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Sequential Production of Fatty Liver, Hepatitis, and Cirrhosis in Sub-Human Primates Fed Ethanol with Adequate Diets

(alcoholism/fibrosis/microsomes/mitochondria/liquid diets)

CHARLES S. LIEBER*†, LEONORE M. DECARLI*†, AND EMANUEL RUBIN‡

* Section of Liver Disease and Nutrition, Veterans Administration Hospital, Bronx, New York 10468; and † Departments of Medicine and ‡ Pathology, Mt. Sinai School of Medicine of the City University of New York, New York, N.Y. 10029

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ABSTRACT **This study reproduces in experimental animals the sequential development of all the liver lesions seen in the human alcoholic: in 15 baboons fed ethanol, all developed fatty liver, five progressed to hepatitis, and five had cirrhosis. Maintenance of a nutritionally adequate regimen despite the intake of inebriating amounts of ethanol (50% of total calories) was achieved by incorporation of the ethanol in a totally liquid diet. Upon ethanol withdrawal, signs of physical dependence, such as seizures and tremors, developed. Ultrastructural changes of the mitochondria and the endoplasmic reticulum were already present at the fatty liver stage and persisted throughout the hepatitis and cirrhosis. The lesions were similar to those observed in alcoholics (including the inflammation and the central sclerosis) and differed from the alterations produced by choline and protein deficiencies. At the fatty liver stage, some "adaptive" increases in activity of microsomal enzymes [aniline hydroxylase (EC 1.14.14.1) and the microsomal ethanol oxidizing system] were observed, but these tended to disappear with the development of hepatitis and cirrhosis. Fat accumulation was also much more pronounced in the animals with the hepatitis as compared with those with simple fatty liver (an 18-fold compared with 3- to 4-fold increase in liver triglycerides). The demonstration that these lesions can develop despite an adequate diet indicates that in addition to correction of the nutritional status, control of alcohol intake is mandatory for the management of patients with alcoholic liver injury.**

With increasing alcohol consumption, the incidence of related complications has been rising steadily, particularly that of associated liver disease, to the extent that at the present time, cirrhosis of the liver, the most severe hepatic complication of alcoholism, is the third cause of all deaths between the ages of 25 and 65 in the city of New York (1). In addition to cirrhosis of the liver characterized by diffuse hepatic scarring, alcohol abuse is also associated with hepatic inflammation and necrosis (alcoholic hepatitis) and excess fat accumulation (alcoholic fatty liver). The relationship of these various liver injuries to each other, however, has been questioned. Furthermore, since not all alcoholics develop liver injury, there has been considerable debate concerning the question whether alcohol itself or some associated factor, such as dietary deficiency, is the main cause for the liver disease. The question has both theoretical and practical implications. The classic belief that liver injury could be prevented in the alcoholic by merely controlling the diet was challenged by evidence that alcohol might exert direct toxic effects upon the liver; it was indeed shown that the fatty liver, the most benign stage of the disease, could be produced in volunteers given alcohol in association with adequate or enriched diets (2–4). Fatty liver, however, is still a fully reversible lesion and the question remained whether alcoholic hepatitis, associated with a high morbidity, and irreversible cirrhosis could also be linked directly to alcohol ingestion itself rather than to a deficient diet. This problem could not be studied in volunteers, in view of the severity of the lesions involved. Previous attempts to produce these lesions in animals failed because of the reluctance of all species used to consume enough alcohol when the latter was given as part of the drinking water. This natural aversion for ethanol was overcome by the incorporation of ethanol in totally liquid diets. This new experimental model for alcohol feeding showed that even when given with adequate diets, ethanol can cause alcoholic hepatitis and cirrhosis in nonhuman primates. The development of this new experimental model clarifies the question of the etiology of liver injury associated with alcohol abuse and represents a new tool for the development of rational forms of prophylaxis and therapy.

