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Alcoholic Liver Injury: Defenestration in Noncirrhotic Livers—A Scanning Electron Microscopic Study

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The fenestration of hepatic sinusoidal endothelial cells in 15 needle biopsies obtained from chronic alcoholics without cirrhosis was studied by scanning electron microscopy. As compared to nonalcoholics, a significant reduction in the number of fenestrae and porosity of the sinusoidal lining wall (fractional area of fenestrae) was observed in acinar Zone 3, both in biopsies with and without Zone 3 fibrosis as judged by light microscopy. A significant reduction of porosity as shown in this study may influence the blood hepatocytic exchange and contribute to the alcohol-induced liver injury.

The normal exchange of metabolites between sinusoidal blood and liver cells is dependent on a delicate barrier consisting of the sinusoidal endothelial cells and the space of Disse. The endothelial cells are fenestrated, and as they lack a basal lamina, there is normally direct access of plasma to the space of Disse and thus to the hepatocytes (1–3). Any morphological alteration concerning the endothelium and the space of Disse may influence the normal exchange of metabolites and may thereby have adverse effects on the function of the parenchymal cells.

Alterations in this hepatocellular microenvironment in the form of deposition of collagen in the space of Disse (4) and the formation of basal laminas may be induced by alcohol (4, 5). Defenestration of the endothelial cells may be another factor in compromising the normal free exchange.

The aim of the present study has been to describe the changes, as evaluated by scanning electron microscopy (SEM), occurring in the sinusoidal fenestration in alcoholic livers without cirrhosis. Since the early alcoholic liver lesions develop in Zone 3 (centrilobularly), we have concentrated the present investigations to this area.

MATERIALS AND METHODS

A consecutive series of liver biopsies from 112 patients was investigated by light microscopy (LM), transmission electron microscopy (TEM) and SEM. Clinical and biochemical data were available in each case, and biopsies were obtained as part of the routine diagnostic work-up.

Patients were categorized as alcoholics when drinking more than 60 gm alcohol every day for a period of more than 3 years. Fifteen biopsies from 15 different alcoholics without cirrhosis as seen by LM comprise the present material. Thirteen biopsies from nonalcoholics with normal parenchyma as judged by LM were examined for comparison.

Liver biopsies were taken with a Menghini needle (diameter-1.6 mm). The biopsy was, when large enough, immediately divided into parts for LM, TEM, SEM and for immunohistochemical investigations.

Tissue for LM was fixed in 4% formaldehyde solution and then embedded in paraffin, cut and stained following our routine protocol (6).

For TEM examination, tissue blocks measuring less than 1 mm^3 were fixed in 3% glutaraldehyde and processed in the conventional way. Semithick sections measuring 1 μm were stained with tolouidine blue for LM to identify the acinar Zone 1 and 3 areas. Thin sections were stained with uranyl acetate and lead citrate.

The specimen for SEM measuring 5 to 10 mm was manually perfused through a needle (0.4 mm) using the technique introduced by Murakami (7) modified by us (8). After placing the specimen in a few drops of 3% glutaraldehyde in a phosphate buffer (pH 7.4, osmolarity = 740 mOsm) and while gently immobilized by forceps, it was perfused with a total volume of 3 to 6 ml 3% glutaraldehyde. Following perfusion, specimens were fixed for a further 24 hr in 3% glutaraldehyde at 4°C. After washing, samples were postfixed for 2 hr in 2% osmium tetroxide, washed, dehydrated and critical point-dried. The specimens were fractured under the stereomicroscope, mounted on metal stubs and coated with gold before examination in a JEOL JSM 25 scanning electron microscope.

The evaluation of the fenestration was based on the SEM investigation. In SEM specimens, portal tracts were recognized by the presence of abundant connective tissue, and terminal hepatic veins (central veins) by a limited amount of connective tissue and by the numerous sinusoidal connections giving the appearance of a perforated vein wall (8, 9).

