Thrombosis and Haemostasis – © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 62 (2) 694–698 (1989)

Effect of Heparin and/or Antithrombin III on the Generation of Endotoxin-Induced Plasminogen Activator Inhibitor

C. Gómez, J. A. Páramo, M. Colucci*, and E. Rocha

From the Haematology Service, University Clinic, University of Navarra, Pamplona, Spain and the Institute of General Pathology*, University of Bari, Bari, Italy

Key words

Disseminated intravascular coagulation – Endotoxin – Heparin – Antithrombin III – Plasminogen activator inhibitor

Summary

It has been experimentally shown that endotoxin induces a significant increase in the blood levels of a plasminogen activator inhibitor (PAI). We evaluated the effect of different doses of heparin (5 to 20 IU kg^{-1} h^{-1}), antithrombin III (10 to 40 U kg^{-1} h^{-1} and 240 U/kg as bolus) and of a combination of the two on: 1) the elevation of PAI activity, 2) fibrin deposition in kidneys and 3) mortality in rabbits infused with *E. coli* lipopolysaccharide. Our results show that heparin plus AT III is able to significantly reduce the generation of endotoxin-induced PAI activity in rabbits' circulation. Low dose of heparin and a bolus injection of AT III both cause a decrease in the generation of PAI at 2 but not at 6 hours of endotoxin infusion. Moreover, fibrin deposits in kidneys of animals receiving heparin plus AT III or a bolus injection of AT III were significantly reduced as compared to control rabbits. The association between low levels of PAI and decreased fibrin deposits is strengthened by the significant correlation ($p < 0.05$) found between these two parameters. Finally, the plasma levels of PAI activity at 2 and 6 hours of endotoxin infusion in surviving animals were lower than those observed in animals that died within 2 hours after the end of treatment. We conclude that heparin plus AT III partially prevents the endotoxin-induced generation of PAI activity which seems to correlate with the reduced presence of fibrin deposits in kidneys and with a reduced mortality.

Introduction

Although disseminated intravascular coagulation (DIC) is a common clinical syndrome related to sepsis (1), its mechanism has not been established. Endotoxin, which is released from the cell wall of gram negative bacteria, is thought to play a relevant role in the pathogenesis of DIC associated with septicemia. Endotoxin may trigger blood coagulation by causing endothelial damage and/or by stimulating the generation of procoagulant activity of endothelial cells (2–4). Impairment of the fibrinolytic system may also contribute to DIC by retarding the clearance of fibrin from circulation (5). It has been recently shown that endotoxin induces a significant increase in the blood levels of a fast-acting plasminogen activator inhibitor (PAI) (6), which is thought to play a role in different clinical conditions related to thrombotic phenomena (7–12). Whether reduction in the PAI concentration represents a therapeutic approach to endotoxin-induced DIC has yet to be clarified.

We studied the effect of heparin and/or antithrombin III (AT III) on the generation of PAI activity in the blood of rabbits infused with endotoxin and the possible relationship between PAI levels, fibrin deposition and mortality.

Correspondence to: E. Rocha, Haematology Service, University Clinic of Navarra, Apartado 192, 31080-Pamplona, Spain

Materials and Methods

Materials

Escherichia coli lipopolysaccharide (LPS) was purchased from Sigma Chemical Co., St. Louis, MO. Plasminogen was prepared by affinity chromatography as described (13). Fibrinogen fragment was obtained by digestion of fibrinogen with CNBr as described (14). Two-chain melanoma cell tissue-type plasminogen activator (t-PA) was kindly provided by Dr. Collen (Leuven, Belgium). The preparation used in the present study had a specific activity of 500,000 IU/mg by comparison with the International Reference preparation of t-PA. Chromogenic substrate S-2251 was obtained from Kabi Diagnostica, Sweden. Urokinase (UK) was purchased from Roger Lab, Barcelona, Spain (a preparation of 100,000 IU was used). Sodium heparin was purchased from Roger Lab, Spain. Human antithrombin III was provided by Landerlan Lab, Madrid, Spain and prepared by the method of Camacho et al. (15).

