Acta Medica Okayama

Volume 40, *Issue* 6 1986 *Article* 3

DECEMBER 1986

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Abstract

The effects of ethanol on rat Kupffer cells were studied functionally and morphologically. Eight g ethanol per kg body weight per day was intragastrically administered to rats for 7 days. An isocaloric glucose solution was administered to control rats. The phagocytic activity of the reticuloendothelial system was measured by the carbon clearance method (57 mg carbon particles per kg body weight) on the 7th day. Kupffer cells having phagocytized carbon particles were counted under the light microscope. Kupffer cells were also observed by scanning electron microscopy. Both the carbon clearance and Kupffer cell number were lower in ethanol-administered rats (32 +/- 8 X 10(-4) mg/ml; 0.6 +/- 0.3/0.01 mm2 liver lobule) as compared to control rats (63 +/- 15; 3.1 +/- 1.0). Microvilli and filopodia of Kupffer cells were fewer in ethanol-administered rats than in control rats. Carbon clearance correlated with Kupffer cell number per 0.01 mm2 liver lobule and liver weight. These results suggest that the decrease in carbon clearance induced by ethanol is due mainly to the decrease in Kupffer cell number and partly to the decrease in Kupffer cell activity as demonstrated by the disappearance of microvilli and filopodia.

KEYWORDS: Kupffer cells, ethanol, carbon clearance, scanning electron microscopy, rats

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*PMID: 3825592 [PubMed - indexed for MEDLINE]

Acta Med Okayama 40 (6) 291-299 (1986)

Injurious Effects of Ethanol on Rat Kupffer Cells

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The effects of ethanol on rat Kupffer cells were studied functionally and morphologically. Eight g ethanol per kg body weight per day was intragastrically administered to rats for 7 days. An isocaloric glucose solution was administered to control rats. The phagocytic activity of the reticuloendothelial system was measured by the carbon clearance method (57 mg carbon particles per kg body weight) on the 7th day. Kupffer cells having phagocytized carbon particles were counted under the light microscope. Kupffer cells were also observed by scanning electron microscopy. Both the carbon clearance and Kupffer cell number were lower in ethanol-administered rats ($32\pm8\times10^{-4}$ mg/ml; $0.6\pm0.3/0.01$ mm^2 liver lobule) as compared to control rats (63 ± 15; 3.1 ± 1.0). Microvilli and filopodia of Kupffer cells were fewer in ethanol-administered rats than in control rats. Carbon clearance correlated with Kupffer cell number per 0.01 mm^2 liver lobule and liver weight. These results suggest that the decrease in carbon clearance induced by ethanol is due mainly to the decrease in Kupffer cell number and partly to the decrease in Kupffer cell activity as demonstrated by the disappearance of microvilli and filopodia.

Key words : Kupffer cells, ethanol, carbon clearance, scanning electron microscopy, rats

Effects of ethanol on the reticuloendothelial system (RES) have been disclosed (1-3) since Ali and Nolan reported in 1967 that ethanol induced depression of RES (4). Heavy drinkers are liable to bacterial infections such as lung tuberculosis, pneumonia, and sepsis (5, 6), probably because of the depression of RES induced by ethanol (1, 4). Patients with alcoholic liver cirrhosis often show poor uptake of technetium sulfur colloid on their liver scintigram, which is also explained by the depression of RES in the liver (2, 7). However, effects of ethanol on Kupffer cells have scarcely been studied from the aspects of function or morphology. In this paper, the effects were investigated functionally by the carbon clearance method and morphologically under the scanning electron microscope (SEM).

Materials and Methods

Ethanol administration. Twenty-eight male Sprague-Dawley rats, weighing 250-300 g each, were divided into 4 groups as follows: Ethanol 1 (E1) group, 8 rats given 4 g ethanol (20 vol. %) per kg body weight once daily (28 Cal per kg body weight); Ethanol 2 (E2) group, 8 rats given 4 g ethanol (20 vol. %) per kg body weight twice daily (56 Cal per kg body weight); Glucose (G) group, 6 rats given a 25 vol. % glucose solution isocaloric to the E1 group twice daily, and Starvation (S) group, 6 rats given nothing except water. Ethanol and glucose were administered to rats through a stomach tube under light ether anesthesia for 7 days. Rats were deprived of all food, but allowed free access to water.