All ultrastructural evaluations were done without knowing the clinical history, light microscopical features or biochemical data. Furthermore, all measurements on micrographs were done blindly.

The number of fenestrae was counted in an area of 4 μm^2 at 20,000×. Three areas adjacent to each other were examined in 3 to 5 different sinusoids in both Zones 1 and 3. Thus, the fenestrae in a total area of 36 to 60 μm^2 were counted in both Zones 1 and 3 in each specimen. Two independent counts were performed in five cases with a variation of less than 2%. Only fully exposed sinusoids were examined, and areas with more than 1% large fenestrae [some of these appearing as obvious artifacts (8)] were excluded.

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The size of fenestrae was determined on micrographs taken in eucentric positions at 20,000×. The resolution on micrographs taken at 20,000× did not allow an exact measurement of especially the smallest fenestrae. Therefore, we allocated the fenestrae according to their largest diameter to 1 or 3 groups: (i) fenestrae with a diameter of less than 100 nm; (ii) fenestrae with a diameter between 100 and 200 nm, and (iii) fenestrae with a diameter larger than 200 nm. Two independent estimations were done showing a variation of less than 5%.

The porosity (fenestrated area in per cent of total area of sinusoidal lining wall) was measured on micrographs using a texture analyzing system (Leitz, TAS 1978). This system allows a computerized contrasting of fenestrae on micrographs, and by combining the texture analyzing system to a Hewlett-Packard 9815A calculator, the percentage area of fenestrae as compared to the total area could be measured. Measurements were done on the same micrographs as used above, and a total number of 133 micrographs were analyzed blindly.

Statistics. The significance of differences between medians of paired or grouped data was tested by the Wilcoxon or Mann-Whitney rank sum tests, respectively. Heterogeneity between more than two groups was determined by the Kruska Wallis one-way analysis of variance or the Friedman two-way analysis of variance. Comparisons of medians of more than two data sets were performed by a nonparametric application of Shaffer's extension of Dunnett's procedure (10).

Correlations between independently determined variables were determined by the Spearman rank correlation test (rho). $P < 0.05$ was considered significant.

RESULTS

LM. The main histological diagnosis for the 28 liver biopsies from nonalcoholics (13 biopsies) and from chronic alcoholics (15 biopsies) are listed in Table 1.

There was no parenchymal fibrosis in the 13 biopsies from the nonalcoholics.

TABLE 1. Main histological diagnosis

	Diagnosis	No. of patients
Non-alcoholics[a]	Normal	4
	Slight steatosis	3
	Slight nonspecific changes	3
	Portal inflammation suggestive of primary biliary cirrhosis	3
	Total	13
Alcoholics	No Zone 3 Fibrosis	
	Steatosis	5
	Steatosis and Mallory bodies	2
	Steatosis and lipogranulomas	1
	Total	8
	Zone 3 Fibrosis	
	Steatosis	2
	Steatosis and alcoholic hepatitis	4
	Steatosis and lipogranulomas	1
	Total	7
	Total	15

[a] Biopsy was performed because of obesitas, in search for metastases or as control for hepatitis or primary biliary cirrhosis.

With regard to the alcoholics, there was no fibrosis in Zones 1 and 2 in any of the cases. *Eight biopsies* showed no Zone 3 fibrosis as revealed by the van Gieson stain. In this group, a few liver cells with Mallory bodies, but without simultaneous occurrence of neutrophils, were present in two cases and a few large lipogranulomas in another case. Steatosis in varying degrees was seen in all eight biopsies. *In seven biopsies*, Zone 3 fibrosis in varying degrees was noted partly along the sinusoids (perisinusoidal fibrosis) and partly pericellularly. Four of these biopsies contained scattered liver cells, with Mallory bodies surrounded by neutrophils (alcoholic hepatitis). One biopsy contained a few large lipogranulomas, and all seven cases showed varying degrees of steatosis.

TEM. *In biopsies from nonalcoholics*, the sinusoidal endothelial cells were thin and delicately fenestrated without any underlying basal lamina (Figure 1). No difference in ultrastructural morphology was seen between Zones 3 and 1.

