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Pancreatitis-Associated Ascitic Fluid: Effect on the Oxygen Consumption of Liver Cells (41372)

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Abstract. We examined the effect of pancreatitis-associated ascitic fluid (PAAF) on the oxidative activity of rat liver cells and found a depression with eight of nine dog PAAF samples. Pancreatitis was induced in dogs by retrograde pancreatic duct infusion of bile salts plus trypsin. The resultant PAAF was collected, passed through a sterile 0.22-μm Micropore filter to remove bacteria and other debris, and stored at −18° until used. Liver cells were obtained from minced Sprague–Dawley rat livers by gentle homogenization and filtration through a nylon mesh filter to produce a cell suspension. The oxygen utilization of liver cells incubated at 37° with a 1:50 dilution of PAAF was compared to controls incubated with normal dog plasma. The oxygen uptake of PAAF-treated cells was significantly lower with seven of nine ascitic fluids and the depression in oxygen consumption was found to be dose dependent over the range of 10 to 80 μl PAAF. One human PAAF sample was tested and it caused a 72% decrease in liver cell oxygen uptake as compared to controls. The observed depression in rat liver cell oxygen uptake may be an expression of the cytotoxic component of PAAF that leads to cell damage and multiple organ failure in acute hemorrhagic pancreatitis.

Acute hemorrhagic pancreatitis (AHP), an inflammatory disease of the pancreas which is associated with a high mortality rate, results in destruction of the pancreas and multiple organ failure (1). The most serious systemic sequelae of AHP are an acute respiratory distress syndrome (2) and renal insufficiency (3). Less common problems are direct cardiac depression (4), hepatic abnormalities (5), and disseminated intravascular coagulation (6). The classic treatment of AHP is fluid replacement for intravascular losses into the interstitial space and peritoneum. Unfortunately, multiple organ failure of AHP occurs in the face of adequate fluid resuscitation.

In 1966, Rodgers and Carey (7) noted that dogs suffering from an experimental model of severe AHP did not die as frequently, if their abdomen was washed with Ringer's lactate solution to remove the pancreatitis-associated ascitic fluid (PAAF) which collected there in large volume. They suggested that peritoneal lavage removed some of the toxic properties associated with PAAF. This observation was verified by subsequent clinical studies which indicated that peritoneal lavage, early in the course of AHP, reversed the systemic sequelae and decreased the mortality (8, 9).

The toxic potential of PAAF has been demonstrated by several *in vivo* bioassays. Amundsen *et al.* (10) showed that canine PAAF caused transient hypotension when injected into normal dogs, and similar results were obtained by Lankisch *et al.* (11) with a rat model of pancreatitis. Takada *et al.* (12) injected PAAF subcutaneously in dogs and demonstrated evidence of capillary permeability changes at the peri-injection site. Ellison *et al.* (13) injected human and canine PAAF intraperitoneally into rats and noted an increase in the tail vein hematocrit 2 hr postinjection. These studies all utilized *in vivo* assays to test for the presence of the toxic property of PAAF. In order to isolate the substance or substances responsible for PAAF's toxic effects, a more sensitive and specific assay is necessary. The present study describes a specific rat liver cell *in vitro* bioassay in which cell oxygen consumption can be used to evaluate the toxic effects of PAAF.

Methods. Nine female mongrel dogs

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weighing 20–30 kg each were fasted for 18 hr and anesthetized with sodium pentobarbital (25 mg/kg) followed by additions to maintain anesthesia. All dogs were prepared with femoral artery catheter, a Swan–Ganz pulmonary artery catheter via the jugular vein, an intravenous catheter in the foreleg, and an endotracheal tube. AHP was induced by a modification of Elliott's (14) model of pancreatitis. The main pancreatic duct was localized extraduodenally and cannulated with a 22-gauge Teflon catheter and a phosphate buffer solution (30 ml at pH 7.4) containing 1.8 g of sodium taurocholate and 2.5×10^5 or 2×10^6 IU of crystalline trypsin was infused under 30 cm of water pressure. During the infusion, the lesser pancreatic duct was occluded to prevent extravasation of fluid into the duodenum and after completion of the infusion, the main duct was ligated.

