

# Combined treatment with C1 esterase inhibitor and antithrombin III improves survival in severe acute experimental pancreatitis

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## Abstract

**Background**—Patients with severe acute pancreatitis die of complications closely related to the systemic activation of protease cascades.

**Aim**—To examine the effects of human C1 esterase inhibitor (C1 INH) and antithrombin III (AT III) on two experimental models of acute pancreatitis.

**Methods**—Oedematous pancreatitis was induced by continuous intravenous infusion of caerulein and haemorrhagic pancreatitis by retrograde injection of sodium taurocholate into the biliary pancreatic duct. C1 INH and AT III were given intravenously, either before or after the induction of pancreatitis. Treatment with C1 INH and AT III had no beneficial effect on oedematous pancreatitis. On the other hand, combined C1 INH and AT III therapy improved the survival in haemorrhagic pancreatitis compared with treatment with human serum albumin. This reduction in mortality was found regardless of whether the treatment was given prophylactically or therapeutically.

**Conclusions**—treatment with C1 INH and AT III represents a promising therapeutic concept for patients with severe haemorrhagic pancreatitis.

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Keywords: experimental acute pancreatitis, C1 esterase inhibitor, antithrombin III, kallikrein-kinin system, coagulation system.

Patients with acute pancreatitis can be divided into two groups. Between 80% and 85% develop a benign self limiting disease not requiring any specific treatment. The other 15% to 20% of patients develop a severe haemorrhagic and necrotising variety of pancreatitis. Some 10% to 20% of these die of multiorgan failure despite intensive care treatment or surgery. The multiorgan failure of severe acute pancreatitis is often associated with sepsis and disseminated intravascular coagulation. Systemic activation of various protease cascades may contribute to these severe complications.

Plasma C1 esterase inhibitor (C1 INH) is a single chain glycoprotein with a molecular weight of 105 kDa. C1 INH is the only plasma inhibitor of C1r and C1s, two components of the classic pathway of complement.<sup>1</sup> The activation of the complement cascade produces

the membrane attack complex (C5b-9 complex) and anaphylatoxins (C3a and C5a). In addition, C1 INH is regarded as a major physiological inhibitor of plasma kallikrein<sup>2 3</sup> and of factor XIIa<sup>4</sup> of the contact system. The activation of the kallikrein-kinin system produces bradykinin. Both protease cascades increase vascular permeability and may contribute to the hypovolaemic shock found in lethal acute pancreatitis.<sup>5</sup> On the other hand, antithrombin III (AT III) is the major inhibitor in the coagulation system of thrombin, factor Xa, IXa, and XIIa. The activities of kallikrein, plasmin, and trypsin are also inhibited by AT III.<sup>6</sup> Overactivation of the coagulation system induces the formation of thrombi within the vascular system, and may cause organic hypoperfusion. We have used two unrelated experimental models of pancreatitis which mimic a clinically mild and a clinically severe disease variety. We have tested whether C1 INH or AT III can influence the course and outcome of pancreatitis in these models.

## Methods

### MATERIALS

Human C1 INH (Berinert) and AT III (Kybernin) were obtained from Behringwerke AG, Marburg, Germany. Caerulein (Takus) was purchased from Pharmacia GmbH (Erlangen, Germany). Sodium taurocholic acid and human serum albumin (HSA) were purchased from Sigma Chemical Co, St Louis, Missouri, USA.

Male Wistar rats (body weight 250–300 g) were obtained from the breeding colony of Ulm University Animal Facilities. They were kept on a 12 hour light/dark cycle with free access to standard diet and water. The experiments were approved by the institutional animal care committee and carried out according to the National Animal Welfare Law.

### EXPERIMENTAL PROTOCOL

#### Experiment I

The animals were equipped with jugular vein catheters (PE 10) under general anaesthesia (60 mg/kg intraperitoneal pentobarbitone). Oedematous pancreatitis was induced by continuous intravenous infusion of caerulein (10 µg/kg/h) via this catheter.<sup>7</sup> C1 INH, AT III, and HSA were given as boluses through this catheter as follows:

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**Experiment Ia: prophylactic treatment with C1 INH and AT III** – Rats were divided into four groups (n=5). Animals of each group were injected with C1 INH (250 U/2 ml/kg), AT III (250 U/2 ml/kg), both, or HSA (50 mg/2 ml/kg) intravenously. The injections were given 15 minutes before, and two hours and four hours after the start of caerulein infusion. The rats were killed after a six hour infusion of caerulein.