Experimental Studies

Male New Zealand rabbits, weighing 2–2.5 kg were anaesthetized by intravenous infusion of 10 mg/kg body wt of Nembutal (Abbot Laboratories) via a marginal ear vein, where a catheter was placed to administer treatment. DIC was induced in 76 rabbits by intravenous infusion, via the contralateral marginal ear vein, of 20 μg kg^{-1} h^{-1} of endotoxin during 6 hours. Blood samples were taken via a catheter inserted into a femoral vein.

Surviving rabbits were sacrificed 2 hours after endotoxin infusion, by IV injection of 60 mg/kg body wt of Nembutal, for histological examination of kidney sections.

Treatment Schedules

Treatment was started simultaneously with endotoxin infusion. Four different groups were established: (1) Control group. Eleven animals were used as controls and were infused with saline solution (10 ml/h) during 6 hours. (2) Heparin group. Twenty-five rabbits were infused with different doses of sodium heparin (Roger Lab, Barcelona), diluted in 60 ml of saline, during 6 hours: 9 received 5 IU kg^{-1} h^{-1}, 8 received 10 IU kg^{-1} h^{-1} and 8 received 20 IU kg^{-1} h^{-1}. (3) AT III group. Thirty-two rabbits were treated with different doses of human AT III diluted in 60 ml of saline: 8 received a bolus dose of 240 U/kg immediately before endotoxin infusion. The remaining were divided into three groups of 8 animals which received 10, 20 or 40 U kg^{-1} h^{-1} during 6 hours. (4) Heparin plus AT III group. Eight rabbits received an infusion of heparin (10 IU kg^{-1} h^{-1}) plus AT III (20 U kg^{-1} h^{-1}) diluted in 60 ml of saline for 6 hours.