Animals received lactated Ringer's solution in order to maintain a baseline pulmonary capillary wedge pressure of at least 5 mm Hg as measured by the Swan–Ganz catheter. Normothermia was maintained by a K-pad water mattress (Gorman-Rupp Industries, Belleville, Ohio) and PAAF was collected hourly. The infusion was terminated at 6 hr and the total volume of PAAF collected was determined at this time. Following each experiment, a biopsy specimen of the pancreas was fixed in 10% formalin, embedded in paraffin, sectioned, stained, and examined microscopically.

Severity of pancreatitis was judged by a grading system involving six categories: peripancreatic fat necrosis, pancreatic edema, pancreatic necrosis, pancreatic hemorrhage, percentage of gland involvement, and volume of ascitic fluid collected. These were individually evaluated as: 0 = minimal, 1 = moderate, and 2 = severe and summated. The maximal degree of pancreatitis was scored as 12/12.

Human PAAF. PAAF was collected from a single patient with fatal postoperative pancreatitis. The patient was admitted in shock with multiple organ failure and underwent emergency operation whereby 500 ml of brown PAAF was removed. The diagnosis of hemorrhagic pancreatitis was confirmed, and intraoperative and postoperative peritoneal lavage failed to reverse the patient's moribund state.

Human and experimental dog PAAF was collected through sterile plastic tubing inserted into the abdominal cavity and passed through a 0.22-μm Millipore filter before collection in sterile containers. PAAFs were stored at −18° and to insure sterility, aerobic and anerobic cultures were run before the liver cell bioassay and contaminated samples were discarded. Fluids were analyzed for trypsin activity by spectrophotometric measurement of consumption of a trypsin substrate, benzyl-arginine ethyl ester (15), and osmolarity determined by a method described by Henry *et al.* (16).

Liver cell preparation. Sprague–Dawley rats (200–300 g) were fasted for 24 hr with water *ad lib.* After decapitation and exsanguination, the livers were rapidly removed and placed in ice-cold 0.9% sodium chloride. All preparatory steps were carried out at 4°. The livers were rapidly minced and washed free of blood in four 30-ml aliquots of 0.9% sodium chloride. Care was taken to remove blood clots, large blood vessels, and connective tissue. The minced liver was placed in 18 ml of 0.25 *M* sucrose–Tris buffer solution (pH 7.4) and poured into an homogenizer tube. After 8–10 passes of the loosely fitted Teflon pestle, the liver mash was filtered through a siliconized nylon filter (gauge 500) and centrifuged at 500*g* for 10 min. The supernatant was discarded and the sediment resuspended in 0.25 *M* sucrose–Tris solution to produce a dilution of one volume of liver tissue to 32 vol of ice-cold buffer with the liver cells kept uniformly suspended by a magnetic stirrer.

Oxygen consumption. Determinations were carried out at 37° in a modified YSI Model 53 Biological Oxygen monitor (17). Two milliliters of liver cell suspension was pipetted into a reaction chamber and oxygen uptake was determined over 10 min after a 4-min equilibration period to allow the contents of the reaction chamber to reach a constant temperature. To determine the effect of PAAF, 40 μl of PAAF was added to the liver cell suspension and oxy-

gen uptake determined. The activity of the PAAF-treated tissue was compared to control samples which were simultaneously exposed to 40 μl of normal dog plasma. The effect of the length of exposure to PAAF was determined by exposing liver tissue at 37° to PAAF for 5, 10, 20, and 40 min before measuring the oxygen consumption of the tissue as described previously. Control samples were exposed to normal dog plasma at 37° for the same time periods. Results are expressed as microliters (mean ± SD) of oxygen consumed per milligram of liver per hour (μl/mg/hr). The dry weight of each sample was carefully determined by quantitative removal of the liver sample from each reaction chamber into a tared aluminum weighing dish which was dried to constant weight and weighed with a semimicrobalance. Corrections were made for the additional dry weight of the sucrose–Tris medium, PAAF, or dog plasma.

