Alpha-2-Macroglobulin Functions as an Inhibitor of Fibrinolytic, Clotting, and Neutrophilic Proteinases in Sepsis: Studies Using a Baboon Model


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Alpha-2-macroglobulin (α2M) may function as a proteinase inhibitor in vivo. Levels of this protein are decreased in sepsis, but the reason these levels are low is unknown. Therefore, we analyzed the behavior of α2M in a baboon model for sepsis. Upon challenge with a lethal (4 baboons) or a sublethal (10 baboons) dose of Escherichia coli, levels of inactivated α2M (iα2M) steadily increased, the changes being more pronounced in the animals that received the lethal dose. The rise in iα2M significantly correlated with the increase of thrombin-antithrombin III, plasmin-α2-antiplasmin, and, to a lesser extent, with that of elastase-α1-antitrypsin complexes, raising the question of involvement of fibrinolytic, clotting, and neutrophilic proteinases in the inactivation of α2M. Experiments with chromogenic substrates confirmed that thrombin, plasmin, elastase, and cathepsin G indeed had formed complexes with α2M. Changes in α2M similar to those observed in the animals that received E. coli occurred in baboons challenged with Staphylococcus aureus, indicating that α2M formed complexes with the proteinases just mentioned in gram-positive sepsis as well. We conclude that α2M in this baboon model for sepsis is inactivated by formation of complexes with proteinases, derived from activated neutrophils and from fibrinolytic and coagulation cascades. We suggest that similar mechanisms may account for the decreased α2M levels in clinical sepsis.

There is abundant evidence for a role of cytokines in the pathogenesis of sepsis (5, 7, 12, 19). In addition to these mediators, the generation of proteinases may play an important role in the development of sepsis (8, 14, 29). Excessive activity of proteinases in vivo is counteracted by inhibitors, including serine-proteinase inhibitors and alpha-2-macroglobulin (α2M). This latter protein inhibits proteinases of all four catalytic classes (4, 23, 49). Proteinases inhibited by α2M include the coagulation proteinases thrombin and factor Xa (13, 16), the fibrinolytic enzymes urokinase-type and tissue-type plasminogen activators as well as plasmin (24, 31, 36, 49), kallikrein of the contact system (22, 35, 50, 60), the neutrophilic proteinases elastase, cathepsin G, and collagenase (49, 62), and several bacterial proteinases (40). Thus, α2M may play an important role in regulating the hemostatic and inflammatory reactions that occur in sepsis.

The inhibition of proteinases by α2M is due to a unique mechanism: the proteinases cleave α2M at the bait region, which contains a number of peptidyl bonds that are easily hydrolyzed by various proteinases (4, 23). Cleavage of the bait regions then induces the exposure of internal thiolester bonds, which are subsequently hydrolyzed (52, 53). This latter process coincides with a change in conformation of α2M, which results in entrapment of the proteinase (4, 20, 21). A variable amount of the proteinase entrapped is not bound to α2M via its active site and, therefore, retains some activity against small synthetic substrates (4, 42, 49). This property of proteinases bound to α2M has been used to identify the proteinases in complex with α2M (25–27).

Plasma levels of α2M are reduced in patients with sepsis (14, 15, 34, 41, 51) and associated with a fatal outcome in some studies (14, 51) but not in others (1). The formation and subsequent clearance of α2M-proteinase complexes may explain the decrease of α2M levels in sepsis (33, 45, 46). However, increased plasma levels of α2M-proteinase complexes occur only infrequently in patients with sepsis (3). To clarify the role of α2M in sepsis, we have studied the state of α2M in a baboon model for sepsis by using a recently developed assay to quantify the total amount of inactivated α2M (i.e., chemically inactivated α2M and α2M in complex with a proteinase) in plasma. Our results show that, in this animal model for sepsis, α2M is inactivated because of the formation of complexes with proteinases.

**MATERIALS AND METHODS**

**Preparation of Escherichia coli organisms.** Pathogenic E. coli organisms type B were isolated from stool specimens at Children’s Memorial Hospital, Oklahoma City, Okla., as described previously (30). The serotype of this strain is O8K6H1; the lipopolysaccharide phenotype is part rough. The strain produces no hemolysin. After growth in tryptic soybean agar, the microorganisms were lyophilized and stored at 4°C. Shortly before the experiment, the bacteria were reconstituted, washed, and characterized as described by Hinshaw et al. (30).

