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Liver Biopsy in Human Leptospirosis: A Light and Electron Microscopy Study*

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Introduction

Our previous work on human leptospirosis (kidney biopsies) (BRITO et al., 1965; PENNA et al., 1963) and experimental disease (kidney and liver) (BRITO et al., 1966) lead us to believe that changes in the cell membrane are the first elements of the cellular lesion. The findings are consistent with previous work which support the hypothesis that circulating toxin or toxins are involved in the mechanism of leptospiral pathogenicity (AREAN, 1964; STAVITSKY, 1945).

A further step was to study human liver biopsies, and this is the subject of this paper.

Material and Methods

Sixteen patients were studied, all of them having clinical features of the disease and significative ascending titers in the agglutination test for Leptospira (15 cases for *L. icterohaemorrhagiae* and 1 for *L. hebdomadis*). Biopsies were performed through a small laparotomy, according to the technique of MONTANS and BARRETO (to be published), usually on the second week of hospitalization. This technique allowed us to obtain liver fragments $1 \times 0{,}5$ cm of average diameter. The patients were submitted to the biopsy conveniently hydrated, in normal circulatory conditions and after the acute toxemic hemorrhagic phase of the disease has subsided. Care was taken to carry out the biopsy while clinical and laboratory evidences of active disease were still present; in this sense, considerable importance was given to the serum mucoprotein levels. At the time of the biopsy, blood was taken from the patients, liver function tests, bilirrubin level, mucoproteins, transaminases and serum electrophoresis being done.

Part of the liver fragment obtained was fixed in Bouin's fluid, embedded in paraffin, and treated by the usual histological methods.

The remainder of the liver fragment was studied as follows: part was fixed in cold buffered formalin, pH 7.2, plus sucrose, during 24—48 hours at 5°C, then transfered to a mixture of gum accacia-sucrose for at least 24 hours. Afterwards, they were cut 5 micra thick in a cryostat microtome and alkalyne and acid phosphatase as well as non specific esterase activities, were tested according to techniques described by PEARSE. The remainder of the liver fragment was immediatelly cut with the cryostat microtome without fixation and succino dehydrogenase, using as substrate Nitro B.T. (ditetrazolium chloride) and M.T.T. (3-(4,5-dimethylthiazolil-2)-2,5 diphenyl tetrazolium bromide) and citochrome oxidase (using as substrate the para-amino-diphenylamine) (BURSTONE) activities, were studied.

Finally, 0.3—0.5 mm thick slices of liver were cut with a razor blade and, in eight cases, fixed during 2 hours at 5°C in one per cent osmium tetroxide buffered to pH 7.3 with veronal acetate buffer plus 0.045 g of sucrose for each ml of fixative. In the remainder eight cases fixation was performed with a 6 percent solution of glutaraldehyde in phosphate buffer,

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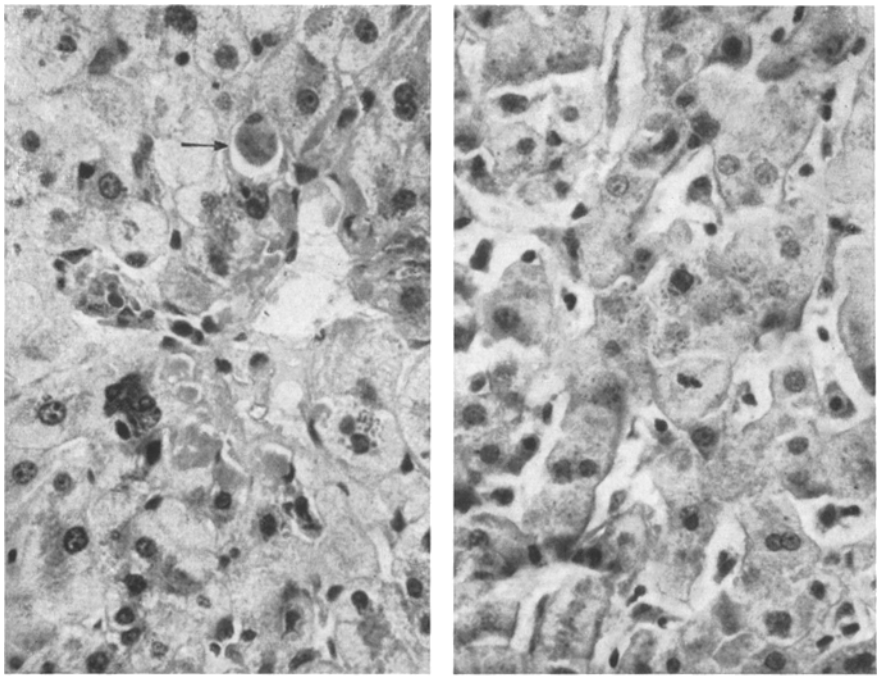
pH 7.2 during 2 hours at 5°C. Fragments were then washed in phosphate buffer and refixed in osmium tetroxide as described above. Afterwards, in all cases, tissues were dehydrated in a graded series of ascending alcohols and embedded in Epon 812, by a method similar to LUFT's. Thin sections were cut in a Porter Blum microtome equipped with glass and diamond knives. The sections were doubly stained first in uranyl acetate (WATSON) and then in lead citrate (REYNOLDS). The preparations were examined either in a Siemens Elmiskop I, or in a Zeiss EM9 electron microscope.