MATERIALS AND METHODS

The detailed composition of the liquid diet fed to the baboons is given elsewhere (5). The protein content (18% of total calories) corresponds to that of commonly used commercial diets that are satisfactory for the baboon and is almost twice the amount recommended for human diets (6). The mineral and vitamin content of the diet exceeded the requirements formulated for the monkey (7–9). Its caloric value was 1 calorie/ml. The diet was prepared by Bio Serv Inc., Frenchtown, N.J., and was given to the baboons twice a day in standard drinking bottles equipped with an outlet valve. Each alcohol-fed animal was matched with a control, the dietary intake of which was identical except for the isocaloric substitution of carbohydrate by ethanol to the extent of 50% of total calories. This technique of daily pair feeding was adopted to assure a strictly equal caloric intake in both ethanol-treated animals and in their individual pair-fed controls.

The 30 adolescent or young animals used for this study were either *Papio hamadryas* or olive and yellow baboons. Twelve animals were raised in this country, whereas the remainder were imported from Africa and were studied after prolonged quarantine periods. They were housed in individual cages at the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP), Tuxedo, N.Y. Until the actual study period, they were given a routine regimen of Purina® monkey chow *ad libitum*, supplemented with a daily vitamin prep-

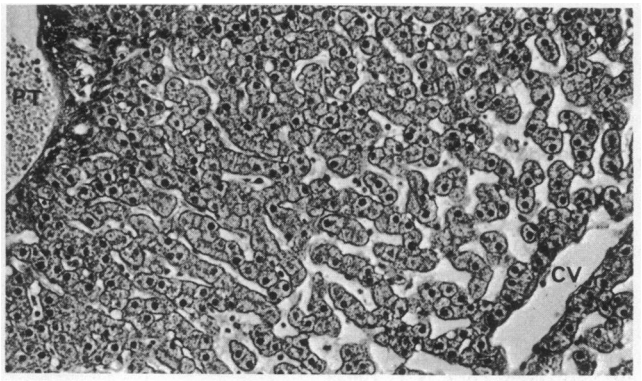

FIG. 1. Section of liver of baboon no. 536 before administration of ethanol. The architecture is normal, with the usual relation of portal tracts (PT) and central veins (CV). No fat, cell degeneration, or inflammation is present. Hematoxylin and eosin (magnification: ×111).

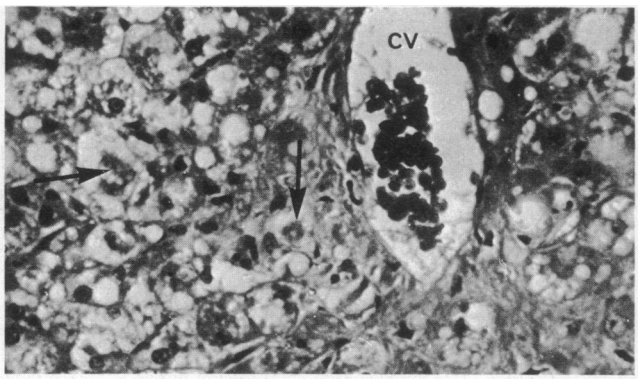

FIG. 3. High power view of liver shown in Fig. 2. Numerous ballooned hepatocytes are located around a thickened central vein (CV). Irregular eosinophilic cytoplasmic inclusions, resembling human alcoholic hyaline, are present (arrows). Hematoxylin and eosin (magnification: ×222).

aration. The animals entered the study after prolonged observation and after repeated hematological and stool examinations had indicated the absence of disease.

Surgical biopsies of the liver were performed at regular intervals under anesthesia. Samples were taken for analysis of total lipids and triglycerides, light and electron microscopy, and alcohol dehydrogenase activity, as described (10). The activity of the microsomal ethanol-oxidizing system (11) and that of aniline hydroxylase (12) were determined in liver microsomes. Blood samples were taken for the measurement of ethanol (13) and of cholesterol, albumin, bilirubin, alkaline phosphatase activity, serum glutamic-oxaloacetic transaminase (EC 2.6.1.1; L-aspartate:2-oxoglutarate aminotransferase) activity, creatinine, urea, and glucose in a Technicon Autoanalyser. The absence of hepatitis-associated antigen in the blood was verified by radioimmunoassay.