**Experiment Ib: therapeutic treatment with C1 INH and AT III** – Rats were divided into four groups (C1 INH, AT III, both, or HSA in the doses mentioned above). The injections were given two hours and four hours after the start of caerulein infusion. The rats (n=5) were killed after a 12 hour infusion of caerulein by exsanguination with blood sampling from the abdominal aorta. The blood was used for determination of serum amylase. The pancreas was removed and was prepared for the measurement of pancreatic wet weight and histological examination.

### Experiment II

The animals were equipped with a jugular vein catheter. C1 INH, AT III, and HSA in the above mentioned concentrations were injected through this catheter. After laparotomy the common bile duct was clamped at the liver hilus. Taurocholic acid (5%, 2 ml/kg) was injected for a period of three minutes into the common biliopancreatic duct with a 27 G needle inserted through the duodenal wall into the duct. After removal of the needle and the clip the abdomen was closed in two layers. The boluses of C1 INH, AT III, and HSA were given as follows:

**Experiment IIa: prophylactic treatment with C1 INH and AT III** – Rats were randomly divided into four groups (C1 INH, AT III, both, or HSA). The injections were given 15 minutes before and two hours and four hours after the injection of taurocholate (n=10).

**Experiment IIb: therapeutic treatment with C1 INH and AT III** – Rats were randomly divided into three groups (C1 INH, AT III, or both). The injections were given one hour, three hours, and five hours after the induction of pancreatitis (n=8).

The survival of the rats was noted for 72 hours after the induction of pancreatitis, a time interval that has previously been determined to allow for the best discrimination between treatments.<sup>8</sup>

### Experiment III

Pancreatitis was induced by taurocholate in rats as in experiment II. After the induction of pancreatitis, rats were randomly divided into two groups. In one group both C1 INH (250 U/kg) and AT III (250 U/kg) were given intravenously as a bolus (one hour, three hours, and five hours) after the induction of pancreatitis. In the control group, HSA (50 mg/kg) was given instead of C1 INH and AT III. Twenty four hours after the induction of pancreatitis, ascites fluid was collected from

the surviving rats (n=6) under light ether anaesthesia, and its volume was measured. Blood (1.6 ml) was sampled from the abdominal aorta by a chilled plastic syringe containing 0.4 ml citrate (3.13% sodium citrate). Residual blood was collected for the measurement of serum amylase activity. Plasma C1 INH and AT III activity and lactate dehydrogenase (LDH) and lactate measurements were performed. After the exsanguination of the rats, the pancreas, lungs, liver, and kidneys were removed for histological examination.

The concentrations of C1 INH and AT III used in this study were higher than those in clinical usage, which adjusts for the shorter biological activity of human C1 INH and AT III in rats.<sup>5 9 10</sup>

### HISTOLOGICAL EVALUATION

For light microscopy, tissue of the pancreas and the above mentioned organs were fixed in 4% paraformaldehyde and embedded in paraffin wax for routine sections. Histological specimens (5 µm) were stained with haematoxylin and eosin and morphological alterations were graded as previously reported by an observer familiar with pathological alterations, but unaware of the treatment groups.<sup>11 12</sup>

### BIOCHEMICAL DETERMINATIONS

Amylase, LDH, and lactate concentrations were measured with test kits for a photometric autoanalyser (Cobas Bio, Hoffmann-La Roche AG, Basel, Switzerland). Activities of C1 INH and AT III in plasma were measured by their abilities to inactivate human C1 esterase and  $\alpha$ -thrombin respectively, with commercial test kits (Behringwerke AG, Marburg, Germany). The activities of C1 INH and AT III in standard human plasma were defined as 100%.

