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Effect of the administration of recombinant hirudin and/or tissue-plasminogen activator (t-PA) on endotoxin-induced disseminated intravascular coagulation model in rabbits

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Summary. We evaluated the effect of r-hirudin and/or tissue-plasminogen activator (t-PA) in a model of DIC in rabbits induced by i.v. infusion of 100 μg/kg/h/6 h endotoxin. Rabbits were treated with saline (endotoxin control group), r-hirudin at 0·3 mg/kg/h/6 h, t-PA at 0·3 mg/kg for 90 min and r-hirudin plus t-PA at the doses described above. The best results were achieved when r-hirudin and t-PA were infused together. This treatment reduced the consumption of platelets and protein C and attenuated the increase of PAI-1 more efficiently than r-hirudin or t-PA alone. r-Hirudin plus t-PA also resulted in the lowest formation of fibrin deposits in the kidneys. Finally, mortality at 24 h dropped from 70% in the endotoxin control group to 40%, 10% and 0% in the t-PA, r-hirudin and r-hirudin plus t-PA groups respectively. None of the t-PA-infused rabbits which had died by 24 h showed macroscopic signs of haemorrhage. r-Hirudin alone was better than t-PA alone, as was shown by fibrin deposits and mortality. We conclude that r-hirudin and t-PA given simultaneously were more efficient than either given alone in this model of DIC. Effective thrombin inhibition, which could influence other pathophysiological mechanisms apart from coagulation, together with the improvement in fibrinolysis, would explain these results.

Keywords: DIC, sepsis, endotoxin, hirudin, t-PA.

Gram-negative bacteria-induced sepsis frequently results in disseminated intravascular coagulation (DIC) (Bick, 1992). Despite the use of potent antibiotics and intensive supportive care, the mortality remains close to 60% (Páramo & Rocha, 1994).

Endotoxin or lipopolysaccharide (LPS), a constituent of the outer membrane of Gram-negative bacteria, leads monocytes and endothelial cells to generate several cytokines which activate coagulation (Warr *et al*, 1990). The high amounts of thrombin generated transform fibrinogen into fibrin and stimulate platelet aggregation, leading to the raising of stable microthrombi. On the other hand, the continuous thrombogenic stimuli induce the exhaustion of antithrombin III (AT III) and protein C, the main coagulation inhibitors, thus perpetuating fibrin generation (Marlar *et al*, 1982; Fenton, 1986) and allowing the appearance of microvascular thrombi in various organs which subsequently results in multiple organ failure (MOF) (Robboy *et al*, 1972). The dramatic increase in plasminogen activator inhibitor-1 (PAI-1) in these patients, with the subsequent inhibition of the main fibrinolysis activator, tissue-type plasminogen activator (t-PA) (Páramo *et al*, 1988; Páramo *et al*, 1990), leads to a hypofibrinolytic state which could contribute to the persistence of fibrin deposits.

Recombinant hirudin (r-hirudin), cloned from the medicinal leech, *Hirudo medicinalis*, is a direct and selective thrombin inhibitor able to effectively inhibit clot bound thrombin (Weitz *et al*, 1990; Markward, 1991). r-Hirudin, whose efficacy in venous and arterial thrombosis has been widely studied (Ten-Cate, 1996), could be an attractive therapeutic agent in LPS-induced DIC, as is suggested by previous work in several experimental models (Ishikawa *et al*, 1980; Hoffmann *et al*, 1990; Zawilska *et al*, 1993; Dickneite & Czech, 1994; Dickneite *et al*, 1994; Hermida *et al*, 1998). On the other hand, fibrinolytic activators have been found to be useful in different animal models of thrombosis and DIC (Matsuo *et al*, 1981; Korninger *et al*, 1982; Collen *et al*, 1983; Agnelli *et al*, 1985; Schneider, 1993; Paloma *et al*, 1995; Hardaway *et al*, 1996). Therefore a treatment which combines an antithrombotic agent and a thrombolytic could be a promising therapeutic strategy in DIC. The aim of the present work was to study the

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beneficial effects, if any, of treating LPS-induced DIC rabbits with r-hirudin and t-PA simultaneously, when compared with rabbits treated with r-hirudin alone or t-PA alone. Mortality and kidney fibrin deposition are evaluated and the changes in some coagulation and fibrinolytic parameters are assessed as secondary end-points.