**Animals.** A mixed breed of Papio c. cynocephalus and Papio c. anubis baboons (Charles River Breeding Laboratories Inc., Wilmington, Del.) were housed at the University of...
Oklahoma Health Sciences Center Animal Facility at the Oklahoma City Zoo. The animals were tuberculosis free, weighed 5 to 17 kg, and had leukocyte concentrations of 5,000 to 10,000 cells per µl and hematocrits exceeding 36%. They were observed for a minimum of 30 days to ensure adequate equilibration prior to experimentation. During recovery from shock, the baboons were observed daily and medically treated as deemed appropriate. Surviving animals were euthanized with sodium pentobarbital after a minimum of 7 days. The clinical and laboratory data of the animals used for this study have been described elsewhere (10).

**Experimental procedures.** Experimental and infusion procedures on the baboons were performed as described previously (9, 56, 57). Briefly, baboons were fasted overnight prior to each experiment and given water ad libitum. After immobilization and proper anesthesia with sodium pentobarbital, the baboons were challenged with a 2-h intravenous infusion of either a lethal dose of *E. coli* (4 baboons), i.e., 4 × 10^10 CFU/kg of body weight, or a sublethal dose of *E. coli* (10 baboons), i.e., 0.4 × 10^10 CFU/kg. The animals receiving the lethal dose died between 6 and 10 h. During the experiments, heart rate, mean systemic arterial pressure, respiration rate, and rectal temperature were monitored hourly for 6 h and daily for 7 days. Hematologic parameters were assessed in blood samples collected at T+0, +30, +60, +120, +180, +240, +300, +360, and +1,440 min as described previously (56). In addition, at each of these time points, 2.5-ml blood samples were collected in 10 mM EDTA and 0.05% (wt/vol) Polybrene (final concentrations) as described previously (3). Hematologic and proper laboratory data of the animals challenged with *E. coli* were euthanized at T+480 min.

All experimental procedures described in this manuscript were performed as described in the NIH guidelines for the use of experimental animals.

Activated Sepharose 4B and zinc chelating Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), hexadimethrine bromide (i.e., Polybrene) was from Janssen Chimica (Beerse, Belgium), and Tween 20 and dimethyl sulfoxide were from J. T. Baker Chemicals Co. (Phillipsburg, N.J.). Purified human neutrophil elastase and cathepsin G were purchased from Elastin Products Co. Inc. (Pacific, Mo.). Human thrombin was a kind gift from K. Mertens of the Department of Coagulation of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, human kallikrein was generously provided by G. Tans from the Department of Biochemistry of the University of Limburg, Maastricht, the Netherlands. Human plasmin and the chromogenic substrates H-d-Pro-Phe-Arg-p-nitroanilide (H-d-Pro-Phe-Arg-pNA; S2302), H-d-Val-Leu-Lys-pNA(S2251), and H-d-Phe-Pip-Arg-pNA(S2238) were purchased from Kabi Vitrum (Stockholm, Sweden); the substrates M4765 (MoO-Suc-Ala-Ala-Pro-Val-pNA) and S7388 (N-Suc-Ala-Ala-Pro-Phe-pNA) were from Sigma Chemical Co. (St. Louis, Mo.). Monospecific rabbit antisera against human α2M (KH-08-P01) was obtained from the Department of Immune Reagents, Red Cross Blood Transfusion Service.

**Purification of human α2M.** Human α2M was purified from human plasma by a modification of the method of Kurecki et al. (37). After differential (NH₄)₂SO₄ precipitation, the α2M preparation was loaded onto a zinc-chelated Sepharose column (2.5 by 11 cm). The column was washed with 0.02 M sodium phosphate–0.15 M NaCl (pH 6.0) at a flow rate of 50 ml/h until the A₂₈₀ was 0.04. Bound proteins were eluted with 0.02 M NaOAc–0.15 M NaCl (pH 5.0). Fractions containing protein were pooled and concentrated. The purified α2M migrated as a homogeneous band with an Mₜ of 184,000 on a sodium dodecyl sulfate (SDS)–4 to 15% polyacrylamide gel under reducing conditions. The preparation was stored at 4°C in the presence of 0.02% sodium azide.