### STATISTICAL ANALYSIS

The results are expressed as means (SEM). Statistical evaluations of the difference between the mean values of the various groups of experiments were made with non-paired Student's *t* test and one way analysis of variance (ANOVA). Survival data were analysed using a  $\chi^2$  test.

## Results

### EXPERIMENT I

Serum amylase activity increased from 3118 (220) to 19920 (2200) U/l after infusion of caerulein. In the prophylactic treatment group C1 INH and AT III either alone or together had no effect on serum amylase activity (C1 INH 25700 (6120) U/l; AT III 26790 (4240) U/l; C1 INH+AT III 25200 (3500) U/l).

Pancreatic oedema was expressed as wet weight per body weight and was increased from 3.28 to 10.42 (0.53) mg/g after infusion of caerulein. Treatment with C1 INH (10.21

(0.61 mg/g), or AT III (9.27 (0.62) mg/g), or both substances together (8.80 (0.85) mg/g), had no effect on pancreatic oedema.

As in the prophylactic treatment group, therapeutic administration of C1 INH or AT III did not alter serum amylase activity and pancreatic oedema compared with the HSA group (data not shown). In the histological examination, C1 INH or AT III did not have any beneficial effects on oedema formation, neutrophil infiltration, or vacuolisation of acinar cells in pancreatitis.

#### EXPERIMENT II

In taurocholate induced pancreatitis, prophylactic treatment with C1 INH or AT III increased the survival rate at 72 hours after the induction of pancreatitis from 10% to 40% and 50% (Fig 1A). This result indicates a positive (but non-significant) trend for both treatment regimens. The combination therapy with C1 INH and AT III improved survival significantly (Fig 1A). Administration of C1 INH or AT III alone after the induction of pancreatitis did not

improve survival, whereas the combination therapy with both inhibitors significantly improved survival compared with the HSA treated control group (Fig 1B).

#### EXPERIMENT III

Combined treatment with C1 INH and AT III did not reduce ascites formation or amylase exudation into ascites in taurocholate induced pancreatitis. Combined administration of the two inhibitors significantly prevented the increases in serum amylase and plasma LDH activities, but not the increase in plasma lactate (Fig 2). Plasma C1 INH activity did not change at 24 hours in the group treated with HSA compared with the untreated group, but in the group treated with C1 INH and AT III, plasma C1 INH activity was significantly higher (Fig 3). Plasma AT III activity decreased after the induction of pancreatitis, but treatment with AT III prevented this reduction at 24 hours (Fig 3). On histological examination, severe necrosis and haemorrhage of the pancreas were found in both groups, without difference in the severity of pancreatitis (Fig 4). Focal alveolar wall oedema in the lung and cell infiltration and focal necrosis under the surface of the liver were found in some rats of both groups. We did not see any notable changes in the kidneys in any animals (data not shown).