MATERIAL AND METHODS

Material. LPS (*E. coli* 0111:B4) was obtained from Difco Laboratories (Detroit, U.S.A.). Recombinant desulphatohirudin variant 1 (Revasc™, specific activity 115 000 ATU/mg) was from Ciba-Geigy (Switzerland). t-PA (Actylise™), was purchased from Boehringer Ingelheim (Germany).

Experimental model. Male New Zealand white rabbits (weight 2–3 kg) were used. Animals were anaesthetized by an intramuscular injection of 30 mg/kg ketamin hydrochloride and 0·002 mg/kg xylacine hydrochloride followed by intramuscular boosts of ketamin hydrochloride throughout the experiment. 10 rabbits were infused with saline at 20 ml/h for 6 h as a first saline control group. DIC was induced in rabbits by intravenous infusion of 100 μg/kg/h LPS for 6 h in 60 ml (10 ml/h) saline through the marginal ear vein.

Treatments started simultaneously with LPS infusion through the contralateral marginal ear vein. Four different groups (10 rabbits each) were established: LPS group: saline as placebo (10 ml/h) for 6 h; r-hirudin group: 0·3 mg/kg/h r-hirudin for 6 h; t-PA group: 0·3 mg/kg t-PA for 90 min; r-hirudin + t-PA group: 0·3 mg/kg/h r-hirudin for 6 h + 0·3 mg/kg t-PA for 90 min.

Surviving rabbits were killed 24 h after the start of treatment by intravenous injection of 60 mg/kg Nembutal (Abbot Laboratories, U.S.A.). Kidneys were extracted from all animals (survivors and non-survivors) for subsequent histological studies.

Laboratory methods. Blood samples were taken through a catheter inserted into a femoral artery immediately before LPS infusion, and 2 and 6 h after the start. Blood for platelet counts was collected in tubes containing K_3-EDTA. Stabilyte tubes (Biopool, Umea, Sweden) were used in order to avoid inhibitor interference to collect blood for t-PA activity. The rest of the samples were collected in 3·2% citrate to determine fibrinogen, AT III, protein C, plasminogen, PAI-1 and t-PA activity: blood was kept on ice for no longer than 2 h and platelet-poor plasma obtained by centrifugation at 1600 *g* for 20 min at 4°C and stored at −70°C until assay.

A Counter STKS automatic analyser (Coulter Corp., Hialeah, Fla., U.S.A.) was used to count platelets. Fibrinogen was measured by Clauss's method (Clauss, 1957). Commercially available assays based on chromogenic substrates were used to determine AT III (Abilgaard *et al*, 1976), protein C (Vinazzer & Pangraz, 1987), plasminogen (Krishnamurti *et al*, 1987), t-PA activity (Verheijen *et al*, 1982) and PAI-1 activity (Páramo *et al*, 1985) (Coamatic AT III, Coamatic Protein C, Coamatic Plasminogen, Coaset t-PA and Coatest PAI, Chromogenix, Stockholm, Sweden).

Histological examination. Kidney sections were fixed in formalin, embedded in paraffin, stained with Masson's trichrome and examined for the presence of fibrin microthrombi by a pathologist unaware of the experimental design. Tissue sections were scored on a scale from 0 to 4 as previously described (Gómez *et al*, 1989). Briefly: (0) no fibrin; (1) partial fibrin deposits in some glomeruli; (2) partial deposits in all glomeruli; (3) large quantities of fibrin in all glomeruli; (4) fibrin thrombi in glomerular capillaries and in non-capillary vessels.

Data analysis. Within groups: The Student *t*-test for paired data and the Wilcoxon test for paired data were used to compare baseline values of the haemostatic parameters with values at 2 and 6 h. Between groups: Results of the haemostatic parameters at 2 and 6 h were converted to percentages assuming a value of 100% for baseline data, and were expressed as mean ± standard error of the mean (SEM). The Kruskal-Wallis test followed by the Mann-Whitney U test was applied for purposes of group comparison. Differences in mortality rate at 24 h were assessed by the Fisher's exact test. Differences in kidney fibrin deposits were determined by the Kruskal-Wallis test followed by the Mann-Whitney U test.

