Evidence of Complement Catabolism in Experimental Acute Pancreatitis

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Serum specimens from guinea pigs with experimentally induced acute pancreatitis were examined for evidence of protease-antiprotease imbalance and complement catabolism. Pancreatitis was induced in 22 male Hartley guinea pigs by the injection of sodium taurocholate into the pancreatic parenchyma. Only a laparotomy was performed in 6 control animals. In 10 experimental animals that survived for less than 24 hours, there was a significant, early reduction of serum trypsin inhibitory capacity (a measure of antiprotease activity). Levels of total hemolytic complement as well as titers of hemolytic C3 and C4 fell significantly in all experimental animals during the first 24 hours. Factor B activity, however, did not change. Only serum from experimental animals contained chemotactic activity for human polymorphonuclear leukocytes. These findings indicate that circulating complement components are cleaved during the course of experimental acute pancreatitis. As a consequence, complement-derived peptides are generated that may mediate local and systemic tissue injury. (Am J Pathol 1980, 101:205-216)

ACUTE PANCREATITIS is usually a benign, self-limited disease. At times, however, the disorder progresses rapidly with multisystem involvement despite application of all known methods of therapy.[1] One of the most serious systemic sequelae of acute pancreatitis is respiratory insufficiency.[1-3] Despite numerous attempts to identify factors involved in the pathogenesis of this potentially fatal complication, the precise mechanism responsible for injury to the lung in pancreatitis is still obscure.

One factor that may be relevant to the pathogenesis of respiratory insufficiency in patients with acute pancreatitis is the low-molecular-weight cleavage product of the fifth component of complement, C5a.[4] C5a, which can be generated by activation of either the classic or alternative complement pathways as well as by direct proteolytic cleavage of native C5,[4,5] possesses anaphylatoxin activity [4] and potent chemotactic activity for polymorphonuclear leukocytes (PMNs).[6] C5a also has been found capable of increasing the adhesiveness of PMNs *in vitro* (causing the cells to aggregate) and of provoking pulmonary capillary leukostasis (and hypox-

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emia) *in vivo*.[7,8] It has been proposed that these and other biologic activities of C5a can mediate injury to the lung and account for respiratory insufficiency in patients undergoing hemodialysis as well as in patients with other disorders associated with complement catabolism.[7,8]

Evidence of complement catabolism in acute pancreatitis has been reported by several investigators.[9–11] For example, we have found decreased levels of total hemolytic complement, C3, C4, or some combination thereof, as well as degradation products of C3 (revealed by crossed immunoelectrophoresis) in acute-phase serum specimens from 8 of 13 patients with acute pancreatitis.[9] Neither the significance of complement catabolism nor the mechanism responsible for cleavage of complement components in patients with pancreatitis could be determined from the results of these preliminary studies. It seems unlikely that additional clinical studies would serve to resolve these issues, for the following reason. First, patients with pancreatitis often have other diseases (eg, alcoholism, cirrhosis) in which complement metabolism may be altered. Second, it is difficult to determine the precise onset and severity of the disease in most patients. Third, tissue is rarely available from patients for histopathologic studies. And finally, modern therapeutic measures introduce a number of variables that cannot be easily controlled. We have therefore resorted to an experimental animal model of acute pancreatitis in an attempt to answer some of the questions raised by previous studies.

In this report, evidence will be presented indicating that transient protease–antiprotease imbalance can occur during the course of acute pancreatitis induced in guinea pigs by intraglandular injection of sodium taurocholate. Evidence also will be presented that in this experimental model there is cleavage of complement proteins and generation of complement-derived peptides that are capable of mediating local and systemic tissue injury.