Carbon clearance. Phagocytic activity of Kupffer cells was measured by the carbon clearance method (8, 9). On the 7th day of the experiment, India ink (Drafting ink, Pilot Co., Tokyo) containing 57 mg of carbon particles per ml was diluted ten fold in saline, and 10 ml per kg body weight of the diluted ink was injected into the inguinal vein. Blood samples (0.03 ml) were obtained from the jugular vein before and 5, 10, 15, 20 and 25 minutes after the carbon injection. The carbon concentration was determined by measuring the absorbance of the samples diluted in 3 ml of 0.1 % Na_2CO_3 at 660 nm in an electrophotometer (Hitachi, FPW-1, Japan)(10). The carbon clearance rate was calculated from the regression line determined by the carbon concentration.

Microscopy. After blood sampling for carbon clearance, heparin was injected into the heart to prevent coagulation. The liver was irrigated with 500 ml of Ringer solution, perfusion-fixed with 200 ml of 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) through the thoracic aorta (150 mm Hg perfusion pressure), and cut into small blocks. The blocks were further fixed overnight in glutaraldehyde, stained by the revised tannin-osmium method (11), dehydrated in a graded series of ethanol, and dried by the critical point method using liquid carbon dioxide. The specimens were fractured under a stereomicroscope (12, 13), and thinly sputter-coated with platinum-palladium in an ion coater. The fractured surfaces of liver lobules were observed with special attention to Kupffer cells by SEM (Hitachi, model JSM-U3, Japan) at an accelerating voltage of 15 kv. For light microscopy, the perfusion-fixed blocks were transferred to 10% formalin, embedded in paraffin, sectioned to 5 μm, and stained with hematoxylin-eosin. Kupffer cells were counted in 10 areas of 0.1 mm× 0.1 mm at 200×magnification under a light microscope (LM), considering phagocytized carbon particles as a specific indicator of Kupffer cells. The number of Kupffer cells without microvilli and filopodia per 20 Kupffer cells was determined under the SEM.

Table 1 Weight changes[a] of body, liver and spleen by ethanol administration.

		Glucose	Starvation	Ethanol 1	Ethanol 2
Body weight (g)	before	277±8	279±8	277±7	282±8
	7th day	213±17	194±17	192±16	197±15
Body weight loss (%)		23.3±4.4	30.7±4.3	30.6±4.6	30.2±4.4
Liver weight (g)		8.7±0.5	6.1±0.7	6.5±0.9	7.7±1.3
Liver weight/Body weight		4.1±0.5	3.2±0.6	3.4±0.7	3.9±0.5
Spleen weight (g)		0.51±0.1	0.27±0.05	0.29±0.05	0.24±0.04
Spleen weight/Body weight		0.24±0.05	0.14±0.01	0.15±0.03	0.12±0.01
Liver weight/Spleen weight		17.5±3.2	23.4±5.8	23.0±4.3	32.8±4.7

a: Mean±SD

b: ※p < 0.05, ※※p < 0.01, ※※※p < 0.001

Analysis of important parameter of carbon clearance. Eleven variables were used to select important parameters of carbon clearance: body weight at the starting and end points of the experiment, body weight loss, ratio of body weight loss to the body weight at the start, liver weight, liver/body weight ratio, spleen weight, spleen/body weight ratio, ratio of the sum of liver and spleen weight to body weight, total Kupffer cell number per 0.01 mm^2 and number of Kupffer cells without microvilli and filopodia per 0.01 mm^2. The number of Kupffer cell without microvilli and filopodia per 0.01 mm^2 was obtained by using the total Kupffer cell number per 0.01 mm^2 and the ratio of injured Kupffer cells.

Chi square test, analysis of variance and multiple regression analysis (14) were performed by microcomputer (NEC 9801, Japan).

