Acute Hemorrhagic Pancreatitis (Massive Necrosis) With Fat Necrosis Induced in Mice by DL-Ethionine Fed With a Choline-Deficient Diet

Benito Lombardi, MD, Larry W. Estes, MS, and Daniel S. Longnecker, MD

Female, albino mice were fed a choline-deficient diet containing 0.5% dl-ethionine. All animals died within 5 days due to the development of an acute hemorrhagic pancreatitis with fat necrosis throughout the peritoneal cavity. The pancreatitis was characterized by a massive necrosis of the exocrine parenchyma with intense hemorrhage and inflammatory reaction of the stroma. The sequence of histologic and ultrastructural alterations occurring in the acinar cells of the pancreas were studied in mice fed the diet for 1, 2, and 3 days. Major findings consisted of accumulation of zymogen granules, vacuolation due to foci of cytoplasmic degradation, and alterations in the morphology of the zymogen granules. The pancreatitis appears to be due to intraparenchymal activation of zymogens, resulting from a synergistic action of choline deficiency with the basic toxicity of ethionine toward the acinar cells of the pancreas. The experimental model simulates closely the acute hemorrhagic pancreatitis with fat necrosis occurring in humans and may prove useful for exploring the pathogenesis of this condition. (Am J Pathol 79:465–480, 1975)

Induction of acute pancreatitis in rats treated with ethionine was first reported in 1950 by Farber and Popper and Goldberg et al. Since then, ethionine-induced pancreatitis has been shown to occur readily in other species of experimental animals, including the mouse, hamster, cat, dog, and monkey. In one instance, death of some of the animals due to the development of an acute hemorrhagic pancreatitis with fat necrosis was observed to result after various periods of treatment. Usually, however, death of the animals did not occur, and the lesions consisted mainly of focal necrosis and atrophy with progressive fibroblastic and fibrous substitution of the parenchyma, along with regenerative attempts of the acinar cells. For these reasons, the

From the Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa, and the Department of Pathology, Dartmouth Medical School, Hanover, NH.

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Address reprint requests to Dr. Benito Lombardi, Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261.
ethionine model has been used frequently to investigate not only the pathogenesis of acute pancreatitis, but also to study the regenerative power of the pancreatic parenchymal cells.\textsuperscript{11-13}

We have recently observed that death from acute hemorrhagic pancreatitis with diffuse necrosis of the fat tissues of the abdominal cavity results, consistently, in 100% of mice after 4 days of feeding ethionine with a choline-deficient diet. A description of the experimental conditions and of the anatomicopathologic and ultrastructural alterations observed in the pancreas of these mice forms the subject of this paper.

Materials and Methods

Young 30-day-old female mice of the Swiss Webster strain (Hilltop Laboratory Animals, Inc, Scottdale, Pa) were used. They were kept in individual suspended wire-mesh cages in an air-conditioned room. After arrival in the laboratory, they were fed laboratory chow (Purina, Ralston Purina Co, St. Louis, Mo) for 2 to 3 days, and then one of two diets: either a choline-deficient diet prepared according to Basal B diet of Young et al,\textsuperscript{14} or the same diet in which 0.5% DL-ethionine replaced an equal amount of sucrose. The animals had access to the diets and to water \textit{ad libitum}. Mice fed the choline-deficient diet (control diet) will be referred to as control animals, and those fed the ethionine-supplemented diet (experimental diet) as experimental animals.

The appearance of various organs was noted at autopsy. Pieces of pancreas and of other organs were fixed in Stieve's solution and processed for histologic examination of hematoxylin- and eosin-stained sections. Frozen sections were stained with oil red O. Small pieces of pancreas were fixed in buffered osmium tetroxide \textsuperscript{15} for 2 hours at 4 C and then dehydrated also at 4 C. The tissue samples were embedded in an Epon-Araldite mixture.\textsuperscript{16} Ultrathin sections were cut with glass or diamond knives, stained with uranyl acetate,\textsuperscript{17} and poststained with lead citrate.\textsuperscript{18} The sections were lightly carboned, either before or after staining, and were examined and photographed with a Philips EM-200 or 300 electron microscope of 60 or 80 kV.