RESULTS

Nine pairs of animals were pair-fed alcohol-containing or the control liquid diet for 8–22 months. The average duration of the treatment was 15 months, and the mean intake was 80.0 ± 2.24 ml/kg per day. The alcohol-fed baboons and their controls had average initial weights of 10.6 ± 0.35 and 10.6 ± 0.33 kg, respectively. The baboons fed alcohol maintained their weight (10.2 ± 0.46 kg) throughout the study, whereas the controls increased their weight to an average of 11.8 ± 0.34 kg ($P < 0.01$). Liver biopsies obtained prior to the start of the study revealed normal morphology (Fig. 1), and no abnormalities developed in the controls. In the animals drinking the alcohol-containing diet, inebriation was commonly observed. Blood ethanol concentrations in inebriated animals were 262 and 258 mg/100 ml on two occasions in one baboon and 358 and 376 on one occasion in two other animals. Alcohol consumption resulted in the development of a fatty liver, with an average triglyceride content of 144 ± 36 compared to 10 ± 2 mg/g in the control ($P < 0.01$). Four pairs of animals were biopsied sequentially after 8.5 and 21 months; whereas the triglyceride was only 62 ± 19 mg/g after 8.5 months of alcohol treatment, this value increased to a mean of 165.3 ± 39.8 mg/g of liver at the end of 21 months. A case of obvious steatosis is shown in Fig. 2. In addition to the fat, mild inflammation, cellular degeneration, and some fibrosis were noted. In three animals fed ethanol for 9 months and one animal fed ethanol for 12 months, alcoholic hepatitis developed, as defined by cell degeneration, inflammation, and central sclerosis (Figs. 2–4).

In the four animals that developed hepatitis, triglyceride accumulation was more pronounced (180.4 ± 55.7 mg/g) than in the five animals that had only a fatty liver (33.0 ± 5.7 mg/g). Two of the four animals that had developed hepatitis after 9 months were again subjected to biopsy after

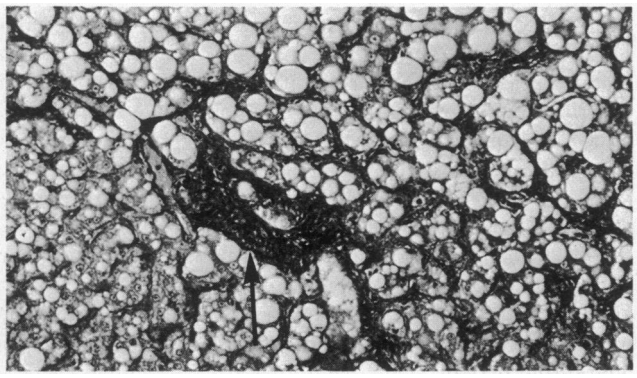

FIG. 2. Section of liver of baboon no. 536 after 9 months of ethanol administration. Severe fatty liver is present. Diffuse interstitial fibrosis and central sclerosis (arrow) are prominent. Chromotrope-aniline blue (magnification: ×44).

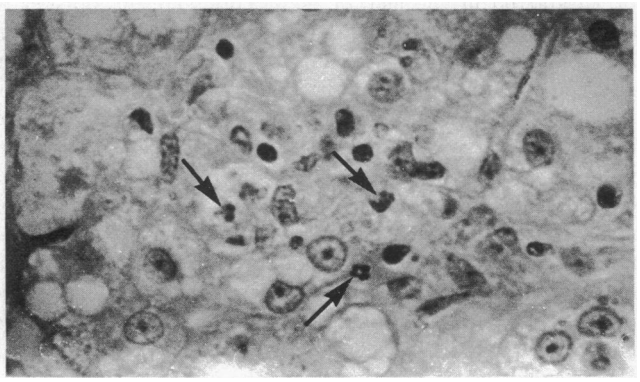

FIG. 4. High power view of liver shown in Fig. 2. Area of central necrosis and fibrosis, showing polymorphonuclear leukocytes (arrows). Hematoxylin and eosin (magnification: ×444).