In biopsies from alcoholics, the endothelial cells in Zone 3 appeared thickened, and a discontinuous or continuous basal lamina separating the cells from the perisinusoidal space was often seen (Figure 2). A basal lamina consisting of a lamina densa between two layers of fine fibrillar material was observed in all seven cases with Zone 3 fibrosis and in 5 of the 8 biopsies without Zone 3 fibrosis. Only occasionally were fenestrae seen in areas where a basal lamina was present.

SEM. In specimens from both nonalcoholics and alcoholics, sinusoids were generally empty, and only few erythrocytes and platelets were seen in the lumen. In nonalcoholics, the sinusoidal lining cells (endothelial cells) showed laminar extensions, with a varying number of fenestrae often arranged in sieve plates (Figure 3). In the biopsies from alcoholics, the number of fenestrae in Zone 3 was obviously reduced, and they only occasionally formed sieve plates (Figure 4, A to C). In Zone 2 and 1 areas, fenestrae showed a higher tendency to sieve plate arrangements.

Fenestrae appeared oval to round in shape, with diameters of 50 to 300 nm. Large fenestrae with a diameter exceeding 300 nm were rare, and some appeared as

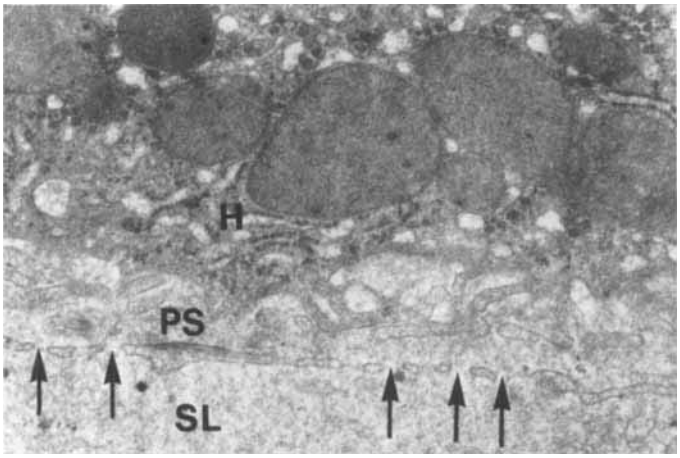

FIG. 1. *Nonalcoholic.* TEM micrograph showing several fenestrae (*arrows*) in the sinusoidal endothelium. Hepatocyte = H; perisinusoidal space = PS; sinusoidal lumen = SL. Original magnification, × 13,000.

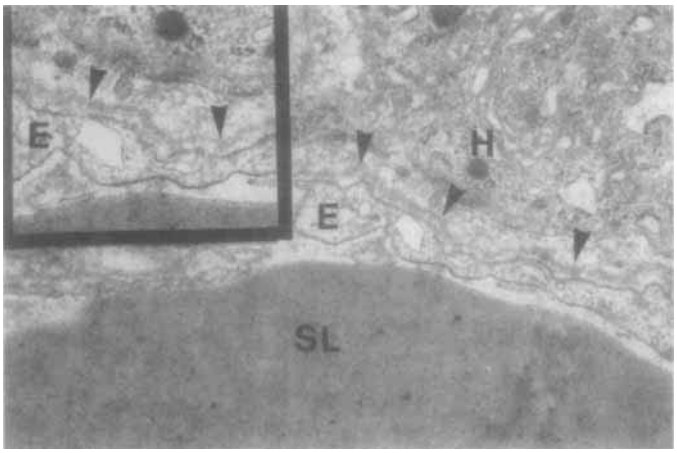

FIG. 2. *Alcoholic.* TEM micrograph showing a basal lamina (*arrowheads*) underlying the sinusoidal endothelium (E). Hepatocyte = H; sinusoidal lumen = SL. Original magnification, × 13,000. (*Inset*) High power view. Original magnification, × 26,000.