Results

Several experiments were performed on groups of 10 to 15 mice fed either the control or the experimental diet at 9 to 10 AM. During the fourth day of feeding, mice fed the experimental diet developed signs of acute sickness: They had staring fur, became motionless, were cold, and displayed fine tremors. An occasional animal died at this time, and, invariably, all the animals were dead by the morning of the fifth day after the beginning of the experiment, having been fed the diet for 4 days. No death ever occurred in mice fed the control diet, and these animals were usually killed on the fifth day. At autopsy, the latter animals showed no abnormality in the gross appearance of the abdominal organs except for a somewhat yellowish (fatty) liver. On the other hand, two major abnormalities (Figure 1) were consistently observed in the experimental animals: a) an intensely hemorrhagic and
swollen pancreas of very friable and gelatinous consistency, and b) a whitish-gray, opaque color of the fat tissues of the abdominal cavity, the tissues having small, coarse nodules scattered throughout their surface.

On histologic examination, the pancreas of control animals showed a normal architecture and no alteration in the appearance of the acinar cells. In experimental animals, a massive colliquative necrosis of the parenchyma was present (Figure 2). Throughout the organ, acinar cells were reduced mainly to indistinct masses of eosinophilic, granular debris, with an occasional karyolytic nucleus visible. In all cases, however, a few acinar cells could still be discerned, retaining not only their boundary but also a moderate degree of basophilia and an intact nucleus. Ductular and islet cells appeared to be unaffected (Figure 3). There was also severe congestion and edema of the stroma, which contained large quantities of extravasated red cells as well as numerous inflammatory cells, mostly neutrophils (Figure 3). No histologic alteration was evident in adipose tissues of control animals, while in experimental animals acute inflammation, necrosis, and areas of early calcification were present in the interior of the tissues as well as near their surface. Only small variations in the degree of these findings, both anatomic and histologic, were seen from animal to animal and between various experiments. The similarity of these anatomic and histologic changes with those found in the counterpart disease in humans is remarkable. Fatty infiltration of the liver was present in all the mice but appeared to be more severe in control animals than in those fed ethionine with the choline-deficient diet, in agreement with observations previously reported by Sidransky and Verney.

In order to study the progression of the pancreatic lesions, groups of 6 to 8 mice were killed after having been fed either the control or the experimental diet for 1, 2 or 3 days. The appearance of the pancreas and of the abdominal fat tissues was noted, and pancreas sections were examined at both the light and electron microscopic level.

No abnormality was seen in the gross appearance of the pancreas and fat tissues of all the mice fed the control diet and of those killed after having been fed the experimental diet for 1 day. In mice fed the experimental diet for 2 days the fat tissues appeared normal, but the pancreas was white, opaque, enlarged, and soft. After 3 days of feeding, the color of the pancreas became reddish and that of the fat tissues, white and opaque.

No histologic alteration was evident in the pancreas of mice fed the control diet (Figure 4). In mice fed the experimental diet for 1 day,
Acinar cells were intensely eosinophilic, with the cytoplasm crowded by zymogen granules. There was also a considerable reduction in the amount of basal and perinuclear basophilia, but no nuclear change (Figure 5). After 2 days of feeding, the loss of basophilia in the acinar cells was almost complete, and numerous, small, cytoplasmic vacuoles appeared which were localized mostly, but not exclusively, in the basal part of the cells (Figure 6). The vacuoles did not stain for fat. Nuclear changes were absent. The stroma of the organ showed a moderate degree of edema and congestion but contained neither red cells nor inflammatory cells. An eosinophilic, amorphous material filled the lumen of some of the ducts. The vacuolation of the acinar cells became more extensive after 3 days, but many cells at this time appeared to be already undergoing necrosis, as evidenced by the presence of a pyknotic nucleus, clumps of eosinophilic material in the cytoplasm, and indistinct cell boundaries. The interstitial edema and the congestion were more severe than those seen after 2 days of feeding, and hemorrhage and inflammatory reaction were present. No visible alteration was apparent in ductular cells or islet cells.

