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The effect of defibrotide on thromboembolism in the pulmonary vasculature of mice and rabbits and in the cerebral vasculature of rabbits

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1 Administration of bovine thrombin (100 u kg^{-1}) into the carotid artery of rabbits induces a sustained accumulation of ^{111}Indium-labelled platelets within the cranial vasculature over the subsequent 3 h.

2 Intracarotid (i.c.) administration of defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} for 1 h) prior to i.c. thrombin (100 u kg^{-1}) significantly reduces the ability of thrombin to induce cranial thromboembolism in rabbits.

3 Intravenous (i.v.) administration of thrombin (20 u kg^{-1}) in rabbits induces a reversible accumulation of radiolabelled platelets into the thoracic circulation which is significantly reduced by i.v. administration of defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} for 1 h) prior to i.v. thrombin. In contrast, platelet accumulation in response to adenosine diphosphate (ADP; 20 μg kg^{-1}, i.v.) or platelet activating factor (PAF; 50 ng kg^{-1}, i.v.) is not significantly affected by this treatment.

4 Intravenous administration of the nitric oxide (NO)-synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 10 mg kg^{-1}) potentiates platelet accumulation induced by low dose thrombin (10 u kg^{-1}, i.v.) within the pulmonary vasculature of rabbits. The potentiated response is significantly abrogated following pretreatment with defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} for 1 h, i.v.).

5 Intravenous injection of human thrombin (1250 u kg^{-1}) to mice induces death within the majority of animals which is significantly reduced by pretreatment with defibrotide (150–175 mg kg^{-1}, i.v.). In contrast, death induced by i.v. collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}) is not significantly affected by defibrotide pretreatment.

6 The inhibitory effect of defibrotide in mice is abolished following concomitant treatment with the inhibitor of fribrinolysis, tranexamic acid (100 mg kg^{-1}, i.v.), but is unaffected following treatment with the cyclo-oxygenase inhibitor, aspirin (300 mg kg^{-1}, i.p.).

7 The protective effect of defibrotide against thrombin-induced thromboembolism in the mouse is potentiated by recombinant tissue-plasminogen activator (rt-PA; 1 mg kg^{-1}, i.v.) or unfractionated heparin (10 u kg^{-1}, i.v.) administration.

8 The results suggest that defibrotide may possess antithrombotic activity on thrombin-induced thromboembolism which, at least in the mouse, may be partially mediated via induction of the fibrinolytic pathway.

Keywords: ^{111}-Indium; platelets; defibrotide; thrombosis; fibrinolysis

Introduction

Defibrotide, a single stranded polydeoxyribonucleotide obtained by controlled depolymerisation of mammalian DNA, is a cluster of chains of different length with a mean molecular weight ranging between 15 and 30 kDa. This substance displays a multiple pharmacological profile in animals, by being profibrinolytic (Pescador *et al.*, 1983), anti-thrombotic (Niada *et al.*, 1986) and by preventing post-ischaemic organ failure in experimental models (Thiemermann *et al.*, 1989; Berti *et al.*, 1991). Defibrotide has also been reported to possess some beneficial effects in patients with thrombotic disorders (Coccheri *et al.*, 1988; Strano *et al.*, 1991). Defibrotide exhibits a number of pharmacological effects on the vascular endothelium and on circulating blood elements. It favourably modulates the release of tissue plasminogen activator (t-PA) and its inhibitor(s) (plasminogen activator inhibitor, PAI) from the vascular wall (Nunziata *et al.*, 1991), enhances the release of prostaglandins E_2 and I_2 from isolated organ preparations (Bianchi & Berti, 1992) and inhibits activation and adherence of polymorphonuclear leukocytes (Di Perri & Laghi Pasini, 1988).

We have recently described an automated isotope monitoring system to investigate the behaviour of platelets and blood elements *in vivo* in a variety of circumstances (May *et al.*, 1990; 1992). In studies in the rabbit, it was shown that intravenous (i.v.) or intracarotid (i.c.) injection of any one of a variety of platelet agonists caused transient, reversible accumulation of platelets within the pulmonary vasculature with a concomitant decrease in platelets in the vascular beds of the hind limb or head (May *et al.*, 1990). Among the agents tested, thrombin was unique in its ability to produce a significant, sustained accumulation of platelets in the cerebral vasculature following its injection directly into a carotid artery (May *et al.*, 1990). Platelet accumulation within the cranial vasculature was accompanied by increased localization of radiolabelled fibrinogen (May *et al.*, 1990). These changes were not merely reflections of alterations in local blood flow or blood pooling, since they were not seen in experiments using radiolabelled red blood cells or serum albumin (May *et al.*, 1990). Interestingly, the aggregation of platelets within the pulmonary (but not the cerebral) circulation could be increased by pretreating animals with N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO)-synthase (May *et al.*, 1991), suggesting that the