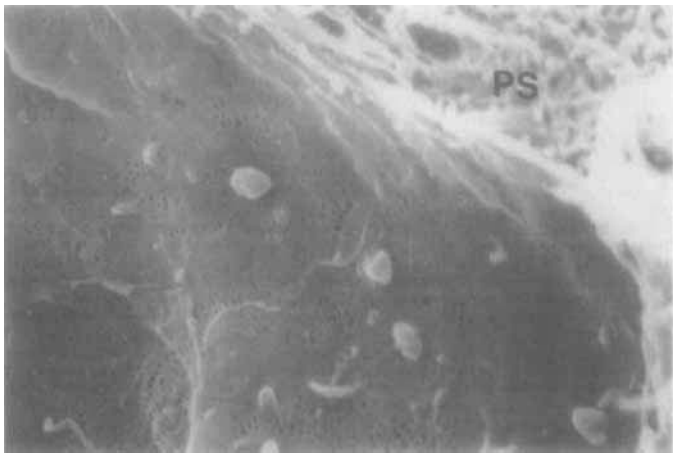

FIG. 3. *Nonalcoholic.* SEM micrograph of sinusoidal lining wall in Zone 3 demonstrating numerous fenestrae most often arranged as sieve plates. The perisinusoidal space (PS) is exposed. Original magnification, × 7,800.

obvious artifacts caused by the coalescence of smaller fenestrae (2).

The number of fenestrae in acinar Zones 3 and 1 in alcoholics and in the reference material is summarized in Table 2. In normal livers, the number of fenestrae was higher in Zone 3 than in Zone 1 (medians = 23.5 vs. 19 per μm^2, respectively, $p < 0.005$). In alcoholics, both with and without Zone 3 fibrosis, the number was significantly reduced in Zone 1 ($p < 0.05$) and in Zone 3 [$p < 0.01$ (biopsies without fibrosis) and $p < 0.005$ (biopsies with fibrosis)]. While the number of fenestrae was below the normal range in all biopsies showing Zone 3 fibrosis, three biopsies with no fibrosis revealed a number of fenestrae within the normal range (Table 2). These three biopsies were the same as mentioned above, without occurrence of subendothelial basal laminas in Zone 3. When comparing the alcoholics without fibrosis and the alcoholics with fibrosis, a significant difference in Zone 3 was achieved by the Mann-Whitney test ($p < 0.05$).

The distribution of fenestrae according to their *sizes* is illustrated in Figure 5. In Zone 3 areas, the numbers