Dose effect. Effect of ascitic fluid concentration was determined by varying the amount of PAAF added to cells in the reaction chamber (10, 20, 40, 80 μl) and the oxygen uptake determined as described above. Control samples were treated identically with similar doses of normal dog plasma.

Osmolarity controls. To study the effect of osmolarity of medium on the oxygen consumption of liver cells, we used 40 μl of a hyperosmolar solution (Dineal 137, 486 mosm/liter). Dineal 137 (a solution used in peritoneal lavage) was added to six samples of liver cells in the sucrose–Tris medium and their oxygen consumption compared to simultaneously run control samples exposed to 40 μl of dog plasma.

Statistics. The two-tailed Student's *t* test was used to compare the mean of the test group to the mean of the control groups and $P < 0.05$ was considered significant. Linear regression and correlation coefficients were used to evaluate whether or not the changes in liver tissue oxygen consumption were dependent on the dose of PAAF used or correlated with the time of exposure of liver cells to PAAF.

Results. Retrograde infusion of the trypsin–bile salt medium induced AHP in all dogs. During the initial postperfusion period, the pancreas became slowly edematous. After 15 to 20 min the gland became grossly edematous, with diffuse areas of hemorrhage and PAAF could be seen oozing from the surface of the pancreas. During the 6-hr experimental period, hourly samples of PAAF were collected from the abdomen. Dogs with mild pancreatitis produced 200 to 400 ml of PAAF while those with severe pancreatitis exuded 500 to 1000 ml in 6 hr. A lipid layer was seen on top of the PAAF when the abdomen was opened, and the fluid appeared thick and brown with a hematocrit of about 5%. There was usually peripancreatic and mesenteric fat necrosis.

Throughout the course of the experiment, the dogs became mildly hypotensive following the trypsin–bile salt infusion, but their blood pressure was maintained near normal with adequate fluid resuscitation. This required an infusion of 1 to 4 liters of Ringer's lactate solution over 6 hr.

Five of nine of the animals studied developed the maximal grade of pancreatitis (12/12) while the other four showed a lesser grade of pancreatitis (Table I). The grade of pancreatitis was predominantly dependent on the anatomy of the dog's ductal system. Dogs developing maximal grades of pancreatitis had a large single ductal system, which was easy to perfuse. Those animals with small multibranched pancreatic ductal systems developed lesser grades of pancreatitis. When more than 500,000 IU of trypsin was infused, a maximal grade of pancreatitis was observed, while lesser amounts of trypsin caused maximal pancreatitis only in animals with large ductal systems. The dose of trypsin infused did not influence the amount of active trypsin measured in the PAAF, nor the ability of the fluid to decrease liver cell oxygen consumption. As noted in Table I, several of the PAAFs with little or no active trypsin caused a significant decrease in oxygen consumption of liver cells.

Histology. Light microscope examination of hematoxylin/eosin-stained sections of pancreas from animals with experimentally induced pancreatitis showed evidence

TABLE I. COMPARISON OF PAAF SAMPLES: BIOACTIVITY AND TRYPSIN CONTENT

PAAF	IU trypsin infused	IU trypsin in PAAF	Severity of pancreatitis[a]	Significant fall in O_2 uptake[b]
Canine				
1	2.5×10^5	120	6/12	–
2	2.5×10^5	147	8/12	–
3	2.5×10^5	150	5/12	++
4	2.5×10^5	nm[c]	8/12	–
5	2.5×10^5	nm	12/12	++
6	2.5×10^5	213	12/12	++
7	5.0×10^5	nm	12/12	++
8	2.5×10^5	0	12/12	++
9	2.0×10^6	nm	12/12	++
Human	—	0	12/12	++

[a] 12/12 = maximum degree of pancreatitis.
[b] See Table II for data.
[c] Not measured.

of edema and the presence of an inflammatory infiltrate throughout the gland. Intermittent areas demonstrated frank necrosis of islet and parenchymal cells. Fat necrosis and blood vessel necrosis with subsequent hemorrhage were also common findings.