**Assays.** (i) **Quantification of total α2M.** Antigenic levels of α2M in baboon plasma were determined with a Behring nephelometric analyzer (Behringwerke AG, Marburg, Germany) with antisera against human α2M (Behringwerke AG) and as described in the manufacturer's instructions. Results were expressed as levels relative to the baseline. Prechallenge levels of each individual baboon were arbitrarily set at 1.0.

(ii) **Radioimmunoassay for α2M.** Inactivated α2M (iα2M) in baboon plasma was measured by the M1 assay as described by Abbink et al. (2, 3). Briefly, monoclonal antibody (MAb) M1, which binds iα2M (i.e., chemically inactivated and/or complexed α2M) but not native α2M, was coupled to Sepharose and incubated with 50 µl of baboon plasma for 4 h at room temperature by head-over-head rotation. Bound iα2M was then quantified by a subsequent incubation with 153I-anti-α2M antibodies as described previously (2, 3). Levels of iα2M in baboon plasma were expressed as percentages of that in normal baboon plasma in which α2M was completely inactivated by incubation with methylamine chloride (final concentration, 0.2 M; methylamine-treated normal baboon plasma [MA-NBP]) similarly as described for human plasma (3).

(iii) **Chromogenic assay for α2M-proteinase complexes in plasma.** The MAB M1-Sepharose suspension (0.5 ml) was incubated with 50-µl plasma samples as described previously (2, 3). The Sepharose was washed five times with saline and, finally, once with the appropriate substrate buffer (50 mM Tris–100 mM NaCl at pH 7.8 for S2302, at pH 7.4 for S2251, and at pH 8.4 for S2328; and 0.1 M HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]–10% [vol/vol] dimethyl sulfoxide at pH 7.5 for M4765 and S7388). The Sepharose suspensions were brought to 100 µl and incubated with 100 µl of substrate solution in the appropriate buffer (final concentration, 2 mM) under continuous shaking. After 240 min, substrate hydrolysis was stopped by the addition of 200 µl of 50% acetic acid. The Sepharose was pelleted, 200 µl of each supernatant was transferred into enzyme-linked immunosorbent (ELISA) plates, and the A₄₉₀ was measured in microtiter plates (Titertek; Flow Laborato-

(iv) **Other assays.** Plasmin-α2-antiplasmin complexes were measured with a radioimmunoassay that originally was developed for use in human studies (38) and modified for use in
baboons (10). In this assay, MAAb AAP-11, which is directed against a neoepitope on complexed α2-antiplasmin, is used as a catching antibody, and polyclonal radiolabeled rabbit antibodies against human plasmin(ogen) is used as a detecting antibody. Baboon plasma in which a maximal amount of plasmin-α2-antiplasmin was generated by the addition of methylamine and urokinase (10, 38) was used as a standard.

Elastase-α1-antitrypsin complexes were measured by a radioimmunoassay as described previously (43). In this assay, polyclonal rabbit antibodies against human elastase and a radiolabeled MAAb against complexed α1-antitrypsin were used as catching and detecting antibodies, respectively. Results were expressed in nanomoles per liter by reference to a standard curve of normal baboon plasma in which 66.6 nmol of elastase-α1-antitrypsin complexes per liter was generated by incubation of 1 volume of purified human elastase (10 μg/ml in phosphate-buffered saline [PBS]) with 4 volumes of normal baboon plasma for 15 min at room temperature (11). Thrombin-antithrombin III complexes were measured with a commercial ELISA obtained from Behringwerke AG (10).

Analysis of data. In the lethal group of animals, three samples at T+180, one at T+240, and two at T+360 min, and in the sublethal group, six samples at T+240, eight at T+360, and seven at T+440 min were available for the present investigation. At all other time points, 10 and 4 samples were available in the sublethal and lethal groups, respectively.

The parameters which were assessed in the baboons were expressed as means ± standard errors of the means. Most of the parameters studied were not normally distributed. Therefore, the Kruskall-Wallis test was used to assess significant changes in parameters during the observation period. Subsequently, the Wilcoxon-Mann-Whitney rank sum test was used to assess the differences between levels at individual time points and baseline values and to determine the significance of the differences between the animals challenged with a lethal and with a sublethal dose of E. coli. A difference was considered significant, by using a two-tailed probability test, at a P < 0.05 and highly significant at a P of <0.005. Spearman's rank correlation analysis was used to assess the correlation between the parameters.