Materials and Methods

Animal Studies

Twenty-two male Hartley guinea pigs (400–600 g) were anesthetized after an overnight fast with intraperitoneal sodium pentobarbital (35 mg/kg). The right atrium was cannulated with a 19-gauge polyethylene catheter. Blood withdrawn through this catheter was restored volume-for-volume with Ringer's lactate solution. After midline laparotomy, acute pancreatitis was induced by injection of 1.0 ml sterile normal saline containing 100 mg sodium taurocholate (Sigma Chemical Co., St. Louis, Mo.) and 50 mg cephalothin (Eli Lilly Co., Indianapolis, Ind) into the pancreatic parenchyma.[12]

Six control animals underwent cannulation and laparotomy but were not given injections of the bile salt. After recovery, the animals were deprived of food and water throughout the experiment. Venous blood samples (0.5 ml) were collected at regular time intervals

until the animals died or were killed at 96 hours. Blood was allowed to clot at room temperature. Serum was separated by centrifugation and stored at −70 C for further analysis.

When guinea pigs were killed, or at necropsy, samples of pancreas were fixed in 10% buffered formalin. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic study.

In order to determine the extent to which sodium taurocholate was absorbed into the systemic circulation, 4 additional animals were given injections as described above of 1.0 ml of sterile saline containing 100 mg sodium taurocholate and 10 μCi of uniformly labeled ^{3}H-taurocholic acid (New England Nuclear Corp., Boston, Mass.). Serial samples of serum (0.1 ml) were added to 10 ml Bray's solution and counted in a Beckman LS 7000 liquid scintillation counter.

Serum Trypsin Inhibitory Capacity and Amylase Activity

Serum trypsin inhibitory capacity (which reflects serum antiprotease activity) was assayed by employing a minor modification of the method of Erlanger et al.[13] This assay measures the ability of serum to inhibit cleavage of the chromogenic substrate, benzoyl-DL-arginine-p-nitroanilide by purified bovine trypsin (Sigmal Chemical Co.). We assayed serum amylase activity by employing 1.0% (wt/vol) starch as substrate.[14]

Complement Determinations

Sheep erythrocytes in Alsever's solution were obtained from the Public Health Research Institute of the City of New York. The cells were washed three times and sensitized as described previously[15] with rabbit anti-Forssman antibody (Difco Laboratories, Detroit, Mich.). Functionally pure guinea pig complement components, stable cellular intermediates and cobra venom factor were obtained from Cordis Laboratories, Miami, Florida. C4-deficient guinea pig serum was generously provided by Dr. Irma Gigli, of New York University Medical Center.

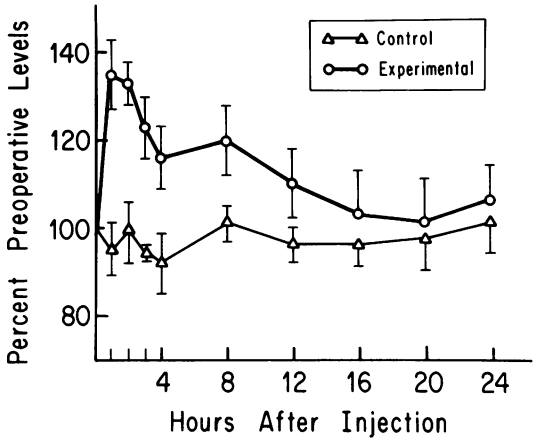
Total hemolytic complement activity (CH_{50}) was measured according to the method of Mayer,[15] employing sensitized sheep erythrocytes and serial dilutions of guinea pig serum. Hemolytic C3 was assayed as described previously,[16] employing appropriate stable cellular intermediates and functionally pure guinea pig complement components. The method of Gaither et al[17] was used to measure hemolytic C4. Functional titers of factor B (of the alternative complement pathway) were measured as described by Brai and Osler.[18] "Cobra venom factor activable hemolysis titers" ($CVFAH_{50}$) were determined with the use of purified cobra venom factor (100 U/ml), unsensitized sheep erythrocytes, and guinea pig complement.

