Treatment with neutralising antibody against cytokine induced neutrophil chemoattractant (CINC) protects rats against acute pancreatitis associated lung injury

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Abstract

Background—Lung injury manifest clinically as adult respiratory distress syndrome (ARDS) is a common cause of morbidity and mortality following acute pancreatitis (AP). Neutrophils play a critical role in the progression of AP to ARDS. C-x-C chemokines are potent neutrophil chemoattractants and activators and have been implicated in AP.

Aims—To evaluate the effect of blocking the C-x-C chemokine, cytokine induced neutrophil chemoattractant (CINC), in AP on pancreatic inflammation and the associated lung injury in rats.

Methods—AP was induced by hourly intraperitoneal injections of caerulein. Goat anti-CINC antibody was administered either prophylactically (lung MPO (fold increase over control): 1.53 (0.21) p<0.05; microvascular permeability (L/P%): 0.31 (0.05)) or therapeutically (lung MPO —Treatment with anti-CINC antibody (fold increase over control): 2.13 (0.10) v 4.42 (0.65), p<0.05; microvascular permeability (L/P%): 0.31 (0.05) v 0.77 (0.11), p<0.05) or therapeutically (lung MPO activity as a measure of neutrophil sequestration in the pancreas). Lung injury was determined by measurement of pulmonary microvascular permeability and lung MPO activity.

Results—Treatment with anti-CINC antibody had little effect on caerulein induced pancreatic damage. However, it reduced the caerulein mediated increase in lung MPO activity as well as lung microvascular permeability when administered either prophylactically (lung MPO (fold increase over control): 1.53 (0.21) v 3.30 (0.46), p<0.05; microvascular permeability (L/P%): 0.42 (0.07) v 0.77 (0.11), p<0.05) or therapeutically (lung MPO (fold increase over control): 2.13 (0.10) v 4.42 (0.65), p<0.05; microvascular permeability (L/P%): 0.31 (0.05) v 0.79 (0.13), p<0.05).

Conclusion—Treatment with anti-CINC antibody afforded significant protection against pancreatic and associated lung injury. These results suggest that CINC plays an important role in the systemic inflammatory response in AP.

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Keywords: chemokines; acute pancreatitis; caerulein; adult respiratory distress syndrome

Acute pancreatitis (AP) is a common disease; the incidence in the UK is about 40 cases per 100 000 population and has been increasing over recent years with an overall mortality of approximately 7.5%.1 2 AP has many different causes; activation of digestive enzymes within pancreatic acinar cells is thought to be a critical initiating event. Pancreatic damage then leads to a localised inflammatory response. Leucocyte derived products contribute to local damage and to the subsequent systemic inflammatory response syndrome (SIRS), which if marked leads to multiple organ dysfunction syndrome (MODS), and is the major cause of death.

The chemokines are a family of small (8–10 kDa) inducible secreted cytokines with chemotactic and activating effects on leucocyte subsets. They can be divided into two major subgroups on the basis of the orientation of the first two cysteines. C-C chemokines principally affect monocytes while C-x-C chemokines which contain the tripeptide sequence ELR at the N-terminal tend to act on neutrophils.4 5 Very little work has as yet been undertaken to evaluate the role of chemokines in AP. Interleukin 8 (IL-8) is the best characterised of the ELR positive C-x-C chemokines. Plasma levels of IL-8 are elevated early in the course of an attack in patients with AP and correlate with disease severity.6 7 An anti-human IL-8 antibody was recently shown to reduce lung injury in a rabbit model of AP induced by retrograde injection of 5% cheno-deoxycholic acid.8 However, this model is not a well established model of AP and the criteria for lung injury were entirely histological, which can be very subjective. There is little published work examining the role of C-C chemokines in AP although knockout mice deficient in the C-C receptor CCR-1 have recently been shown to be protected against AP associated lung injury.9

We have recently shown that circulating levels of the human ELR positive C-x-C chemokine GRO-α are elevated in AP and that levels correlate with disease severity (Shokuhi S, Bhatia M, Slavin J, et al, unpublished). There is no direct homologue of IL-8 in the rat. The

Abbreviations used in this paper: AP, acute pancreatitis; ARDS, adult respiratory distress syndrome; MPO, myeloperoxidase; MODS, multiple organ dysfunction syndrome; SIRS, systemic inflammatory response syndrome IL-8, interleukin-8; CINC, cytokine induced neutrophil chemoattractant; FITC, fluorescein isothiocyanate.
best characterised of the rat C-x-C chemokines is cytokine induced neutrophil chemoattractant (CINC), the homologue of the human GRO-α–11. We have recently shown an increase in systemic CINC levels in rats following induction of AP.12 A neutralising antibody against CINC has been used to establish its importance as a mediator in a range of inflammatory conditions, including peritonitis, shock associated lung injury, and cerebral ischaemia.13–16 This antibody is specific for CINC and does not cross react in vivo with the related chemokines CINC2 and CINC3 (MIP2).12 Caerulein induced pancreatitis in the rat is a widely used model for AP. The aim of this study was to examine the effect of an anti-CINC neutralising antibody on pancreatic inflammation and the associated lung injury in AP induced by caerulein in rats.