RESULTS

Haemostatic parameters during the experiment in the different groups

No changes in the saline control group were observed during the experiment (data not shown). Table I shows the plasma

Table I. Haemostatic parameters at baseline and 2 and 6 h after the start of the LPS infusion in the LPS group ($n = 10$).

	Baseline	2 h	6 h
Platelets ($\times 10^9$/l)	454 ± 40	226 ± 33***	97 ± 21***
Fibrinogen (g/l)	2·78 ± 0·31	2·48 ± 0·25	1·58 ± 0·17**
AT III (U/ml)	1·17 ± 0·03	1·03 ± 0·05**	0·85 ± 0·06**
Protein C (U/ml)	1·42 ± 0·05	0·84 ± 0·08***	0·23 ± 0·05***
Plasminogen (U/ml)	0·84 ± 0·03	0·75 ± 0·04*	0·64 ± 0·04***
t-PA (U/ml)	0·60 ± 0·02	0·41 ± 0·03***	0·35 ± 0·04**
PAI-1 (U/ml)	30·5 ± 0·7	172 ± 3***	361 ± 10***

* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$ when compared with basal values.

Table II. Haemostatic parameters 2 and 6 h after the start of the LPS infusion in rabbits in the LPS and treatment groups. Data shown as mean ± SEM percent of the basal value.

Group	*n*	Time (h)	Platelets ($\times 10^9$/l)	Fibrinogen (g/l)	AT III (U/ml)	Protein C (U/ml)	Plasminogen (U/ml)	t-PA (U/ml)	PAI-1 (U/ml)
LPS	10	2 h	49 ± 5***	0·91 ± 0·04	0·88 ± 0·04**	0·59 ± 0·06***	0·89 ± 0·04*	70 ± 4***	563 ± 11***
		6 h	20 ± 3***	0·58 ± 0·05**	0·73 ± 0·06**	0·16 ± 0·03***	0·75 ± 0·03***	62 ± 7**	1178 ± 31***
Hirudin	10	2 h	63 ± 5***	0·92 ± 0·08	0·89 ± 0·05*	0·74 ± 0·04**	1·01 ± 0·04	82 ± 7*	384 ± 15**
		6 h	44 ± 5***	1·00 ± 0·11	0·8 ± 0·05**	0·43 ± 0·04***	0·91 ± 0·05	86 ± 8	1284 ± 70***
t-PA	10	2 h	62 ± 5***	0·9 ± 0·03**	0·71 ± 0·08*	0·83 ± 0·05*	0·89 ± 0·07	607 ± 102**	267 ± 76*
		6 h	31 ± 5***	0·76 ± 0·04**	0·95 ± 0·04	0·31 ± 0·04***	0·84 ± 0·05*	137 ± 28	1141 ± 149***
Hirudin + t-PA	10	2 h	75 ± 2***	0·93 ± 0·04	0·85 ± 0·08	0·89 ± 0·04*	0·91 ± 0·04*	383 ± 93*	251 ± 47**
		6 h	49 ± 3***	0·81 ± 0·08*	0·89 ± 0·05	0·46 ± 0·06***	0·88 ± 0·05*	84 ± 10	1529 ± 249***

* $P<0·05$, ** $P<0·01$, *** $P<0·001$ when compared to the basal value.

levels of haemostatic parameters during the experiment in the LPS group and Table II shows the haemostatic parameters at 2 and 6 h in the LPS and treatment groups.

Effect of treatments on LPS-induced changes in coagulation parameters

The simultaneous addition of r-hirudin and t-PA was the most efficient treatment to reduce platelets and protein C consumption; r-hirudin, alone as well as with t-PA, partially prevented the platelet drop observed in the LPS group at 2 and at 6 h and the simultaneous infusion of r-hirudin and t-PA was more efficient than r-hirudin alone at 2 h ($P<0·05$) and than t-PA alone at both 2 ($P<0·05$) and 6 h ($P<0·01$) (Fig 2A); moreover, although all treatments significantly attenuated the protein C consumption assessed in the LPS group at 2 and 6 h (Fig 2B), r-hirudin and t-PA given simultaneously were more efficient than r-hirudin alone at 2 h ($P<0·05$). The decrease in the AT III consumption

