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Alpha-2-macroglobulin as the major defence in acute pseudomonal septic shock in the guinea-pig model*

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Summary. An intravenous injection of 1.2 mg/kg of *Pseudomonas aeruginosa* elastase induces immediate lethal shock in guinea-pigs. In the present study, alpha-2-macroglobulin (α_2M) was shown to be the major factor in guinea-pig plasma that inhibits the enzymatic activity of elastase *in vitro*. Depletion of circulating α_2M by injecting anti-guinea-pig α_2M rabbit IgG F(ab′)$_2$ rendered the animals sensitive to a dose of elastase of 0.05 mg/kg. When the α_2M-depleted guinea-pigs were reconstituted with human α_2M, this sensitivity was reversed. Lethal shock did not occur in α_2M-depleted animals even at an elastase dose of 0.2 mg/kg when Hageman factor was simultaneously depleted, indicating that elastase induces shock through activation of the Hageman factor-dependent system.

Similar results were obtained when the culture supernatants of an elastase-producing strain, IFO-3455, were used instead of the purified elastase, whereas no cardiovascular changes occurred, even in the α_2M-depleted guinea-pigs, when the culture supernatants were pretreated with an elastase specific inhibitor (zincov) or when the culture supernatants of an elastase non-producing strain, PA-103 were used.

Keywords: alpha-2-macroglobulin, pseudomonal elastase, septic shock

As the term 'endotoxin shock' is often used to represent 'septic shock', endotoxin has been thought to be the major or sole cause of Gram-negative septic shock (McCabe & Olans 1981; Sanford 1985). However, the effectiveness of septic shock treatment with anti-endotoxin monoclonal antibodies E5 (Greenman *et al.* 1991) and HA-1A (Ziegler *et al.* 1991) is still controversial (Warren *et al.* 1992). In addition, circulating endotoxin levels correlate poorly with clinical findings, microbiologic status and clinical outcome (Brock *et al.* 1988; Andersen *et al.* 1987; Rocke *et al.* 1987). For these reasons investigators have come to think that bacterial products other than endotoxin may also be important contributors to the toxicity and

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mortality of Gram-negative septic shock (Danner *et al.* 1990; Parker *et al.* 1987).

We have been paying special attention to some pathological functions of bacterial proteinases. We have reported that an intravenous injection of a Gram-negative bacterial proteinase, *Pseudomonas aeruginosa* elastase at a dose of 1.2 mg/kg, induced lethal shock in guinea-pigs (Khan *et al.* 1993a) through activation of the Hageman factor/kallikrein–kinin pathway (Khan *et al.* 1993b). The pattern of the shock, with low vascular resistance and low cardiac output, resembled that of clinical septic shock at the hypotensive crisis stage (Abraham *et al.* 1983). Since an intravenous injection of pseudomonal endotoxin at a dose of 2.0 mg/kg in guinea-pigs did not cause cardiovascular failure, we assumed that one of the major mechanisms that cause clinical septic shock might be the activation of the Hageman factor/kallikrein–kinin pathway by bacterial proteinases.

On the other hand, the major inhibitor against bacterial proteinases is thought to be α_2-macroglobulin (α_2M) (Northemann *et al.* 1988). α_2M is a plasma proteinase inhibitor with a molecular size of 720 kDa; α_2M forms a complex with proteinases by a unique trapping mechanism. This mechanism is initiated by proteolytic cleavage of the 'bait' region and subsequent activation of the internal β-cysteinyl-γ-glutamyl thiol-ester in the inhibitor molecules (Gonias *et al.* 1982). This induces a conformational change in α_2M, causing the entrapment of the proteinase and the exposure of previously hidden recognition sites for the receptors which are present on several cell types (Van Leuven *et al.* 1982), followed by the rapid clearance of the α_2M-proteinase complex by these highly discriminating receptors (Travis & Salvesen 1983).

As a logical consequence, we suggested an important role for α_2M in the prevention of septic shock. This assumption was challenged experimentally in the present study by the use of the pseudomonal elastase induced shock model. Since there is a single type of α_2M in the guinea-pig (Ishimatsu *et al.* 1984) in contrast to the rat and mouse, we used the guinea-pig as the experimental animal in this study.

Materials and methods

All surgical and experimental procedures were conducted only after prior approval from the Animal Care and Use Committee of Kumamoto University School of Medicine. Guidelines set by the National Institute of Health and the Public Health Service policy on the humane use and care of laboratory animals were followed at all times.

Animals

Albino Hartley strain guinea-pigs of both sexes (250–320 g) were used in these experiments.

Substances

IFO-3455, a proteinase producing strain, and PA-103, a proteinase non-producing strain of *Pseudomonas aeruginosa*, were obtained from Kitasato Institute (Tokyo, Japan). Trypto soy broth was purchased from Eiken Chemical Company (Tokyo, Japan). Crystallized preparations of pseudomonal elastase were obtained from Nagase Biochemicals (Fukuchiyama, Japan). Aminobenzyl (Abz)-Ala-Gly-Leu-Ala-nitrobenzylamide (Nba) and $HONHCOCH(CH_2C_6H_5)CO$-Ala-Gly-NH_2 (zincov), which were synthesized as reported previously (Nishino & Powers 1980), were gifts from Dr Nishino of Kyushu Institute of Technology, Kitakyushu, Japan. Anti-human α_2M rabbit antiserum was purchased from Behring-Werke company (Marburg, Germany). Other chemicals were purchased mostly from Wako Pure Chemicals (Osaka, Japan) and Nakarai Chemicals (Kyoto, Japan).