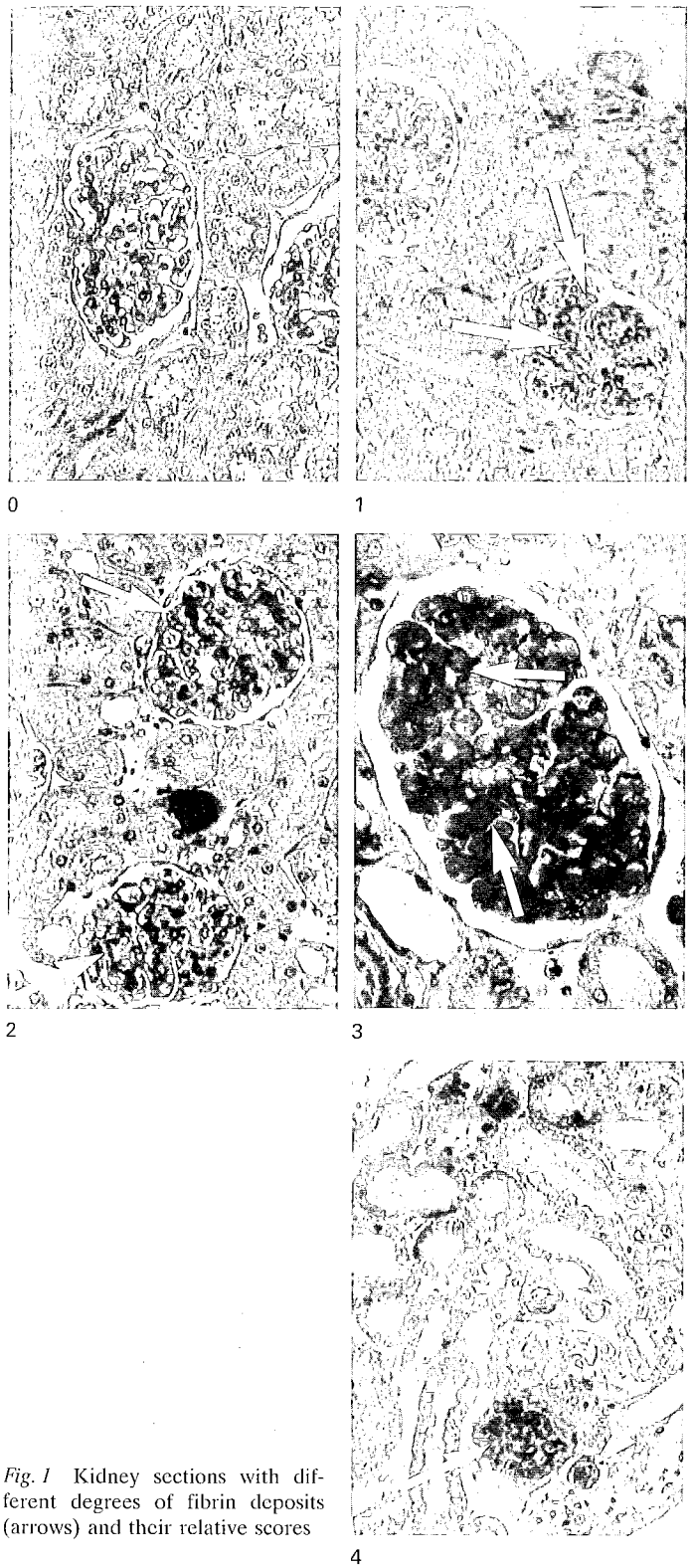

Fig. 1 Kidney sections with different degrees of fibrin deposits (arrows) and their relative scores

Laboratory Methods

Blood samples were taken before endotoxin, at 2 and at 6 hours of infusion. Plasma was immediately prepared by centrifugation and kept on ice or frozen at −70° C until tested. Platelet count and fibrinogen concentration were determined according to standard laboratory assays. Fibrinogen degradation products (FDP) were assayed by the method of Merskey et al. (16) using rabbit fibrinogen (Sigma Chemical Co.) and specific antiserum (Nordic Immunology, Belgium).

t-PA inhibitor capacity of rabbit plasma (refer to as PAI activity) was measured by an amidolytic assay as previously described (6). Two-chain t-PA (10 IU/ml final concentration) was added to diluted plasma sample and incubated during 5 min at 37° C. Samples were then acidified with M/6 HCl, neutralized with N/6 NaOH and tested for t-PA activity by the addition of purified human plasminogen, CNBr fibrinogen and S-2251. t-PA standard curve was processed in the same way. PAI activity is expressed in units of t-PA inhibited per mililiter. Assays performed with single-chain t-PA gave identical results.

Plasma samples to be analyzed by SDS-PAGE were first incubated at 37 °C for 5 min in the presence of t-PA (250 IU/ml final concentration). Ten µl of 1/20 diluted sample was then subjected to electrophoresis. SDS-PAGE on mini slab gels (Min Protean II, BIO-RAD, Richmond, CA) was carried out using resolving gels of 10% acrylamide and stacking gels of 4% acrylamide. Fibrin autography was performed as reported (17).

Histological Examination

Sections from kidneys used for histological examination were stained with hematoxylin-cosin and Masson's Trichrome for fibrin analysis. Tissue sections were examined in blind by a histologist and scored on a scale from 0 to 4 as follows: (0) no fibrin, (1) partial fibrin deposit in some glomeruli, (2) partial deposit in all glomeruli, (3) large quantity of fibrin in all glomeruli, (4) fibrin thrombi in the glomerular capillaries and in other non-capillary vessels (Fig. 1).