Liver cell respiration. Eight of nine dog PAAF samples decreased the oxygen consumption of liver tissue when compared to controls. The depression in oxygen uptake was statistically significant with six of nine fluids (Table II). The human PAAF sample decreased the oxygen consumption of liver cells by 73% as compared to the six canine PAAFs which reduced the oxygen uptake 39 ± 8% (PAAFs which decreased oxygen consumption to a significant degree).

Dose–time effect. The depression of oxygen consumption varied directly with the dose of PAAF (Fig. 1). Ten microliters of PAAF caused no reduction in oxygen uptake when compared to control activity but a statistically significant decrease in oxygen consumption of liver cells was found after adding 20, 40, or 80 μl of PAAF. There was a strong negative correlation between the dose of PAAF and the oxygen consumption of liver cells ($P < 0.01$, $r = -0.85$).

PAAF-treated samples incubated for up to 40 min had a reduction in oxygen consumption compared to those incubated for 10 min. Control samples also showed a parallel fall in respiratory activity but PAAF-treated samples always showed diminished oxygen uptake as compared to controls. There was no correlation between the exposure time and the reduction in oxygen consumption of liver tissue treated with PAAF (after correction for the fall in oxygen consumption of the control samples).

Osmolarity. The osmolarity of several PAAF samples was measured. The human PAAF was 290 mosm/liter and the canine PAAFs ranged from 300 to 400 mosm/liter. The hyperosmolar solution Dineal 137 (486 mosm/liter) added to the liver cell preparation in sucrose–Tris solution caused no significant change in oxygen consumption

TABLE II. OXYGEN CONSUMPTION OF LIVER CELLS EXPOSED TO 40 μl HUMAN PAAF, CANINE PAAF, OR NORMAL DOG PLASMA

PAAF used	O_2 uptake[a] with PAAF	O_2 uptake[a] normal dog plasma
Canine		
1	1.52 ± 0.27	2.01 ± 0.55
2	1.53 ± 0.27	2.01 ± 0.55
3	1.43 ± 0.28*	2.01 ± 0.55
4	0.99 ± 0.25	0.81 ± 0.26
5	0.73 ± 0.10*	1.17 ± 0.22
6	0.67 ± 0.13*	1.17 ± 0.22
7	0.63 ± 0.12*	1.08 ± 0.09
8	0.72 ± 0.11*	1.46 ± 0.15
9	0.91 ± 0.30*	1.31 ± 0.10
Human	0.35 ± 0.03*	1.31 ± 0.10

[a] μl O_2/mg dry wt/hr; $\bar{X}$ ± SD ($n = 5$).
* Significantly less than controls ($p < 0.05$).

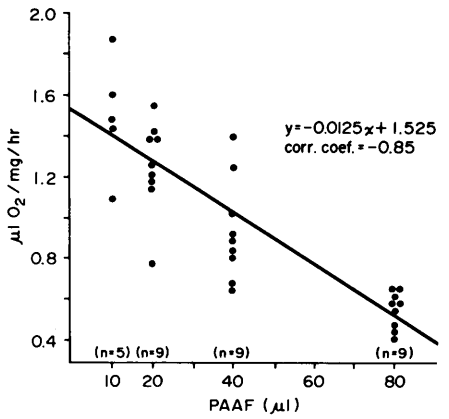

FIG. 1. Dose effect of PAAF on the oxygen consumption of rat liver cells. Varying amounts of different PAAF samples were added to a liver cell suspension and oxygen consumption was determined after a 4-min equilibration period. Control samples incubated with similar amounts of normal dog plasma showed little or no change. The curve was fitted by linear regression analysis.

of liver cells as compared to control samples.