Preparation of culture supernatants of *Pseudomonas aeruginosa*

Strains IFO-3455 and PA-103 of *Pseudomonas aeruginosa* (10^8 colony forming unit/ml) in 20 ml of trypto soy broth were cultured for 18 hours at 30°C in a shaking water bath according to the method of Morihara and Homma (1985). The supernatants obtained by centrifuging for 20 minutes at 8000 ***g*** and passage through a sterile filter (0.22 μm pore size; Millipore, Bedford, MA) were stored at −85°C until used.

Preparation of α_2M, antibodies against α_2M, and inactive α_2M

Human or guinea-pig α_2M were purified from their respective plasmas as described previously (Ishimatsu *et al.* 1984). The preparations demonstrated single protein bands in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate with the apparent molecular size of 360 kDa or of 180 kDa in the absence or presence of a reducing reagent, respectively. From the trapping ability for trypsin, which was measured by the method described previously (Ishimatsu *et al.* 1984), more than 95% of the purified α_2M molecules were recognized to be active as the proteinase inhibitor (data not shown).

The preparative methods and the qualities of anti-

guinea-pig α_2M rabbit IgG F(ab′)$_2$ and of normal rabbit IgG F(ab′)$_2$ were described previously (Ishimatsu *et al.* 1984).

To prepare inactive molecules of human α_2M, a part of the purified preparation (1 mg/ml) was heated for 120 minutes at 80°C as described previously (Howard *et al.* 1980). The complete loss of inhibitory activity against pseudomonal elastase was confirmed by the enzymatic method with pseudomonal elastase as described below.

Immunoquantitation of α_2M and albumin

The concentration of α_2M in the plasma samples was measured by the rocket immunoelectrophoresis as described by Laurell (1966) or by the single radial immunodiffusion method according to Mancini *et al.* (1965). Albumin was measured by the single radial immunodiffusion method.

Measurement of pseudomonal elastase and treatment of it with synthetic inhibitor

The concentration of pseudomonal elastase was determined from the hydrolytic activity towards Abz-Ala-Gly-Leu-Ala-Nba by the use of the k_{cat} value as described previously (Nishino & Powers 1980). Elastase activity was inhibited with the synthetic inhibitor, $HONHCOCH(CH_2C_6H_5)CO$-Ala-Gly-NH_2 (zincov) as described previously (Nishino & Powers 1980).

Depletion of α_2M from human and guinea-pig plasmas in vitro

Normal human or guinea-pig plasmas (1000 μl) were mixed with one-fifth of their volume of a solution of the respective anti-α_2M IgG (F(ab′)$_2$ antibody ($A_{280} = 37.5$) and incubated for 30 minutes at 37°C. The antibody-treated plasmas were centrifuged for 10 minutes at 10 000 ***g*** as described previously (Ishimatsu *et al.* 1984). The supernatants were used as the α_2M-depleted plasma and contained no α_2M as detected by the immunoquantitation techniques described above. In control experiments, plasmas were pretreated with normal rabbit IgG F(ab′)$_2$ or saline instead of the anti-α_2M antibody.

Assay of elastase-trapping activity of α_2M

When the α_2M-attributed inhibitory capacity of plasma to pseudomonal elastase was measured, pseudomonal elastase (20 μl in saline) was treated for 30 minutes at 37°C with 180 μl of either saline, the α_2M-depleted plasma or the control plasmas which had been pre-treated with saline or with normal rabbit IgG F(ab′)2. The samples treated with the saline or the control plasmas were then divided equally into three parts, A, B and C, and mixed with one-fifth of their volume of either saline (A), the solution of anti-α_2M F(ab′)$_2$ antibody (B), or the solution of normal rabbit IgG F(ab′)$_2$ (C). To the group treated with the α_2M-depleted plasma, one-fifth of their volume of saline was added. All were incubated for 30 minutes at 37°C and centrifuged for 10 minutes at 10 000 ***g***. The elastase activity remaining in the supernatants was then measured as described above.

When the concentration of functionally active α_2M in plasma was measured, pseudomonal elastase (final concentration 5 μg/ml) was initially treated with serially diluted plasma samples. After removing the elastase–α_2M complex with the anti-α_2M antibody as described above, the peptidolytic activity of untrapped elastase was measured. From the calibration curve obtained with purified α_2M or normal plasma, where untrapped elastase activity was plotted as a function of dose of α_2M used, the concentration of α_2M in the plasma samples was calculated.

Measurement of systemic arterial blood pressure

Guinea-pigs were anaesthetized with a single intraperitoneal injection of sodium pentobarbital at a dose of 30 mg/kg. The right carotid artery was cannulated for the continuous measurement of blood pressure (BP) through a pressure transducer (TP-200T, Nihon Koden, Japan) as described previously (Haung & Leenen 1992; Khan *et al.* 1993a). The right external jugular vein was cannulated with a catheter for the intravenous injections.

Immunological depletion of circulating α_2M from guinea-pigs

Under continuous monitoring of BP, a total amount of 220 mg/kg of the anti-guinea-pig α_2M IgG F(ab′)$_2$ antibody in 2 ml of phosphate-buffered saline (PBS, pH 7.4) was infused slowly (0.66 ml/min) into the jugular vein of guinea-pigs. In the control animals, the same amount of normal rabbit IgG F(ab′)$_2$ in 2 ml of PBS was infused.