All hemolytic assays were modified to accommodate small volumes (0.01 ml) of test serum and were performed in duplicate. Sheep erythrocytes and stable cellular intermediates were used at a final concentration of 10^8 cells/ml. For all assays, absorbance at 415 nm was measured to determine extent of hemolysis (y).[15] Titers yielding 50% hemolysis were calculated by linear regression of log serum dilution versus log y/(1-y).[15]

Chemotaxis Assays

Serum specimens from control experimental animals were examined for their content of chemotactic factors for human PMNs. PMNs were isolated from venous blood of healthy adult volunteers as described previously[19] and suspended in phosphate(10 mM)-buffered saline, pH 7.4, containing 2.0% (wt/vol) bovine serum albumin, 0.6 mM $CaCl_2$, and 1.0 mM $MgCl_2$.

PMN random motility and directed migration (chemotaxis) were assessed by employing the "leading front" technique of Zigmond and Hirsch.[20] The responses of PMNs either to buffer alone (random motility) or to chemotactic stimuli are reported as the distance (μ/35



TEXT-FIGURE 1—Serum amylase activity in control (△—△) and experimental (○—○) animals. Results are expressed as a percentage of preoperative levels. Each point represents data (mean ± SEM) obtained from at least 6 animals.

min) that the leading front of cells migrated into 3.0 μ (pore diameter) cellulose nitrate micropore filters (Sartorius, Brinkmann Instruments, Inc. Westbury, NY) separating the upper, or cell, compartments from the lower, or stimulus, compartments of modified Boyden chambers (Nucleopore Corp., Pleasanton, Calif). Triplicate chambers were employed in each experiment, and 10 fields were examined in each filter.

Statistical Analysis

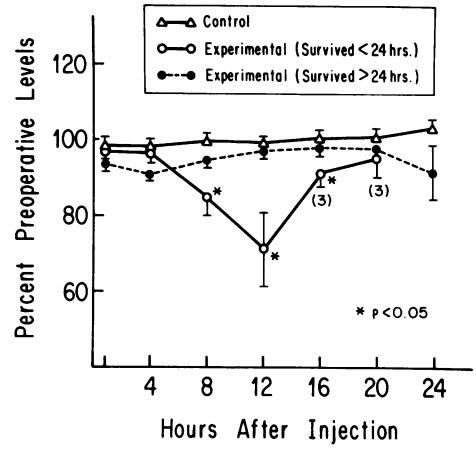
All complement titers and enzyme activities are expressed as a percentage of preoperative levels. A two-tailed Student t test was used to determine significance between the mean values of experimental and control data.

Results

Experimental Pancreatitis

The median duration of survival of the 22 animals with experimentally induced pancreatitis was 22 hours (range, 6–96 hours). Ten animals succumbed within 24 hours (median, 12 hours; range, 2–24 hours), while 12 animals survived for periods of 68–96 hours. All 6 sham-operated control animals survived for 96 hours.

Animals with experimentally induced pancreatitis exhibited significant (but transient) elevations of serum amylase activity and had gross as well as histologic evidence of pancreatic necrosis with edema, hemorrage, and fat necrosis at the time of death or when killed. Serum amylase activity rose by ± 8% during the first hour after induction of pancreatitis ($P < 0.005$) returned slowly to preoperative levels during the initial 24 hours of observation (Text-figure 1). Animals that survived for less than 24 hours could not be distinguished from animals that survived longer with respect to changes in serum amylase activity. Amylase activity did not change significantly in sham-operated control animals (Text-figure 1).



TEXT-FIGURE 2—Tryspin inhibitory capacity in control (△—△) and experimental animals (○—○, survived <24 hours; ●—●, survived >24 hours). Except as indicated in parentheses, each point represents data (mean ± SEM) obtained from at least 6 animals.

