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Inhibition of Proteinases with Recombinant Eglin C during Experimental *Escherichia coli* Septicemia in the Pig

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Key Words. Septic shock · *Escherichia coli* · Pigs · Neutrophil elastase · Eglin C · Proteinase inhibitors · Vascular permeability

Abstract. Administration of the proteinase inhibitor eglin C reduces the symptoms of capillary leakage in a porcine model of septic shock. This was assessed by measurements of blood pressure, plasma protein concentration, hematocrit, and duration of urine production. Eglin C plasma levels around 1.2 μ*M* resulted from a dose of 1.9 $mg \cdot kg^{-1} \cdot h^{-1}$ for 4 h and were therapeutically effective. A higher dose of eglin C (7.7 $mg \cdot kg^{-1} \cdot h^{-1}$ for 4 h) induced levels of approximately 5.6 μ*M* in plasma and was not superior. This observation indicates that lysosomal proteases from neutrophils or degranulation of mast cells play a crucial role in the increase of capillary permeability during septicemia.

Introduction

Eglin C is a protein proteinase inhibitor originally derived from the medical leech [10]. It contains 70 amino acid residues in a single polypeptide chain without disulfide bridges and its relative molecular weight is 8,100. It is a rapid and potent inhibitor of human granulocytic elastase, cathepsin G, and chymotrypsin, and it is unusually stable against denaturation by heat, acid, and proteolysis.

In a previous study, we examined possible pathophysiologic mechanisms in septic shock which may be initiated by lysosomal proteinases released into the extracellular space [4] using r-eglin C in a dose of 3.85 $mg \cdot kg^{-1} \cdot h^{-1}$. The present study was conducted to determine the effect of a higher (2-fold increased) and a lower (50% reduced) dose of r-eglin C on experimental septicemia in a similar model of septic shock.

Materials and Methods

A synthetic gene coding for the sequence of eglin C was expressed in transformed *Escherichia coli* and the expression product, recombinant eglin C (r-eglin C) was purified [9]. r-Eglin C was found to possess the same inhibitory properties as the natural protein from the leech.

Weaned domestic pigs were purchased from the Lehr- und Versuchsgut Oberschleissheim, Veterinary

School, University of Munich. The experiments were performed with 18 pigs with body weights ranging from 17 to 25.5 kg (mean: 19.7 kg). Acepromazine maleate, 50 mg, was given intramuscularly for premedication after fasting overnight and anesthesia was induced with pentobarbital, 15 mg/kg, given intravenously. Narcosis was maintained by repeated injections of pentobarbital, 80 mg, as needed. Pentobarbital injection was avoided during the 10 min preceding aortic blood pressure measurements.

Left groin vessels were cannulated with saline-filled polyethylene catheters. The blood pressure in the aorta was measured with a Bentley Trantec Model 800 transducer and a Siemens Sirecust 404 monitor. Two femoral venous lines were used for infusions. Blood was sampled with a short, large bore cannula in the left external jugular vein. A suprapubic cystostomy was performed to enable collection of urine. The animals were allowed to rest for 60 min after the operation and remained in the right lateral position during the experiment. They were breathing spontaneously room air.

All animals received a suspension of freshly cultured *E. coli* (014 B26), $3 \cdot 10^{10}$ cells, as assessed by turbidimetry, in 24 ml saline over 2 h. Each animal was randomly assigned to 1 of 3 treatment protocols: 6 animals received *E. coli* and 0.9% saline, 200 ml, and served as controls; 6 animals were given *E. coli* and a 'low' dose of r-eglin C, 1.9 $mg \cdot kg^{-1} \cdot h^{-1}$; and 6 animals *E. coli* and a 'high' dose of r-eglin C, 7.7 $mg \cdot kg^{-1} \cdot h^{-1}$. The r-eglin C (or saline) infusion was started simultaneously with the *E. coli* infusion (time zero) and was given for 4 h in a volume of 200 ml.

