The potential role of Clostridium perfringens alpha toxin in the pathogenesis of acute pancreatitis

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Abstract

Background—Clostridium perfringens is a bowel commensal that can colonise the biliary tract. It produces the alpha toxin (phospholipase C), which can induce spontaneous tissue necrosis.

Aims—To investigate whether there is any evidence that Clostridium perfringens alpha toxin can be detected in acute pancreatitis.

Methods—Serum samples from 21 patients with acute pancreatitis and 22 controls were assayed for C perfringens phospholipase C as well as anti-phospholipase C IgG and IgM; IgG and IgM anti-toxins were measured by enzyme linked immunosorbent assay.

Results—In normal healthy controls there is a very high level of natural anti-toxin of both the IgG and IgM class. Of the 21 patients with acute pancreatitis alpha toxin was detected in five (23.8%). Levels of both IgG and IgM anti-toxin were significantly reduced in acute pancreatitis.

Conclusions—The results suggest that there is an abnormality of the immune status to C perfringens alpha toxin in patients with acute pancreatitis. This may be the result of a release of alpha toxin, although it is difficult to state whether this is a primary or secondary phenomenon in these patients. These preliminary results merit further investigation.

Keywords: Clostridium perfringens, alpha toxin, phospholipase C, acute pancreatitis.

Acute pancreatitis is a common condition which carries significant morbidity and mortality. Most cases run a self-limiting course, but up to 10% develop severe complications.1,2 These complications are directly related to the severity of the disease and the highest mortality is seen in those patients in whom the pancreas undergoes necrosis or in those who get a superimposed pancreatic infection.3,4 Both chemical/irritant and infective aetiologies have been suggested for acute pancreatitis. However, the factors that influence the pathogenesis of pancreatitis and determine which patients will develop subsequent complications remain poorly understood.1 It is clear that the severest forms of acute pancreatitis are associated with a systemic illness in which pancreatic inflammation is only one feature.

Clostridium perfringens is a bowel commensal in a large proportion of the population.3 This organism is implicated in many cases of intra-abdominal sepsis when mixed bowel flora are identified, although little significance is usually attributed to this finding.3,4 It is also an organism that readily colonises the bile ducts and can be identified in 10–18% of patients undergoing biliary surgery.1,5 The commonest strain pathogenic to humans is type A which produces, amongst other aggressins, the alpha toxin or phospholipase C. This toxin has a number of cytopathic effects including the ability to degrade cell membranes directly, which leads to tissue necrosis.6-8

It is our hypothesis that the alpha toxin of C perfringens may play a role in the development of some cases of acute pancreatitis. The aim of this pilot study was to test this hypothesis by looking for any evidence of C perfringens alpha toxin in patients with acute pancreatitis.

Methods

Patients presenting with acute pancreatitis had a serum sample taken as part of their routine biochemical screening on admission. Samples were stored at –70°C pending analysis. Control serum samples from unknown healthy blood donors were supplied by the Regional Blood Transfusion Service.

C perfringens alpha toxin (phospholipase C) was assayed by an enzyme linked immunosorbent assay (ELISA) which we have described previously for use with bacterial cultures.11 The serum was assayed at a dilution of 1 in 10 in phosphate buffered saline (PBS). Although this assay is very sensitive in cooked meat medium, detecting concentrations as low as 0.005 units/ml,11 its performance in serum is less sensitive and could only reliably detect toxin at concentrations of 0.5 units/ml.

Total IgG and IgM anti-phospholipase C were also assayed by ELISA using a method described previously by ourselves for anti-pneumococcal antibody12 and shown to be specific for C perfringens type A alpha toxin.13 Briefly, C perfringens type A (NCTC type 11229) was grown overnight at 37°C in Robertson’s cooked meat medium (Bacto cooked meat medium, Difco Laboratories, Detroit, Illinois, USA). The culture tubes were centrifuged and the neat supernatant containing the toxin was coated onto 96-well microtitre plates (Nunc type F, Gibco Life Technologies, Paisley, UK) using a carbonate/bicarbonate coating buffer, incubated for four hours at 37°C and then overnight at 4°C. Although the method of preparation of C...
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Alpha toxin seems rather crude, we have previously shown this to be a cheap, simple and effective method.11 The plates were washed three times with PBS with 0.1% Triton X-100 (PBST) and 100 μl aliquots of each serum sample were pipetted into separate wells. For IgG assays the serum was diluted 1 in 100 in PBS and for IgM assays the serum was diluted 1 in 10 in PBS. Plates were incubated at 37°C for 45 minutes and washed three times with PBST. Alkaline phosphatase linked goat anti-human IgG and IgM (types A-3150 and A-3275; Sigma, Poole, Dorset, UK) were used as detecting antibodies; 100 μl of a 1 in 1000 (in PBS) dilution was added to each well and incubated for a further 45 minutes at 37°C, and washed three times before the addition of alkaline phosphatase substrate (104-105 phosphatase substrate; Sigma). The optical densities were read at 405 nm using an automated plate reader.