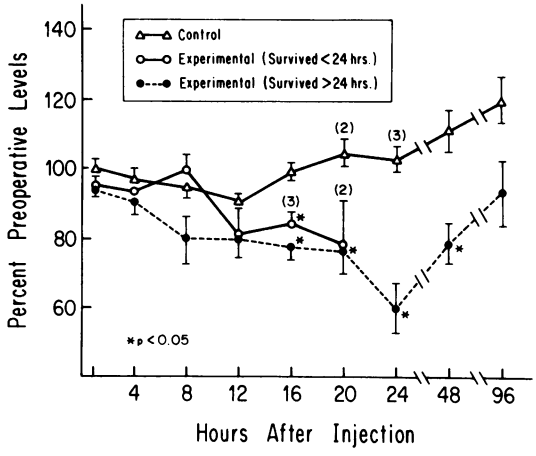
Trypsin Inhibitory Capacity

Serum trypsin inhibitory capacity fell significantly during the first 12 hours after induction of pancreatitis only in the animals that survived for less than 24 hours (Text-figure 2). The earliest measurable decrement occurred at 8 hours. By 24 hours after induction of pancreatitis, trypsin inhibitory capacity was comparable to levels measured preoperatively. Although a subsequent fall was noted in animals surviving for more than 24 hours, this change was not statistically significant. Sham-operated control animals did not exhibit significant changes in serum trypsin inhibitory capacity.

Complement Measurements

Serum total hemolytic complement activity (CH_{50}) fell in all animals with experimental pancreatitis during the first 24 hours of observation (Text-figure 3). In animals that survived for more than 24 hours, serum hemolytic complement slowly returned toward preoperative levels. Changes in hemolytic titers of C3 and C4 paralleled the changes in CH_{50} (Table 1). Factor B activity ($CVFAH_{50}$), however, did not change significantly. Sham-operated control animals exhibited only a small decrement in total hemolytic complement activity during the first 12 hours of observation. Total hemolytic complement in control animals returned to preoperative levels by 24 hours and increased during the following 48 to 72 hours (Text-figure 3).

Since experiments *in vitro* in which sodium taurocholate was added to fresh guinea pig serum revealed evidence of inhibition by the bile salt of total hemolytic complement activity, it was imperative to determine the extent to which sodium taurocholate was absorbed into the circulation of



TEXT-FIGURE 3—Total hemolytic complement activity in control (Δ—Δ) and experimental animals (○—○, survived <24 hours; ●—●, survived >24 hours). Except as indicated in parentheses, each point represents data (mean ± SEM) obtained from at least 6 animals.

experimental animals. Text-figure 4 summarizes the results of experiments in 4 animals in which trace amounts of uniformly labeled ^{3}H-taurocholic acid were injected into the pancreas. The peak serum concentration of bile salt observed in these experiments *in vivo* (48 μg/ml) was more than 200-fold lower than that required to inhibit hemolytic complement activity *in vitro* (>10 mg/ml).

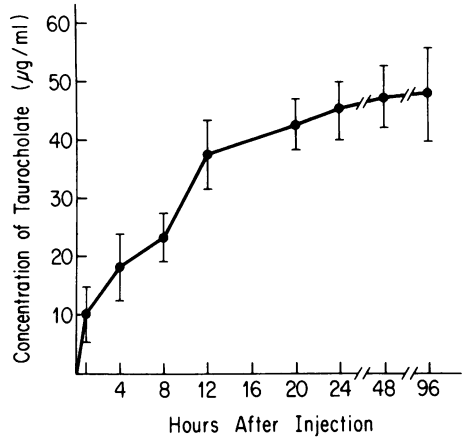
Chemotaxis

Incubation of fresh guinea pig serum with zymosan results in activation of the alternative complement pathway and generation of complement (C5)-derived peptides with chemotactic activity.[21] As demonstrated in Table 2, human PMNs were stimulated to migrate in a directed fashion

Table 1—Complement Levels in Control Animals and Animals with Pancreatitis

		Control animals*	Animals with pancreatitis*
CH_{50}	12 hours	90 ± 2 (6)	81 ± 4 (18)
	24 hours	103 ± 4 (6)	62 ± 6 (14)†
C3	12 hours	95 ± 5 (6)	84 ± 13 (8)
	24 hours	105 ± 11 (6)	75 ± 7 (6)‡
C4	12 hours	94 ± 2 (5)	81 ± 10 (8)
	24 hours	92 ± 9 (5)	53 ± 10 (7)‡
Factor B§	12 hours	101 ± 4 (4)	93 ± 7 (6)
	24 hours	113 ± 3 (4)	96 ± 12 (6)

* Results are expressed as a percentage of preoperative levels.
Mean ± SEM (number of animals studied).
† *P* versus control < 0.001 (two-tailed Student *t* test).
‡ *P* versus control < 0.05 (two-tailed Student *t* test).
§ $CVFAH_{50}$.