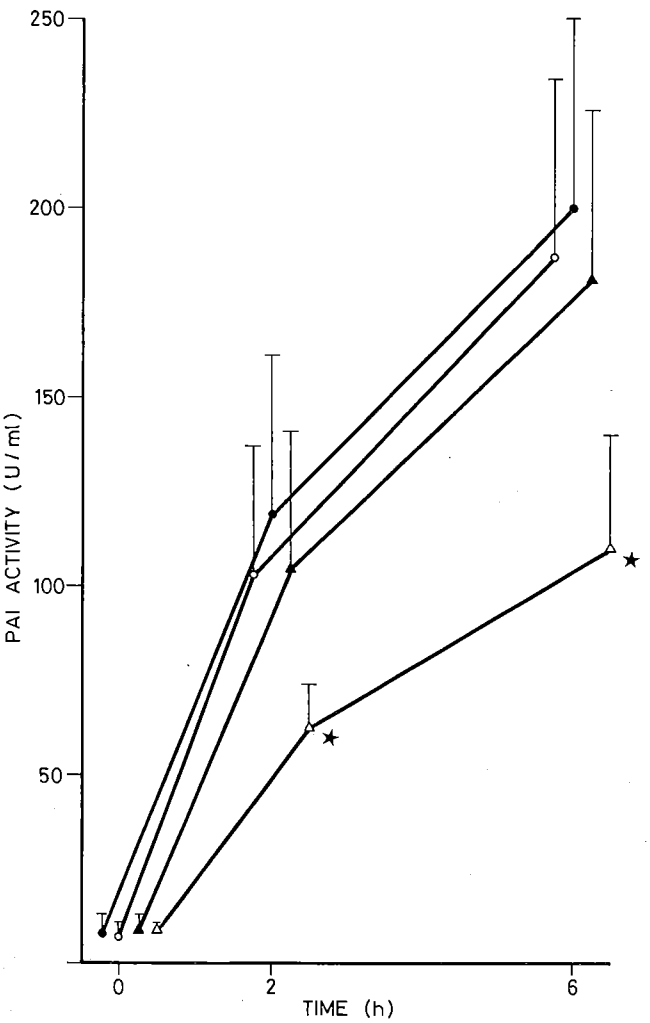

Fig. 2 Generation of PAI activity in rabbit plasma during LPS infusion. (●) control, (○) heparin 5–20 IU kg^{-1} h^{-1} (▲) AT III 10 to 240 U kg^{-1} h^{-1}, (△) heparin 10 IU kg^{-1} h^{-1} plus AT III 20 U kg^{-1} h^{-1}. * $p < 0.01$ as compared to the control group

Table 1 PAI response related to the different treatment schedules. Results are expressed in U/ml as mean ± SD

Group	Number	Basal	2 h increase	6 h increase
Control	11	8.7 ± 3.6	118.9 ± 41.3	200.6 ± 54.6
Heparin 5 IU kg^{-1} h^{-1}	9	7.3 ± 2.2	75.4 ± 15.5*	155.4 ± 36.5
Heparin 10 IU kg^{-1} h^{-1}	8	8.0 ± 3.8	118.8 ± 32.1	170.6 ± 42.4
Heparin 20 IU kg^{-1} h^{-1}	8	7.6 ± 2.1	95.7 ± 51.2	216.1 ± 61.4
AT III 10 U kg^{-1} h^{-1}	8	8.9 ± 3.7	117.0 ± 31.4	176.8 ± 45.8
AT III 20 U kg^{-1} h^{-1}	8	8.0 ± 5.4	87.6 ± 17.0	174.6 ± 46.9
AT III 40 U kg^{-1} h^{-1}	8	9.5 ± 2.0	113.4 ± 48.3	191.3 ± 52.4
AT III 240 U/kg (bolus)	8	9.2 ± 1.6	70.1 ± 12.4*	150.1 ± 29.1
Heparin 10 IU kg^{-1} h^{-1} + AT III 20 U kg^{-1} h^{-1}	8	9.1 ± 1.7	63.11 ± 11.2**	110.5 ± 30.1*

* $p < 0.05$, ** $p < 0.01$ as compared to the control group

Statistical Methods

Results are expressed as mean ± SD. Two-way analysis of variance followed by Tukey's multiple comparison test for all pairs was applied for the purposes of group comparison.

Results

Effect of AT III and/or Heparin Treatments on the PAI Induced by Endotoxin

Fig. 2 shows the plasma levels of PAI activity, at 2 and 6 h of endotoxin infusion in rabbits grouped according to treatment type, regardless of drug dosage.

Although a marked and time-related increase in plasma PAI activity was observed in all groups, analysis of variance revealed that the intensity of the PAI response to endotoxin in rabbits receiving heparin plus AT III was significantly lower as compared to the control group (about 50% reduction). Similar results were obtained when the PAI levels were expressed as percentage of preinfusion values.

As shown in Table 1, the PAI activity observed after 2 hours of endotoxin infusion in rabbits treated with low doses of heparin (5 IU kg^{-1} h^{-1}) was lower than that found in controls ($p < 0.05$). A similar partial effect was observed when a bolus injection of AT III was given to the animals prior to endotoxin infusion. Neither heparin (10 and 20 IU kg^{-1} h^{-1}) nor AT III (10, 20 and 40 U kg^{-1} h^{-1}) appeared to significantly affect the generation of PAI.