The ultrastructure of the pancreatic acinar cells of mice fed the choline-deficient diet was similar to that described previously, in mice and other experimental animals fed laboratory chow, by several authors. Typically, numerous zymogen and prozymogen granules of various diameters were present in the apical zone of the cells, while most of the cytoplasm was occupied by rough endoplasmic reticulum and mitochondria. The Golgi apparatus was well defined, and an occasional, small secondary lysosome was seen. In mice fed the experimental diet for 1 and 2 days, alterations in the ultrastructure of the acinar cells were notable mostly for their overall paucity. No abnormality was seen in the structure of nuclei, nucleoli, mitochondria and plasma membrane, and there was no increase in the number of lysosomes. The Golgi apparatus was still well defined even though, frequently, it appeared as less developed than that in cells of control mice. There was, however, a considerable increase in the number of zymogen and prozymogen granules (Figure 7) which occupied a much larger area of the cells than was the case in control mice. The most evident alterations involved the rough endoplasmic reticulum. After 1 day of feeding, there was a slight dilatation of the cisternae, which appeared to be real rather than artifactual since it was present in some cells and completely absent in contiguous ones (Figure 8). Furthermore, the dilatation became more pronounced and widespread after 2 and 3 days of feeding. However, no loss of ribosomes attached to the membranes was apparent. A second,
more conspicuous, alteration of the endoplasmic reticulum was represented by numerous, small foci of membrane damage and degradation (Figures 9 and 10). After 1 day of feeding, these foci were not bounded by a membrane and were located, almost exclusively, in the basal part of the cells, close to the interstices. Subsequently, they were present also in the marginal regions and apical parts of the cells, and some of them were in the form of membrane-bounded vacuoles containing fragments of rough endoplasmic reticulum and, occasionally, mitochondria or zymogen granules. Beside those already mentioned, other changes were present in mice fed for 3 days. In some of the acinar cells there was a more extensive breakdown of the endoplasmic reticulum, areas of which were replaced by accumulations of electron-dense membrane profiles, flocculent or granular dense particles, or an amorphous electronlucent material (Figure 11). In other areas, myelin figures were present. The Golgi apparatus was also affected: it lacked the broad stacks of cisternae and was composed instead of smooth membrane vesicles of varying size. Extensive scalloping of the zymogen granules became a prominent feature (Figures 11 and 12). In other cases, the central portion of the granules had a normal appearance but there was loss of density at the periphery with a space between the dense core and the granule membranes (Figure 13). The latter was often wrinkled and discontinuous, and a slightly granular, electronlucent material occupied the intragranular space. In some of the acinar cells, however, the disorganization and degradation of the various organelles was such to indicate that they were either necrotic or undergoing necrosis. Ductular and islet cells, even in mice fed for 3 days, appeared to have a normal size and configuration. Acinar lumina often contained a granular, electron-dense material representing, most likely, extruded debris of damaged cells. In a few instances, a separation of the basement membrane from the base of the cells was noted with accumulation of granular and membranous debris in the interposing space or in the interstitium. It seems likely that this extrusion of cellular debris in the interstitium may play a role in eliciting the inflammatory component of the process, as it has been suggested recently after a similar observation in puromycin-induced pancreatitis.

Discussion

Feeding DL-ethionine with a choline-deficient diet has been found to lead, after 4 days, to a total mortality of mice. The immediate cause of death appears to be a massive hemorrhagic necrosis of the pancreas with concomitant fat necrosis throughout the peritoneal cavity.
The toxicity of ethionine toward the acinar cells of the pancreas is a well-documented phenomenon, and indeed, several of the anatomic and cellular findings described in this paper have been reported previously by other investigators. However, three features seem to be unique to the present model, namely, a) the severity of the pancreatitis, b) the rapidity of its development, and c) the total mortality of the animals. These features are not present, or as marked, when ethionine is fed to mice with a choline-supplemented diet or with laboratory chow. They appear, therefore, to result from a synergism of choline deficiency with the basic toxicity of ethionine toward the pancreas.