All samples were assayed in quadruplicate and the assay measurement was the average of the four wells per specimen. Pooled serum from a number of individuals was used as a reference control in all assays. Units for each assay were defined arbitrarily such that 1000 units gave a reading of 1.000 at 405 nm in a standardised assay. The units for the IgG and the IgM assays are not comparable.

The severity of the pancreatitis in each patient was graded using the acute physiology score (APS) and age components of the APACHE II scoring system.14

**Results**

Serum samples from 21 patients with acute pancreatitis and 22 controls were assayed for *C. perfringens* phospholipase C as well as anti-phospholipase C IgG and IgM. The aetiology of pancreatitis was gallstones in eight patients, alcohol excess in six, idiopathic in six, and carcinoma of the pancreas in one. The mean (SEM) APS was 12 (2) (range 4–32) and the mean serum amylase activity on admission was 1193 (262) U/l (range 219–4020 U/l). One patient in the alcohol excess group died of necrotising pancreatitis.

**PHOSPHOLIPASE C ASSAYS**

The results of assay of phospholipase C are shown in table 1. The mean concentration of toxin detected in patients with acute pancreatitis was 81 units. Although this was not statistically significantly different from the mean concentration in the control group (p = 0.052, Student's t test), this may be due to the fact that one very high sample was biasing the standard deviation. The mean + 2 SD for the control group was 60 units. In seven patients the toxin concentration was greater than 60 units and in five it was greater than 100 units (mean + 3 SD). In one patient the toxin concentration was exceptionally high (739 units). This was in a patient with alcoholic pancreatitis with an antibody level of 10 who subsequently recovered. The patient who died had the highest APS of 32 but no detectable toxin.

**ANTI-PHOSPHOLIPASE C IgG AND IgM ASSAYS**

IgG anti-toxin levels were very high in the controls and serum had to be diluted at least 100 times before the levels were within the detectable range of the assay. The results are shown in table 1. The mean (SEM) IgG anti-phospholipase C level in controls was 412 (19) units in contrast with a mean level of 317 (27) units in patients with pancreatitis. Thus, the levels in those with pancreatitis were significantly lower than in controls (p < 0.001, Student's t test). Circulating IgM anti-toxin was much lower in controls than IgG and the assays were easier to quantitate. The results for patients and controls are summarised in table 1. The level of IgM antibody in patients with pancreatitis was significantly lower than in controls (p < 0.0000001, Students t test).

There was no inverse or direct correlation between the level of phospholipase C and the level of IgG or IgM anti-phospholipase C in any individual patient. In addition, there was no correlation between the severity of the pancreatitis as determined by the APS, the serum amylase activity and the amount of toxin and anti-toxin detected. The numbers for each aetiological group were small and it was impossible to state whether there were differences between the various causes of pancreatitis with respect to toxin and anti-toxin concentrations.

**Discussion**

In this paper we have carried out a preliminary study to determine whether there is any evidence of *C. perfringens* toxin release in patients with acute pancreatitis. This has been done by measuring both the amount of phospholipase C in the serum, and by comparing the levels of antibody to the toxin in both patients and controls. Phospholipase C was not detected in all cases of acute pancreatitis although there was a suggestion that toxin was measurable in some patients. In this context the performance of the phospholipase C assay may be relevant. We had previously developed this method for use in bacterial culture media but had not been as successful at adapting the method to serum. This difficulty resulted from competition between the binding antibody on the plate and natural anti-phospholipase C activity within the serum for the binding of the toxin. On the basis that a high anti-phospholipase C activity is found in normal serum it is likely that the detection of any phospholipase C by the assay method should be regarded as highly significant. However, a more accurate assay method would be required if smaller amounts of toxin are to be detected and it would be important that this could detect both protein bound and "free" toxin.

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**Table 1** Alpha toxin (phospholipase C), IgG and IgM anti-toxin levels in controls and patients with acute pancreatitis. The units for each assay are not comparable and are defined arbitrarily. Results are expressed as mean (SEM) (range).