Reconstitution of α_2M-depleted guinea-pigs with human α_2M

A group of the α_2M-depleted guinea-pigs was infused with the purified human α_2M to a total amount of 7.1 mg/kg in 2 ml of PBS. In controls, 2 ml of either PBS or the heat-inactivated human α_2M in PBS were infused in the same way.

Artificial increment of circulating α_2M in guinea-pigs

Animals were given the purified guinea-pig α_2M ($A_{280} = 6.0$) to a total dose of 43.6 mg/kg in 3 ml of PBS intravenously (0.66 ml/min). As a control, 3 ml of PBS was infused in the same way.

Depletion of Hageman factor from guinea-pigs

Hageman factor in the blood stream was immunologically depleted as described previously (Khan *et al.* 1993b).

Statistical analysis

Results of the in-vitro experiments are described by mean ± standard deviation.

Results

α_2M as the major inhibitor of pseudomonal elastase in plasma

The inhibitory capacity of guinea-pig or human plasma against pseudomonal elastase was examined with respect to α_2M. As shown in Figure 1, both plasmas strongly inhibited the elastase. In contrast, the remaining activity of elastase after treatment with the α_2M-depleted plasmas was the same as that of the elastase treated with saline (data not shown in the figure). These results indicate that the whole inhibitory capacity of plasma against pseudomonal elastase was due to α_2M.

The inhibitory capacity of α_2M in the guinea-pig or human plasmas was quantitatively measured. In this experiment, pseudomonal elastase at various concentrations (10, 20, 30 and 60 μg/ml) was treated with the neat plasmas. In the case of human plasma, the inhibition of elastase was incomplete when the concentration of elastase was 20 μg/ml or more. At 60 μg/ml, the remaining activity of elastase was 31.3 ± 2.2% of its initial level (Figure 1). As shown in Figure 1, the inhibitory capacity of the guinea-pig plasmas used was slightly greater than that of the human. However, at an elastase concentration of 60 μg/ml, inhibition was incomplete as observed with the human plasma; the remaining activity of elastase was 6.5 ± 3.1% of its initial level.

Consumption of circulating α_2M in shock caused by intravenous pseudomonal elastase injection

Consistent with the previous report (Khan *et al.* 1993a),

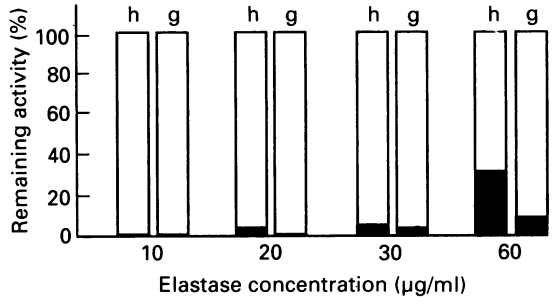

Figure 1. Inhibitory capacity of human (h) or guinea-pig (g) plasma against pseudomonal elastase. Different concentrations of elastase (10, 20, 30 or 60 μg/ml) were treated for 30 minutes at 37°C either with saline (100%); □, α_2M-depleted plasma or ■, control plasmas which were pretreated with saline or with normal rabbit IgG $F(ab')_2$. Then one-fifth of the volume of either saline or respective solution of anti-α_2M IgG $F(ab')_2$ or the solution of normal rabbit IgG $F(ab')_2$ was mixed. The samples treated with the α_2M-depleted plasma were mixed with one-fifth of their volume of saline. All samples were then incubated for 30 minutes at 37°C, centrifuged and measured for the remaining activity of elastase in the supernatants (see Materials and methods).

when 1.2 mg/kg of pseudomonal elastase in 0.5 ml saline was injected slowly (0.66 ml/min), all the guinea-pigs ($n = 10$) developed severe hypotension within 5 minutes (Figure 2a) and died by 45 minutes after the injection. Blood samples (maximum 0.5 ml) were taken from four guinea-pigs before and during shock, and the plasma concentrations of α_2M and of albumin were measured. In shock, the concentration of α_2M decreased to 30.6 ± 2.0% of its initial level (data not shown) when measured by the immunological technique. When the same samples were subjected to enzymatic assay to measure functional α_2M, the α_2M level observed was 26.7 ± 1.5% of the initial. Thus, most of the remaining α_2M in the circulation seemed to be functionally active. Since the plasma albumin level was found to be unchanged during shock (data not shown), the loss of α_2M was interpreted as the result of specific consumption by the elastase injection.

Effects of modulation of circulating α_2M level on pseudomonal elastase-induced shock

To confirm the essential role of α_2M in preventing the pseudomonal elastase-induced shock, circulating α_2M was depleted from guinea-pigs by intrajugular injections of the monospecific antibody prior to the elastase injection. As a control, $F(ab')_2$ of normal rabbit IgG was injected. After treatment with the rabbit IgG

$F(ab')_2$, plasma levels of α_2M as well as of albumin were measured by immunological means. Complete depletion of the circulating α_2M was achieved in all of the 12 guinea-pigs treated with the monospecific antibody without a significant change of the albumin level, whereas no change in these proteins was observed in the control animals ($n = 3$) (data not shown). The depleted state of α_2M lasted for more than 2 hours (data not shown). The infusion of the anti-α_2M $F(ab')_2$ antibody but not of the normal $F(ab')_2$ had some effect on the animals. A weak but evident fluctuation of the systemic blood pressure was observed, within a range between 5 and 10 mmHg, in association with a hurried respiration. These untoward reactions started 5–6 minutes after the infusion, continued for 15–20 minutes, and then disappeared. When the condition of the animals became stable around 30 minutes after the final injection of the antibody, pseudomonal elastase was injected intravenously.