TEXT-FIGURE 4—Concentrations of taurocholate in serum after intrapancreatic injection. Each point represents the mean ± SEM of data obtained from 4 animals.

toward 1.0% (vol/vol) zymosan-treated guinea pig serum. Chemotaxis was not observed when dilutions of untreated guinea pig serum were added to the stimulus compartment of modified Boyden chambers. Serum obtained preoperatively from experimental animals and serum obtained at regular intervals from sham-operated control animals also failed to stimulate PMN directed migration. In contrast, serum obtained from animals as early as four hours after induction of experimental pancreatitis exhibited significant chemotactic activity (Table 2).

Table 2—Chemotactic Activity in Guinea Pig Serum

Stimulus	Number of experiments	PMN migration* (μ/35 min)
Buffer alone (random motility)	7	94.4 ± 0.6
Fresh guinea pig serum (GPS) (1.0%, vol/vol)	7	94.1 ± 0.4
Zymosan-treated GPS (1.0%, vol/vol)†	4	119.1 ± 2.0‡
GPS—control animals (1.0%, vol/vol)		
4 hours	4	94.9 ± 0.7
8 hours	4	95.5 ± 1.0
12 hours	4	95.0 ± 0.6
24 hours	4	95.0 ± 0.5
GPS—experimental animals (1.0%, vol/vol)		
4 hours	7	96.9 ± 1.1§
8 hours	6	103.6 ± 0.3‡
12 hours	5	106.6 ± 0.6‡
24 hours	5	114.9 ± 0.9‡

* Results represent the mean ± SEM.
† Fresh GPS was incubated in zymosan (1.0 mg/ml) at 37 C for 15 minutes.
‡ *P* versus fresh GPS < 0.001 (Student *t* test).
§ *P* versus fresh GPS < 0.05 (Student *t* test).

Discussion

As reported previously,[12] intraglandular injection of sodium taurocholate proved to be an effective method for inducing experimental pancreatitis in guinea pigs. Exogenous proteases (eg, trypsin) were not required, and it was possible to determine the precise onset and severity of the disease. Furthermore, most experimental animals survived sufficiently long to allow serial measurements of serum complement activity, and so forth. Although all animals were subjected to the same experimental procedure, we did observe considerable variability in the duration of survival. Although it cannot be concluded with certainty, it is likely that this variability reflected the extent of pancreatic necrosis (which was not quantified). This variability was advantageous, however, since it permitted us to compare data obtained from short- and long-term survivors.

As expected, animals with pancreatitis exhibited significant, but transient, elevations of serum amylase activity (Text-figure 1). Serum amylase activity, however, did not correlate with the severity of the disease, since animals that survived for less than 24 hours could not be distinguished from animals that survived for periods of up to 96 hours. Failure of serum amylase activity to reflect the severity of experimental pancreatitis has been noted previously.[12]

