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


Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands; Chiron Corporation, Emeryville, California 94608; and Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Received 24 May 1993/Returned for modification 21 July 1993/Accepted 23 September 1993

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 catalytic activity against 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 forms 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 region 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 O6K6:H1; the lipopolysaccharide phenotype is por Aa 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, weighted 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). After centrifugation, the resulting plasma samples were stored in aliquots at −70°C and used in the present study.

One baboon was challenged with 7.3 × 10^10 live CFU of *Staphylococcus aureus* per kg of body weight, and another baboon was challenged with 6.5 × 10^10 heat-inactivated CFU of *S. aureus* per kg. The infusion procedure, monitoring of the animals, and sampling of the blood were identical to those procedures for the animals challenged with *E. coli*. These two animals 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.

Chemically inactivated 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 Elastine Products Co. Inc. (Pacific, Mo.). Human thrombin was kindly given by 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 (MoOC-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 Mₐ of 184,000 on a sodium dodecyl sulfate (SDS)–4% 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 antiserum 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 (iox2M) in baboon plasma was measured by the M1 assay as described by Abbink et al. (2, 3). Briefly, monoclonal antibody (Mab) M1, which binds iox2M (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 iox2M was then quantified by a subsequent incubation with 151I-anti-α2M antibodies as described previously (2, 3). Levels of iox2M 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 S2238; and 0.1 M HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], pH 7.0 for S2351). 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 A₄₀₀ was measured in microtiter plates (Titertek; Flow Laboratories, Inglewood, Calif.). Finally, the amount of pNA released from the chromogenic substrate was calculated (ε = 10,000 mol−1 liter−1). Results were expressed as micromoles of substrate hydrolyzed per hour. Preformed complexes of α2M and elastase, kallikrein, plasmin, thrombin, and cathepsin G were prepared by incubating these proteinases at a concentration of 1 μM with a twice-molar excess of α2M for 90 min at room temperature. Thereafter, 50 μl of serial dilutions of each α2M-proteinase complex mixture was tested in the chromogenic assay for α2M-proteinase complexes as described above.

(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, MAb 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 methylvamine 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 MAb 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 37°C (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+1,440 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 levels 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 pm³ 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 C5b-9 complex levels increased 12- and 3-fold in the lethal and sublethal groups, respectively [see references 10 and 11]).

Detection of io2M in baboon plasma. Initially, we established whether io2M in baboon plasma could be detected by the M1 assay, which was developed to measure human io2M (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 methylvamine was also tested as a control. Although the assay for baboon io2M was approximately 10-fold less sensitive than that for human io2M, 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 io2M in baboons. The amount of io2M was expressed as a percentage of that in MA-NBP, which was arbitrarily set at 100%. io2M 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 io2M differed markedly between both groups of animals. During the first 6 h, io2M 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 io2M 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
and thereafter. Thus, a significant inactivation of α2M 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 α2M.

**Correlation between levels of iα2M 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-α2-antiplasmin, and elastase-α1-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 α2M was related to the action of these proteinases, we correlated (by Spearman’s rank correlation analysis) the levels of iα2M with those of plasmin-α2-antiplasmin, thrombin-antithrombin III, and elastase-α1-antitrypsin complexes. All three complexes correlated significantly with iα2M (Table 1). In particular, there was a strong correlation between iα2M and thrombin-antithrombin III (Fig. 3A) and between iα2M and plasmin-α2-antiplasmin (Fig. 3B) complexes in the lethal group.

