequences of four tryptic peptides indicated by an asterisk in SI Table 1 were deduced from the series of *b* and *y* fragment ions produced by CID. The masses of tryptic peptides and fragment ions from CID were used to search the GenBank at National Center for Biotechnology Information (NCBI) using the Protein Prospector MS-Fit and MS-Tag software (http://prospector.ucsf.edu).

Statistical Analysis. Data are reported as mean ± SEM and are representative of two or three independent experiments with five animals in each group. The survival curves are reported as percentage of survival ($n = 10–12$), and a log–rank test (χ^2 test) was used to examine differences between groups. Differences between two unpaired groups were compared by the Student t test. The mean differences between three or more groups within an experiment were compared by ANOVA. When significant differences were identified, individual comparisons were subsequently tested with the Bonferroni t test. Statistical significance was set at $P < 0.05$.

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1. Feihl F, Waeber B, Liaudet L (2001) *Pharmacol Ther* 91:179–213.
2. Yamashiro S, Kamohara H, Wang JM, Yang D, Gong WH, Yoshimura T (2001) *J Leukocyte Biol* 69:698–704.
3. Benjamim CF, Ferreira SH, Cunha FQ (2000) *J Infect Dis* 182:214–223.
4. Crosara-Alberto DP, Darini AL, Inoue RY, Silva JS, Ferreira SH, Cunha FQ (2002) *Br J Pharmacol* 136:645–658.
5. Tavares-Murta BM, Zaparoli M, Ferreira RB, Silva-Vergara ML, Oliveira CH, Murta EF, Ferreira SH, Cunha FQ (2002) *Crit Care Med* 30:1056–1061.
6. Benjamim CF, Silva JS, Fortes ZB, Oliveira MA, Ferreira SH, Cunha FQ (2002) *Infect Immun* 70:3602–3610.
7. Tavares-Murta BM, Cunha FQ, Ferreira SH (1998) *Br J Pharmacol* 124:1369–1374.
8. Rice TW, Bernard GR (2005) *Annu Rev Med* 56:225–248.
9. Eisenfeld L, Krause PJ, Herson VC, Block C, Schick JB, Maderazo E (1992) *Am J Perinatol* 9:5–8.
10. Pereira MA, Sannomiya P, Leme JG (1987) *Diabetes* 36:1307–1314.
11. Fiuza C, Salcedo M, Clemente G, Tellado JM (2000) *J Infect Dis* 182:526–533.
12. Kuritzkes DR (2000) *Clin Infect Dis* 30:256–260.
13. Shirai R, Kadota J, Iida K, Kawakami K, Abe K, Yoshinaga M, Iwashita T, Matsubara Y, Oka M, Kohno S (1998) *Lung* 176:363–370.
14. Brinkman-van der Linden EC, van Ommen EC, van Dijk W (1996) *Glycoconj J* 13:27–31.
15. Barrabes JA, Garcia-Dorado D, Mirabet M, Inserte J, Agullo L, Soriano B, Massaguer A, Padilla F, Lidon RM, Soler-Soler J (2005) *J Am Coll Cardiol* 45:293–299.
16. Chauvet P, Bienvenu JG, Theoret JF, Latour JG, Merhi Y (1999) *J Cardiovasc Pharmacol* 34:597–603.
17. Gunnarsson P, Levander L, Pahlsson P, Grenegard M (2007) *FASEB J* 2:1–11.
18. Solomkin JS, Cotta LA, Brodt JK, Hurst JW, Ogle CK (1984) *J Surg Res* 36:407–412.
19. Jarstrand C, Holmquist L, Wiernik A, Akerlund B, Carlson LA (1990) *J Clin Lab Immunol* 33:69–73.
20. Fournier T, Medjoubi NN, Porquet D (2000) *Biochim Biophys Acta* 1482:157–171.
21. Schmid K, Kaufmann H, Isemura S, Bauer F, Emura J, Motoyama T, Ishiguro M, Nanno S (1973) *Biochemistry* 12:2711–2724.
22. Alves-Filho JC, Benjamim C, Tavares-Murta BM, Cunha FQ (2005) *Mem Inst Oswaldo Cruz* 100(Suppl 1):223–226.
23. Kubes P, Suzuki M, Granger DN (1991) *Proc Natl Acad Sci USA* 88:4651–4655.
24. Spiecker M, Darius H, Kaboth K, Hubner F, Liao JK (1998) *J Leukocyte Biol* 63:732–739.
25. Seidelin JB, Nielsen OH, Strom J (2002) *Intensive Care Med* 28:1613–1618.
26. Logdberg L, Wester L (2000) *Biochim Biophys Acta* 1482:284–297.
27. Knaus WA, Draper EA, Wagner DP, Zimmerman JE (1985) *Crit Care Med* 13:818–829.
28. Clark D (1996) *Guide for the Care and Use of Laboratory Animals* (Natl Acad Press, Washington, DC).
29. Wichterman KA, Baue AE, Chaudry IH (1980) *J Surg Res* 29:189–201.
30. Fraticelli A, Serrano CV, Jr., Bochner BS, Capogrossi MC, Zweier JL (1996) *Biochim Biophys Acta* 1310:251–259.
31. Gomes DA, Reis WL, Ventura RR, Giusti-Paiva A, Elias LL, Cunha FQ, Antunes-Rodrigues J (2004) *Brain Res* 1016:33–39.
32. Bidlingmeyer BA, Cohen SA, Tarvin TL (1984) *J Chromatogr* 336:93–104.
33. Atherton ND (1989) *Clin Chem* 35:975–978.
34. Travis J, Bowen J, Tewksbury D, Johnson D, Pannell R (1976) *Biochem J* 157:301–306.
35. Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) *Electrophoresis* 9:255–262.
36. Castellanos-Serra LR, Fernandez-Patron C, Hardy E, Huerta V (1996) *Electrophoresis* 17:1564–1572.