Seven liver controls from patients with duodenal ulcer obtained during the gastrectomy were studied in a similar manner. At the time of the biopsy, blood was also taken from these patients and the same tests described in the leptospirotic patients were carried out.

Results

Light Microscopy Findings. The lobular architecture of the liver is preserved. Pathological findings are restricted to the central zone and characterized by the presence of enlarged liver cells with clear cytoplasm, a few mitotic figures and multinucleated cells (Fig. 1a and b).



a b

Fig. 1. a Liver centro lobular area showing hepatic cells, some multinucleate, with a light cytoplasm containing bile pigment granules. An acidophilic round corpuscle (Councilmans' like body) appears loose at the liver trabecula, nearby the centrolobular vein (arrow). HE. 220 ×. b Mitotic figure in a hepatic cell. Kupffer's cell show hypertrophy. PAS positive granules are seen in their's and in the cytoplasm of few hepatocytes. PAS after salive digestion and hematoxylin. 220 ×

Also present, are atrophic liver cells with acidophilic cytoplasm and degenerated nuclei, some morphologically similar to the Councilman-like bodies' seen in virus hepatitis (Fig. 1a). In only two cases there is focal fatty change. Bile granules are seen, both in the cytoplasm of the cells with regenerative aspect, and Kupffer's cells. Bile thrombi are also seen, inside the bile capilaries. Hepatic cells around the portal spaces are normal. Kupffer's cells throughout the lobule show hypertrophy and hyperplasia, sometimes appearing in groups inside the sinusoidal lumina, an aspect resembling the "residual nodules" seen in virus hepatitis. Few portal areas in a given specimen show mild inflammatory infiltrate made up of histiocytes and lymphocites.

Histochemistry. Glycogen disappeared partially in individual or in small groups of hepatic cells in three out of eight cases. They were clinically the more severe cases of our series. It is worth mentioning that glycogen was demonstrated in the light cytoplasm of the hepatic cells with regenerative characteristics. PAS positive granules were present both in the cytoplasm of Kupffer cells and in individual hepatic cells after salive digestion (Fig. 1b). Iron was demonstrated as few small granules in the cytoplasm of Kupffer's cells in 5 of the 16 cases studied. Alkaline phosphatase activity was generally increased. In two cases enzyme activity was diminished when compared with controls. One of these cases was, clinically, the most severe of our series. Acid phosphatase activity was more marked in Kupffer's cell when compared with controls. Esterase activity showed slight deviation from the normal. In most of the cases a diminution of the enzyme activity was seen at the centrolobular region. Both oxydative enzymes, succino and cytochrome oxydase showed no decreased activity when compared with the controls.