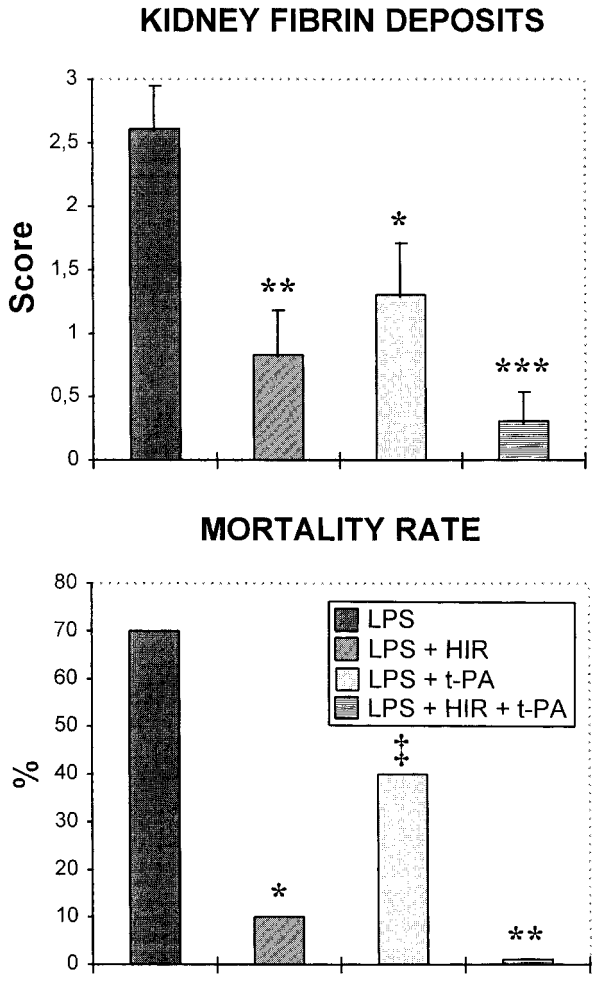

Fig 1. Kidney fibrin deposits (score values expressed as mean ± SEM) and mortality rate (expressed as the percentage of non-survivors rabbits) in the LPS group and all treatment groups. * $P<0·05$, ** $P<0·01$ and *** $P<0·001$ as compared to the LPS group; § $P<0·05$ as compared to the r-hirudin + t-PA group.