In contrast to amylase activity, changes in serum trypsin inhibitory capacity did appear to be related to the severity of pancreatitis. Only in the 10 animals that survived for less than 24 hours was there evidence of a significant reduction of antiprotease activity. This reduction occurred during the first 12 hours after the induction of pancreatitis (Text-figure 2). Serum trypsin inhibitory capacity reflects the ability of whole serum to inhibit the activity of a known quantity of bovine trypsin.[13] Consequently, it is only an indirect measure of serum antiprotease activity. Nevertheless, small (10–15%) decrements in serum trypsin inhibitory capacity can reflect the presence in the circulation of active proteases.[22] Our data suggest, therefore, but by no means prove, that active proteases (eg, trypsin, chymotrypsin) were released from the damaged pancreas early in the course of the experimentally induced disease. Evidence of transient protease–antiprotease imbalance during the course of acute pancreatitis has been provided by other investigators.[22–24] Perhaps most relevant are studies in man that have disclosed evidence (by radioimmunoassay) that pancreatic elastase and carboxypeptidase appear in the circulation during the course of acute pancreatitis.[23,24] Thus, active proteolytic enzymes may not only be confined to the gland parenchyma during episodes of acute pancreatitis, but also gain access to the circulation. It remains to be determined whether changes in serum trypsin inhibitory capacity accom-

panying pancreatitis accurately reflect the extent of pancreatic necrosis or whether such changes can be relied on to herald the onset of extrapancreatic sequelae.

Levels of total hemolytic complement as well as levels of hemolytic C3 and C4 fell significantly during the first 24 hours after the induction of pancreatitis (Text-figure 3 and Table 1). Factor B activity, however, did not change significantly. Complement activity fell to a minimal degree in sham-operated control animals. It should be noted that small decrements (5–10%) in hemolytic complement activity have been observed during the early postoperative period following various surgical procedures in man.[25]

There are several possible explanations for these data. First, the changes in complement activity may have been due to absorption into the circulation of large amounts of sodium taurocholate. Appropriate experiments, however, revealed that maximal concentrations of the bile salt *in vivo* were well below the concentrations required to inhibit complement activity *in vitro*. Second, it is possible that the changes in complement activity occurred not as a consequence of pancreatitis directly but rather as a consequence of sepsis and endotoxemia.[25] This possibility is unlikely, however, since activation of the alternative complement pathway by intact bacteria or by bacterial endotoxins would have been reflected by changes in serum factor B activity, which were not observed. Furthermore, the significant decreases in C4 activity observed in experimental animals are not consistent with activation of the alternative complement pathway. Third, the changes in complement activity may have been due to alterations in rates of synthesis rather than catabolism. The rapidity with which the changes occurred and the evidence, albeit indirect, of complement-derived chemotactic activity in experimental animals argue against this possibility. Finally, it is possible that pancreatitis led to either activation of the classic complement pathway or to direct proteolytic cleavage of native complement components. The experimental data described in this report do not help to distinguish between these two possibilities. Nevertheless, it is intriguing to speculate that at least some complement catabolism during the course of pancreatitis occurs as a consequence of protease–antiprotease imbalance. Pancreatic proteases have been demonstrated previously to be capable of cleaving C3, C4, and C5.[4,27] In fact, cleavage of native C5 by trypsin is an efficient way of generating peptides (eg, C5a) with potent chemotactic activity. Our findings of chemotactic activity in serum from experimental animals are consistent with this mechanism of degradation of C5. Indeed, the appearance of chemotactic activity in serum was perhaps the most sensitive indicator of complement catabolism in experimental animals. Furthermore, circulat-

ing chemotactic activity suggests a mechanism for the pathogenesis of some systemic sequelae of acute pancreatitis. Complement-derived chemotactic peptides exhibit a variety of biologic activities and may be responsible for the production of respiratory insufficiency.[4,6-8] Further studies are warranted to determine whether such peptides are, in fact, responsible for provoking the lung injury that can occur as a complication of pancreatitis.

Evidence for involvement of complement in experimental acute pancreatitis has been reported previously by Seelig et al.[28-29] In the course of experimental sodium-deoxycholate- and sodium-taurocholate-induced pancreatitis in the rat, these investigators observed a rapid and significant fall in serum total hemolytic complement activity. Seelig et al [28,29] have proposed that complement-mediated cytolysis is an early event in acute pancreatitis, leading secondarily to proteolytic "autodigestion." Whatever the role be of complement in acute pancreatitis, our data are consistent with the view that complement components are degraded and that proinflammatory, complement-derived peptides are generated during the course of this disease.

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