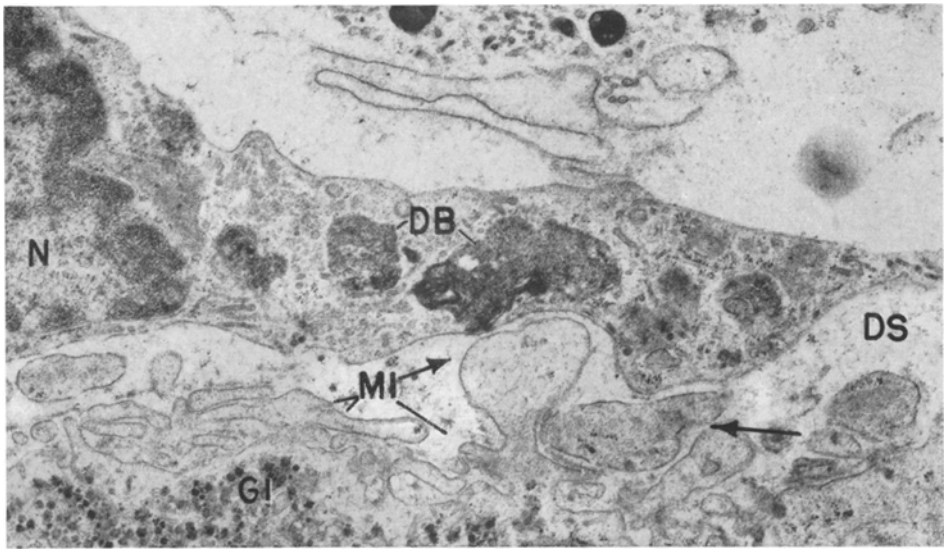


Fig. 2. Liver cell sinusoidal pole showing irregular, edematous microvilli (MI-arrow) projecting in the Disse's (*DS*) space. Glycogen granules (*Gl*) are observed in the liver cell cytoplasm. A hypertrophic Kupffer's cell is seen with irregular dense bodies (*DB*), in the cytoplasm and a normal nucleus (*N*). 14500 × (reduced to 19/20)

Electron microscopy findings. Electron microscopy findings are focal and more intense in the more severe cases.

Kupffer's cells are enlarged, with irregular contour and with many irregular dense bodies in the cytoplasm. Some of them, which have an irregular contour and a light central area are probably lipofuscin granules.

Hepatic cells with definite lesions were found throughout the hepatic lobule. These cells alternate irregularly with normal cells displaying no definite pattern of distribution. Hepatic cells microvilli are irregular, edematous, some showing a bullous, less electron dense free surface (Fig. 2). In few hepatic cells microvilli are scarce. Some Disse's spaces contain cell debris and altered organelles (Fig. 3). The attachment between hepatic cells is usually preserved. Enlargement of the intercellular space was rarely seen (Fig. 3). Parts of cells, probably macrophages, are observed sometimes inside the enlarged space. The junctional complexes around bile capillaries are preserved (Fig. 4). One of the most conspicuous and frequent lesion was total or partial disappearence of the *bile ductules* micro-