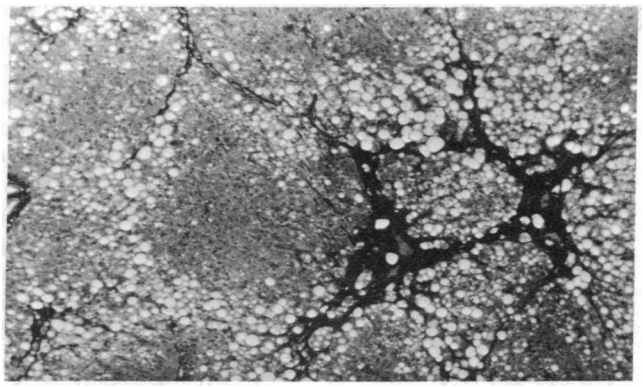

FIG. 5. Section of liver of baboon no. 536 after 20 months of ethanol administration. Connective tissue septa have formed, and in one area have already circumscribed a nodule. This case was classified as incomplete cirrhosis. Chromotrope-aniline blue (magnification: ×44).

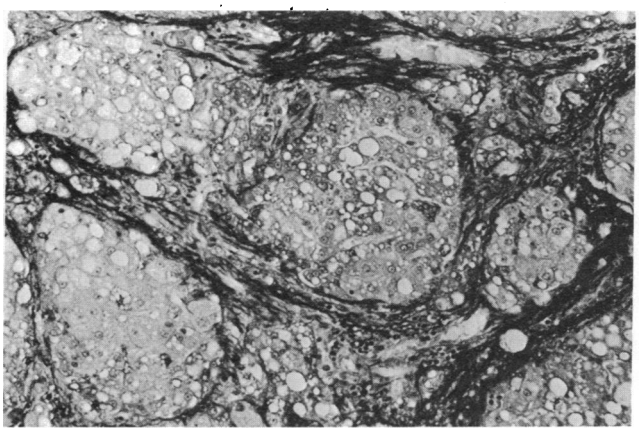

FIG. 6. Complete cirrhosis of liver of baboon no. 536 after 24 months of ethanol administration. Broad connective tissue septa circumscribe nodules containing irregularly arranged hepatocytes and fat. Chromotrope-aniline blue (magnification: ×44).

20 months. Each showed the development of extensive fibrosis, corresponding to a diagnosis of incomplete cirrhosis. The progress of the lesions from the alcoholic hepatitis to cirrhosis is shown in Fig. 5. One of the animals died after 2 years of treatment because of withdrawal symptoms (convulsions) which developed when the intake of ethanol decreased because of an intercurrent infection. The autopsy revealed typical complete alcoholic Laennec's cirrhosis of the liver (Fig. 6). Another animal that showed alcoholic hepatitis also died after 18 months of treatment because of a similar complication. The autopsy revealed only fatty liver. When alcohol intake was decreased for reasons of intercurrent upper respiratory infection, withdrawal symptoms (such as tremor and seizures) were observed in at least four animals.

Ultrastructural changes were already pronounced in the fatty livers; they remained present throughout the stages of hepatitis and cirrhosis. The mitochondrial lesions of the fatty livers were characterized by enlargement, irregular forms, and disoriented cristae. The rough endoplasmic reticulum was decreased, and the smooth endoplasmic reticulum was vesicular and proliferated (Fig. 7). Similar lesions were found in the livers displaying alcoholic hepatitis and cirrhosis.