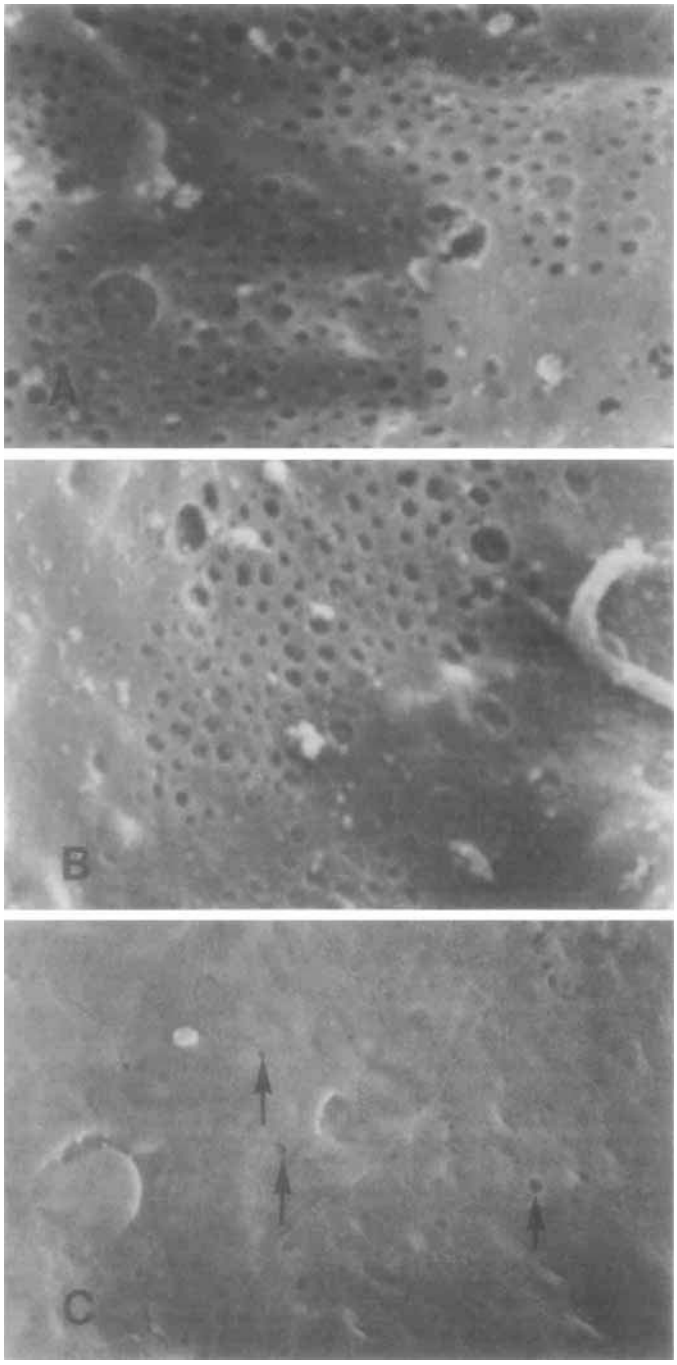

FIG. 4. (A) *Nonalcoholic.* SEM of normal sinusoidal lining wall in Zone 3 with numerous fenestrae. Original magnification, × 20,000. (B) *Alcoholic.* SEM of sinusoidal lining wall in Zone 3 showing a slight reduction in the number of fenestrae as compared to (A). LM biopsy showed no Zone 3 fibrosis. Original magnification, × 20,000. (C) *Alcoholic.* Sinusoidal lining wall in Zone 3 with only occasional fenestrae (*arrows*). LM biopsy showed Zone 3 fibrosis. Original magnification, × 20,000.

of fenestrae measuring less than 100 nm, between 100 and 200 nm, and more than 200 nm in diameter were reduced equally as compared to nonalcoholics ($p < 0.005$). No significant difference was found in Zone 1. Micrographs from Zone 3 in one alcoholic and from Zone 1 in two alcoholics were excluded because of unsatisfac-

TABLE 2. Number of fenestrae per square micron and porosity[a] of sinusoidal lining wall in acinar Zones 3 and 1

	No. of fenestrae per μm^2		Porosity (%)	
	Median	Range	Median	Range
Zone 3				
Nonalcoholics	23.5 (n = 13)	15–25	9.3 (n = 13)	4.8–16.2
Alcoholics	8.5[b] (n = 15)	2–21	3.0[c] (n = 14)	0.2–7.8
Without fibrosis	12.5[b] (n = 8)	5–21	4.6[b] (n = 7)	1.2–7.8
With Zone 3 fibrosis	6.5[c] (n = 7)	2–12	2.7[b] (n = 7)	0.2–4.8
Zone 1				
Nonalcoholics	19 (n = 10)	10–24	7.6 (n = 10)	3.8–12.3
Alcoholics	12[d] (n = 9)	2–18	3.5 (n = 7)	0.2–9.9

[a] Fenestrated area in per cent of total area of sinusoidal lining wall. Significantly different from nonalcoholics: [b] $p < 0.01$; [c] $p < 0.005$; [d] $p < 0.05$.