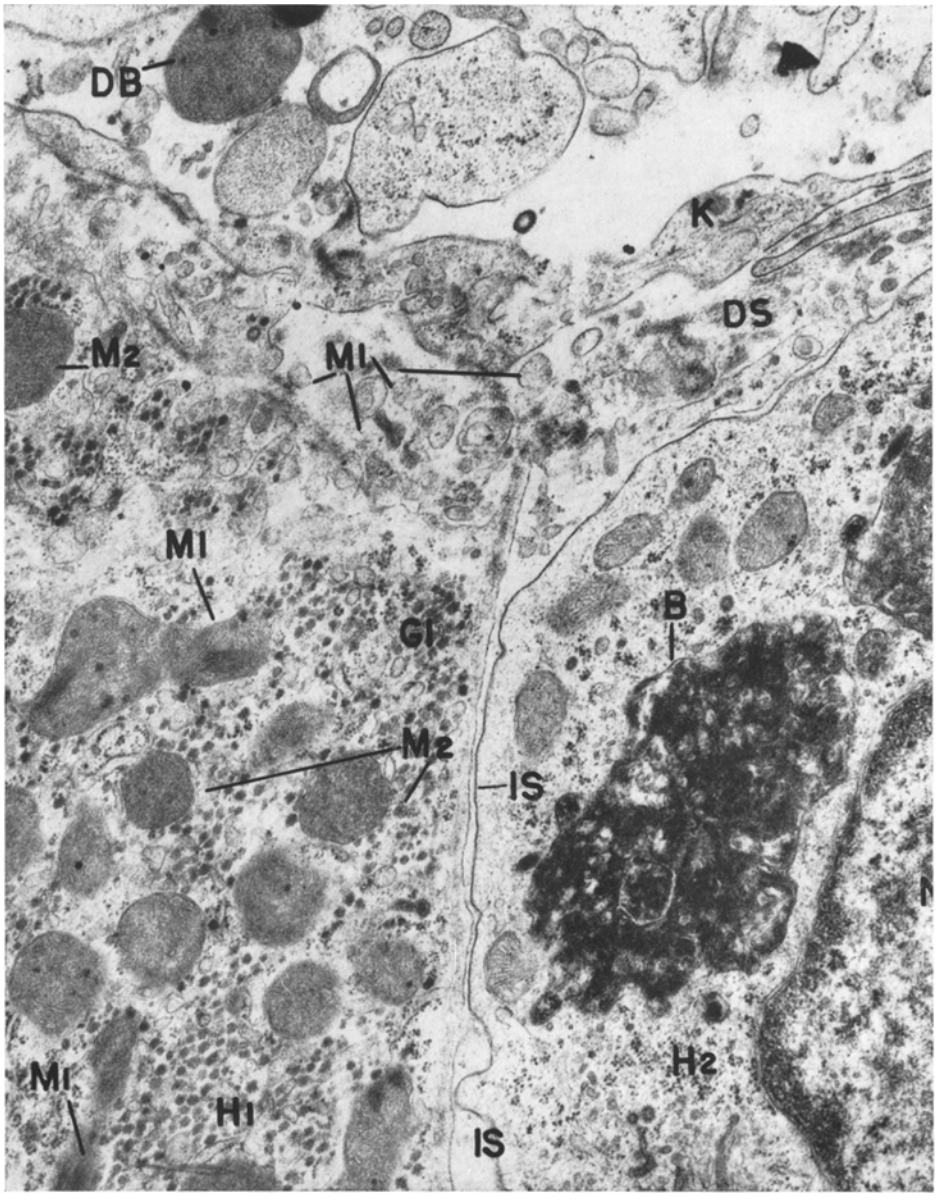


Fig. 3. Vascular pole of two hepatic cells (H_1—H_3). Their attachment is loose and an enlargement of the intercellular space is seen (*IS*). One hepatic cell (H_1) show glycogen (*Gl*) and few mitochondria (M_1) disclose "fibrillar degeneration". Others (M_3) show a dense matrix. The other hepatic cell (H_2) shows glycogen depletion and an irregular electron dense structure (*B*) which could be interpreted either as bile or as segregated degenerated cytoplasm (cytosegregosome or autophagic vacuole). Nucleus (*N*) is intact. Hypertrophic Kupffer's cells (*K*) cytoplasms are seen overlapping in areas. A dense body (*DB*), probably an altered organelle, is seen inside the sinusoidal lumen. 14500 ×

villi (Fig. 4). The microvilli present are irregular in size and shape. When bile thrombi are present the ductules are distended and devoid of microvilli. Severe cases show marked *glycogen* depletion (Fig. 3 and 4) and a predominance of smooth

over rough reticulum (Fig. 5). In areas, ribonuclein particles sometimes display a spiral disposition.

Peribiliary dense bodies are more numerous than in the controls. While many have the morphological characteristics of lipofuscin (Fig. 4) and are similar to some of the dense bodies seen inside Kupffer's cells, others are smaller, round, with an electron dense mathrix surrounded by single or double membrane. These dense bodies are not only in the peribiliary zone but also in different areas of the hepatic cell and even inside Disse's space (Fig. 3). Some show remnants of