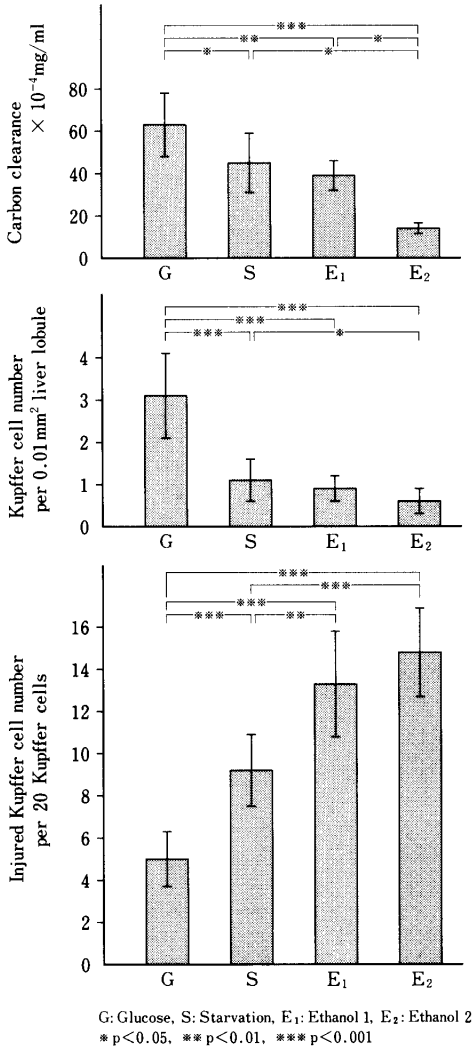
Results

Mortality. In the E1 and E2 groups, 2 rats (25%) in each group died before the end of the experiment. No rat died in the S and G groups. However, there was no significant difference in the mortality rate between the ethanol-treated groups and control groups.

Body weight loss and liver/spleen weight ratio. Body weight loss of the G group was smaller than that of the S ($p<0.01$), E1 ($p<0.01$) and E2 ($p<0.05$) groups (Table 1). There was no significant difference among the S, E1 and E2 groups. Liver weight of the G and E2 groups was greater than that of the S and E1 groups. Spleen weight of the G group was greater than that of the other groups. The liver was 18 to 33 times heavier than the spleen, and the liver/spleen weight ratio of the E2 group was greater than that of the other groups ($p<0.01$).

Carbon clearance. Carbon clearance was decreased in the E2 (15 ± 2; $p<0.001$), E1 (39 ± 6; $p<0.01$) and S (45 ± 14; $p<0.05$) groups as compared to the G (63 ± 15) group, but there was no difference between the S and E1 groups (Fig. 1). Carbon clearance of the E2 group was reduced to a quarter of the G group, and was lower than that of the S and E1 ($p<0.05$) groups.

LM evaluation. Kupffer cell number was less in the S (1.1 ± 0.5), E1 (0.9 ± 0.3) and E2 (0.6 ± 0.3) groups than in the G group (3.1 ± 1.0; $p<0.001$; Fig. 1). Kupffer cell number was less in the E2 than in the S group ($p<0.05$). However, there was no significant difference between



G: Glucose, S: Starvation, E_1: Ethanol 1, E_2: Ethanol 2
* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Fig. 1 Carbon clearance and Kupffer cell number.