The α_2M-depleted guinea-pigs fell into lethal shock when only 0.05 mg/kg of pseudomonal elastase was injected (Figure 2c), some even dying at as little as 0.025 mg/kg of elastase. In contrast, the normal rabbit IgG $F(ab')_2$-injected control guinea-pigs ($n = 2$) had no cardiovascular response to 0.05 or 0.2 mg/kg of pseudomonal elastase, nor did the normal guinea-pigs ($n = 3$) (Figure 2b). When the pseudomonal elastase was pretreated with the synthetic inhibitor of elastase (zincov) and injected into the α_2M-depleted animals ($n = 2$), no cardiovascular alteration occurred (data not shown).

Human α_2M had negligible reactivity to the anti-guinea-pig α_2M rabbit $F(ab')_2$ antibody when examined with the double immunodiffusion technique (not shown). Therefore, a group of the α_2M-depleted guinea-pigs were reconstituted with the purified human α_2M. The reconstitution was achieved up to 1.58 mg/ml of α_2M in the plasma 5 minutes after the intravenous infusion. The reconstituted guinea-pigs were not affected at all by the injection of elastase at 0.05 mg/kg (data not shown). Even at 0.3 mg/kg, they developed only mild and reversible hypotension (Figure 2d). In contrast, reconstitution with the heat-inactivated human α_2M did not restore resistance. The guinea-pigs ($n = 2$) died at 0.3 mg/kg of pseudomonal elastase (data not shown).

A converse experiment was also carried out. When normal guinea-pigs ($n = 2$) were first given purified guinea-pig α_2M intravenously to raise the plasma α_2M level to 150% of the initial, injection of a lethal dose of

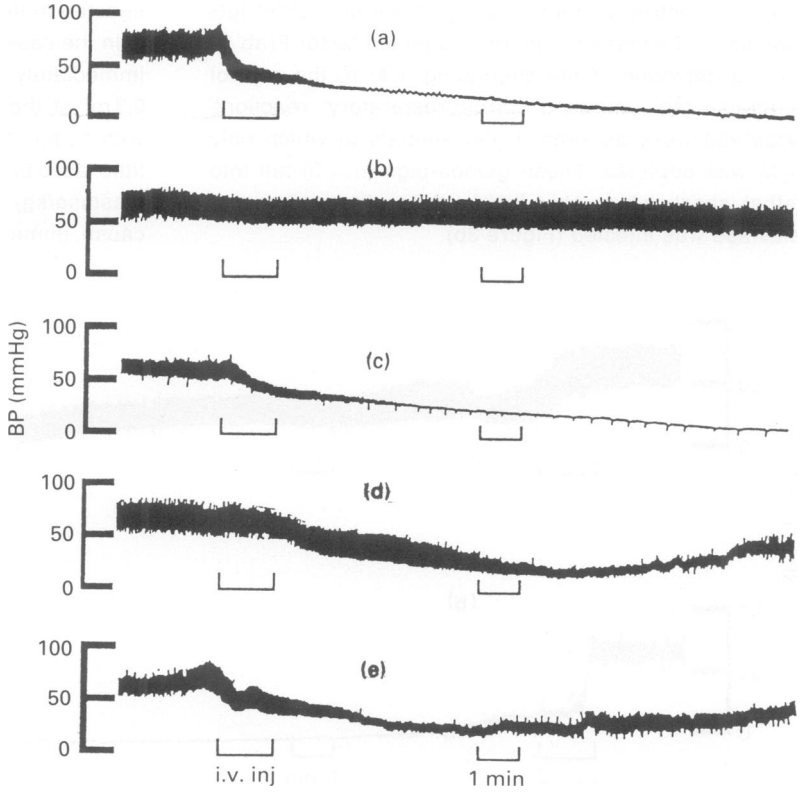

Figure 2. Role of circulating α_2M in prevention of shock caused by administration of purified pseudomonal elastase. Typical tracings showing the changes of arterial blood pressure (BP). The horizontal and vertical axes indicate time period (min) and BP (mmHg), respectively. i.v.inj. denotes the period of intravenous injection of elastase. Purified pseudomonal elastase was dissolved in saline. a, Elastase (0.5 ml of 800 μg/ml, 1.2 mg/kg) was injected into a normal guinea-pig. b, Elastase (0.5 ml of 135 μg/ml, 0.2 mg/kg) was injected into a normal guinea-pig. c, Elastase (0.5 ml of 35 μg/ml, 0.05 mg/kg) was injected into an α_2M-depleted guinea-pig. d, Elastase (0.5 ml of 200 μg/ml, 0.3 mg/kg) was injected into a guinea-pig which was initially depleted, the circulating α_2M then reconstituted with human α_2M. e, Elastase (0.5 ml of 800 μg/ml, 1.2 mg/kg) was injected into a guinea-pig in which plasma α_2M level was previously raised up to 150% of the normal by intravenous injection of purified guinea-pig α_2M.

pseudomonal elastase (1.2 mg/kg) caused only a mild and reversible hypotension (Figure 2e).