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control of the interaction of blood elements with the vessel wall may vary in different vascular beds. While established fibrinolytic agents such as urokinase, eminase or recombinant tissue-plasminogen activator (rt-PA) could effectively reverse thrombin-induced embolisation within the cerebral vasculature, defibrotide only weakly affected this response (May et al., 1992), an observation which conflicted with previously reported data showing that defibrotide was able to reduce the size of pre-formed thrombi (Fumagalli et al., 1989). The reason for this discrepancy is not clear and remains to be explained. The aim of the present study was to understand better the mechanism of the anti-thrombotic activity of defibrotide. Therefore, in addition to investigating the effects of defibrotide pretreatment on thrombin-induced embolisation within the cerebral and pulmonary vasculature of the rabbit, we have also investigated thrombin-induced pulmonary embolism in the mouse, a convenient method for elucidating the site(s) at which drugs act to prevent thrombus formation (Gresele et al., 1990a,b; Momi et al., 1992).

Methods

Experimental animals

The study was carried out on rabbits (New Zealand White; Froxfield Farms, Petersfield, Hants, UK and Charles River, Milan, Italy) of either sex weighing 1.8–2.8 kg or male Swiss CD-1 mice (Charles River, Calco, Italy) weighing 20–25 g. Animals received a standard diet and water *ad libitum*.

Preparation of rabbits

Neuroleptic analgesia was induced in rabbits with a combination of diazepam (5 mg kg^{-1}, i.p.) followed 10 min later by fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1} (Hypnorm; 0.4 ml kg^{-1}, i.m.). Neuroleptic analgesia was maintained by i.m. injections of Hypnorm (0.1 ml kg^{-1}) as necessary (approximately 30 min intervals). The left common carotid artery was cannulated in the direction of blood flow for intracarotid bolus injections and infusions. Intravenous bolus injection and infusions were made into a marginal ear vein.

^{111}Indium labelling of rabbit platelets

Full details of the protocol for isolation and radiolabelling of rabbit platelets with ^{111}indium-oxine have been described elsewhere (May et al., 1990). Briefly, 9 ml blood obtained from a marginal ear vein was collected into 1 ml of 3.8% (w/v) trisodium citrate and centrifuged at 200 *g* (10 min) to obtain platelet-rich plasma (PRP). The platelets were then washed by centrifugation (640 *g*, 10 min) in a calcium- and magnesium-free Tyrode solution containing prostaglandin E_1 (PGE_1) 300 ng ml^{-1} (CFTP). After removing the supernatant, the surface of the platelet pellet was carefully rinsed with 1 ml CFTP. The platelets were gently resuspended in 1 ml CFTP and incubated for 1.5 min at 37°C with ^{111}indium-oxine (25–50 μCi). After a further centrifugation (640 *g*, 10 min) to remove any excess, unbound ^{111}indium, the platelet pellet was carefully rinsed and then resuspended in 2 ml CFTP. The labelled platelets were administered (1 ml/recipient) to anaesthetized rabbits via a marginal ear vein.

Monitoring of radiolabelled platelets in vivo

Radioactivity associated with ^{111}indium-labelled platelets was continuously monitored by means of one inch crystal scintillation detectors placed over the thorax (to measure changes in the pulmonary circulation) and head (to measure changes in the cerebral circulation). Counts were estimated with a multi-channel spectrometer (Nuclear Enterprises NE461) and logged, with the aid of a special application interface (AIMS 8000, Mumed Ltd), by a microcomputer (Dell System 200).

Following i.v. administration of radiolabelled platelets, counts were monitored for 1 h until they had stabilised. Each animal then received a bolus dose of adenosine diphosphate (ADP; 100 μg kg^{-1}, i.c. or 10 μg kg^{-1}, i.v.) in order to determine whether the platelets were functioning normally, and also to eliminate any that might have been damaged during labelling (May et al., 1990).

After a further 30 min stabilisation, responses to treatment infusions and to administration of platelet agonists were recorded as changes in radioactivity from these later stable baseline values. Agonist-induced responses have been plotted as % change from baseline counts and expressed in the text as % change from counts immediately prior to injection of the agonist. The area under the time curve (AUC) of agonist induced % change in counts has been calculated to give a measure of the response duration.