The output of urine was measured hourly. Anuria was diagnosed when the urine production fell below 3 ml/h. Survival periods and duration of urine production were calculated from time zero. The experiments were terminated 24 h after time zero if death due to septic shock was not imminent. Plasma samples were made as follows: blood was anticoagulated with 3.8% citrate 1:10 and centrifuged at 4 °C at 3,000 rpm for 20 min. The supernatant was aliquoted and stored at – 80 °C. Arterial blood gas analyses were performed with an AVL Gas Check 939 on heparinized samples. Blood for cell counts was anticoagulated with EDTA. Hematocrit was measured with microcapillaries and a Hettich Hämatokrit centrifuge, hemoglobin was measured with a Coulter Haemoglobinometer, white blood cells were counted with a Coulter Counter Model D, and platelets in a Neubauer hemocytometer chamber. r-Eglin C concentrations in plasma were quantitated by a competitive enzyme-linked immunosorbent assay [8] using peroxidase-conjugated eglin and affinity purified antibodies from sheep directed against leech eglin [Müller-Esterl et al., unpubl. results]. The total protein concentration in plasma was measured by the Biuret method. Creatinine in plasma was measured kinetically on an automated analyzer using picric acid (Merck, Darmstadt).

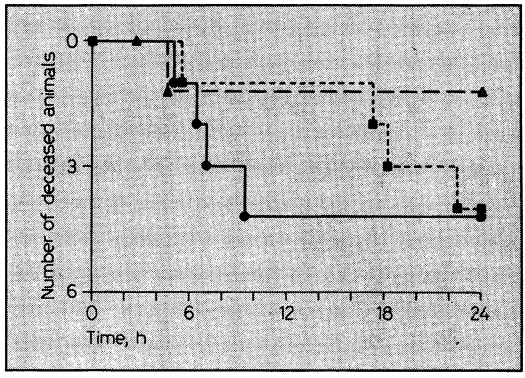

Fig. 1. Cumulative mortality after *E. coli* and r-eglin C administration (n = 6 in each group).
E. coli, $3 \cdot 10^{10}$ cells + NaCl, 0.9% (control group) (●——●).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 1.9 $mg \cdot kg^{-1} \cdot h^{-1}$ (▲– –▲).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 7.7 $mg \cdot kg^{-1} \cdot h^{-1}$ (■----■).

All data are presented as arithmetic mean ± sample standard deviation. Survival curves for the events death and anuria were analyzed with the generalized Mantel-Haenszel test followed by Scheffé type multiple comparisons ($\alpha = 0.05$). All other parameters were compared using a one-way analysis of variance followed by Scheffé's multiple comparison test with $\alpha = 0.05$. For statistical significance testing we selected the time 4 h after start of the infusions for two reasons: the eglin infusions were discontinued after 4 h, and the case numbers in the three groups declined thereafter due to mortality from septicemia. Since there was considerable variation of the hematocrit starting values between the three groups we selected the hourly rate of increase of hematocrit of each individual animal during the first 4 h, as assessed by linear regression over time, as a measure of hemoconcentration.

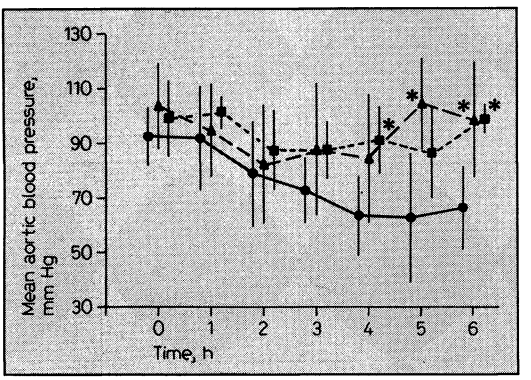

Fig. 2. Mean aortic pressure after *E. coli* and r-eglin C administration. Means and standard deviation. Asterisk indicates a significant ($p < 0.05$) difference from the control group ($n = 6$ in each group).
E. coli, $3 \cdot 10^{10}$ cells + NaCl, 0.9% (control group) (●——●).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 1.9 $mg \cdot kg^{-1} \cdot h^{-1}$ (▲--▲).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 7.7 $mg \cdot kg^{-1} \cdot h^{-1}$ (■----■).