When heparin, AT III or a combination of the two were added in vitro to PAI-rich plasma samples obtained from LPS-treated rabbits, 6 hours after the start of infusion, no effect on PAI activity was demonstrated (not shown).

Fibrin autography of plasma samples taken at 6 hours of LPS infusion did not reveal the presence of fibrinolytic activity, neither in the region corresponding to free activators nor in that corresponding to activator-inhibitor complex (not shown). However, when samples were supplemented with t-PA (250 IU/ml) the formation of enzyme-inhibitor complex with an apparent M_r 100 kDa was observed (Fig. 3). The lysis zone of this complex was greater in LPS-treated animals than in animals simultaneously treated with AT III-heparin, confirming a reduction in inhibitor activity in this latter sample.

Laboratory Parameters of DIC

Infusion of endotoxin produced overt intravascular coagulation in control animals as indicated by the marked changes in platelet count, fibrinogen and FDP levels. All treatments significantly reduced the generation of FDPs without affecting drops in platelet count and fibrinogen levels (Fig. 4).

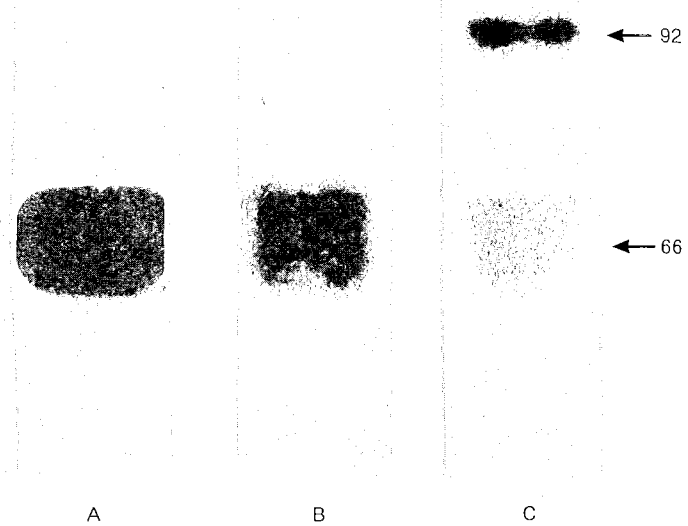

Fig. 3 t-PA binding capacity of rabbit plasma at 6 hours of LPS infusion. A: control without LPS infusion; B: treated with AT III 20 U kg^{-1} h^{-1} plus heparin 10 IU kg^{-1} h^{-1} and LPS infusion; C: control with LPS infusion. Molecular weights are shown as $M_r \times 10^{-3}$

Histological Findings

Intense fibrin thrombi were observed in the majority of glomeruli of kidney sections from control animals. Infusion of heparin or AT III had little effect on the extent of fibrin deposition while both a bolus injection of AT III and an infusion of AT III-heparin significantly reduced it (Table 2). In tissue sections from these latter groups only a small proportion of glomeruli were stained with fibrin-specific dye, and only faintly so.

When plasma inhibitor levels at 6 hours of endotoxin infusion were plotted against the score of fibrin deposits, a positive correlation ($r = 0.27$, $p < 0.05$) was found, suggesting that the two phenomena may be related.

Mortality Rate

In the control group, 5 out of 11 rabbits died shortly after the endotoxin infusion while all "treated" animals, except 1 in the heparin group, survived until the end of the experiment (8 h),