There was an increase in the activity of the microsomal ethanol-oxidizing system in the animals fed ethanol: 23.0 ± 2.5 nmol/min per mg of protein compared to 13.7 ± 1.5 in the controls ($P < 0.01$). It is noteworthy, however, that of the four animals that had the hepatitis, only two had a significant increase in the activity of the microsomal ethanol-oxidizing system, whereas the two others had values comparable to that of the corresponding controls. We have reported previously that other microsomal enzymes also increase in activity after ethanol feeding, both in rats (14, 15) and in baboons (10). This was confirmed for microsomal aniline hydroxylase activity measured in 4 pairs of baboons: whereas the mean value in the animals fed ethanol was 0.874 nmol/min per mg of microsomal protein, the corresponding controls had activities of 0.299.

Serum cholesterol was moderately increased in the alcohol-treated baboons (214.1 ± 22.2 compared to 153.5 ± 11.0 mg/100 ml, $P < 0.05$). In all pairs of animals, the values of serum glutamic-oxaloacetic transaminase were higher in the ethanol-fed animals than in the corresponding controls. In the five animals with a simple fatty liver, however, the increase was small. By contrast, in the four animals that had hepatitis, there were striking elevations of 2650, 227, 123, and 75 IU/ml, as compared to values of 40, 37, 35, and 38 in the corresponding controls. No significant abnormalities were noted in albumin, bilirubin, alkaline phosphatase activity, creatinine, urea, and glucose. Hemoglobin and hematocrit values had a tendency to be lower in the alcohol-fed animals, but no meaningful decrease has been noted thus far.

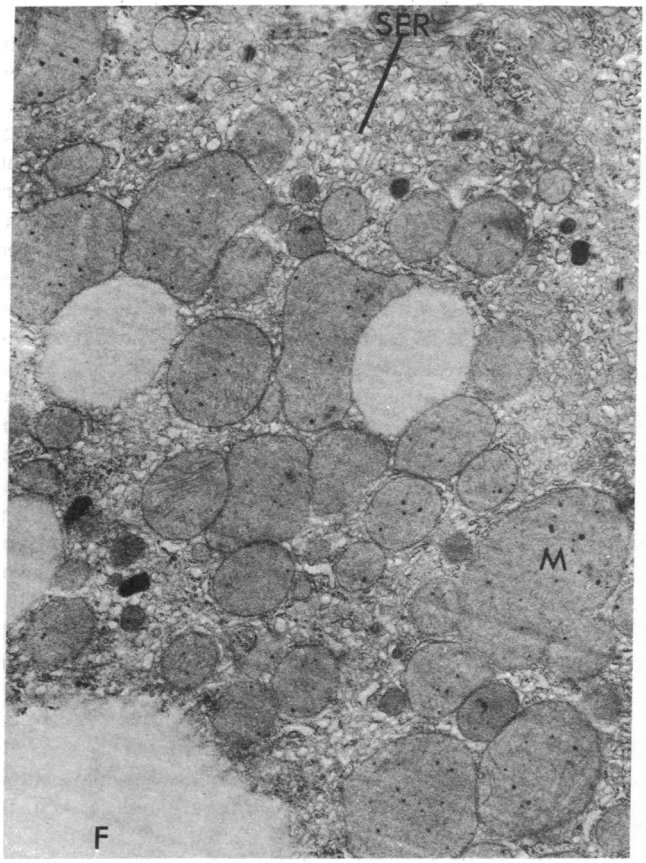

FIG. 7. Electron micrograph of liver during fatty liver stage. Mitochondria (M) are enlarged and misshapen, and exhibit disoriented cristae. Rough endoplasmic reticulum is sparse, while smooth endoplasmic reticulum (SER) is increased. Fat, F (magnification: ×7992).