Thrombin-induced pulmonary embolism in mice

The method has been described elsewhere (Gresele et al., 1990a,b; Momi et al., 1992). Mice were caged and fed a regular diet for at least 1 week before use. The drugs to be tested, or their vehicles, were administered either by the intraperitoneal (i.p.) route in a volume of 20 ml kg^{-1} or i.v. in a fixed volume of 100 μl in a tail vein, prior to the thrombotic challenge. In the majority of these experiments, the thrombotic challenge was induced by 100 μl (containing 1250 u kg^{-1}) of thrombin from human plasma. In some experiments, animals were challenged with collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}) i.v. Mice were accustomed to handling by the investigators and the i.v. injections were carried out by skilled investigators with minimal disturbance to the animals. The total duration of each experiment was 15 min and all surviving animals were immediately killed by exposure to ether vapours. The majority of animals injected with thrombin died within 5 min. Any animals which did not die within this time or which were obviously distressed were killed after 15 min and were recorded as survivors. No anaesthesia was used during the experiments because of the short duration and because it has been shown previously that anaesthesia interferes with the effects of thrombin on pulmonary embolism and mortality (Di Minno & Silver, 1983; Gresele et al., unpublished observations). In each experimental session, at least five animals per treatment group were tested; control groups were run at the beginning and at the end of every session. There was no significant difference in thrombin-induced mortality between any of the control groups and hence these have been pooled. The data have been presented as number of animals dead/total number of animals tested or as % mortality.

Tissue histology in mice

Two minutes after i.v. injection of thrombin or saline, mice were rapidly killed with ether and the lungs, kidneys, liver, spleen and brain removed, rinsed with cold saline and immediately fixed in 10% formalin. The wet tissues were blocked in a uniform manner and paraffin sections, cut at 5–6 μm, were stained with haematoxylin and eosin. The specimens were examined under light microscopy by a pathologist unaware of the treatment administered to the animals. At least four fields, at a magnification of 400×, were observed for every specimen using a micrometer eye piece in order to assess vessel wall diameters.

Materials

Drugs and reagents were obtained as follows: adenosine diphosphate (ADP, Sigma, Poole), adrenaline bitartrate (Mascia Brunelli, Milan), aspirin, lysine salt (Italseber, Milan), bovine serum albumin (BSA, Fraction V), bovine thrombin (Sigma, Poole), soluble collagen from bovine ten-

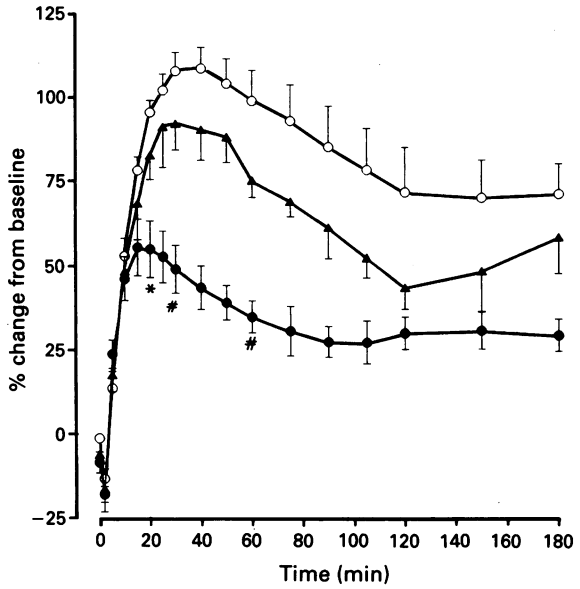

Figure 1 ^{111}In-labelled platelet accumulation in the cerebral circulation of rabbits following intracarotid injection of thrombin (100 u kg^{-1}) at time 0. Animals were pretreated, via a carotid artery, with a bolus injection, followed by 1 h continuous infusion, terminating at time 0, of saline (0; $n = 8$), defibrotide 32 mg kg^{-1} plus 32 mg kg^{-1} h^{-1} (▲; $n = 3$) or defibrotide 64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1} (●; $n = 6$). Results are expressed as mean ± s.e.mean percentage changes from baseline values recorded prior to commencement of the infusions. *$P < 0.05$; #$P < 0.01$ compared with saline pretreated controls.

don (Hormon Chemie, Munich), defibrotide (batch no. 82, Crinos SpA, Villa Guardia), diazepam (Valium, Roche), unfractionated heparin (Novo Nordisk, Denmark), Hypnorm (Janssen), L-NAME (Sigma, Poole), PAF (C16 PAF; Novabiochem, Nottingham), PGE_1 (Sigma, Poole), rt-PA (Boehringer Ingelheim, Ingelheim), thrombin from human plasma (Sigma, St. Louis) and tranexamic acid (Malesci, Italy). All materials were dissolved in saline except for human plasma thrombin (distilled water), PAF (saline containing 0.25% BSA) and collagen (isotonic glucose buffer, pH 2.7).