Effect of simultaneous depletion of Hageman factor on the elastase-induced shock in α_2M-depleted guinea-pigs

To confirm that the mechanism of the pseudomonal elastase-induced shock in the α_2M-depleted guinea-pigs was due to activation of the Hageman factor dependent pathway, as in normal guinea-pigs (Khan *et al.* 1993b), the effect of pseudomonal elastase was examined by double depletion of both α_2M and Hageman factor.

Initially, the circulating Hageman factor was depleted by the intravenous infusion of monospecific anti-guinea-pig Hageman factor goat IgG $F(ab')_2$, as described previously (Khan *et al.* 1993b). By this treatment the Hageman factor-depleted state continued for at least 2 hours (Khan *et al.* 1993b). After 20 minutes, the circulating α_2M was then depleted. In animals simultaneously depleted of Hageman factor, the untoward reactions which were observed when they were depleted of α_2M alone were not seen. These guinea-pigs ($n = 2$) developed only a mild and reversible hypotension but did not fall into shock after the pseudomonal elastase injection, even at 0.2 mg/kg (Figure 3a). They survived more than 6 hours without any adverse reactions.

In the control animals, $F(ab')_2$ of normal rabbit IgG was infused instead of the anti-Hageman factor $F(ab')_2$. During depletion of the circulating α_2M in the control animals, the untoward cardio-respiratory reactions observed were as seen in the animals in which only α_2M was depleted. These guinea-pigs ($n = 3$) fell into lethal shock when only 0.025 mg/kg of pseudomonal elastase was injected (Figure 3b).

Induction of shock with pseudomonal culture supernatants, and its prevention by α_2M or synthetic elastase inhibitor

In the next series of experiments, pseudomonal culture supernatants were used instead of purified elastase. A representative sample of the culture supernatant of IFO-3455 contained elastase and alkaline proteinase at concentrations of 100 μg/ml and 0.059 μg/ml, respectively. Concentrations of endotoxin and exotoxin A were 162 μg/ml and 3.0 μg/ml, respectively (Khan *et al.* 1993a). In a typical supernatant of PA-103, no proteinase activity was detected. Concentrations of endotoxin and exotoxin A were 81 μg/ml and 10 μg/ml, respectively (Khan *et al.* 1993a).

Consistent with the previous report (Khan *et al.* 1993a), immediate hypotension (Figure 4a) with other symptoms of shock was observed when 2 ml (7.14 ml/kg) of the culture supernatant of the elastase producing strain, IFO-3455, corresponding to an elastase dose of 0.73 mg/kg, were injected intravenously into normal guinea-pigs. All the guinea-pigs ($n = 6$) died around one hour after the injection. When 0.3 ml of the culture supernatant was injected after dilution with 1.70 ml of saline (elastase, 0.10 mg/kg), no significant cardio-respiratory change was observed ($n = 3$) (Figure 4b). The dilution with saline was to adjust the sample volume injected to the same final volume of 2 ml.

In the case of α_2M-depleted guinea-pigs, the animals immediately fell into the same lethal shock at a dose of 0.3 ml of the culture supernatants of IFO-3455 diluted with 1.7 ml of saline ($n = 2$) (data not shown). A dose as little as 0.02 ml of the culture supernatant (0.0073 mg elastase/kg) diluted with 1.98 ml of saline was enough to cause immediate hypotension (Figure 4c), with other

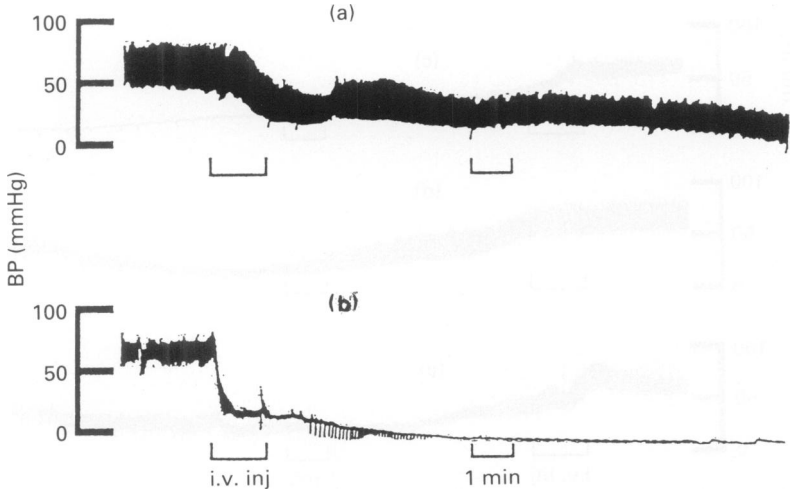

Figure 3. Typical tracings showing the defensive role of the Hageman factor depletion on the elastase-induced shock in α_2M-depleted guinea-pigs. Notations are the same as those in Figure 3.
a, Elastase (0.2 mg/kg) was injected into a guinea-pig of which the circulating Hageman factor and α_2M had been depleted. b, Elastase (0.025 mg/kg) was injected into a guinea-pig which had been treated with normal rabbit IgG $F(ab')_2$ and depleted α_2M.

severe shock symptoms followed by death within one hour ($n = 5$), when the minimum requirement was examined in the depleted animals.