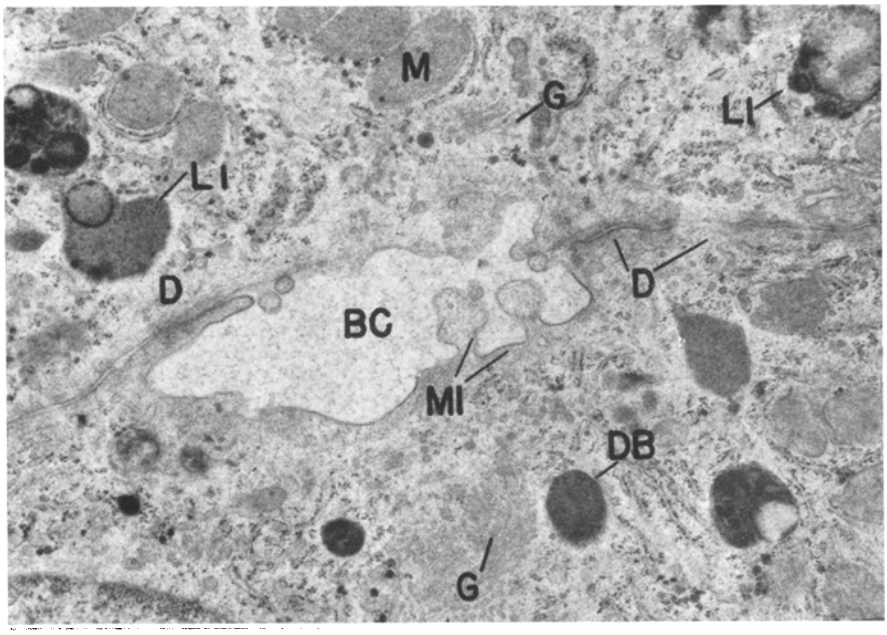
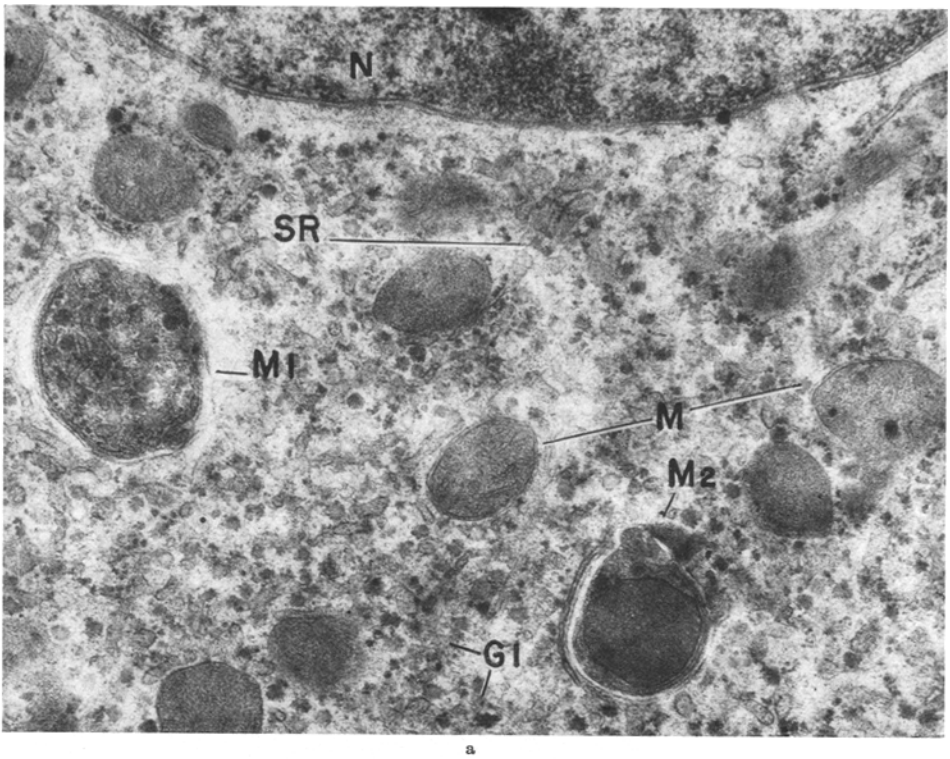


Fig. 4. Bile capillary (*BC*) showing almost total microvilli absence. The few still remaining (*MI*) are irregular and edematous. Mitochondria (*M*) and nuclei of both hepatic cells are normal. Glycogen granules are scarce. Junctional complexes (*D*) around the bile capillary are intact and no enlargement of the intercellular space is seen. Dense bodies with small light areas are seen, interpreted as lipofuscin granules (*LI*). Golgi (*G*) complex appears intact. *DB* designates a pericapillary dense body. 12000 ×

cristae and were interpreted as altered *mitochondria* (Fig. 5c). This mitochondrial alteration and another in which irregular electron dense areas are seen in the mitochondrial matrix (Fig. 5a) are among the more frequently found. Sometimes, altered mitochondria are surrounded by extra membranes, suggesting a process of organelle sequestration (Fig. 5a and c). A less frequent mitochondrial lesion is characterized by areas in the mathrix where many parallel striations are seen (so called "fibrillary degeneration") (Minio et al., 1965) (Fig. 5b and 3). Mitochondrial changes are more intense in severe cases; many intact mitochondria are seen in the majority of the hepatic cells. Ovoidal corpuscles made up of faintly electron dense mathrix surrounded by a single membrane are seldom seen among the peribiliary dense bodies. They were interpreted as cytosomes.