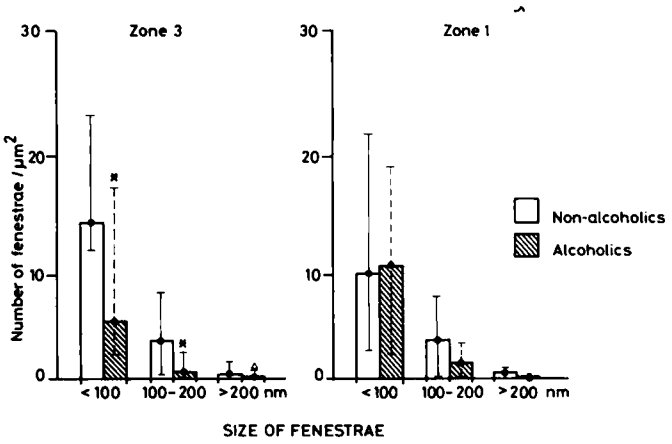

FIG. 5. Diagram illustrates the difference in the distribution of fenestrae according to their size in Zone 3 and Zone 1 in nonalcoholics and alcoholics, respectively. Number of biopsies analyzed in Zone 3: nonalcoholics = 13; alcoholics = 14. Number of biopsies analyzed in Zone 1: Nonalcoholics = 10; alcoholics = 6.

tory resolution. (This accounts for the difference in the number of cases in Table 2 and Figure 7, A and B).

The porosity is illustrated in Figure 6 and Table 2. There was a significant difference in Zone 3 porosity between nonalcoholics (mean = 9.3%) and alcoholics (median = 3.0%, $p < 0.005$). All biopsies with Zone 3 fibrosis revealed a porosity less than 5% (normal = 4.8 to 16.2%). In the group of alcoholics without fibrosis, four biopsies had a porosity higher than 5% (6.0, 7.5, 7.6 and 7.8%), i.e., within the normal range. Three of these four biopsies are the same as mentioned above without basal lamina. No significant difference was found in Zone 1 porosity.

Relationship between porosity and number of fenestrae is illustrated in Figure 7, A and B. Highly significant correlations were found between these variables, which were calculated independently in Zone 1 as well as Zone

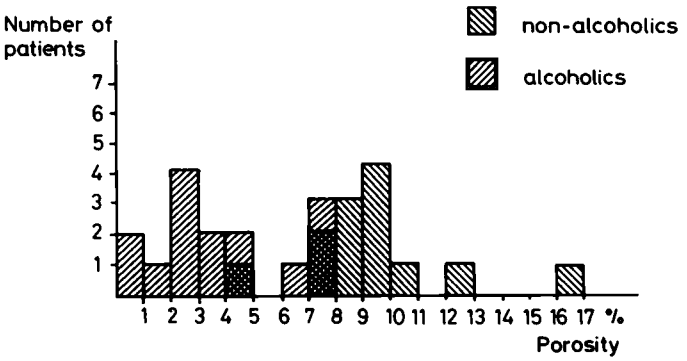

FIG. 6. Diagram showing the distribution of the porosity of sinusoids in Zone 3 in nonalcoholics and alcoholics. Four biopsies from alcoholics show a porosity higher than 5%.

3 areas (rho = 0.80, $p < 0.01$; rho = 0.90, $p < 0.001$, respectively).

DISCUSSION

The literature reveals only a few SEM investigations on human hepatic sinusoidal lining cells (1, 2, 7, 8, 11–15). Nopanitaya et al. (11) examined a large series of human needle biopsies fixed by immersion and found the sinusoidal surface covered by blood cells and precipitates. Thus, in order to investigate the walls of the sinusoids, it is necessary to remove the blood, and this is only possible using perfusion.