When α_2M-depleted guinea-pigs were reconstituted with human α_2M as described above, the guinea-pigs ($n = 3$) exhibited no significant cardio-respiratory responses, even to 0.3 ml of the culture supernatant diluted with 1.7 ml of saline (Figure 4d). When the culture supernatant was pretreated with the specific elastase inhibitor (zincov) at a concentration of 5 mM as described previously (Khan *et al.* 1993a), even 2 ml did not cause any cardio-respiratory changes in the α_2M-depleted guinea-pigs ($n = 2$) (Figure 4e).

In contrast, when the culture supernatants of the elastase non-producing strain PA-103 were injected at 2 or 4 ml (to adjust the dose of endotoxin to that in 2 ml of the IFO-3455), no significant cardio-respiratory change was observed in either normal ($n = 4$) (Khan *et al.* 1993a) or α_2M-depleted guinea-pigs ($n = 3$) (Figure 4f).

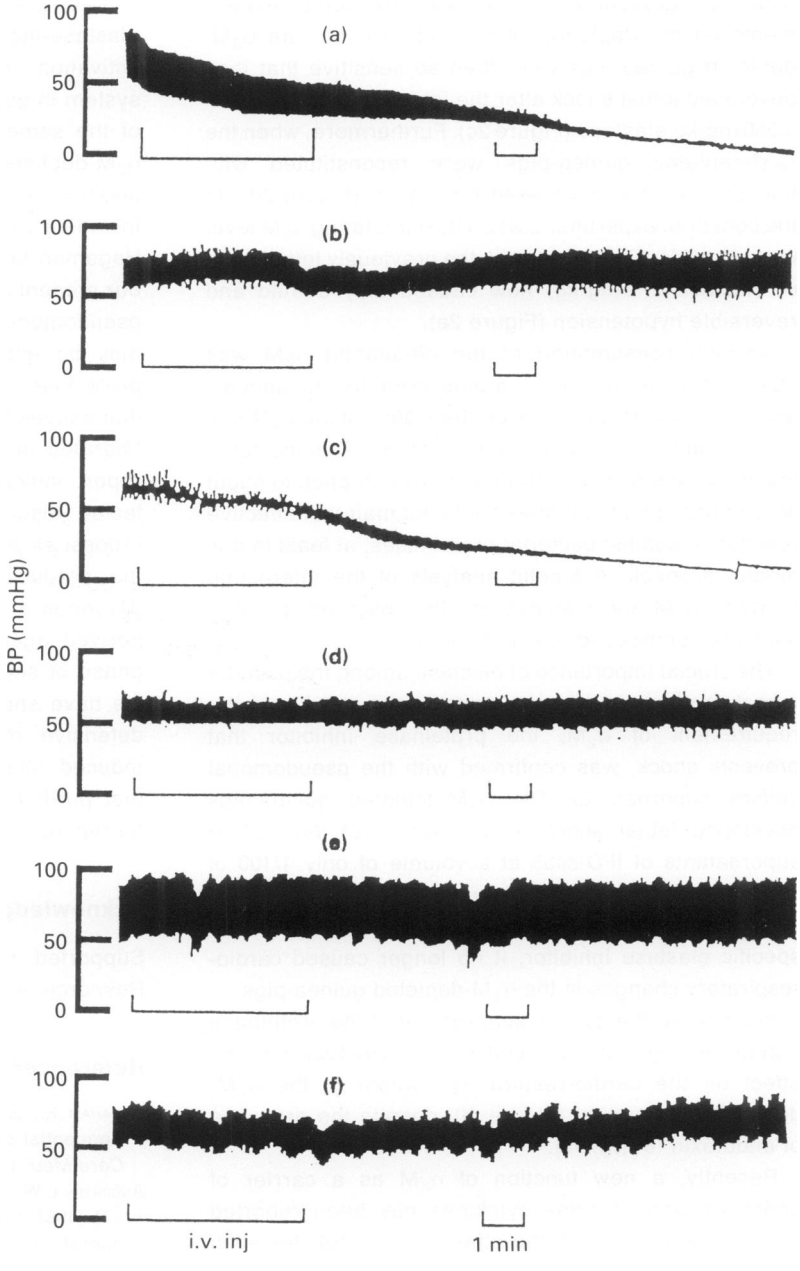

Figure 4. Typical tracings showing role of α_2M in the prevention of shock caused by intravenous injection of culture supernatants of *Pseudomonas aeruginosa*. Notations are the same as those in Figure 3. a, 2 ml (7.14 ml/kg) of the culture supernatant were injected intravenously into a normal guinea-pig; b, 2 ml (0.3 ml of the culture supernatant mixed with 1.7 ml of saline) were injected into a normal guinea-pig; c, 2 ml (0.02 ml of the culture supernatant mixed with 1.98 ml of saline) were injected into an α_2M-depleted guinea-pig; d, 2 ml of the culture supernatant were injected into a guinea-pig in which the circulating α_2M had been initially depleted and then reconstituted with human α_2M; e, 2 ml of the culture supernatant of IFO-3455 pretreated with the specific elastase inhibitor (zincov, final concentration 5 mM) were injected into an α_2M-depleted guinea-pig; f, 2 ml of a culture supernatant obtained from the elastase non-producing strain PA-103 were injected into an α_2M-depleted guinea-pig.