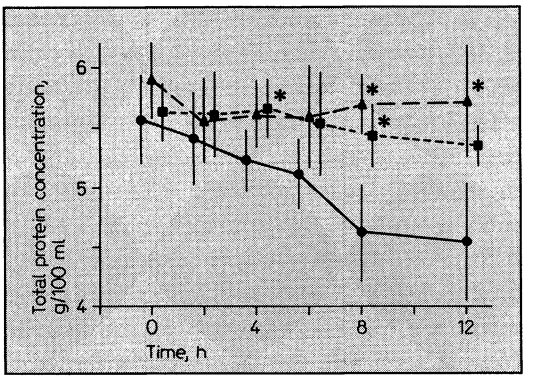

Fig. 3. Total protein concentration in plasma after *E. coli* and r-eglin C administration. Means and standard deviation. Asterisk indicates a significant ($p < 0.05$) difference from the control group ($n = 6$ in each group).
E. coli, $3 \cdot 10^{10}$ cells + NaCl, 0.9% (control group) (●——●).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 1.9 $mg \cdot kg^{-1} \cdot h^{-1}$ (▲--▲).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 7.7 $mg \cdot kg^{-1} \cdot h^{-1}$ (■----■).

Results

Mortality (fig. 1)

One animal that had received *E. coli* and r-eglin C in the low dose, 1.9 $mg \cdot kg^{-1} \cdot h^{-1}$ for 4 h, died after 2 h 40 min of nonseptic cardiac failure and was eliminated thereafter from anuria analysis. Total mortality due to septicemia was 9/17 (≙ 53%). The 24-hour survival rate was 2/6 in the septic reference group, 4/5 in the low-dose and 2/6 in the high-dose eglin group.

Arterial Hypotension (fig. 2)

In nearly all animals a marked reduction in mean arterial blood pressure was observed at the end of the *E. coli* infusion. All animals of the septic reference group had a severe hypotension (a fall in mean aortic pressure by more than 25 mm Hg) during the first 4 h. Two animals of the low-dose eglin group and 4 pigs of the high-dose group did not show this response. In the surviving animals, a recovery of blood pressure was noted, in some of them even exceeding the starting value. Group means differed significantly after 4 h with the blood pressure in the septic reference group being 20-30 mm Hg lower than in the two eglin groups (in the control group 63.5 ± 14.7 mm Hg, in the low-dose group 84.6 ± 23.7 mm Hg, and 91.2 ± 12.5 mm Hg in the high-dose group). In the Scheffé comparison only the high dose group differed significantly from the control group.

Hypoproteinemia (fig. 3)

The total plasma protein concentration decreased steadily in the septic reference group from 5.6 ± 0.4 to 4.6 ± 0.5 g/100 ml, whereas it remained more or less constant in both eglin groups (slight decrease from 5.9 ±

0.3 to 5.7 ± 0.5 in the low-dose and from 5.6 ± 0.2 to 5.4 ± 0.2 in the high-dose eglin group). Group means differed significantly after 4 h with the total plasma protein concentration in the septic reference group being about 0.4 g/100 ml lower than in the two eglin groups (in the control group 5.23 ± 0.26 g/100 ml, in the low-dose eglin group 5.62 ± 0.28 g/100 ml, and 5.67 ± 0.24 g/100 ml in the high dose group). In the Scheffé comparison only the high-dose group differed significantly from the control group.

Hemoconcentration (fig. 4)

Hematocrit increased rapidly (from 30 ± 3 to 38 ± 3%) during the first 4 h in the septic reference animals. The rise was less pronounced under eglin administration (from 32 ± 4 to 36 ± 4%). In the septic reference animals, the hemoconcentration expressed as the mean rate of rise of hematocrit during the first 4 h by linear regression over time was 1.5 ± 0.9% per hour as opposed to 0.7 ± 0.5% per hour in all eglin-treated animals. The hemoglobin concentrations behaved similarly.

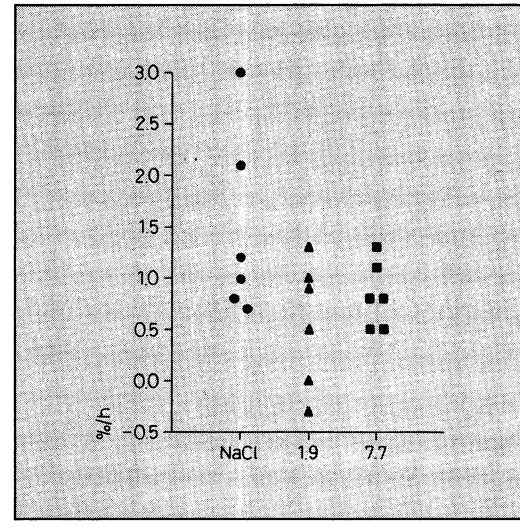

Fig. 4. Hemoconcentration during the first 4 h of *E. coli* and r-eglin C administration. Abscissa = Experimental groups; ordinate = hourly rise of hematocrit by linear regression over time (%/h) (n = 6 in each group).
E. coli, $3 \cdot 10^{10}$ cells + NaCl, 0.9% (control group) (●).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 1.9 mg·kg^{-1}·h^{-1} (▲).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 7.7 mg·kg^{-1}·h^{-1} (■).