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 22)</th>
<th>Patients (n = 21)</th>
<th>p value</th>
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<tbody>
<tr>
<td>Alpha toxin (units)</td>
<td>5 (6) (0-72)</td>
<td>81 (38) (0-772)</td>
<td>0.052*</td>
</tr>
<tr>
<td>IgG anti-toxin (units)</td>
<td>412 (19) (262-593)</td>
<td>317 (27) (194-593)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgM anti-toxin (units)</td>
<td>571 (32) (258-868)</td>
<td>307 (30) (76-554)</td>
<td>&lt;0.0000001</td>
</tr>
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*Not significant.
This paper has also confirmed our previously unpublished finding of high levels of natural serum anti-phospholipase C activity in normal controls. This is particularly the case in the IgG class, although the relatively high levels of IgM are harder to explain. It is reasonable to assume that these both represent the "normal" antibody status of the population and it would seem logical that this is because of the commensal or frequent carriage of the organism as part of the colonic flora. Constant exposure to the organism and its alpha toxin must lead to continuous antibody formation. The only other explanation for the relatively high level of IgM would be that the antibody assay is detecting non-specific IgM anti-phospholipase C activity in the serum.

If C. perfringens toxin is released in acute pancreatitis it would bind to available IgG and IgM antibodies in the serum. This would be compatible with our finding of significantly lower levels of IgG and IgM when compared with controls. Unfortunately, we were unable to demonstrate either an inverse or direct relation between the toxin and anti-toxin levels. Even with the use of an immune complex assay it may be impossible to demonstrate a direct relation because we are uncertain whether the anti-phospholipase C activity represents a neutralising antibody or an opsonophagocytic antibody. It would be more appropriate to look at convalescent serum samples to establish whether the serum antibody level returns to normal or remains raised as a result of exposure to the toxin.

C. perfringens is an ideal candidate to play a role in the pathogenesis of acute pancreatitis and in a few instances the organism has been directly linked with the disease. The organism can be identified in many pathological situations such as soft tissue infections, post-partum and post-abortion sepsis. In the majority of cases it is generally regarded as a bowel contaminant and not a primary pathogen of clinical importance. Escherichia coli is the usual organism identified in infections associated with acute pancreatitis. Nevertheless, 57% of the infections seen in conjunction with acute pancreatitis are polymicrobial and it is difficult to determine which, if any, is the principal pathogen. Although anaerobic bacteria are identified in up to 10% of infections associated with acute pancreatitis, the individual organisms are rarely characterised. Widdison and Karanja stress that the anaerobic bacteria which predominate in the colon are rarely isolated from patients with pancreatic infection and the distribution of bacterial species more closely resembles that found in the bile ducts rather than that found in the large intestine. For this reason, the well recognised association of C. perfringens with the biliary tract may be of significance. Up to 18% of patients undergoing biliary surgery will yield the organism from the bile, it is the principal organism identified in cases of emphysematous cholecystitis and biliary surgery is one of the commonest circumstances leading to post-operative cholangial myonecrosis.

Several of the more unusual and serious C. perfringens infections arise in the absence of primary tissue infarction. These include post-operative myonecrosis, emphysematous cholecystitis and necrotising jejunitis (Pig bel). A feature of both emphysematous cholecystitis and necrotising jejunitis is that tissue necrosis is a secondary event to the infection and occurs in the absence of vascular infarction. It is likely that this spontaneous tissue necrosis is the result of toxins produced by the organism. In necrotising jejunitis this is thought to be the beta toxin of C. perfringens type C. However, neither this strain nor the beta toxin is identified in all cases.

The clinical manifestations of acute pancreatitis closely resemble those of necrotising jejunitis. Both have a spectrum of severity from very mild to very severe, they can both be recurrent, can develop chronic forms, and both can have a variable amount of tissue fibrosis as an end result. Lastly, both can develop fulminant, necrotising forms which can prove rapidly fatal.

With this background it is highly plausible that C. perfringens has a role in acute pancreatitis. For example, type A, which is the strain usually identified in humans, the alpha toxin or phospholipase C would be the most probable agent responsible for any locally destructive effects as a result of its cytopathic activity against cell membranes. The toxin is also known to be able to elicit superoxide production from neutrophils and to induce toxic effects on cardiac muscle, both of which can be associated with the severest forms of acute pancreatitis.

This preliminary study has supplied some evidence that C. perfringens may be implicated in some cases of acute pancreatitis. Certainly, there would appear to be some evidence of altered antibody levels to the toxin. However, at this stage it would be difficult to be certain whether the defects in immunity and the result of the organism acting as a primary initiating event or are a consequence of translocation of colonic bacteria secondary to intra-abdominal inflammation. In order to prove an association it would be important to undertake more detailed and sequential immune analysis of the serum of patients with acute pancreatitis. We feel that this hypothesis merits further investigation.

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