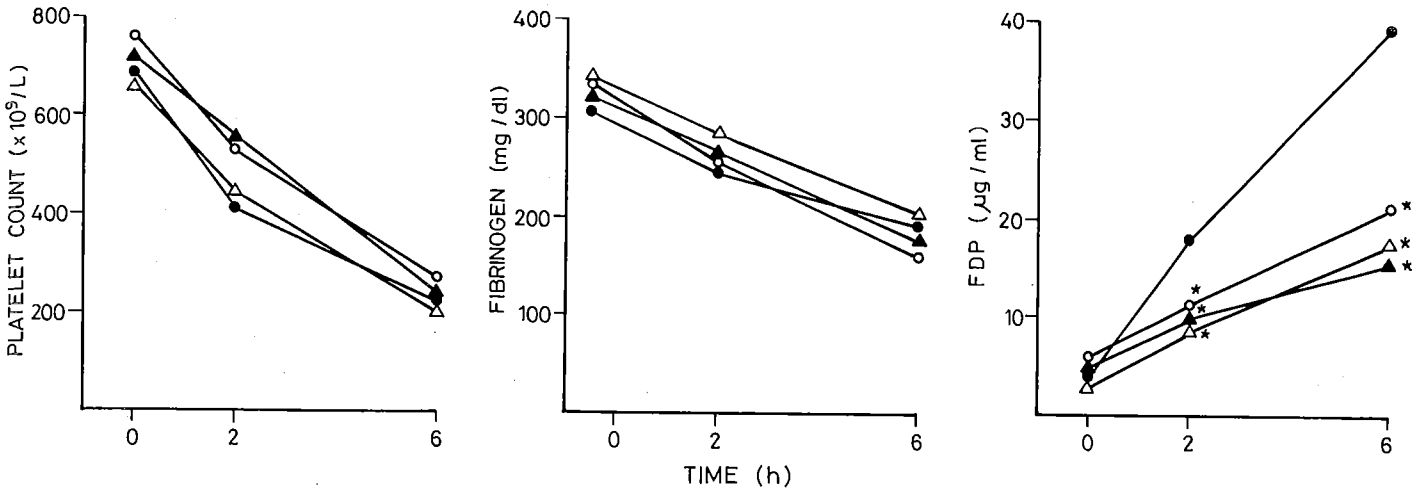

Fig. 4 Evolution of platelet count, fibrinogen and FDP levels in the different groups during LPS infusion. (●) control, (○) heparin 5–20 IU kg^{-1} h^{-1}, (▲) AT III 10–240 U kg^{-1} h^{-1}, (△) heparin 10 IU kg^{-1} h^{-1} plus AT III 20 U kg^{-1} h^{-1}. * $p < 0.001$ as compared to the control group

Table 2 Score of fibrin deposits in kidneys. Results are expressed as mean ± SD

Group	Number	Kidney
Control	11	2.9 ± 1.0
Heparin 5 IU kg^{-1} h^{-1}	9	2.1 ± 1.4
Heparin 10 IU kg^{-1} h^{-1}	8	2.4 ± 1.1
Heparin 20 IU kg^{-1} h^{-1}	8	2.7 ± 0.5
AT III 10 U kg^{-1} h^{-1}	8	2.5 ± 1.3
AT III 20 U kg^{-1} h^{-1}	8	2.1 ± 1.0
AT III 40 U kg^{-1} h^{-1}	8	2.1 ± 1.1
AT III 240 U/kg (bolus)	8	1.2 ± 0.7*
Heparin 10 IU kg^{-1} h^{-1} + AT III 20 U kg^{-1} h^{-1}	8	0.9 ± 0.8**

* $p < 0.05$, ** $p < 0.01$ as compared to the control group

when they were sacrificed for the histological studies. In spite of the very short follow up, mortality rate in the control group was significantly higher than in the other groups ($p < 0.01$). Moreover, the increase in plasma PAI activity in the six animals that died following endotoxin infusion was significantly higher ($p < 0.05$) than in the survivors (212.6 ± 53.6 U/ml vs 165.3 ± 51.1 U/ml).

Discussion

Bacterial endotoxin causes disseminated intravascular coagulation and fibrin deposits in various organs. Impairment of the fibrinolytic system as a contributing factor in the pathogenesis of endotoxin-induced DIC has already been suggested (18) and the recent observation that endotoxin induces a very marked and prolonged elevation of a circulating fast-acting inhibitor of plasminogen activator supports that hypothesis (6). The endotoxin-induced PAI seems to be of endothelial type (PAI-1) according to cell culture studies and physicochemical analysis (6, 19). It cannot be excluded that other PA-inhibitors, like the PAI-2 produced by peripheral monocytes may partially contribute to plasma PAI activity in experimental endotoxemia (20).