Renal Function

Figure 5 shows a life table plot for the time intervals between time zero and the onset of anuria for the three groups. After 24 h, only 6 of all animals (≙ 35%) were not anuric: 1/6 (≙ 17%) of the septic reference animals but 3/5 (≙ 60%) and 2/6 (≙ 33%) of the low- and high-dose eglin-treated pigs, respectively. The difference between the three groups was not significant. When data from both eglin groups were pooled renal function was significantly protected ($p = 0.048$) when compared to the septic reference animals by the generalized Mantel-Haenszel test. Creatinine concentration in plasma increased from 1.06 ± 0.22 mg/100 ml at time zero to 2.6 ± 1.6 mg/100 ml (last available value for each animal). Plasma creatinine concentration was noticeably higher in controls than in the other groups after 6 h (1.2 ± 0.34 mg/100 ml in the low-dose group and 1.26 ± 0.26 mg/100 ml in the high-dose group as compared to 1.78 ± 0.29 mg/100 ml in the control group) and later on.

Eglin C Plasma Levels (fig. 6)

Maximum eglin C plasma levels were 9.6 ± 1.3 µg/ml (≙ 1.2 µ*M*) after 4 h of low-dose eglin C infusion, 1.9 mg·kg^{-1}·h^{-1}. In the group with the high eglin dose, 7.7 mg·kg^{-1}·h^{-1} for 4 h, 5 of 6 animals reached maximum plasma levels of 44.7 ± 13.6 µg/

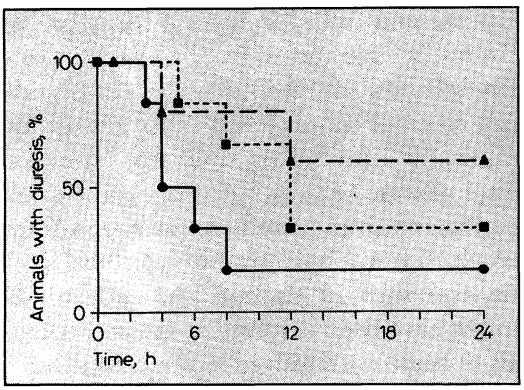

Fig. 5. Cumulative probability plot for the occurence of anuria after *E. coli* and r-eglin C administration (n = 6 in each group).
E. coli, $3 \cdot 10^{10}$ cells + NaCl, 0.9% (control group) (●—●).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 1.9 mg·kg^{-1}·h^{-1} (▲--▲).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 7.7 mg·kg^{-1}·h^{-1} (■----■).

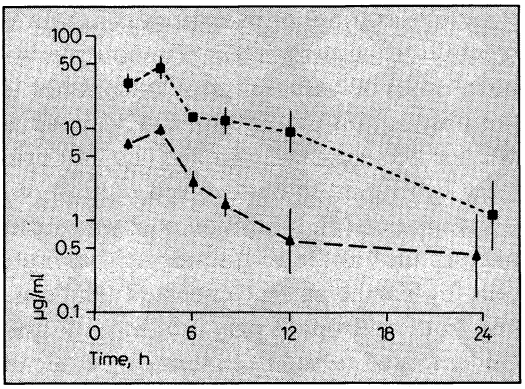

Fig. 6. Eglin C concentration in plasma after *E. coli* and r-eglin C administration. Ordinate: eglin C concentration in plasma (µg/ml, logarithmic scale, means ± SD) (n = 6 in each group).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 1.9 mg·kg^{-1}·h^{-1} (▲--▲).
E. coli, $3 \cdot 10^{10}$ cells + r-eglin C, 7.7 mg·kg^{-1}·h^{-1} (■----■).

ml (≙ 5.6 µ*M*) and 1 animal 100 µg/ml. The data of this latter experiment were not used in figure 6. Eglin plasma levels decayed rapidly after the end of the infusion.