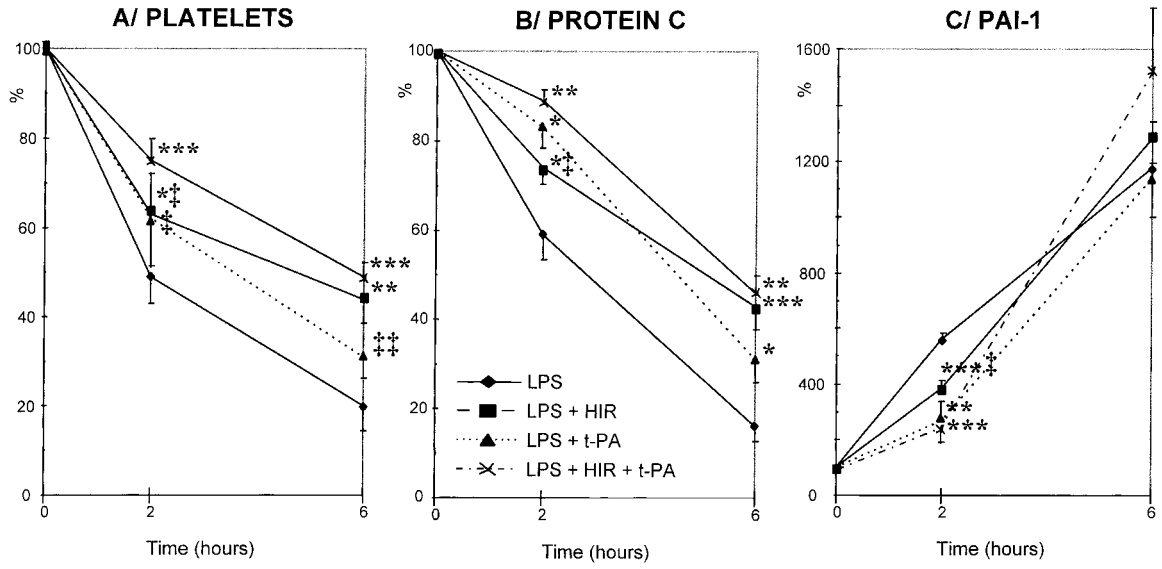

Fig 2. Haemostatic parameters at 2 and 6 h in the LPS group and all treatment groups. Values are expressed as the mean ± SEM percent of the initial value before LPS infusion. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ and *** $P < 0{\cdot}001$ as compared to the LPS group; ‡ $P < 0{\cdot}05$, ‡‡ $P < 0{\cdot}01$ as compared to the r-hirudin + t-PA group.

shown by the LPS group at 6 h was partially prevented both by the r-hirudin and t-PA treatment and by t-PA alone, but not by r-hirudin alone. Finally, there were no differences between groups in fibrinogen levels at 2 h and all treatments significantly reduced its consumption at 6 h.

Effect of treatments on LPS-induced changes in fibrinolytic parameters

As expected, t-PA was markedly increased ($P < 0{\cdot}001$) at 2 h in both t-PA treatment groups and was still raised at 6 h with respect to the LPS group. Rabbits treated with r-hirudin alone also showed higher t-PA levels than the LPS group at 6 h, although the amounts of activator measured were not as high as in the t-PA-infused rabbits. Although a reduction in the increase of PAI-1 levels at 2 h was achieved with all treatments (Fig 2C), such reduction was significantly higher ($P < 0{\cdot}05$) in the rabbits given r-hirudin and t-PA simultaneously than in the rabbits treated with r-hirudin alone. However, the changes were never dramatic. Finally, there were no changes in plasminogen between groups along the experiment.

Effect of treatments on kidney fibrin deposits and mortality rate

Rabbits infused with saline did not show kidney fibrin deposits. Intense deposits of fibrin within glomerular capillaries were observed in the LPS group rabbits (score $2{\cdot}61 \pm 0{\cdot}34$, expressed as mean ± SEM). As shown in the top panel of Fig 1, although all treatments significantly reduced the kidney fibrin deposits with respect to the LPS group, a higher reduction was achieved when r-hirudin and t-PA were infused simultaneously (score $0{\cdot}31 \pm 0{\cdot}21$, $P < 0{\cdot}001$) than when r-hirudin and t-PA were given alone (scores $0{\cdot}83 \pm 0{\cdot}35$, $P < 0{\cdot}01$ and $1{\cdot}30 \pm 0{\cdot}42$, $P < 0{\cdot}05$ respectively). However, the scores of the r-hirudin alone and t-PA alone groups were not significantly different from the score of the r-hirudin plus t-PA group, which is probably because of the relatively small number of rabbits included in the study.

As far as mortality is concerned, it must be noted firstly that none of the rabbits had died at the end of the 6 h of endotoxin and treatment infusion, which enabled us to rule out the possibility that death by 24 h, when it occurred, was anaesthesia-related. All rabbits infused with saline survived. 7/10 rabbits which were infused with LPS without treatment had died by the first 24 h after the start of the experiment, as shown in the bottom panel of Fig 1. 4/10 rabbits died when t-PA was given alone, which produced in a mortality rate not significantly lower than that obtained in the LPS group. However, mortality significantly decreased when rabbits were treated with r-hirudin, and the results were similar when r-hirudin was infused alone (only 1/10 died, $P < 0{\cdot}05$) to when it was given with t-PA (none of them died, $P < 0{\cdot}01$). Mortality in the latter group was also significantly lower ($P < 0{\cdot}05$) than that achieved in the rabbits infused with t-PA alone, which, on the other hand, did not show macroscopic signs of haemorrhage (nor did the rabbits from the other groups) in the post-mortem examination.