**Methods**

**INDUCTION OF ACUTE PANCREATITIS**

All experiments were performed under a current Home Office project licence. Caerulein was obtained from Research Plus (Bayonne, New Jersey, USA). Wistar rats (200–250 g) were randomly assigned to control or experimental groups (n=8 for each group). Animals were given hourly intraperitoneal injections of saline (control) or saline containing a supramaximally stimulating dose of caerulein (50 µg/kg) for six hours. Anti-CINC antibody was produced as previously described13 and administered to rats at a dose of 8 mg/kg intraperitoneally either 30 minutes before (to determine prophylactic effects) or one hour after (to determine therapeutic effects) the first caerulein injection. This dose has been used in a number of previous studies in other models of inflammation in the rat to neutralise CINC.

**Figure 1** Effects of prophylactic treatment with anti-cytokine induced neutrophil chemoattractant antibody (anti-CINC antibody) on acute pancreatitis. The results shown are mean (SEM) for eight animals in each group. The results are as follows: plasma amylase (U/l)—control 1593 (54); placebo (Pbo) followed by caerulein (Caer) 20 266 (2374); anti-CINC antibody (Ab) followed by Caer 24 266 (1391); pancreatic water content (% of wet weight)—control 69.7 (1.2); Pbo followed by Caer 86.4 (1.0); Ab followed by Caer 84.1 (1.4); pancreatic myeloperoxidase (MPO) activity (fold increase over control)—control 1.0 (0.26); Pbo followed by Caer 9.72 (1.43); Ab followed by Caer 3.67 (0.38). *p<0.05, antibody treated animals with acute pancreatitis compared with placebo group.

**Figure 2** Effects of therapeutic treatment with anti-cytokine induced neutrophil chemoattractant antibody (anti-CINC antibody) on acute pancreatitis. The results shown are the mean (SEM) for eight animals in each group. The results are as follows: plasma amylase (U/l)—control 1593 (54); caerulein (Caer) followed by placebo (Pbo) 23 860 (3377); Caer followed by anti-CINC antibody (Ab) 25 046 (5337); pancreatic water content (% of wet weight)—control 69.7 (1.2); Caer followed by Pbo 86.1 (0.5); Caer followed by Ab 83.8 (0.9); pancreatic myeloperoxidase (MPO) activity (fold increase over control)—control 1.0 (0.26); Caer followed by Pbo 8.86 (1.8); Caer followed by Ab 2.38 (0.3). *p<0.05, antibody treated animals with acute pancreatitis compared with placebo group.
activity. One hour after the last caerulein injection, animals were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone and samples rapidly prepared for storage.

Harvested heparinised blood was centrifuged (3000 g, 15 minutes, 4°C), plasma removed, and stored at −70°C. Random cross sections of the head, body, and tail of the pancreas and samples of the right lung were fixed in 4% neutral phosphate buffered formalin for 48 hours and then embedded in paraffin wax. A sample of pancreas was weighed and dried for 72 hours at 60°C and reweighed to determine pancreatic water content. Samples of pancreas and lung were removed, weighed, and stored at −70°C for subsequent measurement of tissue myeloperoxidase (MPO) activity, as described below.

| Table 1 Pancreatic injury (histological evidence) in caerulein induced pancreatitis on prophylactic/therapeutic treatment with anti-cytokine induced neutrophil chemoattractant antibody (anti-CINC antibody) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Prophylactic     |                 | Therapeutic     |                 |
|                 | Control          | Caerulein+      | Control          | Caerulein+      |
|                 | placebo          | placebo         | antibody         | antibody         |
| Oedema (<0.5)   | 1.9 (0.2)*       | 1.4 (0.3)*      | 1.7 (0.3)*       | 1.9 (0.3)*      |
| Inflammation (<0.5) | 1.3 (0.2)*      | 1.6 (0.3)*      | 1.4 (0.2)*       | 1.1 (0.1)*      |
| Necrosis (<0.5) | 0.5             | >0.5            | <0.5             | <0.5            |

*p<0.05 v control, Mann Whitney U test.

**AMYLASE ESTIMATION**

Amylase activity was measured using a kinetic spectrophotometric assay. Plasma samples were incubated with the substrate, 4,6-ethylidene (G7)-p-nitrophenyl (G1)-1-D-maltoheptoside (Sigma, St Louis, Missouri, USA) for two minutes at 37°C and absorbance measured every minute for the subsequent two minutes at 405 nm. The change in absorbance was used to calculate amylase activity.