Nuclei are normal except in the severe cases where a certain degree of depletion of ribonuclein granules is observed. The Golgi apparatus seldom contained ovoidal electron dense corpuscles inside the vesicles.



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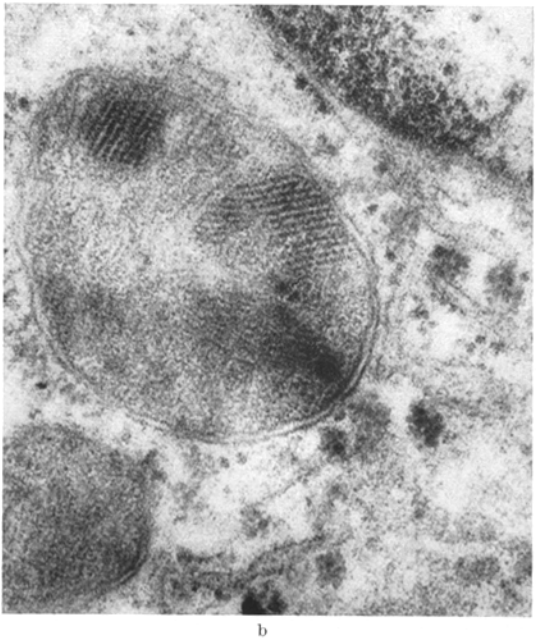

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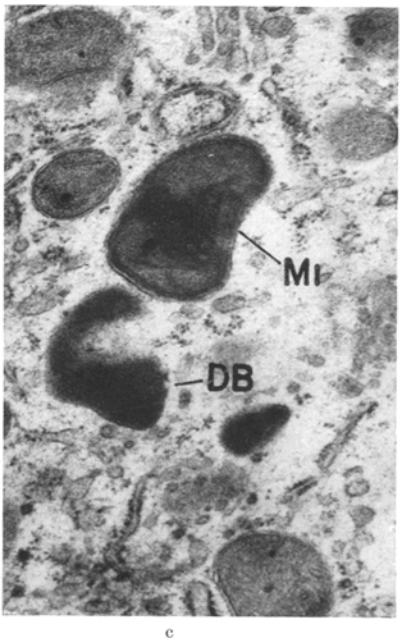

c

Fig. 5. a Hepatic cell with an intact nuclei (*N*) showing side by side with intact mitochondria (*M*) others in which irregular dense areas are seen in the mathrix (M_1 and M_2). Around them membranes are seen, suggesting that the altered organelles are being sequestrated. Smooth (*SR*) predominates over the rough reticulum. Few glycogen granules (*Gl*) are also seen. 29000×. b Mitochondria showing "fibrillar degeneration". 50000 ×. c Mitochondria (M_1) showing remnants of cristae and a dense matrix. An extramembrane is seen around the altered organelle. *DB* designates a dense body. 29000 × (reduced to 19/20)

Comments

Previous studies of biopsied human liver in leptospirosis (DOTTI and SABBIONI, 1959; OSTERTAG, 1950) show essentially the same findings described by us using larger biopsy specimens. "Loose hepatic cells" as described in necropsy material (BEITZKE, 1916; KOPPISCH and BOND, 1953; AREAN, 1962), or in the terminal phase of the experimental disease (BRITO et al., 1966) were not seen in biopsy specimens. It seems that the aspect seen at necropsy appears only at the more acute phase of the disease or during the agonal period.

DOTTI and SABBIONI (1959) failed to observe any significant alteration of the glycogen in the liver cells. In our patients in the more severe phase of the disease, groups or isolated hepatic cells showed decrease of the glycogen content, a finding confirmed by electron microscopy and in accordance with the necropsy finding of DRÄGERT (1934).

Worth commenting is the PAS positive granules of nonglycogenic character, which are considered a non-specific finding resulting either from the liver cell damage or from decreased metabolic activity, possibly connected with faulty biliary excretion (POPPER et al., 1960). These granules were also observed by us in the experimental disease (BRITO et al., 1966).

Electron microscopy showed definite changes of the sinusoidal lining, a finding also observed by us in the experimental disease (BRITO et al., 1966). Altered microvilli were seen by STEINER in the allergic liver injury (1961) who ascribed them to a toxic effect of antigen-antibody conjugates. Altered sinusoidal microvilli were observed by HAENNI (1964) in the experimental intoxication by allyl formiate. He interpreted the presence of organelles and dense bodies in the Disse's space as indirect evidence of cell wall breakdown.