DISCUSSION

The effect of the simultaneous administration of r-hirudin and t-PA to rabbits given LPS to induce DIC was compared with the effect of the infusion of r-hirudin or t-PA alone. As in experiments previously performed by our group (Gómez *et al*, 1989; Paloma *et al*, 1992, 1995; Hermida *et al*, 1998), a significant impairment in the haemostatic parameters as well as an intense kidney fibrin deposition and a high mortality rate were assessed following LPS infusion in rabbits.

r-Hirudin given alone, in agreement with previous reports (Ishikawa *et al*, 1980; Hoffmann *et al*, 1990; Dickneite *et al*, 1994; Hermida *et al*, 1998), reduced the consumption of platelets, fibrinogen, AT III and protein C but also attenuated the increase of PAI-1 at 2 h, which could be due to a lower stimulation of its thrombin-mediated release from the endothelium (Gelerther & Sznycer-Laszuk, 1986; Bevilacqua *et al*, 1986; Van Hinsberg *et al*, 1988). PAI-1 was not reduced at 6 h which was probably due to the action of tumour necrosis factor-α (TNF-α) which would induce a retarded, sustained PAI-1 increase, although some controversy about the relationship between TNF-α and PAI-1 exists (Emeis *et al*, 1995). The overall result of treating rabbits with r-hirudin was a significant reduction in kidney fibrin deposits and a significant decrease in the mortality rate. Taking into account the fact that a previous report showed that the mortality reduction could not be explained merely by an effect on the haemostatic system (Taylor *et al*, 1991), it is tempting to speculate that the improvement in some haemostatic markers that we have assessed in this study could prevent other inflammatory responses apart from coagulation (Ten-Cate *et al*, 1996).

When rabbits were treated with t-PA alone, the availability of this fibrinolytic activator in plasma at 2 h was greatly improved, in agreement with our previous experiments (Paloma *et al*, 1995). As a result, the PAI-1 increase subsequent to LPS infusion was partially reduced 2 h after the start of the experiment due to the formation of t-PA–PAI-1 complexes. However, since t-PA was given only for the first 90 min and taking into account the fact that the activator is rapidly removed from the circulation (Krause, 1988), this treatment was not able to reduce the notable PAI-1 increase at 6 h. On the other hand, these changes in the fibrinolytic profile were accompanied by a reduction in the consumption of protein C, AT III and fibrinogen. Kidney fibrin deposits at 24 h were decreased, in agreement with our previous data (Paloma *et al*, 1995). However, the mortality rate was not significantly reduced, suggesting again that lethality cannot be explained merely by the haemostatic imbalance.

The simultaneous administration of r-hirudin and t-PA to rabbits improved the haemostatic markers to a higher degree than any of the other treatments, especially protein C and PAI-1. As these rabbits showed fewer kidney fibrin deposits than the other groups, these data are in agreement with previous reports where we demonstrated a strong direct correlation between protein C consumption or PAI-1 increase and severity of kidney fibrin deposition (Gómez *et al*, 1989; Hermida *et al*, 1998). The remarkable reduction in the renal fibrin deposits assessed in these rabbits would be due, on the one hand, to the thrombin inhibition and, on the other hand, to the fibrinolytic improvement. None of the rabbits infused with t-PA and r-hirudin had died by the first 24 h, in contrast with the animals given t-PA alone (4/10 did not survive). This observation, taken together with the fact that only 1/10 rabbits infused with r-hirudin alone died, seems to indicate that the effective thrombin inhibition by r-hirudin is a key to survival in this model.

In the light of our results we conclude that treatment with r-hirudin and t-PA given simultaneously markedly improved the haemostatic markers and reduced kidney fibrin deposits and mortality in our model of LPS-induced DIC, being more efficient than the treatment with r-hirudin or t-PA alone. Effective thrombin inhibition, together with the fibrinolytic improvement, would explain these results. The mortality assessed in the rabbits treated with t-PA alone indicates that the transient improvement in t-PA availability, although able partially to reduce the renal fibrin deposition, is not enough to reduce lethality in this model. The good results obtained in the rabbits treated with r-hirudin alone show that the efficient thrombin inhibition greatly counteracts the lethal effects of endotoxaemia, probably not merely by an effect on the coagulation system but also by an effect on other host responses in which some coagulation markers (e.g. protein C) may be involved, although further work is needed to assess this point.

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