PAI-1 seems to play an important role in different clinical conditions related to thrombosis (7–12), although there is no evidence of a cause and effect relationship. In the present study we report that infusion of heparin plus AT III significantly reduces the generation of PAI activity in the plasma of endotoxin-treated animals as assessed by functional (amidolytic) assay and fibrin autography. This effect seems to be related to a decrease in PAI and not to the release of t-PA since no evidence of t-PA-PAI complex was obtained by fibrin autography of plasma samples not supplemented with t-PA. AT III or heparin given alone showed no remarkable change in the evolution of PAI activity during endotoxin infusion. The finding that a low dose of heparin and a bolus injection of AT III caused a reduction in the plasma levels of PAI activity at 2 hours but not at 6 hours of endotoxin infusion is unclear. One possible explanation could be that these two regimens have only delayed the rise in PAI activity following endotoxin stimulation (normally occurring at about 2 hours) without affecting the maximal response, which is better evaluated at 6 hours when the plateau level of PAI is reached. We do feel, however, that this effect is not relevant.

The effect of AT III-heparin on the generation of PAI correlated with a reduction in fibrin-like material precipitated in kidneys. Simple microscopic examination of tissue sections did not allow for accurate quantification of microthrombosis in renal tissue; however, the striking difference in microscopic appearance between kidney sections from control and treated rabbits leaves no doubts as to the beneficial effect of AT III-heparin on the amount of fibrin accumulation. Among the remaining treatment schedules only the AT III bolus dose clearly reduced fibrin deposits in kidneys. Infusion of low heparin doses, which partially prevented the generation of PAI, had only a limited non-statistically significant effect.

Apart from a decreased generation of FDPs, none of the treatments prevented laboratory changes of DIC associated with endotoxin infusion. The fact that FDP levels are unrelated to PAI activity may be due to a plasminogen-independent fibrin(ogen) degradation mechanism (21, 22). It has also been shown that the t-PA mediated lysis of fibrin may occur, at least in particular conditions, even in the presence of high levels of PAI (23).

All treatment schemes seemed to reduce mortality regardless of their influence on PAI evolution. It should be stressed, however, that the design of the experiment was not the most suitable for evaluation of mortality due to the very short follow up. Consequently, any possible association between PAI levels and mortality could not be properly evaluated. Nevertheless, a significant difference in PAI activity was observed between survivors and non-survivors.

The mechanism by which AT III-heparin attenuates the PAI response to endotoxin infusion is unknown. Recently, Gelehrter et al. (24) reported that thrombin induces PAI activity in cultured

endothelial cells. Available in vivo data however, do not support the hypothesis that AT III-heparin exerts its action on PAI, via neutralization of thrombin. Firstly, the dose of endotoxin capable of inducing a maximal PAI response is much lower than that required to produce laboratory signs of DIC (6). Secondly, an infusion of thrombin (from 1 to 5 IU kg^{-1} min^{-1}, for 60 min) produces no significant elevation of the plasma levels of PAI (25). One possibility might be the that the elevated AT III-heparin concentrations neutralizes one or more proteases, generated during endotoxin infusion, which may contribute to the induction of PAI.

In conclusion, our data indicate that the combination of AT III and heparin significantly reduces the generation of PAI activity and the deposition of fibrin in tissues, induced by endotoxin infusion. The relationship observed between these two phenomena suggests that PAI may play a role in the regulation of fibrinolysis. Whether the control of PAI generation represents an alternative treatment of experimental DIC is under investigation.

Acknowledgements

This work was supported by Grant PB85-0035 from the DGICYT of the Ministerio de Educación y Ciencia, Spain. We are grateful to I. Paz and G. Equiza for their expert technical assistance and to M. A. Iñigo for her expertise in the preparation of this manuscript.