Discussion

The present in-vitro experiments indicated that α_2M is the major inhibitor of pseudomonal elastase in plasma (Figure 1). The inhibitory capacity of α_2M in plasma, however, was not enough to completely block elastase at concentrations of 30–60 μg/ml (Figure 1). This capacity may explain the minimum lethal dose of elastase (1.2 mg/kg) in the guinea-pigs, because the administration of 1.2 mg/kg theoretically resulted in a level of 60 μg/ml of elastase in the circulating plasma.

The most striking evidence for the essential role of α_2M in the prevention of shock was provided by experiments on the depletion of circulating α_2M. The α_2M-depleted guinea-pigs were then so sensitive that they developed lethal shock after the injection of only 0.025–0.050 mg/kg elastase (Figure 2c). Furthermore, when the α_2M-depleted guinea-pigs were reconstituted with human α_2M, they recovered resistance (Figure 2d). In the converse experiment, when the circulating α_2M level was raised to 150% of normal, the previously lethal dose of elastase (1.2 mg/kg) now caused only a mild and reversible hypotension (Figure 2e).

Specific consumption of the circulating α_2M was observed in normal guinea-pigs prior to the appearance of shock. However, even then, 30% of the α_2M still in the circulation was functionally active. This indicates that if the plasma concentration of α_2M dropped to about 30% of normal, the animals could not maintain effective resistance against bacterial proteinases, at least in this model of shock. A kinetic analysis of the interaction between α_2M and elastase or other bacterial proteinases may be needed to explain this.

The crucial importance of elastase among the pseudomonal products in causing acute septic shock and the requirement of α_2M, the proteinase inhibitor that prevents shock, was confirmed with the pseudomonal culture supernatants. The α_2M-depleted guinea-pigs developed lethal shock after injection of the culture supernatants of IFO-3455 at a volume of only 1/100 of the lethal dose for normal guinea-pigs (Figure 4a, c). When the culture supernatant was pretreated with the specific elastase inhibitor, it no longer caused cardio-respiratory changes in the α_2M-depleted guinea-pigs.

In contrast, the culture supernatant of the proteinase non-producing pseudomonal strain (PA-103) had no effect on the cardio-respiratory system in the α_2M-depleted guinea-pigs (Figure 4f), despite the presence of endotoxin (81 μg/ml).

Recently, a new function of α_2M as a carrier of endotoxin and of some cytokines has been reported (Liebl & Koo 1993). On the other hand, cytokines such as interleukin (IL)-1, IL-6 and tumour necrosis factor are proposed as endogenous mediators in septic shock. Therefore, the effect of α_2M-depletion on the sensitivity of the animals to shock observed in the present study may be interpreted as an augmentation of the functions of those cytokines resulting from the absence of the carrier molecule. However, it seems more reasonable to interpret our results as correlated with the function of α_2M as a proteinase inhibitor, because the inducer of the shock is indeed the bacterial proteinase and the cytokine-mediated phenomenon usually takes hours rather than the minutes that were observed in the present study.

It was previously demonstrated that the pseudomonal elastase-induced shock was mediated through the activation of the Hageman factor/kallikrein–kinin system in guinea-pigs (Khan *et al.* 1993b). Participation of the same mechanism in the shock induced in the α_2M-depleted animals was shown here (Figure 3a). In addition, 9 out of 11 microbial proteinases examined, including pseudomonal elastase, activated guinea-pig Hageman factor *in vitro* (Molla *et al.* 1989). Therefore, our present conclusion in regard to the essential role of pseudomonal elastase in the acute septic shock model may be extended logically to many other bacterial proteinases. In this acute model, the chemical mediator that causes the hypotension must be bradykinin finally liberated in the cascade reaction. However, a recent report indicated that an activated form of Hageman factor also stimulated monocytes to release IL-1 (Toossi *et al.* 1992). Furthermore, bradykinin mediated the production of nitric oxide by endothelial cells (Baydoun & Woodward 1991). Therefore, the bacteria-derived agent and endogenous mediator in the late phase of septic shock seems not to be so simple. As we have shown that α_2M acts as the most important defensive molecule against pseudomonal elastase-induced shock in the present study, we now think that purified α_2M should be included in the strategic therapeutic agents for clinical septic shock.

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References

Abraham E., Shoemaker W.C., Bland R.D. & Cobo J.C. (1983) Sequential cardio-respiratory patterns in septic shock. *Crit. Care Med.* **11,** 799–803.

Andersen L.W., Baek L., Degn H., Lehd J., Krasnik M. & Rasmussen J.P. (1987) Presence of circulating endotoxins during cardiac operations. *J. Thorac. Cardiovasc. Surg.* **93,** 115–119.

Baydoun A.R. & Woodward B. (1991) Effects of bradykinin in the rat isolated perfused heart: role of kinin receptors and endothelium-derived relaxing factor. *Br. J. Pharmacol.* **103,** 1829–1833.

Brock U.J.G., Gaffin S.L., Wells M.T., Gathiram P., Sohar E., James M.F., Morrell D.F. & Norman R.J. (1988) Endotoxaemia in exhausted runners after a long-distance race. *S. Afr. Med. J.* **73,** 533–536.

Danner R.L., Natanson C., Elin R.J., Hosseini J.M., Banks S., Macvittie T.J. & Parrillo J.E. (1990) *Pseudomonas aeruginosa* compared with *Escherichia coli* produces less endotoxemia but more cardiovascular dysfunction and mortality in a canine model of septic shock. *Chest.* **98,** 1480–1487.