Other Parameters

White blood cell counts uniformly showed a rapid initial decline by 80–90% with a minimum after 1–6 h followed by the slow development of leukocytosis in all animals that survived more than 12 h. Details on white blood cells and elastase-inhibitor complex levels in plasma have been reported elsewhere [11]. All animals showed a moderate degree of thrombocytopenia but platelet counts below 100,000/µl were found in 1 case only. The decrease was maximum after 8 h (186,000 ± 71,000/µl, i.e. 40% of 459,000 ± 124,000/µl before the start of the *E. coli* infusion). The arterial pO_2 of all animals at baseline was 85.2 ± 8.2 mm Hg. The majority of animals (11/18) experienced severe hypoxemia (decrease in arterial pO_2 by more than 30 mm Hg). The arterial pCO_2 of all animals at baseline was 38.2 ± 4.2 mm Hg. All but 2 animals (both from the high-dose eglin group) developed hypocapnia (arterial pCO_2 below 32 mm Hg). The respiratory rate was 26 ± 11/min at baseline, and all but 4 animals developed tachypnea (respiratory rate above 40/min). Acidosis (pH in arterial blood) was slightly less pronounced in the eglin-treated animals.

Discussion

Treatment of septic pigs with r-eglin C at two different dosages prevented a fall in blood pressure compared to the nontreated controls. Similarly, in a model of traumatic shock of the rat an improvement of the arterial blood pressure after eglin treatment was noted [3]. These findings may indicate the inhibition of the action of vasoactive mediators (with ensuing vascular smooth

muscle relaxation) or of compounds that increase vascular permeability (resulting in hypovolemia) or both. Of great importance is the observation that r-eglin C also inhibits porcine lung mast cell chymase [1]. This protease is believed to be involved in mast cell degranulation [5]. Inhibition of chymase can prevent the release of histamine from mast cells [6]. Furthermore, cathepsin G and elastase from neutrophils may influence the arachidonic acid cascade and thus play a role in vasoregulation [7]. Both enzymes are very rapidly and effectively inhibited by eglin C [10].

The fall in total protein concentration in plasma was significantly attenuated in the eglin-treated animals. Eglin C apparently protected the septic pigs from developing hypoproteinemia. Moreover, the control animals showed more severe hemoconcentration in their hematocrit values than the eglin-treated animals. This supports the view that an increase in permeability to protein was the primary event leading to subsequent loss of fluid from the vascular bed to the interstitial space. There is ample evidence connecting the action of lysosomal proteinases from neutrophils to increased vascular permeability [12]. Mainly involved are peptide fragments from large proteins such as coagulation and complement factors [13].

The influence of r-eglin C on hypovolemia and hypotension following *E. coli* septicemia is of great importance for renal function. However, the effect of r-eglin C treatment on the duration of urine production under this condition may also indicate a direct effect of lysosomal proteinases on kidney function. It was shown that neutrophils stimulated in situ by deposited immune complexes digest glomerular basement membrane [14]. Using an enzyme inhibitor, PMN elastase and cathepsin G were identified as the responsible agents. In 5 of 6 patients suffering from acute renal failure of various origin, elevated plasma levels of the PMN elastase-alpha-1-proteinase inhibitor complex were observed [2]. In blood smears of these patients reduced proteinase activity in granulocytes was found. Hence, lysosomal proteinases such as elastase and cathepsin G might have been involved in the pathogenesis of acute renal failure in these patients.

Although we did not detect statistically significant differences between the two eglin-treated groups, it is interesting to note that the animals in the low-dose group had better survival (fig. 1) and diuresis (fig. 5) curves and less hemoconcentration (fig. 4) than those in the high-dose group. We have to consider contamination of the recombinant gene product r-eglin C with other compounds of bacterial origin as a possible explanation. The batch or r-eglin C used in our experiment contained less than 5 ppm endotoxin [Schnebli H.P., Basel, Switzerland, personal commun.]. It remains questionable if such a low amount of endotoxin is able to influence the therapeutic effect of eglin C in septicemia in the manner that we suspected. Toxic effects of a first batch of r-eglin C given in a dose of 3.85 $mg \cdot kg^{-1} \cdot h^{-1}$ were clearly due to the presence of minute amounts of endotoxin [4].

We conclude that r-eglin C at plasma levels between 1 and 5 µmol/l is able to attenuate in vivo the permeability increase and arterial hypotension induced by systemic bacteremia in pigs. This indicates that liberated PMN elastase significantly contributes, among other factors, to the harmful effects precipitated by bacteria in the circulation. r-Eglin C might therefore be a useful drug for the treatment of septic shock in humans.

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