**Identification of the proteinases responsible for the inactivation of α2M in septic baboons.** The results mentioned above suggested that clotting and fibrinolytic proteinases were involved in the inactivation of α2M in the baboons. However, we considered it necessary to obtain more direct evidence that at least part of iα2M 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 α2M in the septic baboons. iα2M 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 iα2M 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 α2M-proteinase complexes could be detected by using the chromogenic substrates. Levels of complexes between α2M and the neutrophilic proteinases (elastase and cathepsin G) increased immediately after the start of the *E. coli* infusion, reaching peak values at 7 + 2 min (i.e., 5.08 ± 0.42 and 6.08 ± 0.25 [mean ± standard error of the mean] μM substrate conversion per h for α2M-elastase and α2M-cathepsin G complexes, respectively). The course of α2M-elastase complexes is shown in Fig. 4B. That of α2M-cathepsin G was similar. Complexes of α2M with plasmin, kallikrein, and thrombin reached peak values at 7 + 2 min of 19.21 ± 12.81, 31.17 ± 15.86, and 47.00 ± 15.90 μM/h, respectively. The rate of α2M-plasmin complex formation is shown in Fig. 4A. Those of α2M-thrombin and α2M-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 α2M. Initially, we tested the specificity of the chromogenic substrates to be used by incubating them with performed purified human α2M-proteinase complexes (Table 2). Complexes of α2M 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, α2M-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 α2M could be made by using the chromogenic substrate S7388, since this substrate appeared to be rather specific for α2M-cathepsin G complexes (Table 2). Thus, the experiments with purified α2M-proteinase complexes demonstrated that the chromogenic substrates were not entirely monospecific. Nevertheless, we used the results of these experiments to estimate the proteinase(s) in complex with α2M in the lethal group of baboons by assuming that the ratio of hydrolysis of chromogenic substrates for each α2M-proteinase complex would be constant and therefore specific. Thus, the hydrolysis of S2251 for both purified α2M-plasmin as well as α2M-kallikrein complexes. The values obtained with complexed...
\( \alpha2M \) in the baboons were then plotted in the same diagram. As shown in Fig. 5A, values of \( \alpha2M \) from the baboons, in particular at the higher levels, fell around the line of the purified \( \alpha2M \)-plasmin complexes, suggesting that the hydrolysis of S2302 in the baboons was due in part to the presence of \( \alpha2M \)-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 \( \alpha2M \)-plasmin and \( \alpha2M \)-thrombin complexes, indicating that plasmin as well as thrombin complexes contributed to \( \alpha2M \) in the lethal group of baboons. Taken together, these experiments suggested that, in the lethal group of baboons, circulating \( \alpha2M \) consisted at least in part of \( \alpha2M \)-plasmin, \( \alpha2M \)-thrombin, \( \alpha2M \)-cathepsin G, and \( \alpha2M \)-elastase complexes.

\( \alpha2M \) in baboons challenged with \( S. \) \textit{aureus}. We had the opportunity to study the changes of \( \alpha2M \) in two baboons challenged with \( S. \) \textit{aureus}. In one baboon challenged with 7.3 \times 10^{10} \text{CFU/kg of body weight}, plasmin-\( \alpha2M \)-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-\( \alpha2M \)-antiplasmin complexes was generated [10]) at 2 h, whereas thrombin-\( \alpha2M \)-antithrombin III complexes rose to 628 ng/liter at 8 h (Fig. 6A). Thus, like the animals challenged with \( E. \) \textit{coli} (10), the baboon which had received \( S. \) \textit{aureus} developed a procoagulant state 4 h after the challenge, which was characterized by increasing thrombin-\( \alpha2M \)-antithrombin III complexes and decreasing plasmin-\( \alpha2M \)-antiplasmin complexes. Inactivated \( \alpha2M \) and elastase-\( \alpha2M \)-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).

In this study, we demonstrate that in a well-established model for sepsis (30, 55, 58), i.e., baboons challenged with live \( E. \) \textit{coli}, \( \alpha2M \) is inactivated by the formation of complexes with proteinases. In addition, similar results were obtained in two animals challenged with \( S. \) \textit{aureus}, suggesting that the observed inactivation of \( \alpha2M \) is not a particular feature of \( E. \) \textit{coli} sepsis.