Muto et al. (12) examined human needle and wedge biopsies after applying a manual perfusion technique as initially introduced by Murakami (7). Vonnahme (14) and later Burwen et al. (15) improved the perfusion technique; however, in all of these studies, a perfusion with Ringer or other solutions was performed prior to the perfusion with fixative. Delaying the access of fixative to the delicately fenestrated sinusoidal lining wall may cause artifacts induced by hypoxia as described by Frenzel et al. (16). We, therefore, like Wisse et al. (9, 17) omitted the precleaning procedure and found only a few larger (>300 nm in diameter) fenestrae in contrast to both Muto et al. (12) and Motta et al. (13), who found a significant number of fenestrae with a diameter larger than 300 nm. With regard to fenestrae measuring 100 nm or less, Muto et al. (12) found about 25 fenestrae per μm^2, which does not differ much from our results. Comparing the number of fenestrae in Zone 3 (centrilobular) and Zone 1 (periportal), we have previously demonstrated a slight, however significant, difference (2). Although differences between species exist regarding the number of fenestrae per square micron [see (18) for review], a zonal gradient has also been shown in rats (1, 17, 19).

The present study demonstrates a significant reduction of fenestrae in the hepatic sinusoidal lining wall in acinar Zone 3 areas of biopsies taken from chronic alcoholics as compared to nonalcoholics. Concurrent with this reduction, there was a significant decrease in the porosity of the sinusoidal wall. The occurrence of centrilobular fibrosis in the alcoholics was always accompanied by substantial changes in the sinusoidal endothelium, whereas 3 of 8 biopsies without fibrosis showed values