Statistics

Platelet accumulation data are presented as mean values ± standard errors of the means (s.e.mean). Following an analysis of variance, the significance of differences between means was assessed by Student's *t* test (unpaired), Mann Whitney U-test or Wilcoxon test as appropriate. The data for mortality in mice were tested by chi square analysis.

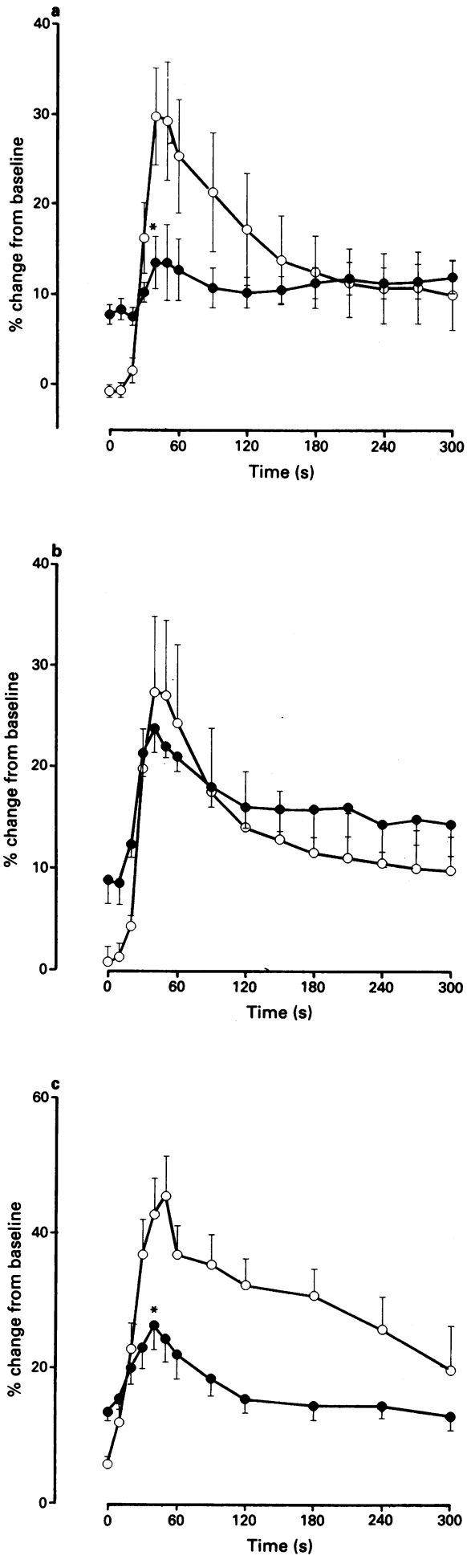

Figure 2 ^{111}In-labelled platelet accumulation in the pulmonary circulation of rabbits following i.v. injection, at time 0, of (a) and (b) thrombin (20 u kg^{-1}) or (c) thrombin (10 u kg^{-1}) 5 min after N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}). Animals were pretreated, via a marginal ear vein, with a bolus injection, followed by 1 h continuous infusion, of saline (○; controls) or defibrotide (●; 64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1}); infusions terminated at time 0 [(a) and (c)] or 1 h prior to thrombin challenge (b). In (a), $n = 6$ per group and in (b) and (c), $n = 4$ per group. Results are expressed as mean (± s.e.mean) percentage changes from baseline values recorded prior to commencement of the infusion. *$P < 0.05$ compared with controls.