RESULTS

Course of sepsis in baboons challenged with a lethal or sublethal dose of E. coli. Clinical and biochemical parameters of the animals used for this study have been described elsewhere (10, 11). Briefly, after the challenge with E. coli, marked changes in mean systemic arterial pressure occurred (decreases from 102 ± 3.7 to 81 ± 5.7 mm Hg at T+120 min in the sublethal group and from 105 ± 4.8 to 61 ± 5.0 mm Hg in the lethal group), leukocyte counts (decreases from 7.1 ± 0.9 to 2.4 ± 0.68 cells per mm³ at T+120 min in the sublethal group and from 8.1 ± 1.24 to 1.6 ± 0.16 per mm³ in the lethal group), and platelet counts (decreases from 289 ± 22 to 154 ± 24 cells per mm³ at T+360 min in the sublethal group and from 380 ± 37 to 187 ± 29 cells per mm³ in the lethal group). In the sublethal group, these parameters returned gradually to almost baseline values, whereas in the lethal group they did not (10). The more severe course after the lethal E. coli challenge was also reflected in the changes of activation parameters of the coagulation, fibrinolytic, and complement systems (i.e., compared with baseline values, thrombin-antithrombin III complexes increased 425- and 33-fold, plasmin-α2-antiplasmin complex levels increased 38- and 20-fold, and CSb-9 complex levels increased 12- and 3-fold in the lethal and sublethal groups, respectively [see references 10 and 11]).

Detection of α2M in baboon plasma. Initially, we established whether α2M in baboon plasma could be detected by the M1 assay, which was developed to measure human α2M (3). Serial dilutions of MA-NBP (3) and of untreated normal baboon plasma were tested in this assay. Similarly, human plasma incubated with or without methylamine was also tested as a control. Although the assay for baboon α2M was approximately 10-fold less sensitive than that for human α2M, the dose-response curve of MA-NBP was very reproducible (Fig. 1). In addition, the results obtained with normal baboon plasma showed that the assay was sufficiently sensitive to assess levels of α2M in baboons. The amount of α2M was expressed as a percentage of that in MA-NBP, which was arbitrarily set at 100%. α2M in normal baboon plasma appeared to be 0.5% of that in MA-NBP, i.e., comparable to the levels found in human plasma (3).

Total and inactivated α2M in septic baboons. During the first 6 h after the start of the E. coli infusion, the levels of total α2M did not change significantly from baseline values both in the animals with a lethal sepsis and those with a nonlethal sepsis (Fig. 2A). At 24 h, however, total α2M levels had increased to 1.62 times the baseline value in the sublethal group. No data from the lethal group of baboons were available at this time point, since all animals of this group had died between 6 and 10 h.

In contrast to total α2M, levels of inactivated α2M differed markedly between both groups of animals. During the first 6 h, inactivated α2M levels in the sublethal group increased gradually from a mean baseline value of 0.42% ± 0.06% of the level in MA-NBP to 1.07% ± 0.09% at T+360 min (Fig. 2B), whereas at 24 h, these levels had decreased to 0.66% ± 0.11%. In the lethal group, the increase of inactivated α2M levels was more pronounced, reaching a maximum value of 9.1% ± 1.9% of the level of MA-NBP at T+360 min (Fig. 2B). This increase differed significantly from that in the sublethal group from T+120 min

FIG. 1. Radioimmunoassay for detection of α2M in baboons. Serial dilutions of MA-NBP, or methylamine-treated human plasma (MA-NHP) were tested as described in Materials and Methods. The symbols represent the mean and standard deviation of three titration curves. Values obtained for normal baboon plasma (NBP) and normal human plasma (NHP), both tested at a 1-to-20 dilution, are also shown. The results are expressed as a percentage of the 125I-anti-α2M antibodies that had bound.

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In both a \(a2M\) (A) and inactivated a2M (B) in baboons that received intravenously a lethal or a sublethal dose of \(E. coli\). The differences in a2M between both groups at 120, 180, and 360 min were significant.

and thereafter. Thus, a significant inactivation of a2M occurred in the animals challenged with a lethal dose of \(E. coli\). The inactivation in this group constituted approximately 10% of the total amount of a2M.