Gonias S.L., Reynolds J.A. & Pizzo S.V. (1982) Physical properties of human α_2-macroglobulin following reaction with methylamine and trypsin. *Biochim. Biophys. Acta.* **705,** 306–314.

Greenman R.L., Schein R.M., Martin M.A., Wenzel R.P., Macintyre N.R., Emmanuel G., Chmel H., Kohler R.B., McCarthy M., Plouffe J. & Russell J.A. (1991) A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of Gram-negative sepsis. *JAMA.* **266,** 1097–1102.

Haung B.S. & Leenen F.H.H. (1992) Brain ouabain and central effects of dietary sodium in spontaneously hypertensive rats. *Circ. Res.* **70,** 430–437.

Howard J.B., Vermeulen M. & Swenson R.P. (1980) The temperature-sensitive bond in human alpha-2-macroglobulin is the alkylamine-reactive site. *J. Biol. Chem.* **255,** 3820–3823.

Ishimatsu T., Yamamoto T., Kozono K. & Kambara T. (1984) Guinea-pig macroalbumin. A major inhibitor of activated Hageman factor in plasma with an alpha-2-macroglobulin-like nature. *Am. J. Pathol.* **115,** 57–69.

Khan M.M.H., Yamamoto T., Araki H., Ijiri Y., Shibuya Y., Okamoto M. & Kambara T. (1993a) Pseudomonal elastase injection causes low vascular resistant shock in guinea-pigs. *Biochim. Biophys. Acta.* **1182,** 83–93.

Khan M.M.H., Yamamoto T., Araki H., Shibuya Y. & Kambara T. (1993b) Role of Hageman factor/kallikrein–kinin system in pseudomonal elastase-induced shock model. *Biochim. Biophys. Acta.* **1157,** 119–126.

Laurell C.B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* **15,** 45–52.

Liebl D.J. & Koo P.H. (1993) Comparative binding of neurotrophins (NT-3, CNTF and NGF) and various cytokines to α_2-macroglobulin. *Biochem. Biophys. Res. Commun.* **193,** 1255–1261.

Mancini G., Carbonarra A.O. & Heremans J.F. (1965) Immunochemical quantitation of antigens by single radial Immunodiffusion. *Immunochemistry.* **2,** 235–254.

McCabe W.R. & Olans R.N. (1981) Shock, III: Gram-negative bacteremia: predisposing factors, pathophysiology and treatment. In *Current Clinical Topics in Infectious Diseases.* Eds J.S. Remington & M.N. Swartz. New York: McGraw-Hill International Book Co. pp. 121–150.

Molla A., Yamamoto T., Akaike T., Miyoshi S. & Maeda H. (1989) Activation of Hageman factor and prekallikrein and generation of kinin by various microbial proteinases. *J. Biol. Chem.* **264,** 10589–10594.

Morihara K. & Homma J.Y. (1985) Pseudomonas proteases. In *Bacterial Enzymes and Virulence.* Ed. I.A. Holder. Florida: CRC Press. pp. 41–79.

Nishino N. & Powers J.C. (1980) *Pseudomonas aeruginosa* elastase. Development of a new substrate, inhibitors, and an affinity ligand. *J. Biol. Chem.* **255,** 3482–3486.

Northemann W., Shiels B.R., Braciak T.A., Hanson R.W., Heinrich P.C. & Fey G.H. (1988) Structure and acute-phase regulation of the rat alpha-2-macroglobulin gene. *Biochemistry.* **27,** 9194–9203.

Parker M.M., Shelhamer J.H., Natanson C., Alling D.W. & Parrillo J.E. (1987) Serial cardiovascular variables in survivors and nonsurvivors of human septic shock: heart rate as an early predictor of prognosis. *Crit. Care Med.* **15,** 923–929.

Rocke D.A., Gaffin S.L., Wells M.T., Koen Y. & Brock U.J.G. (1987) Endotoxemia associated with cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.* **93,** 832–837.

Sanford J.P. (1985) Septic shock. In *Contemporary issues in infectious diseases.* Eds. M.A. Sande & R.C. Root. New York: Churchill Livingstone Inc. pp. 1–11.

Toossi Z., Sedor J.R., Mettler M.A., Everson B., Young T. & Ratnoff O.D. (1992) Induction of expression of monocyte interleukin 1 by Hageman factor (XII). *Proc. Natl. Acad. Sci.* **89,** 11969–11972.

Travis J. & Salvesen G.S. (1983) Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52,** 655–709.

Van Leuven F., Cassiman J.J. & Vandenberghe H. (1982) Functional modifications of alpha-2-macroglobulin by primary amines. Kinetics of inactivation of alpha-2-macroglobulin by methylamine, and formation of anomalous complexes with trypsin. *Biochem. J.* **201,** 119–128.

Warren H.S., Danner R.L. & Munford R.S. (1992) Anti-endotoxin monoclonal antibodies. *N. Engl. J. Med.* **326,** 1151–1157.

Ziegler E.J., Fisher, C.J. Jr., Sprung C.L., Straube R.C., Sadoff J.C., Foulke G.E., Wortel C.H., Fink M.P., Dellinger R.P., Teng N.N., Allen I.E., Berger H.J., Knatterud G.L., Lobuglio A.F. & Smith C.R. (1991) Treatment of Gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double blind, placebo-controlled trial. *N. Engl. J. Med.* **324,** 429–436.