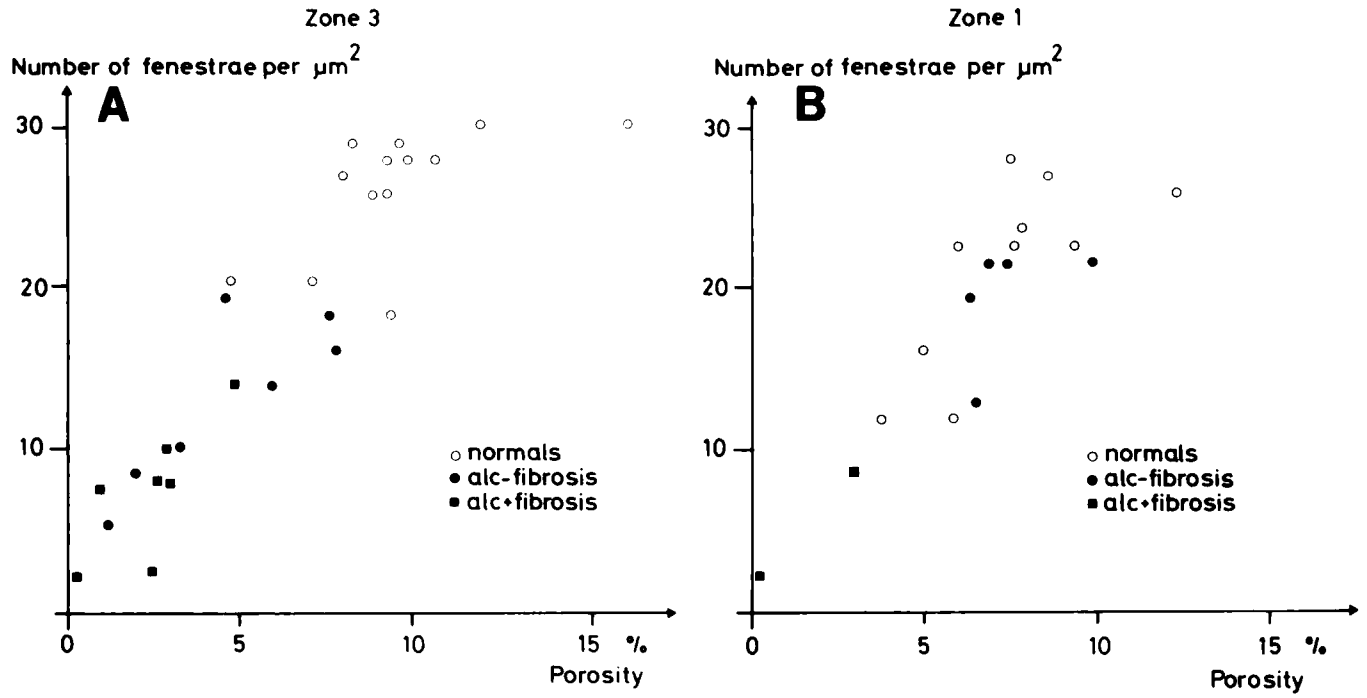

FIG. 7. (A) Diagram illustrating the relationship between number of fenestrae per square micron and porosity in Zone 3 in biopsies from nonalcoholics (*open circles*), alcoholics without fibrosis (*closed circles*) and alcoholics with Zone 3 fibrosis (*closed squares*) ($p < 0.001$). (B) Diagram illustrating the relationship between number of fenestrae per square micron and porosity in Zone 1 in biopsies from nonalcoholics (*open circles*), alcoholics without fibrosis (*closed circles*) and alcoholics with Zone 3 fibrosis (*closed squares*) ($p < 0.01$).

within the lower limit of the normal range, and the endothelial changes in the remaining five were less extensive than those in the biopsies with fibrosis. There was no correlation between endothelial alterations and the occurrence of lipogranulomas or Mallory bodies.

Until now, no information has been obtained on the sinusoidal lining wall in human chronic alcoholics without cirrhosis, but recently Mak et al. (20) have shown a significant decrease in number of fenestrae and porosity of the endothelial cells in alcohol-fed baboons. All alcohol-fed baboons except one developed centrilobular fibrosis, but no correlation was made between the appearance of collagen in the space of Disse and the occurrence of defenestration. The defenestration was followed by an increase in size of existing fenestrae (20). However, the increase in diameter did not compensate sufficiently for the reduction in fenestrae, since the porosity also diminished significantly (20). Although an increase in diameter of fenestrae, especially of those measuring less than 100 nm, is possible in the present material, we found a significant reduction in the porosity (Table 2 and Figure 7 A). Still, a reduction in porosity does of course not exclude a slight effect of alcohol on the size of remaining fenestrae.

The mechanism by which sinusoidal fenestration is controlled is yet unknown. Hepatic sinusoidal endothelial cells have a genuine ability to develop multiple fenestrations arranged as sieve plates (21). The number of fenestrae and their size is influenced by several factors such as oxygen tension and sinusoidal pressure (16, 22), hormones (serotonin and adrenalin (23) and even alcohol (24). Chronic alcohol ingestion in rats was followed by a 20% enlargement of fenestrae as seen by TEM (24). The number of fenestrae was, however, not assessed.

It is a reasonable hypothesis that a modification of the perisinusoidal space causes the described changes of the sinusoidal endothelial cells. There is a positive correlation between defenestration and the occurrence and localization of subendothelial basal laminas and between basal lamina, and the occurrence of collagen in the space of Disse (4, 5). These changes are thought to be induced by activation (transformation) of the fat-storing cells (25, 26). Ethanol or one of its metabolites may thus trigger the fat-storing cells to collagen production with secondary change of the endothelial cells. On the other hand, it cannot be ruled out that ethanol or one of its metabolites induce the ultrastructural alterations in the endothelium, with secondary changes in the perisinusoidal space (fibrosis and formation of basal laminas). The demonstration of a slight defenestration in Zone 1, in areas without the occurrence of either fibrosis or basal laminas, supports the last hypothesis.

Whatever mechanism involved, the reduction of fenestrae in Zone 3 in alcoholics may compromise the exchange of metabolites between the blood and hepatocytes. In the normal sinusoids, the access of small and medium-sized molecules to the perisinusoidal space is flow-limited, and restriction only exists for larger molecules [for review, see Ref. (1)]. A decrease in the number of fenestrae, without significant compensatory enlargement as seen in the present study, may be followed by an alteration in transport capacity and thereby in the composition of the perisinusoidal fluid. The presence of basal laminas and collagen in the perisinusoidal space

(5) would further reduce the exchange possibilities. The development of a significant blood hepatocytic barrier may cause hepatocellular dysfunction and eventually hepatocellular necrosis. A new, potentially pathogenetic mechanism leading to liver injury in chronic alcoholics is thus established.

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