References

1 Mizock B. Septic shock. A metabolic perspective. Arch Intern Med 1984; 144: 579–85.
2 Morrison D C, Ulevitch R J. The effect of bacterial endotoxins on host mediation systems. A review. Am J Pathol 1978; 93: 526–617.
3 Bradley S G. Cellular and molecular mechanisms of action of bacterial endotoxins. Annu Rev Microbiol 1979; 33: 67–94.
4 Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati M B, Semeraro N. Cultured human endothelial cells generate tissue factor in response to endotoxin. J Clin Invest 1983; 71: 1893–6.
5 Collen D. On the regulation and control of fibrinolysis. Thromb Haemostas 1980; 43: 77–89.
6 Colucci M, Paramo J A, Collen D. Generation in plasma of a fast-acting inhibitor of plasminogen activator in response to endotoxin stimulation. J Clin Invest 1985; 75: 818–24.
7 Chmielewska J, Ranby M, Wiman B. Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. Thromb Res 1983; 31: 427–36.
8 Juhan-Vague I, Moerman B, de Cock F, Aillaud M F, Collen D. Plasma levels of a specific inhibitor of tissue-type plasminogen activator (and urokinase) in normal and pathological conditions. Thromb Res 1984; 33: 523–30.
9 Páramo J A, Alfaro M J, Rocha E. Postoperative changes in the plasmatic levels of tissue-type plasminogen activator and its fast-acting inhibitor. Relationship to deep vein thrombosis and influence of prophylaxis. Thromb Haemostas 1985; 54: 713–6.
10 Páramo J A, Colucci M, Collen D, Van de Werf F. Plasminogen activator inhibitor in the blood of patients with coronary artery disease. Br Med J 1985; 291: 573–4.
11 Páramo J A, De Boer A, Colucci M, Jonker J J C, Collen D. Plasminogen activator inhibitor (PA-inhibitor) activity in the blood of patients with deep vein thrombosis. Thromb Haemostas 1985; 54: 725.
12 Rocha E, Alfaro M J, Páramo J A, Cañadell J M. Preoperative identification of patients at high risk of deep venous thrombosis despite prophylaxis in total hip replacement. Thromb Haemostas 1988; 59: 93–5.
13 Deutsch D G, Mertz E T. Plasminogen: purification from human plasma by affinity chromatography. Science 1970; 170: 1095–6.
14 Verheijen J H, Mullaart E, Chang G T G, Kluft C, Wijngaards G. A simple, sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. Thromb Haemostas 1982; 48: 266–9.
15 Camacho Y, Navio G, Acosta J, Rocha E. Purification of pasteurized AT-III. Thromb Haemostas 1985; 54: 90 (Abstr).
16 Merskey C, Lalezari P, Johnson A J. A rapid, simple, sensitive method for measuring fibrinolytic split products in human serum. Proc Soc Exp Biol Med 1969; 131: 871–5.
17 Loskutoff D J, Mussoni L. Interactions between fibrin and the plasminogen activators produced by cultured endothelial cells. Blood 1983; 62: 62–8.
18 Bergstein J M, Michael A F. Renal cortical fibrinolytic activity in the rabbit following one or two doses of endotoxin. Thromb Diath Haemorrh 1973; 29: 27–32.
19 Colucci M, Páramo J A, Collen D. Inhibition of one-chain and two-chain forms of human tissue-type plasminogen activator by the fast-acting inhibitor of plasminogen activator in vitro and in vivo. J Lab Clin Med 1986; 108: 53–9.
20 Schwartz B S, Monroe M C, Levin E G. Increased release of plasminogen activator inhibitor type 2 accompanies the human mononuclear cell tissue factor response to lipopolysaccharide. Blood 1988; 71: 734–41.
21 Egbring R, Schmidt W, Fuchs G, Havermann K. Demonstration of granulocytic proteases in plasma of patients with acute leukemia and septicemia with coagulation defects. Blood 1977; 49: 219–31.
22 Aasen A O, Ohlsson K. Release of granulocyte elastase in lethal canine endotoxin shock. Biol Chem Hoppe-Seyler 1978; 359: 683–90.
23 Colucci M, Páramo J A, Stassen J M, Collen D. Influence of the fast-acting inhibitor of plasminogen activator on in vivo thrombolysis induced by tissue type plasminogen activator in rabbits. Interference of tissue-derived components. J Clin Invest 1986; 78: 138–44.
24 Gelehrter T D, Senyer-Laszuk R. Thrombin induction of plasminogen activator inhibitor in cultured human endothelial cells. J Clin Invest 1986; 77: 165–9.
25 Colucci M, Triggiani R, Cavallo L G, Semeraro N. Thrombin infusion in endotoxin-treated rabbits reduces the plasma levels of plasminogen activator inhibitor (PAI): evidence for a protein C (PC)-mediated mechanism. Fibrinolysis 1988; 2: 153 (Abstr).

Received January 9, 1989 Accepted after revision April 6, 1989