In human leptospirosis we observed altered microvilli and dense bodies in the sinusoidal lumina, suggesting cell membrane alteration in this disease. Previous work (AREAN, 1962; AREAN et al., 1964; STAVITSKY, 1945) called attention to a, so far, hypothetical toxin or toxins as the mechanism of leptospiral pathogenicity. The morphological evidence of the cell membrane alteration could be interpreted as resulting from the action of such a circulating toxin or toxins.

A diminished alkaline phosphatase activity, as previously described in kidneys of guinea-pigs with leptospirosis (AREAN, 1962; BRITO et al., 1966; SQUADRINI et al., 1955) was seen in the human liver only in two cases. On the contrary, most of the cases showed an increased enzymatic activity confirmed by biochemical studies, now in progress. This is in accordance with the faulty biliary excretion, seen in the disease.

Increased acid phosphatase activity was observed in the enlarged Kupffer's cells. Electron microscopy showed in their cytoplasm many dense bodies and some of them could be interpreted as lysosomes.

Electron microscopy disclosed in most of the cases definite alterations of the bile canaliculi microvilli. This is a non-specific finding observed both in the intra and extra hepatic forms of cholestasis (POPPER and SCHAFFNER, 1963). An enlarged intercellular space was seen in a few areas, but the attachment zones of

the hepatic cells nearby the biliary canaliculi system were not damaged. A short-cut between bile canaliculi and Disse's space is not, according to us, the main mechanism of the jaundice in leptospirosis.

Previous light microscopy studies of the liver in leptospirosis (GARNIER and REILLY, 1918; VERNE et al., 1932) showed definite mitochondrial alterations. Electron microscopy disclosed mitochondrial changes in the more severe cases of the disease. Among the mitochondrial alterations described is the „fibrillary degeneration" which is a conspicuous finding in the Rotor-Dubin-Johnson syndrome (MINIO et al., 1965). Similar mitochondrial pathology has been seen in small proportion of the mitochondria in normal livers and in a larger proportion in many different pathological conditions (WILLS, 1965). The pathogenesis of the condition is not well understood. No theory has satisfactorily explained this finding, and its presence in so many diseases has been regarded as a non-specific degenerative phenomenon.

The presence of altered organelles surrounded by extra membranes, could be interpreted as a process of sequestration and digestion by autophagic vacuoles (cytosegregosomes) (TRUMP and ERICSSON, 1965).

The oxydative enzymes cytochemically studied apparently were not abnormal, a finding which awaits confirmation by the quantitative studies now in progress.

The severe cases showed also a predominance of the smooth over the rough reticulum, reflecting a disturbance of protein metabolism.

Summary

Sixteen human liver biopsies of patients with leptospirosis were studied both by light and electron microscopy. The sinusoidal pole of the liver cell show distortion and/or partial disappearence of the microvilli. There is hypertrophy and hyperplasia of Kupffer's cells. These data are suggestive of one or more circulating toxins. Complete cell necrosis is not prominent in human leptospirosis, this finding being in accordance with the low level of serum transaminase seen in the disease. Bile capillaries show microvilli disappearence or distortion, a non specific finding seen both in the extra and intrahepatic forms of cholestasis. No explanation for jaundice mechanism in leptospirosis was found.

Leberbiopsie bei menschlicher Leptospirosis. Licht- und elektronenmikroskopische Untersuchungen

Zusammenfassung

Sechzehn Leberbiopsien wurden untersucht. Der sinusoidale Pol der Leberzellen zeigt Strukturänderungen mit/oder teilweisem Verlust der Mikrovilli, die Kupfferschen Sternzellen weisen Hypertrophie und Hyperplasie auf. Diese Befunde sprechen für das Vorkommen von einem oder mehreren Toxinen. Vollständige celluläre Nekrose wurde nicht oft gefunden, was mit den niedrigen Serum-Transaminase-Werten bei dieser Krankheit übereinstimmt.

Die Gallencapillaren zeigen Formveränderung bis Zerstörung der Mikrovilli, ein unspezifischer Befund der sowohl bei extra- als auch intrahepatischen Formen der Cholestase gesehen wird. Es wurde keine Erklärung für den Ikterus von Leptospirosekranken gefunden.