**Correlation between levels of a2M and activation parameters of neutrophils, coagulation, and fibrinolysis in septic baboons.** In previous studies (10, 11), we observed increased levels of thrombin-antithrombin III, plasmin-a2-antiplasmin, and elastase-a1-antitrypsin complexes in the plasma samples obtained from the same animals described in this article. These findings indicated the formation or release of activated proteinases of both the fibrinolytic and clotting systems and of neutrophils. To investigate whether the inactivation of a2M was related to the action of these proteinases, we correlated (by Spearman’s rank correlation analysis) the levels of a2M with those of plasmin-a2-antiplasmin, thrombin-antithrombin III, and elastase-a1-antitrypsin complexes. All three complexes correlated significantly with a2M (Table 1). In particular, there was a strong correlation between a2M and thrombin-antithrombin III (Fig. 3A) and between a2M and plasmin-a2-antiplasmin (Fig. 3B) complexes in the lethal group.

Identification of the proteinases responsible for the inactivation of a2M in septic baboons. The results mentioned above suggested that clotting and fibrinolytic proteinases were involved in the inactivation of a2M in the baboons. However, we considered it necessary to obtain more direct evidence that at least part of a2M had arisen from the interaction with fibrinolytic, clotting, and neutrophilic proteinases. We used the method developed by Harpel et al. (25–27) and modified by Abbink et al. (2, 3) to identify which proteinases had formed complexes with a2M in the septic baboons. a2M species were purified from baboon plasma by absorption onto MAb M1-Sepharose and incubated with the chromogenic substrates for plasmin (S2251), kallikrein (S2302), thrombin (S2238), elastase (M4765), and cathepsin G (S7388) as described in Materials and Methods. The increase in a2M in the sublethal group was too small to allow a further characterization by these assays (Fig. 4). However, in plasma samples from the lethal group, several a2M-proteinase complexes could be detected by using the chromogenic substrates. Levels of complexes between a2M and the neutrophilic proteinases (elastase and cathepsin G) increased immediately after the start of the \(E. coli\) infusion, reaching peak values at \(T+360\) min (i.e., 5.08 ± 0.42 and 6.08 ± 0.25 [mean ± standard error of the mean] \(\mu\)M substrate conversion per h for a2M-elastase and a2M-cathepsin G complexes, respectively). The course of a2M-elastase complexes is shown in Fig. 4B. That of a2M-cathepsin G was similar. Complexes of a2M with plasmin, kallikrein, and thrombin reached peak values at \(T+120\) min of 19.21 ± 12.81, 31.17 ± 15.86, and 47.00 ± 15.90 \(\mu\)M/h, respectively. The rate of a2M-plasmin complex formation is shown in Fig. 4A. Those of a2M-thrombin and a2M-kallikrein were similar. The known overlap of specificity of the chromogenic substrates (18), however, prompted further experiments to identify more precisely which proteinases had formed complexes with a2M. Initially, we tested the specificity of the chromogenic substrates to be used by incubating them with performed purified human a2M-proteinase complexes (Table 2). Complexes of a2M and thrombin, plasmin, and kallikrein all hydrolyzed to some extent the chromogenic substrates specific for each of these proteinases, whereas they did not convert the substrates for elastase and cathepsin G. Conversely, a2M-elastase and -cathepsin G complexes slightly hydrolyzed their mutual substrates, whereas they did not react at all with the other substrates used (Table 2). Distinction between the two neutrophil-derived proteinases in complex with a2M could be made by using the chromogenic substrate S7388, since this substrate appeared to be rather specific for a2M-cathepsin G complexes (Table 2). Thus, the experiments with purified a2M-proteinase complexes demonstrated that the chromogenic substrates were not entirely nonspecific. Nevertheless, we used the results of these experiments to estimate the proteinase(s) in complex with a2M in the lethal group of baboons by assuming that the ratio of hydrolysis of chromogenic substrates for each a2M-proteinase complex would be constant and therefore specific. Thus, the hydrolysis of S2302 was plotted against that of S2251 for both purified a2M-plasmin as well as a2M-kallikrein complexes. The values obtained with complexed

**FIG. 2.** Mean levels (and standard errors of the mean) of total a2M (A) and inactivated a2M (B) in baboons that received intravenously a lethal or a sublethal dose of \(E. coli\). The differences in a2M between both groups at 120, 180, and 360 min were significant.