**MYELOPEROXIDASE ESTIMATION**

Neutrophil sequestration in pancreas and lung was quantitated by measuring tissue MPO activity. Tissue samples were thawed, homogenised in 20 mM phosphate buffer (pH 7.4), centrifuged (10 000 g, 10 minutes, 4°C), and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 seconds). The sample was then centrifuged (10 000 g, five minutes, 4°C) and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mM tetramethylbenzidine (Sigma), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated at 37°C for 110 seconds, the reaction terminated.
with 0.18 M H₂SO₄, and absorbance measured at 450 nm. This absorbance was corrected for the calculated dry weight of the tissue sample used and results expressed as activity per unit of dry weight (fold increase over control).

MEASUREMENT OF PULMONARY MICROVASCULAR PERMEABILITY
Two hours before sacrifice, each animal received an intravenous bolus injection containing fluorescein isothiocyanate (FITC)-albumin (5 mg/kg, Sigma). Immediately after sacrifice, the trachea was exposed, the right bronchus clamped, and the left lung lavaged three times with 3.3 ml of saline. The lavage fluid was combined, and FITC fluorescence was measured in the lavage fluid and plasma (excitation 494 nm; emission 520 nm). The ratio of fluorescence emission in lavage fluid to plasma was calculated and used as a measure of pulmonary microvascular permeability.

MORPHOLOGICAL EXAMINATION
Paraffin embedded pancreas and lung samples were sectioned (4 µm), stained with haematoxylin/eosin, and examined by light microscopy by an experienced observer who was unaware of the sample identity. Pancreas sections were scored for necrosis, oedema, and inflammation on a scale of 0–3, 0 being the least severe and 3 being the most severe for each parameter. Similarly, lung sections were scored for alveolar thickening (a measure of oedema) and inflammation, again on a scale of 0–3.

STATISTICS
Results are given as mean (SEM). In all figures, vertical bars denote the SEM and the absence of such bars indicates that the SEM was too small to illustrate. Results were compared using an unpaired t test unless stated otherwise; the level of significance was set at 5%.

Results
EFFECT OF ANTI-CINC ANTIBODY TREATMENT ON PANCREATIC INJURY
Administration of supramaximal doses of caerulein to rats resulted in AP. This was confirmed by an increase in plasma amylase, pancreatic water content (a measure of pancreatic oedema), and pancreatic MPO (a measure of neutrophil infiltration in the pancreas) (fig 1). Prophylactic or therapeutic treatment with anti-CINC antibody did not have a significant effect on plasma levels of amylase or pancreatic water content (figs 1, 2). MPO activity in the pancreas was, however, reduced in rats treated with the anti-CINC antibody. The morphological changes in the pancreas on induction of AP and the effect of prophylactic and therapeutic treatment with the anti-CINC antibody are shown in fig 3. The morphological changes in AP, which in this model consisted principally of oedema, inflammation and some necrosis,
showed little difference between rats treated with the anti-CINC antibody and those treated with placebo (fig 3, table 1).

**EFFECT OF ANTI-CINC ANTIBODY TREATMENT ON ACUTE PANCREATITIS ASSOCIATED LUNG INJURY**

Pancreatitis associated lung injury was confirmed by an increase in pulmonary microvascular permeability and an increase in lung MPO activity (figs 4, 5). Prophylactic or therapeutic treatment with anti-CINC antibody significantly reduced pulmonary microvascular permeability and MPO levels in caerulein treated animals (figs 4, 5). The morphological changes in the lung following induction of AP and the effect of prophylactic/therapeutic treatment with the anti-CINC antibody are shown in fig 6. These consist principally of alveolar thickening and infiltration by inflammatory cells, the vast majority of which are neutrophils. After either prophylactic or therapeutic treatment with the anti-CINC antibody, alveolar thickening and inflammation, as determined by the histological scoring of lung sections, was reduced (fig 6, table 2).

**Discussion**

The first sign of MODS in severe AP is often impaired lung function due to ARDS. As a consequence of overactive SIRS, leucocytes and in particular neutrophils become activated within the general circulation and attach to the pulmonary vascular endothelium. As the condition develops, leucocytes migrate into the pulmonary interstitium and increased endothelial permeability leads to tissue oedema. Leucocyte derived products are thought to contribute to pulmonary damage. Leucocyte activation and migration into tissue is a tightly regulated process. Although a number of proinflammatory cytokines such as IL-1, tumour necrosis factor α, and platelet activating factor which have a broad profile of action are implicated, other more specific.