Results

Effects of defibrotide in rabbit cerebral vasculature

A bolus injection of defibrotide (64 mg kg^{-1}) followed by an infusion (64 mg kg^{-1} h^{-1}) for 1 h via the carotid artery had no noteworthy effect on levels of radiolabelled platelets in the cerebral vasculature. However, the peak platelet accumulation induced by i.c. administration of bovine thrombin (100 u kg^{-1}), injected immediately after the infusion of defibrotide ($n = 6$), was significantly ($P < 0.01$) reduced (57.3 ± 7.6% increase above pre-thrombin baseline) compared with animals receiving an infusion of vehicle ($n = 8$; 109.1 ± 5.5% increase; Figure 1) when measured 30 min post thrombin. Platelet accumulation between 20 and 60 min post thrombin was significantly reduced ($P < 0.05$ to $P < 0.01$) in the defibrotide pretreated animals (see Figure 1). The AUC (0–60 min) was also significantly reduced ($P < 0.01$) from 5243.1 ± 257.1 in controls to 3016.8 ± 376.4 in defibrotide-treated animals. When the dose of defibrotide was reduced to 32 mg kg^{-1} plus 32 mg kg^{-1} h^{-1} ($n = 3$), the effect was no longer significant (98.3 ± 6.3% increase at 30 min and AUC (0–60 min) 4751.3 ± 316.8; Figure 1).

Effects of defibrotide in rabbit pulmonary vasculature

Intravenous treatment with defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} infusion for 1 h) led to an increase in platelet-associated radioactivity in the lungs. At the end of the infusion, radioactivity was 7.7 ± 1.1% above baseline ($n = 6$). Thrombin (20 u kg^{-1}, i.v.) administered at the termination of the defibrotide infusion produced a markedly depressed ($P < 0.05$) platelet response ($n = 6$; 7.3 ± 3.7% peak increase and AUC (0–5 min) 17.8 ± 6.7) compared with control animals ($n = 6$; 32.5 ± 5.8% peak increase and AUC (0–5 min) 77.4 ± 21.4; Figure 2a). When thrombin injection was delayed for 1 h after termination of the defibrotide infusion, responses to the thrombin were still reduced (17.5 ± 3.0% peak increase and AUC 37.8 ± 2.8), but were no longer significantly different from control animals (27.5 ± 7.3% peak increase and AUC 64.5 ± 22.9) ($n = 4$; Figure 2b). Responses to low dose thrombin (10 u kg^{-1}, i.v.) following L-NAME (10 mg kg^{-1}, i.v.) were significantly ($P < 0.05$) potentiated (39.8 ± 4.7% peak increase and AUC (0–5 min) 120 ± 17.0; $n = 4$) in comparison with the response to thrombin following saline (18.5 ± 3.1% peak increase and AUC 21.4 ± 3.4; $n = 4$). This potentiated response was significantly ($P < 0.05$) reduced following pretreatment with defibrotide (64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1} for 1 h) (12.8 ± 2.3% peak increase and AUC 16.7 ± 4.3; $n = 4$) (Figure 2c). However, the same dose of defibrotide had no significant effect on responses to PAF (50 ng kg^{-1}) or ADP (20 μg kg^{-1}) measured as peak increase (Table 1) or as AUC (data not shown).

Table 1 Intravenous defibrotide on pulmonary platelet accumulation induced by i.v. ADP or PAF in rabbits

Treatment	*Stimulus*	*Peak change* (%)	n
Control (saline)	ADP	31.6 ± 6.1	3
Defibrotide	ADP	27.4 ± 5.2	3
Control (saline)	PAF	26.5 ± 2.5	3
Defibrotide	PAF	24.0 ± 4.7	3

^{111}In-labelled platelet accumulation in the pulmonary circulation was induced by i.v. injection of ADP (20 μg kg^{-1}) or PAF (50 ng kg^{-1}) immediately after a bolus injection, followed by 1 h continuous infusion, of saline (controls) or defibrotide (64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1}) i.v. Values shown represent mean ± s.e.mean peak % changes from levels recorded at the end of the infusions. Responses following defibrotide pretreatment were not significantly different from controls.

Effects of defibrotide on thrombin-induced pulmonary embolism in mice

Defibrotide at 175 mg kg^{-1}, i.v., 2 min before thrombin (1250 u kg^{-1}, i.v.) protected mice from death (7/52 dead/tested vs 171/210 controls, $P < 0.00001$); the effect was smaller at 150 mg kg^{-1} ($P < 0.05$) and insignificant at 100 mg kg^{-1} (23/51 and 30/38, dead/tested, respectively) (Figure 3). Although the protection decreased when the interval between defibrotide (175 mg kg^{-1}, i.v.) injection and thrombin administration was increased, a significant ($P < 0.01$) protec-