**TABLE 1.** Spearman’s rank correlation analysis of a2M levels with plasmin-a2-antiplasmin, thrombin-antithrombin III, and elastase-a1-antitrypsin complexes in baboons challenged with a sublethal or lethal dose of \(E. coli\)

<table>
<thead>
<tr>
<th>Complexa</th>
<th>Total group of baboons (n = 51)b</th>
<th>Lethal group of baboons (n = 22)</th>
<th>Sublethal group of baboons (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(r)</td>
<td>(P)</td>
<td>(r)</td>
</tr>
<tr>
<td>PAP</td>
<td>0.5780</td>
<td>(8.9 \times 10^{-6})</td>
<td>0.7483</td>
</tr>
<tr>
<td>TAT</td>
<td>0.8421</td>
<td>(9.7 \times 10^{-13})</td>
<td>0.9176</td>
</tr>
<tr>
<td>ELAT</td>
<td>0.7965</td>
<td>(4.6 \times 10^{-12})</td>
<td>0.6968</td>
</tr>
</tbody>
</table>

a PAP, plasmin-a2-antiplasmin; TAT, thrombin-antithrombin III; ELAT, elastase-a1-antitrypsin.

b \(n\), number of observations.
α2M in the baboons were then plotted in the same diagram. As shown in Fig. 5A, values of α2M from the baboons, in particular at the higher levels, fell around the line of the purified α2M-plasmin complexes, suggesting that the hydrolysis of S2302 in the baboons was due in part to the presence of α2M-plasmin complexes. The hydrolysis of S2251 and of S2238 was plotted in a similar graph (Fig. 5B), which shows that almost all baboon data were between the lines of purified α2M-plasmin and α2M-thrombin complexes, indicating that plasmin as well as thrombin complexes contributed to α2M in the lethal group of baboons. Taken together, these experiments suggested that, in the lethal group of baboons, circulating α2M consisted at least in part of α2M-plasmin, α2M-thrombin, α2M-cathepsin G, and α2M-elastase complexes.

α2M in baboons challenged with S. aureus. We had the opportunity to study the changes of α2M in two baboons challenged with S. aureus. In one baboon challenged with $7.3 \times 10^{10}$ CFU/kg of body weight, plasmin-α2-antiplasmin complexes increased to a peak level of 66% of the urokinase-treated MA-NBP standard (i.e., normal baboon plasma in which a maximal amount of plasmin-α2-antiplasmin complexes was generated [10]) at 2 h, whereas thrombin-antithrombin III complexes rose to 628 μg/liter at 8 h (Fig. 6A). Thus, like the animals challenged with E. coli (10), the baboon which had received S. aureus developed a procoagulant state 4 h after the challenge, which was characterized by increasing thrombin-antithrombin III complexes and decreasing plasmin-α2-antiplasmin complexes. Inactivated α2M and elastase-α1-antitrypsin complexes also increased in this animal, reaching peak levels of 19% of that in the MA-NBP standard at 2 h and of 28.9 nmol/liter at 4 h, respectively (Fig. 6B). Similar changes in thrombin-antithrombin III, plasmin-α2-antiplasmin, α2M, and elastase-α1-antitrypsin complexes, although less pronounced, were observed in another baboon that received a challenge, consisting of $6.5 \times 10^{10}$ heat-inactivated S. aureus organisms per kg (data not shown). Thus, the course of these parameters in the baboons challenged with S. aureus was similar to that in the animals that had received E. coli.

**DISCUSSION**

In this study, we demonstrate that in a well-established model for sepsis (30, 55, 58), i.e., baboons challenged with live E. coli, α2M is inactivated by the formation of complexes with proteinases. In addition, similar results were obtained in two animals challenged with S. aureus, suggesting that the observed inactivation of α2M is not a particular feature of E. coli sepsis.