An additional group of 12 animals that had been given a solid diet with either alcohol or carbohydrates in the drinking water as described before (10) for a period varying from 17 to 34 months were then changed to the liquid diet for an average of 17 months. Whereas when alcohol was given with the solid diet no lesions more severe than fatty liver had developed (10), with this new regimen, four of the six animals fed alcohol progressed to a more severe stage: one to alcoholic hepatitis (after 29 months of the solid diet and 19 months on the liquid diet), two to incomplete cirrhosis (after 30 months on the solid diet and 15 months on the liquid diet), and one to complete cirrhosis (after 34 months on the solid and 19 months on the liquid diet). Some of these preliminary findings were previously reported (16).

DISCUSSION

The present study establishes the fact that in subhuman primates, chronic ingestion of alcohol can cause the development of the entire spectrum of liver lesions seen in man, namely, fatty liver, alcoholic hepatitis, and cirrhosis. Out of 15 animals fed alcohol, cirrhosis developed in five. In two animals, this complication appeared as early as 2 years after the administration of a totally liquid diet containing 50% of total calories as alcohol; three other animals developed cirrhosis after this regimen was given for 17 months after administration for 30–34 months of alcohol as part of drinking water (10). Fatty liver was observed in all animals given alcohol. Alcoholic hepatitis, characterized by inflammation and central sclerosis (17), was observed in five animals.

The results of this study are significant, both with regard to the understanding of the pathogenesis of alcoholic liver injury and to its treatment. The experimental reproduction of the lesions of alcoholic hepatitis and the demonstration in an experimental model of its transition to cirrhosis support the hypothesis that alcoholic hepatitis is a precursor of the cirrhotic lesion. Moreover, this study shows that animals that displayed fatty liver with a moderate alcohol intake developed hepatitis and cirrhosis when the alcohol content of the diet was increased; this raises the question whether the fatty liver can be considered as a precursor state for the hepatitis and cirrhosis. This possibility is supported not only by the temporal relationship of the fatty liver, which always preceded the development of hepatitis and cirrhosis, but also by the observation that the ultrastructural changes observed at the fatty liver stage are already as pronounced as those seen when a full blown hepatitis or cirrhosis had developed. In addition, it was found that already in the fatty liver phase there was an increase in chemically detectable collagen, the protein which is the hallmark for the fibrosis characteristic of cirrhosis (18); this was associated with enhanced activity of peptidylproline hydroxylase, an enzyme active in the initial steps of fibrogenesis (18).

The present study also clarifies the respective role of malnutrition and alcohol itself in the pathogenesis of the alcoholic hepatitis and cirrhosis. Our previous observations have shown that the fatty liver can be produced by ethanol *per se* in the absence of dietary deficiencies (2–4). We now find that this also applies to the hepatitis and the cirrhosis. It is noteworthy that although fibrosis and cirrhosis have been produced before in primates after the feeding of deficient diets lacking in protein and/or choline (19–21), these animals did not develop hepatitis, a rather characteristic stage in alcoholic liver injury. Furthermore, the striking ultrastructural changes produced by alcohol in man (3, 4, 22), in rats (23), or in baboons in this study differ strikingly from the ultrastructural changes ascribed to choline (20) and/or protein (24) deficiencies. One may, therefore, conclude from our studies that despite the evidence produced before indicating that malnutrition can cause liver damage, alcohol itself is an indispensable etiologic agent for the development of the typical complications observed in the alcoholic. An important corollary of this finding is the fact that adequate diet did not prevent the development of the alcoholic lesions. The therapeutic implication of this observation is that alcoholics cannot fully prevent the development or the aggravation of liver injury by maintaining an adequate diet unless they also control the degree of alcohol intake. It has been shown in the past by others and our own group that alcohol ingestion results in impaired digestion and in malabsorption and that it produces intestinal injury (25–29). It is unlikely, however, that the effects described are of sufficient magnitude to offset the large excess of nutrients present in our diet. Moreover, preliminary studies have indicated the absence of protein and fat malabsorption under our experimental conditions (J. Lindenbaum and C. S. Lieber, unpublished observation). The possibility that nutritional deficiencies may potentiate the effect of alcohol is presently being investigated in the baboon, since such a phenomenon was observed in the rat (30).

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