Three, overlapping stages can be recognized in the histogenesis of the pancreatitis as observed in mice in the present study. The initial lesion in represented by a decrease in the basophilia of the acinar cells and a concomitant increase in the granularity and acidophilia of the cytoplasm (Figure 5). These changes appear to result from a marked accumulation of zymogen granules, as evidenced not only by electron microscopy (Figure 7) but also by measurement of the enzyme content of the pancreas. The second stage is characterized by a progressive and extensive disorganization and degeneration of the endoplasmic reticulum of the acinar cells. Initial small foci of membrane degeneration are followed by the appearance of large areas of a more complete breakdown of the endoplasmic reticulum. These changes are best seen by electron microscopy (Figures 9–11), but it is very likely that the cell vacuolation seen by light microscopy (Figure 6) is due to the same process. In the third stage, alterations in the morphology of the zymogen granules (Figures 12 and 13) become prominent and diffuse. At the same time, there is a more intense involvement of the stroma with edema, inflammatory reaction, and hemorrhage (Figure 2), but, throughout, the stromal changes appear to be secondary to those occurring in the parenchyma. The outcome of the parenchymal and stromal alterations is a massive hemorrhagic necrosis of the organ. A striking feature of this sequence of lesions is the remarkable overall preservation of cellular structures during the first 24 to 48 hours of treatment and the rapidity with which necrosis ensues.

In a study carried out on six pancreases obtained 8 to 36 hours post-mortem from patients who died of acute hemorrhagic pancreatitis, Geokas et al found that the bulk of elastase and chymotrypsin and a substantial amount of trypsin-like activity were in free form. Destruction of elastic tissue of the intrapancreatic vessels was also observed. The authors concluded that elastase must be responsible for the hemorrhagic nature, and elastase and the other proteases, for the explosive
nature of the necrosis. As reported in another paper, enzyme changes similar to those described by Geokas et al. were found to occur in pancreas of mice fed ethionine with a choline-deficient diet for 2 and 3 days. Therefore, it seems probable that the hemorrhagic necrosis developing in this experimental model has the same or a similar basis as that suggested for the human disease. Another similarity with the latter is the profound shock-like state that precedes the death of the animals. In any case, it appears that the experimental model will be useful for the exploration of factor(s) capable of leading to the intraparenchymal activation of pancreatic zymogens. As for the diffuse necrosis of the peritoneal fat tissues accompanying the pancreatitis, spillage of active pancreatic enzymes in the peritoneal cavity could account for it, although a direct effect of the dietary regimen on the metabolism of these tissues cannot be ruled out.