A protease that is inhibited by α2M becomes physically entrapped in the inhibitor but still retains some of its activity against small synthetic substrates (4, 42, 49). Entrapment of the protease by α2M may prevent its detection in the complex by polyclonal or monoclonal antibodies. Therefore, we used the method developed by Harpel et al. (25-27) and modified by Abbink et al. (2, 3) to identify the proteinases that had formed complexes with α2M in the septic baboons. Conversion of chromogenic substrates was not observed with α2M purified from plasma samples obtained before the challenge or obtained from animals challenged with a sublethal dose of E. coli. In contrast, α2M purified from plasma

<table>
<thead>
<tr>
<th>Chromogenic substrate</th>
<th>α2M-thrombin</th>
<th>α2M-plasmin</th>
<th>α2M-kallikrein</th>
<th>α2M-elastase</th>
<th>α2M-cathepsin G</th>
</tr>
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<tbody>
<tr>
<td>S2238 (thrombin)</td>
<td>2,805</td>
<td>1,355</td>
<td>1,932</td>
<td>92</td>
<td>76</td>
</tr>
<tr>
<td>S2251 (plasmin)</td>
<td>229</td>
<td>847</td>
<td>344</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>S2302 (kallikrein)</td>
<td>1,582</td>
<td>1,452</td>
<td>2,518</td>
<td>66</td>
<td>78</td>
</tr>
<tr>
<td>M4765 (elastase)</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>1,248</td>
<td>520</td>
</tr>
<tr>
<td>S7388 (cathepsin G)</td>
<td>16</td>
<td>ND</td>
<td>ND</td>
<td>5</td>
<td>153</td>
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</table>

*ND, not detected.
DE part of a2M
oa2M-plasmin,
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activation of a2M. How-
ever, comparison of the conversion of the substrates S2302 and S2251 by preformed a2M-kallikrein and a2M-plasmin complexes with that by a2M complexes from the baboons (Fig. 5) suggested that most of the conversion of S2302 by a2M was due to plasmin in complex with a2M. Pixley et al. (47) have shown that a2M-kallikrein complexes, measured by an immunochemical assay (35), also increase in baboons challenged with a lethal dose of E. coli, and therefore, kallikrein very likely also contributes to the inactivation of a2M in these animals. In agreement herewith, conversion of S2302 and S2251 by a2M in baboon plasma samples with moderately increased levels was between that by purified kallikrein- and plasmin-a2M complexes (Fig. 5A), suggesting the presence of both plasmin- and kallikrein-a2M complexes in these samples.

Our results do not allow definite conclusions regarding the proportion of a2M that had arisen by interaction with host proteinases. However, assuming that the concentration of a2M in the blood of humans and baboons is similar, i.e., 20 to 50 μM, and that the chromogenic substrates used are equally well converted by baboon proteinases as they are by their human counterparts, we could estimate (by comparing the conversion rate of the substrates by the preformed human a2M complexes [Table 2] with that by a2M in the

samples obtained from baboons after a lethal challenge with E. coli hydrolized several chromogenic substrates (Fig. 4), demonstrating that proteinases had bound to this a2M and that this a2M at least in part consisted of a2M-protease complexes.

The conversion of chromogenic substrates M4765 (elastase) and S7388 (cathepsin G) by preformed a2M complexes appeared to be rather specific for elastase-a2M and cathepsin G-a2M complexes (Table 2). This led us to conclude that part of a2M in the septic baboons was inactivated by forming complexes with proteinases derived from activated neutro-

FIG. 5. Plot of the conversion of the chromogenic substrates S2251 versus that of S2302 (A) or that of S2238 (B) by a2M-proteinase complexes in the group of baboons challenged with a lethal dose (n = 22). For comparison, results obtained with purified a2M-kallikrein, a2M-plasmin, and a2M-thrombin complexes are also shown.

The nature of the proteinases that were responsible for conversion of the substrates for plasmin, thrombin, and kallikrein was more difficult to establish, since these substrates are all hydrolyzed to a variable extent by these three serine proteinases, as was also observed for preformed complexes with a2M (Table 2). We think that at least thrombin and plasmin contributed to the inactivation of a2M for the following reasons. First, thrombin-antithrombin III complexes as well as plasmin-a2-antiplasmin complexes correlated strongly with total ia2M (Fig. 3). Second, by using a radioimmunoassay for a2M-plasmin complexes (39), we found immunochemical evidence for the presence of these complexes in plasma harvested from animals receiving a lethal dose of E. coli (data not shown). Attempts to further characterize the a2M-proteinase complexes by SDS-polyacrylamide gel electrophoresis and immunoblotting, however, failed because of the limited number of plasma samples available for analysis.