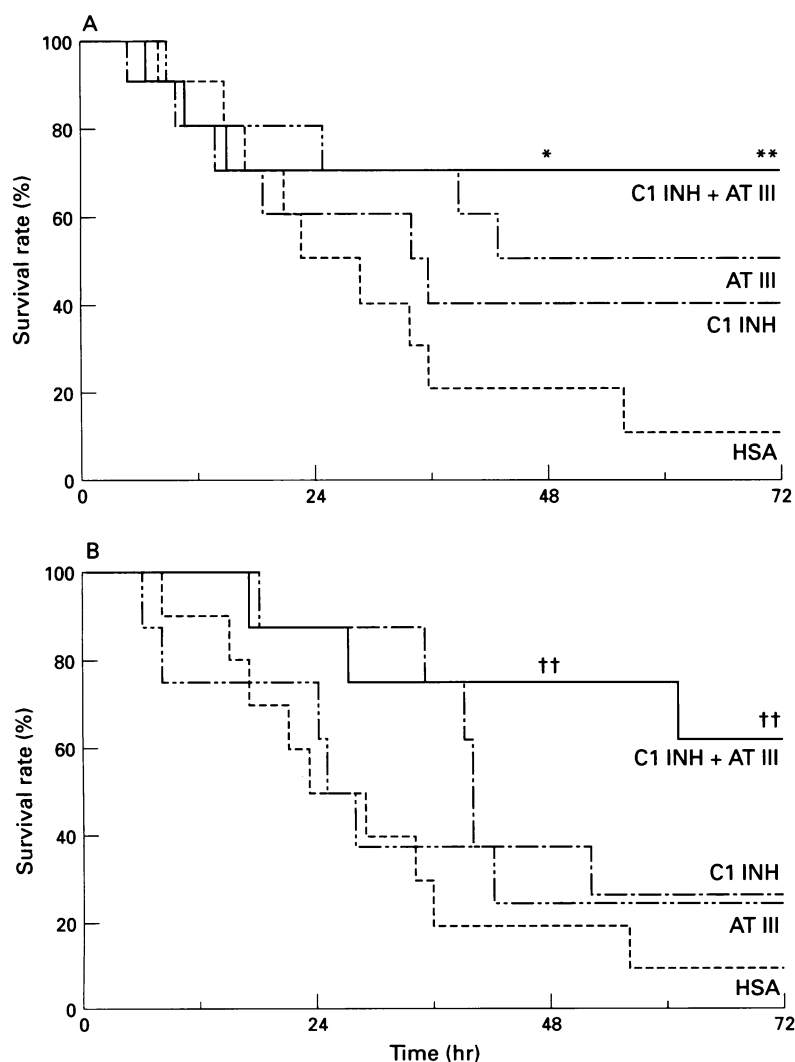


Figure 1: Effects of C1 INH and AT III on survival rate in taurocholate induced pancreatitis. (A) Prophylactic effects. C1 INH (250 U/kg), AT III (250 U/kg), and HSA (50 mg/kg) were given intravenously as a bolus 15 minutes before and two hours and four hours after the injection of taurocholate into the biliopancreatic duct (n=10). Significance v HSA treated group; \* $p < 0.05$ ; \*\* $p < 0.01$ . (B) Therapeutic effects. C1 INH and AT III were given one hour, three hours, and five hours after the injection of taurocholate (n=8). Significance v HSA treated group; †† $p < 0.02$ .

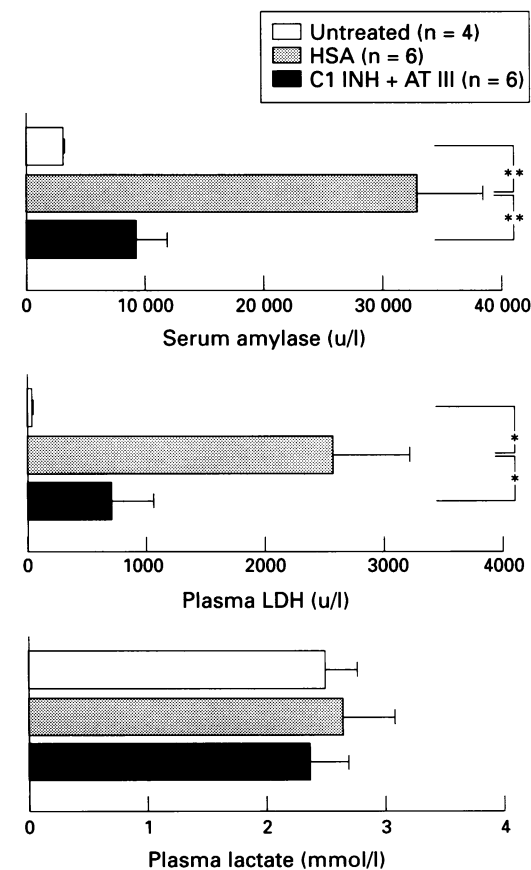


Figure 2: Therapeutic effects of combination therapy with C1 INH and AT III on biochemical variables 24 hours after the injection of taurocholate. C1 INH, AT III and HSA were given one hour, three hours, and five hours after the onset of taurocholate induced pancreatitis. Each bar represents the mean (SEM). \* $p < 0.05$ ; \*\* $p < 0.01$ .