The primary biochemical defect(s) responsible for the pathogenesis of the pancreatic lesions induced by ethionine in several species of experimental animals has not been established. The hypothesis most frequently advanced is that ethionine interferes with the protein and RNA metabolism of the pancreas. This hypothesis is based, essentially, on the known prominence of protein metabolism in the pancreas, and an extrapolation to this organ of the known inhibitory action of ethionine on protein and RNA synthesis in rat liver. However, direct experimental evidence in support of this hypothesis is lacking. One effect of ethionine which is frequently overlooked in considering its toxicity toward mammalian cells is its inhibitory action on phospholipid metabolism. Indeed, ethionine has been shown to inhibit the incorporation of $^{32}$P into phospholipids of rat pancreas, to block the synthesis of lecithins via the transmethylation pathway in rat liver, and to alter the metabolism of phospholipids in liver microsomes. Feeding a choline-deficient diet also induces changes in membrane phospholipids of the endoplasmic reticulum and of other organelles of rat hepatocytes. Furthermore, it reduces the response of the endoplasmic reticulum to phenobarbital administration. Foci of cytoplasmic degradation, mostly involving the endoplasmic reticulum, are one of the earliest ultrastructural changes observed (in this study as well as in others) to develop in the acinar cells of the pancreas of animals treated with ethionine. It seems, therefore, that a more likely primary target of ethionine in the pancreas may be the phospholipid metabolism of the acinar cell, especially of the cytomembranes which are involved in the processes of intracellular transport and secretion of the pancreatic enzymes. Feeding a diet deficient in choline could potentiate this action of ethionine, resulting
in diffuse intraparenchymal activation of zymogens \(^{32}\) and the ensuing massive hemorrhagic necrosis. The basis for the synergism would then be an inhibition of the two main pathways \(^{48,49}\) for the biosynthesis of lecithins, the major phospholipid constituent of mammalian cell membranes. A marked inhibition in the synthesis of these membrane phospholipids could interfere with the intracellular transport and secretion of the pancreatic enzymes, and thus account for the observed accumulation of zymogens in the initial stages of the process. Persistence of the same inhibition could affect not only the functional but also the structural integrity of the cytomembranes, leading to the observed degradation of the endoplasmic reticulum and formation of autophagic vacuoles. Presence of lysosomal enzymes has been shown \(^{7,11,50}\) in the foci of cytoplasmic degradation induced by ethionine in the acinar cells of the pancreas. Thus, involvement of zymogens or of zymogen granules in these foci could very well lead to the activation of zymogens by lysosomal enzymes.\(^{51,52}\) Alternatively, the degenerative processes, or metabolic alterations which accompany them, could bring about changes (especially in the internal pH and \(\text{Ca}^{++}\) concentration of the zymogen granules) conducive to autocatalytic activation of the proenzymes.\(^{53}\) The diffuse alterations observed in the ultrastructure of the granules in mice fed the experimental diet for 3 days (Figures 11–13) could then be the earmark of the zymogen activation. Once started, activation would be promoted further by the active enzymes formed.\(^{53}\) A sequence of events such as this would account also for the contrast between the relative paucity of cellular lesions in the initial stages of the process and the suddenness of onset, rapidity of development, and severity of the ensuing necrosis and inflammatory reaction.

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[Illustrations follow]
Fig 1—Gross appearance of the pancreas and of periovarian and periuterine fat pads of a mouse fed DL-ethionine with a choline-deficient diet for 4 days. Pancreas (P) is enlarged (edematous) and shows dark, hemorrhagic areas. Opaque, white foci of fat necrosis are evident in the fat pads (right, left and bottom).

Fig 2—Massive necrosis of the pancreas parenchyma in a mouse fed DL-ethionine with a choline-deficient diet for 4 days. (H&E, × 50)
Fig 3—Section of pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 4 days shows congestion, hemorrhage, and inflammation in the edematous stroma. Duct and islet cells (center) appear normal. (H&E, × 100)  

Fig 4—Normal histologic appearance of the acinar cells of pancreas of a mouse fed a choline-deficient diet for 3 days. (H&E, × 400)  

Fig 5—Increased cytoplasmic granularity and decrease in the basal and perinuclear basophilia of acinar cells of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 1 day. (H&E, × 400)  

Fig 6—Cytoplasmic vacuolation in acinar cells of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 2 days. (H&E, × 400)
Fig 7—Increased number of zymogen granules in acinar cells of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 1 day. (x 7750)  
Fig 8—Dilation of endoplasmic reticulum cisternae in some acinar cells of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 1 day. (x 5750)  
Fig 9—Foci of endoplasmic reticulum degradation in the basal part of an acinar cell of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 1 day. A basal membrane is visible on the right side of the micrograph. (x 22,600)
Fig 10—Foci of cytoplasmic degradation in an acinar cell of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 2 days. The vacuole contains fragments of rough endoplasmic reticulum and what appears to be a mitochondrion. (× 32,600)

Fig 11—Numerous foci of extensive cytoplasmic degradation in acinar cells of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 3 days. (× 3500)

Fig 12—Scalloping, and discontinuity of the limiting membrane, of zymogen granules in an acinar cell of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 3 days. (× 28,700)

Fig 13—Loss of peripheral density in zymogen granules of acinar cells of the pancreas of a mouse fed DL-ethionine with a choline-deficient diet for 3 days. (× 9075)