References

Arean, V. M., G. Sarasin, and J. H. Green: The pathogenesis of leptospirosis: toxin production by *Leptospira icterohaemorrhagiae*. Amer. J. vet. Res. **25**, 727—738 (1964).

Beitzke, H.: Über die pathologische Anatomie der ansteckenden Gelbsucht (Weilsche Krankheit). Berl. klin. Wschr. **53**, 188—191 (1916).

Brito, T., E. Freymüller, S. Hoshino, and D. O. Penna: Pathology of the kidney and liver in the experimental leptospirosis of the guinea pig: a light and electron microscopy study. Virchows Arch. path. Anat. **341**, 64—78 (1966).

— D. O. Penna, H. S. Santos, E. Freymüller, S. Soares de Almeida, P. A. Ayrosa Galvão, and V. Pereira: Electron microscopy of human leptospirosis (kidney biopsies). Amer. J. trop. Med. **14**, 397—403 (1965).

Dotti, F., e C. Sabbioni: Il quadro istomorfologico del fegato nelle leptospirose benigna: studio bioptico con particolare riguardo alle forme anictériche. Arch. Pat. Clin. med. **36**, 81—101 (1959).

Drägert, E.: Beitrag zur pathologischen Anatomie der Weilschen Erkrankung. Virchows Arch. path. Anat. **292**, 452—464 (1934).

Garnier, M., et J. Reilly: Étude anatomique du foie dans la spirochétose ictérigène chez l'homme. Arch. Méd. exp. **28**, 228—276 (1918).

Haenni, B.: Les effects de l'intoxication aigue au formate d'allyl sur le foie de rat. Étude au microscope electronique. Path. et Microbiol. (Basel) **27**, 974—1002 (1964).

Koppisch, E., and W. M. Bond: The morbid anatomy of human leptospirosis: a report of the thirteen fatal cases. In: Symposium on Leptospirosis (11—12 December 1952). Med. Sci. Publ. No 1, p. 83—105. Washington, U.S. Government Printing Office 1953.

Minio, F., P. Maquenat, D. Gardiol, et A. Gautier: L'ultrastructure du foie human lors d'ictères idiopatiques chroniques. I. Dégénerescence mitochondriale dans un cas d'ictére du type Dubin-Johnson. Z. Zellforsch. **65**, 47—56 (1965).

Ostertag, H.: Leptospirosis icterohaemorrhagica in Bulgarien. Z. Hyg. Infekt.-Kr. **131**, 482—500 (1950).

Penna, D. O., T. Brito, A. Aguiar Pupo, M. Marcondes Machado, P. A. Ayrosa Galvão, and S. S. Almeida: Kidney biopsy in human leptospirosis. Amer. J. trop. Med. **12**, 896—901 (1963).

Popper, H., and F. Schaffner: Fine structural changes of the liver. Ann. intern. Med. **59**, 674—691 (1963).

Schaffner, F., and H. Popper: Morphologic studies of cholestasis. Gastroenterology **37**, 565—573 (1959).

Squadrini, F., E. Bussinello, e F. Bajocchi: Studio istologico e determinazione istochimica dell'attivitá fosfatasica (acida ed alcalina) e lipasica nella cavia in corso di infezione attenuata da leptospira icterohaemorrhagica (ceppo Zaan). G. Clin. med. **36**, 1369—1384 (1955).

Stavitsky, A. B.: Studies on the pathogenesis of leptospirosis. J. infect. Dis. **76**, 179—192 (1945).

Steiner, G. W.: Investigations of allergic liver injury. I. Light, fluorescent and electron microscopic study of the effects of soluble immune aggregates. Amer. J. Path. **3**, 411—436 (1961).

Trump, B. F., and J. L. E. Ericsson: Some ultrastructural and biochemical consequences of cell injury. In: B. W. Zweifach, L. Grant, and R. T. McCluskey, eds., The inflammatory process, p. 35—120. New York: Academic Press 1965.

Verne, J., M. Bariéty et M. Albeaux-Fernet: Étude anatomique du foie dans un cas mortel de spirochétose ictérigène. Ann. Anat. path. **9**, 200—204 (1932).

Wills, E. J.: Crystalline structures in the mitochondria of normal human liver parenchymal cells. J. Cell Biol. **24**, 511—514 (1965).

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