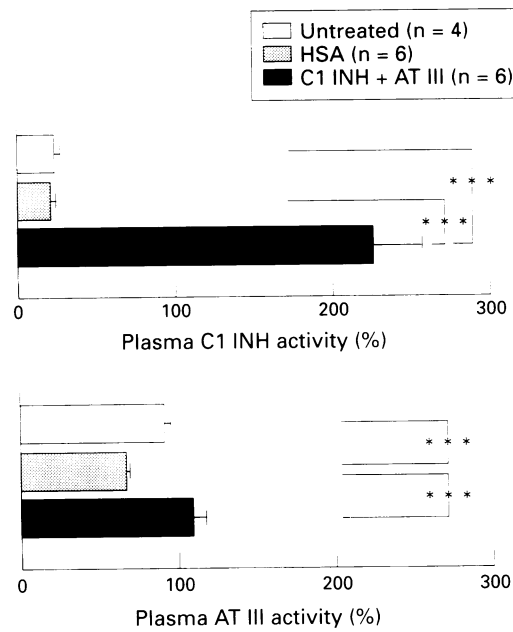


Figure 3: Functional C1 INH and AT III activities in plasma 24 hours after the induction of pancreatitis by taurocholate. Each bar represents the mean (SEM). \*\*\* $p < 0.001$ .

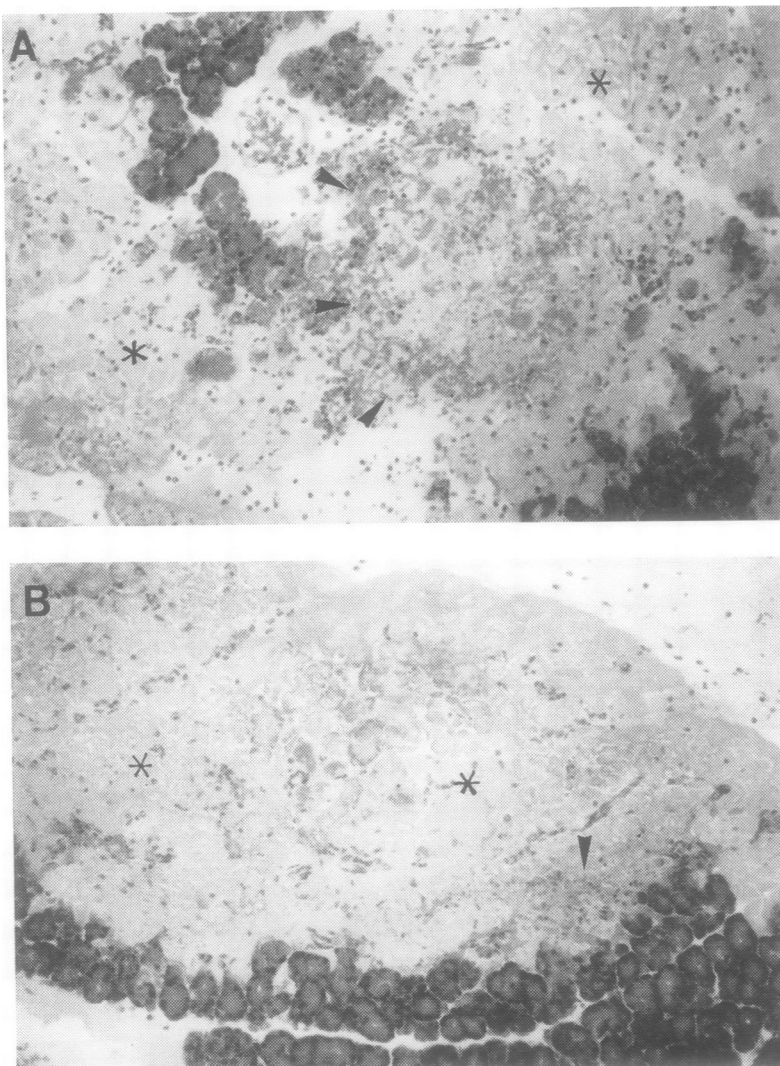


Figure 4: Effects of C1 INH and AT III on morphological alterations in the pancreas. Severe necrosis (asterisks) and haemorrhage (arrowheads) of pancreatic tissue are seen in taurocholate induced pancreatitis treated with C1 INH and AT III (A) as well as in the control group (B). Magnification originally  $\times 20$ .