Discussion. The toxic effects of PAAF have been studied in several bioassay systems. Amundsen *et al.* (10), using PAAF collected from a canine model of AHP similar to the one we used, showed that injections into the pulmonary artery caused a transient hypotension in normal anesthetized dogs. Subsequent studies indicated that the transient decrease in systemic blood pressure was probably due to endogenous histamine release. Lankisch *et al.* (11) reproduced the work of Amundsen and coworkers in rats with AHP induced by retrograde infusion of bile salts into the pancreatic duct. The collected PAAF, given iv into normal rats, produced a fall in systemic blood pressure, as measured by tail artery catheter. The mechanism for this PAAF-induced hypotension has not been characterized, but Takada and collaborators (12) found that subcutaneous PAAF injections in dogs increased capillary permeability in the peri-injection area, and Ellison *et al.* (13) found that ip injections of PAAF into rats significantly increased tail vein hematocrit after 2 hr suggesting that PAAF caused cellular damage that led to fluid shifts and the observed hemoconcentration.

In our experiments, one human and six of the nine experimental dog PAAF samples caused a significant decrease in the respiration of rat liver cells. Most of these were obtained from dogs with maximally graded pancreatitis. This suggests that extensive pancreatic destruction may be required in order to release the toxic substance or substances that cause a depression in cellular oxygen consumption. It seems likely that dogs that develop a milder degree of experimental pancreatitis may produce PAAF with the depressive substances, but they exist at concentrations too low to produce a measurable biologic effect. The depression in cellular oxygen uptake appears to occur in a dose-dependent fashion, at least over the limited range of PAAF dosages used (Fig. 1).

Liver cell suspension incubated at 37° from 5 to 40 min showed a gradual decrease in oxygen consumption, which tended to be correlated with time of incubation and not with the substance under study. In other words, the liver cells either died or had a gradual depression in respiration, possibly due to nutritional deficiencies of the medium. This fact necessitated the use of the simultaneous controls that were employed in our study. There are several possible mechanisms by which a depression of oxygen consumption could occur in metabolizing liver cells. Various parameters of PAAF such as pH, osmolarity, or electrolyte imbalance may have some effect in depressing oxygen consumption but these were controlled within small limits. The question of osmolarity was investigated and in the maximal range found in our PAAF samples there was no alteration in oxygen consumption of liver tissue. Electrolyte and pH changes were minimal in the medium used in our studies. Exogenous trypsin, an essential part of Elliott's (14) model of canine pancreatitis, has known cytotoxic properties. We measured active trypsin in both canine and human PAAF and found no apparent correlation between the amount of trypsin used for the creation of pancreatitis and the amount of measurable active trypsin in each PAAF or in the bioactivity of the PAAF.

Several of the known constituents of PAAF could be responsible for the reduction in liver cell oxygen consumption that we observed. Enzymes other than trypsin, such as elastase and lecithinase, are known to be present in PAAF and their activity cannot be excluded as a possible cause for the decreased oxidative activity noted in our studies. Keynes (18) suggested that bacteria or their end products may be a contributing factor in AHP. The Micropore filtration of our PAAFs insured sterility but did not completely eliminate the possibility of some soluble end product that might affect liver cell metabolism. The free fatty acid (FFA) concentration of PAAF is high (19), and it is known that the presence of fatty acids does influence the oxygen consumption of tissue (20). Borst *et al.* (21) have reported, however, that long-chain fatty acids tend to increase rather than decrease oxygen consumption by uncoupling respiration, an effect opposite to the depression in oxygen uptake we found on addition of PAAF to liver cell preparations.

Since Rogers and Carey (7) showed that peritoneal lavage of dogs with experimentally induced AHP markedly reduced their mortality rate as compared to controls, similar studies were done in humans with AHP showing that peritoneal lavage-treated patients had a decreased incidence of pulmonary, cardiovascular, and other complications (8, 9). There are several implications in our study that may have clinical correlates. Peritoneal lavage apparently decreases the incidence of multiple organ failure in AHP, possibly by removing some toxic substance or substances. The cytotoxic PAAF component may be capable of transperitoneal absorption, thus exerting a detrimental influence on heart, liver, and other vital organ systems. The depressive effect of PAAF on rat liver cell metabolism we observed may be an expression of the toxic factor or factors released during AHP, which results in a depression of cellular oxidative activity (22) that may lead to the multiple organ failure found in AHP. We propose to use the liver cell respiratory assay, coupled with PAAF fractionation, to attempt to identify and isolate the cytotoxic property of PAAF.

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