A proteinase that is inhibited by \( \alpha2M \) becomes physically entrapped in the inhibitor but still retains some of its activity against small synthetic substrates (4, 42, 49). Entrapment of the proteinase by \( \alpha2M \) 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 \( \alpha2M \) in the septic baboons. Conversion of chromogenic substrates was not observed with \( \alpha2M \) purified from plasma samples obtained before the challenge or obtained from animals challenged with a sublethal dose of \( E. \) \textit{coli}. In contrast, \( \alpha2M \) purified from plasma

**TABLE 2. Hydrolysis of chromogenic substrates (final concentration, 2 mM) by 50 \( \mu \text{L} \) of preformed \( \alpha2M \)-proteinase complexes (1 \( \mu \text{M} \)) absorbed onto MAb M1-Sepharose**

<table>
<thead>
<tr>
<th>Chromogenic substrate</th>
<th>( \alpha2M )-thrombin</th>
<th>( \alpha2M )-plasmin</th>
<th>( \alpha2M )-kallikrein</th>
<th>( \alpha2M )-elastase</th>
<th>( \alpha2M )-cathepsin G</th>
</tr>
</thead>
<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>NDa</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>
</tr>
</tbody>
</table>

* ND, not detected.
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 α2M (Table 2). We think that at least thrombin and plasmin contributed to the inactivation of α2M for the following reasons. First, thrombin-antithrombin III complexes as well as plasmin-α2-antiplasmin complexes correlated strongly with total ια2M (Fig. 3). Second, by using a radioimmunoassay for α2M-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 α2M-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 ια2M in the baboons at first glance suggested involvement of kallikrein in the inactivation of α2M. However, comparison of the conversion of the substrates S2302 and S2251 by preformed α2M-kallikrein and α2M-plasmin complexes with that by α2M complexes from the baboons (Fig. 5) suggested that most of the conversion of S2302 by ια2M was due to plasmin in complex with α2M. Paxley et al. (47) have shown that α2M-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 α2M in these animals. In agreement herewith, conversion of S2302 and S2251 by ια2M in baboon plasma samples with moderately increased levels was between that by purified kallikrein- and plasmin-α2M complexes (Fig. 5A), suggesting the presence of both plasmin- and kallikrein-α2M complexes in these samples.

Our results do not allow definite conclusions regarding the proportion of ια2M that had arisen by interaction with host proteinases. However, assuming that the concentration of α2M 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 α2M complexes [Table 2] with that by ια2M in the

FIG. 4. Conversion of the chromogenic substrates for plasmin (S2251; A) and for elastase (M4765; B) by α2M-proteinase complexes purified by absorption onto M1-Sepharose from plasma samples from baboons challenged intravenously with a lethal or sublethal dose of E. coli.

FIG. 5. Plot of the conversion of the chromogenic substrates S2251 versus that of S2302 (A) or that of S2238 (B) by α2M-protei

nase complexes in the group of baboons challenged with a lethal dose (n = 22). For comparison, results obtained with purified α2M-kallikrein, α2M-plasmin, and α2M-thrombin complexes are also shown.
baboon plasma samples [see, for example, Fig. 4]) that the highest concentrations of iα2M 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 iα2M, could be explained by the presence of complexes of α2M and the endogenous proteases plasmin, thrombin, kallikrein, elastase, and cathepsin G. An explanation for this discrepancy between the total levels of iα2M and the levels of α2M complexes actually measured in the baboon samples may be that proteases 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 protease 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 iα2M in the septic baboons may be that α2M was inactivated not only by proteases but also by chemical compounds such as methylene (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 proteases but also by chemical compounds such as oxygen radicals.

In a previous study, we observed increased levels of iα2M in only 4 of 48 patients with sepsis (3). In contrast to these findings, a pronounced increase in iα2M to 10% of the total amount of α2M occurred in the lethal group of baboons. The difference in iα2M levels between the septic patients and the animals with lethal sepsis might be due to species-related differences in the clearance of iα2M 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 iα2M. 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 proteases derived from activated coagulation, fibrinolytic, and contact cascades, and activated neutrophils. These results suggest that α2M regulates the activities of these proteases during the inflammatory response that occurs in sepsis.

ACKNOWLEDGMENTS

We thank Wim Schasberg and Ed Nieuwenhuis for their assistance in the statistical analysis of our data. This study was financially supported in part by grant 900-512-121 from the Netherlands Organization for Scientific Research.

REFERENCES

9. Creasey, A. A., P. Stevent, J. Kenney, A. C. Allison, K. Warren,