The observed conversion of the chromogenic substrate S2302 by ia2M in the baboons at first glance suggested involvement of kallikrein in the inactivation of a2M. However, comparison of the conversion of the substrates S2302 and S2251 by preformed a2M-kallikrein and a2M-plasmin complexes with that by a2M complexes from the baboons (Fig. 5) suggested that most of the conversion of S2302 by a2M was due to plasmin in complex with a2M. Our results (Fig. 5A) suggested the presence of both plasmin- and kallikrein-a2M complexes in these samples.

Our results do not allow definite conclusions regarding the proportion of a2M that had arisen by interaction with host proteinases. However, assuming that the concentration of a2M in the blood of humans and baboons is similar, i.e., 20 to 50 μM, and that the chromogenic substrates used are equally well converted by baboon proteinases as they are by their human counterparts, we could estimate (by comparing the conversion rate of the substrates by the preformed human a2M complexes [Table 2] with that by a2M in the
baboon plasma samples [see, for example, Fig. 4]) that the highest concentrations of ia2M in the baboons receiving the lethal dose of E. coli was approximately 2 μM and that up to 0.2 μM, i.e., 10% of the total amount of ia2M, could be explained by the presence of complexes of α2M and the endogenous proteinases plasmin, thrombin, kallikrein, elastase, and cathepsin G. An explanation for this discrepancy between the total levels of ia2M and the levels of α2M complexes actually measured in the baboon samples may be that proteinases other than those mentioned contributed to the inactivation as well. For example, recent studies suggest that activated protein C also may form complexes with α2M (28, 32). This proteinase is probably activated in the septic baboons (56, 59). To what extent activated protein C contributes to the inactivation of α2M remains to be established.

Another explanation for the discrepancy between levels of α2M complexes and those of the total amount of ia2M in the septic baboons may be that α2M was inactivated not only by proteinases but also by chemical compounds such as methylamine (6, 17, 21) or oxygen radicals (48). Inactivation of α2M by chemical compounds has never been demonstrated to occur in vivo. However, a recent study of the state of α2M in synovial fluid from rheumatoid arthritis patients suggested that up to 90% of the observed inactivation in this condition was due to interaction with oxygen radicals originating from activated neutrophils (2). Activation of neutrophils also occurs in sepsis (43). Therefore, it is conceivable that α2M in the septic baboons was inactivated not only by interacting with proteinases but also by chemical compounds such as oxygen radicals.

In a previous study, we observed increased levels of ia2M in only 4 of 48 patients with sepsis (3). In contrast to these findings, a pronounced increase in ia2M to 10% of the total amount of α2M occurred in the lethal group of baboons. The difference in ia2M levels between the septic patients and the animals with lethal sepsis might be due to species-related differences in the clearance of ia2M or to the shorter intervals between blood sampling, i.e., 1 h in the animal model versus 6 h in the patients (3). In addition, the intensity of the trigger may have also contributed to the generation of ia2M. The activation of the coagulation, fibrinolytic, and complement systems and of neutrophils as well as the concomitant changes in organ functions and hemodynamic parameters occurs within hours after the challenge with E. coli (10, 11, 47). In contrast, in patients with sepsis, these processes tend to develop more slowly, evolving over days. Most studies on the role of coagulation, fibrinolysis, and neutrophils in animal models for sepsis have been done in animals challenged with E. coli or endotoxin. For example, there is no study documenting a procoagulant state, such as that induced by endotoxin (54, 61) or live E. coli (10), in gram-positive sepsis. The observations in the baboons challenged with live S. aureus (Fig. 6) or heat-inactivated S. aureus (data not shown) suggest that such a procoagulant state also occurs in gram-positive sepsis. The inactivation pattern of α2M in these animals was also remarkably similar to that observed in the animals challenged with E. coli. These observations as well as a recent study in which rabbits were challenged with Staphylococcus epidermidis (63) demonstrate that, with respect to pathogenic mechanisms, gram-negative and gram-positive sepsis are similar conditions.

In conclusion, we show that in baboons challenged with E. coli and S. aureus, α2M is inactivated by forming complexes with proteinases derived from activated coagulation, fibrinolytic and contact cascades, and activated neutrophils. These results suggest that α2M regulates the activities of these proteinases during the inflammatory response that occurs in sepsis.

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