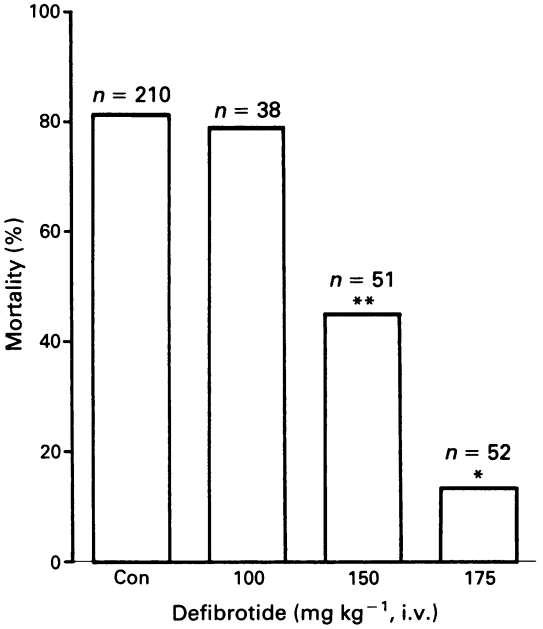

Figure 3 Effect of intravenous injection of defibrotide (100–175 mg kg^{-1}) on mortality in mice injected i.v. 2 min later with thrombin (1250 u kg^{-1}). The number of animals tested per group (n) is shown. *$P < 0.05$; **$P < 0.00001$ compared with controls (Con; saline pretreated).

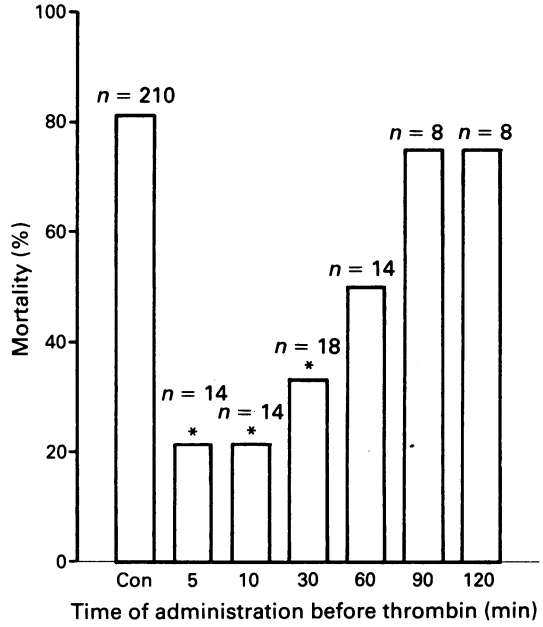

Figure 4 Effect of intravenous injection of defibrotide (175 mg kg^{-1}) at different times (5–120 min) prior to i.v. injection of thrombin (1250 u kg^{-1}) on mortality in mice. The number of animals tested per group (n) is shown. *$P < 0.05$ compared with controls (Con; saline pretreated).

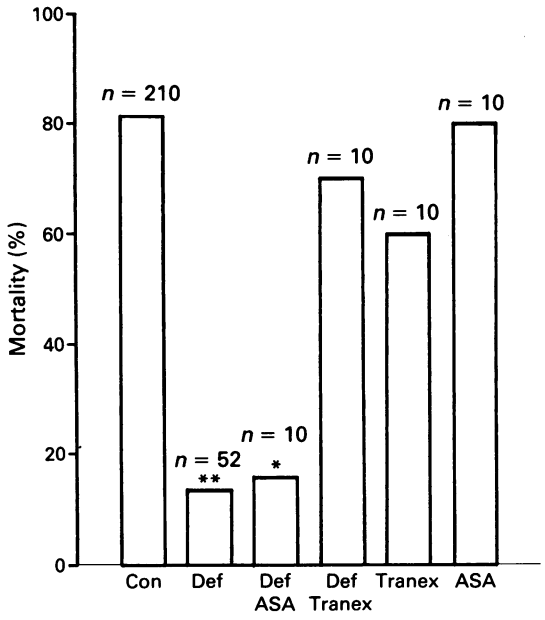

Figure 5 Effect of aspirin (ASA; 300 mg kg^{-1}, i.p.; 1 h before) or tranexamic acid (Tranex; 100 mg kg^{-1}, i.v., 4 min before) pretreatment on the effect of defibrotide (Def; 175 mg kg^{-1}, i.v., 2 min before) on mortality in mice injected i.v. with thrombin (1250 u kg^{-1}). The number of animals tested per group (n) is shown. *$P<0.01$; **$P<0.00001$ compared with controls (Con; saline pretreated).