## Discussion

In the present study high concentrations of C1 INH and AT III did not have any beneficial effects on mild caerulein induced oedematous pancreatitis, even when given before the onset of the disease. However, in severe haemorrhagic pancreatitis, treatment with C1 INH or AT III increased the survival of animals when given prophylactically. Treatment with a combination of C1 INH and AT III improved survival significantly, regardless of whether the administration was started before or after the taurocholate injection.

The present study is the first to investigate the effect of a combined therapy with C1 INH and AT III on acute experimental pancreatitis. C1 INH is regarded as a major inhibitor of kallikrein.<sup>2,3</sup> The activation of the kallikrein-kinin system induces the production of bradykinin, which causes vasodilatation and increased vascular permeability.<sup>13</sup> Some reports showed an increased bradykinin activity in experimental haemorrhagic pancreatitis.<sup>14,15</sup> Protective effects of C1 INH in taurocholate induced pancreatitis may result from the interception of hypovolaemia and hypotension through the inhibition of increased vascular permeability and vasodilatation. There are only a few other studies that have investigated the effect of C1 INH on experimental severe acute pancreatitis. Some reports showed the beneficial effects of C1 INH pretreatment,<sup>16,17</sup> but others failed to do this.<sup>18</sup> In the study by Ruud *et al*, C1 INH pretreatment significantly increased the functional plasma kallikrein inhibition capacity in peritoneal exudate, and improved arterial blood pressure and cardiac output of the animals.<sup>16</sup> Besides pancreatitis, increased concentrations of inactivated C1 INH were found in patients with severe sepsis, and the mortality from sepsis was closely related to the concentrations of inactivated C1 INH.<sup>19</sup> Hack *et al* reported that therapeutic administration of C1 INH can be beneficial in patients with sepsis.<sup>20</sup>

We have recently reported that a specific bradykinin B2 receptor antagonist had no beneficial effect on mortality in taurocholate induced pancreatitis.<sup>8</sup> Furthermore, a bradykinin B2 receptor antagonist reduced pancreatic oedema induced by caerulein, and simultaneously induced haemorrhagic changes in pancreatitis.<sup>21</sup> In the present study C1 INH did not influence the development of caerulein induced pancreatitis. Tissue prekallikrein is abundant in the pancreas and is thought to be activated by trypsin in pancreatitis. The inhibition of tissue kallikrein by C1 INH is not known. Probably, the inhibition of the kallikrein-kinin system by C1 INH is much weaker than that obtained by direct blockage of the bradykinin receptor.

In the present study, we did not succeed in showing the efficacy of treatment with AT III alone on severe pancreatitis. However, Bleeker *et al* reported that not only pretreatment, but also therapeutic treatment with human AT III improved the survival in taurocholate induced pancreatitis in rats.<sup>22</sup> The reason for these different results is not clear, but different

concentrations of substances applied and routes of administration (intraperitoneal AT III) may contribute to these variable findings. Some authors reported an efficacy of AT III in experimental animal models of sepsis and disseminated intravascular coagulation.<sup>23-25</sup> It is thought that intravascular consumption of AT III is accelerated in disseminated intravascular coagulation. Decreased AT III activities in plasma were found in the control group in the present study. Replacement of AT III may reduce active coagulation and improve systemic hypoperfusion and survival. Although our survival studies suggest that the beneficial effect of C1 INH and AT III treatment is due to systemic influences we could not detect differences in histological alterations in lungs, liver, and kidneys between the different groups. This may be due to the difficulty in interpreting the histological evidence of disseminated intravascular coagulation or shock at this early stage.

Our data indicate that combined treatment with C1 INH and AT III can significantly reduce the mortality associated with severe experimental pancreatitis. If this result is applied to a clinical setting it would predict that in patients with early severe pancreatitis the administration of C1 INH and AT III could interrupt the protease cascade activated in this disease. Severe haemorrhagic pancreatitis is still associated with a high mortality and we therefore suggest that a clinical trial that tests treatment with C1 INH and AT III is promising. In mild cases of oedematous pancreatitis no beneficial effect of the treatment with C1 INH and AT III can be expected.

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