Table 2  Lung injury (histological evidence) in caerulein induced pancreatitis on prophylactic/therapeutic treatment with anti-cytokine induced neutrophil chemoattractant antibody (anti-CINC antibody)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Prophylactic</th>
<th>Therapeutic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caerulein +placebo</td>
<td>Caerulein +antibody</td>
<td>Caerulein +placebo</td>
</tr>
<tr>
<td>Alveolar thickening</td>
<td>0.4 (0.2)</td>
<td>2.4 (0.3)*</td>
<td>0.8 (0.3)†</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0.4 (0.2)</td>
<td>2.6 (0.2)*</td>
<td>0.9 (0.3)†</td>
</tr>
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*p<0.05 v control.
†p<0.05 v respective placebo, Mann-Whitney U test.
mediators such as chemokines may play a more central role. Neutrophils also play an important role in local pancreatic damage in AP and more so in the translation of local pancreatic damage to a systemic inflammatory response. Neutrophil depletion partially reduces pancreatic damage in some models of AP but affords almost complete protection against AP-associated lung injury.\textsuperscript{25–27} In an analogous manner, antibodies directed against ICAM-1, which interfere with neutrophil migration, reduce AP-associated lung injury.\textsuperscript{27} Similarly, there is reduced AP-associated lung injury following genetic deletion of ICAM-1.\textsuperscript{29}

Chemokines are thought to influence leukocyte migration into tissues in two main ways. Firstly, binding of chemokine ligand to a leucocyte receptor leads to activation of cell surface integrins and allows strong adhesion to endothelium. Secondly, chemokines promote migration of adherent leucocytes across endothelium and through the extracellular matrix. In addition to leucocyte recruitment, chemokines may be important regulators of leucocyte activation in situ. The profile of chemokines expressed at a site of injury is likely to be one of the major factors that determines the nature of the subsequent leucocyte infiltrate.\textsuperscript{4, 10–34} CINC, the rat homologue of human GRO-\textalpha, is a C-X-C chemokine and a specific neutrophil chemoattractant and activator in vitro.\textsuperscript{10, 35–38} CINC has also been shown to induce neutrophil recruitment in vivo and specific antagonists reduce the inflammatory response in a number of different models of injury.\textsuperscript{35–38} It is therefore reasonable to suggest that neutralising the action of CINC would block neutrophil recruitment and activation in AP.

In this study, we have examined the effect of a neutralising anti-CINC antibody on pancreatic and lung damage using a model of AP induced by the pancreatic secretagogue caerulein. The increase in lung MPO levels and microvascular permeability shows evidence of AP-associated lung injury. Treatment with the anti-CINC antibody resulted in significant protection against lung damage, as measured by pulmonary microvascular permeability. Histological examination of the lungs and lung MPO levels confirm reduced neutrophil infiltration and suggest that the antibody is interfering directly with leucocyte migration. Evidently, CINC plays an important role in neutrophil recruitment to the lungs although the exact molecular mechanism by which CINC acts as a mediator of AP-associated lung injury remains to be investigated.

In patients with AP it is likely that a number of C-X-C chemokines are involved in neutrophil recruitment and activation. For example, plasma levels of IL-8,\textsuperscript{4} ENA-78, and GRO-\alpha (Shokuh S, Bhatia M, Slavin J, et al., unpublished) are elevated in patients with AP and correlate with disease severity. At the present time the range of C-X-C chemokines characterised in the rat is not as large as in humans. Although present evidence suggests that CINC is a major C-X-C chemokine in the rat,\textsuperscript{9–11} other C-X-C chemokines, such as CINC-2, CINC-3, and ENA-78 may conceivably play a role in AP and the associated systemic inflammatory response. For example, ENA-78 has previously been implicated in ischaemia/reperfusion induced liver injury in the rat, a condition similar to pancreatic injury in AP.\textsuperscript{10} A possible involvement of other C-X-C chemokines may, in part, explain the partial rather than complete protection against lung injury following treatment with anti-CINC antibody.

Treatment with anti-CINC antibody did not appear to affect pancreatic damage in AP as determined by an increase in plasma amylase and pancreatic oedema. This is in agreement with previous studies suggesting that neutrophil-mediated damage is not a major factor affecting the pancreas in caerulein induced AP.\textsuperscript{9–20} It is important to acknowledge the distinction between prophylactic and therapeutic use of an agent in AP. Obviously the latter is of relevance in a clinical setting where therapeutic intervention is proposed. Lung injury becomes manifest some hours after the onset of an attack of AP and it is often maximal between 48 and 96 hours. As pain is an early feature, it has been proposed that there is a therapeutic window during which anti-inflammatory therapy might be effective in AP.\textsuperscript{39} It is encouraging that administration of anti-CINC antibody after induction of AP was as effective in reducing lung damage as prophylactic administration, and suggests that strategies that seek to interfere with chemokine function might be of clinical use.