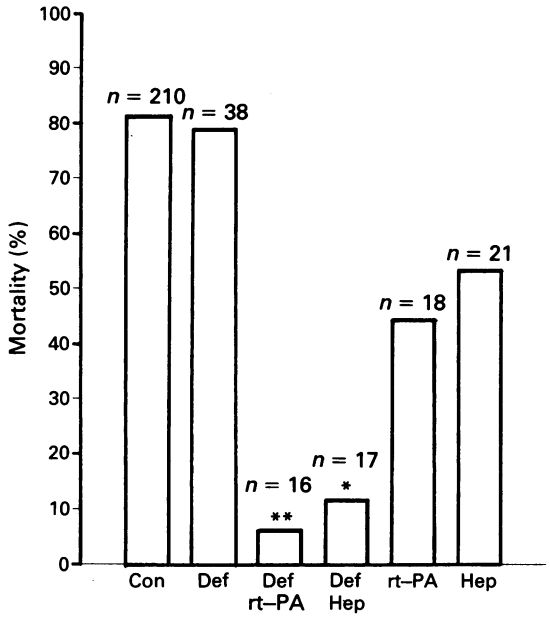

Figure 6 Effect of recombinant tissue-plasminogen activator (rt-PA; 1 mg kg^{-1}, i.v., 4 min before) or unfractionated heparin (Hep; 10 u kg^{-1}, i.v., 4 min before) pretreatment on the effect of defibrotide (Def; 100 mg kg^{-1}, i.v., 2 min before) on mortality in mice injected i.v. with thrombin (1250 u kg^{-1}). The number of animals tested per group (n) is shown. *$P<0.01$; **$P<0.005$ compared with controls (Con; saline pretreated).

Table 2 Intraperitoneal administration of defibrotide on i.v. thrombin-induced mortality in mice

	Dead/Tested	*% mortality*	P
Control	171/210	81.4	–
30 min	14/20	70.0	0.801
60 min	9/20	45.0	0.204
90 min	12/20	60.0	0.524
120 min	15/20	75.0	0.946

Animals were injected i.p. with saline (controls) or defibrotide (350 mg kg^{-1}) at different times (30–120 min) prior to i.v. injection of thrombin (1250 u kg^{-1}). The number of dead animals/total number tested and % mortality are shown for each group.

Table 3 Intravenous defibrotide on i.v. collagen plus adrenaline-induced mortality in mice

	Dead/Tested	*% mortality*	P
Control	64/88	72.7	–
Defibrotide (150 mg kg^{-1})	18/22	81.8	0.881
Defibrotide (175 mg kg^{-1})	17/30	56.6	0.579

Animals were injected i.v. with saline (controls) or defibrotide (150 or 175 mg kg^{-1}) 2 min prior to i.v. injection of collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}). The number of dead animals/total number tested and % mortality are shown for each group.

tion was still present up to 30 min (Figure 4). Defibrotide had some effect when injected intraperitoneally, at a higher dose (350 mg kg^{-1}), though this did not reach statistical significance (Table 2). Aspirin, at a dose known to suppress endogenous PGI_2 synthesis, (300 mg kg^{-1}, i.p., 1 h before thrombin) did not affect the ability of defibrotide (175 mg kg^{-1}, i.v.) to confer protection (3/19 dead/tested), while tranexamic acid (100 mg kg^{-1}), an inhibitor of fibrinolysis, reduced the protective effect (7/10, dead/tested). These doses of aspirin and tranexamic acid did not, by themselves, significantly affect survival (Figure 5). In addition, rt-PA (1 mg kg^{-1}, i.v.) or unfractionated heparin (10 u kg^{-1}, i.v.), at doses which by themselves had no significant effect on thrombin-induced mortality, significantly potentiated the protective activity of defibrotide (100 mg kg^{-1}, i.v.) (Figure 6). Defibrotide (150 or 175 mg kg^{-1}, i.v.) injected 2 min before collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}) had no significant protective effect (Table 3).

Tissue histology in mice

Microscopic observation of kidney, liver, spleen and brain slices prepared from control (saline pretreated) animals injected i.v. with 1250 u kg^{-1} of human thrombin did not show any significant alterations in vessels in terms of patency or diameter. In contrast, the lung slices showed that 61.5% ($n = 182$) of lung vessels were totally occluded by thrombi. In control animals injected with saline instead of thrombin, only 4.4% ($n = 82$) of vessels were occluded in the lung slices. Vessels filled with thrombotic material had a diameter less than 70 μm, although platelet clumps were observed occasionally in larger vessels. In defibrotide pretreated animals injected with thrombin, only 30% ($n = 20$) of lung vessels were occluded by thrombi.

Discussion

Our data demonstrate significant anti-thrombotic activity of defibrotide following i.v. or i.c. administration of the drug with a lesser, non-significant, effect following i.p. treatment. The ability to inhibit the thromboembolism induced by i.v. thrombin within the pulmonary vasculature or by i.c. thrombin in the cerebral vasculature of the rabbit, but the inability to attenuate significantly the platelet aggregation induced by ADP, PAF or collagen plus adrenaline suggests that the anti-thrombotic effect of defibrotide is not a direct effect of

the drug on platelets. This explanation is supported by the observation that the steady state plasma concentrations (100–200 $\mu g\ ml^{-1}$) measured *in vivo* following pretreatment of rabbits with defibrotide were in the same range as the concentrations of defibrotide (108–161 $\mu g\ ml^{-1}$) which inhibit thrombin- but not collagen-induced platelet aggregation in rabbit PRP (Porta & Bianchi, unpublished observations). The small accumulation of labelled platelets in the pulmonary vascular bed following defibrotide infusion cannot account for the decreased response to thrombin since this increase was also observed in experiments with ADP and PAF in the absence of a significant reduction in responses to these agents.

The experiments performed in mice clearly demonstrate that the protective effect of defibrotide against thrombin-induced death from pulmonary embolism in this species is abrogated by prior treatment with a purported inhibitor of fibrinolysis, tranexamic acid. These results would support the concept that part of the anti-thrombotic action of defibrotide may be secondary to the ability of this drug to promote fibrinolysis. Previous experiments using the rabbit heart and kidney (Thiemermann *et al.*, 1989; Berti *et al.*, 1991) have suggested that the action of defibrotide may be secondary to the ability of this drug to release PGI_2 from the vasculature. However, the protective effect of defibrotide in mice against thrombin-induced death from pulmonary embolism was not influenced by aspirin at doses previously reported to inhibit the synthesis of PGI_2 (Gresele *et al.*, 1990a). The observation that defibrotide was able to potentiate the protective effects of low doses of rt-PA or heparin on thrombin-induced thromboembolism in mice might imply that this drug may render blood clots more susceptible to plasmin digestion. Indeed, recently this has been reported to occur with heparin which may modify the structure of thrombi, making them more readily lysable (Nenci *et al.*, 1992). Certainly, the ability of defibrotide to facilitate fibrinolysis is consistent with the effect of this drug against thrombin-induced embolisation of platelets as thrombin is the only one of the agents used in our experiments which also initiates the coagulation cascade with deposition of fibrin and the subsequent activation of the fibrinolytic system. It is likely that, at the dose of thrombin employed, the deposition of fibrin is responsible for the irreversible nature of the embolisation in both the cerebral and pulmonary vasculature, a situation not observed when lower doses of thrombin, ADP or PAF are used to stimulate platelet aggregation (Barrett *et al.*, 1984). We have previously reported that inhibition of NO synthesis with L-NAME can potentiate the platelet aggregation induced by thrombin, converting a fully reversible platelet aggregation within the pulmonary vascular bed into an irreversible aggregation associated with fibrin deposition and often death (May *et al.*, 1992). Therefore, it is particularly noteworthy that defibrotide was effective in protecting animals against this L-NAME-induced potentiation of thrombin-induced embolisation.

In conclusion, our data suggest that the beneficial effects of defibrotide on thrombin-induced thromboembolism could be due to facilitation of fibrinolysis. However, this may not be the only mechanism involved, considering that defibrotide also inhibits the activation of leucocytes in human blood *in vitro* (Di Perri & Laghi Pasini, 1988), as well as in rat (Bianchi *et al.*, 1992) and rabbit blood (Fumagalli *et al.*, 1991) *in vitro* and *ex vivo*. Therefore, it is not surprising that defibrotide could reduce the fibrin network and the number of leucocytes trapped in clots adhering to a collagen-coated prosthesis inserted into a femoral vein in rabbits, both in fresh and in up to 7 day old thrombi (Fumagalli *et al.*, 1987; 1989). These properties may well also contribute to the clinical effects of this drug in peripheral thrombotic diseases (Strano *et al.*, 1991). The exact mechanism through which defibrotide facilitates fibrinolysis in these animal models of thromboembolism remains to be established.

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