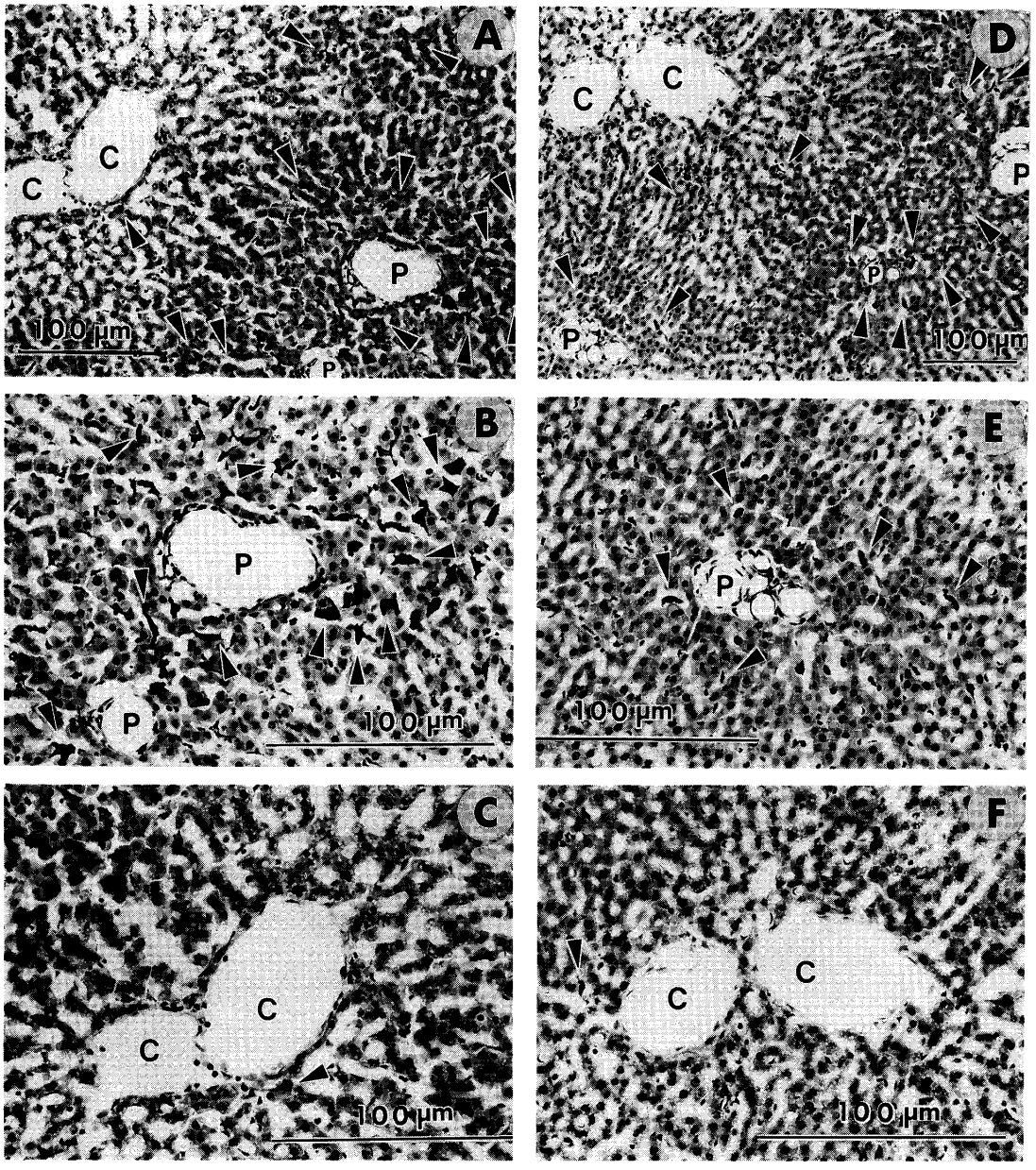


Fig. 2 Light micrographs of the liver of a glucose-fed rat (A, B, C) and an ethanol-treated rat (D, E, F), which were intravenously injected with carbon particles. Kupffer cells (arrow heads) indicated by phagocytized carbon particles are distributed mainly in the periportal zone, slightly in the middle zone; few are found in the central zone. Kupffer cell number of the ethanol-treated rat is less than that of the glucose-fed rat.

Closer views of the periportal zones show that Kupffer cells are numerous and filled by ingested carbon particles in the glucose-fed rat (Fig. 2B), whereas they are decreased in number and size in the ethanol-treated rat (Fig. 2E). Hepatocytes show little change. In the central zones, Kupffer cells are scanty in both glucose-fed and ethanol-treated rats (Figs. 2C & F). Hepatocytes show cell atrophy, steatosis and vacuole formation, which are more marked in the ethanol-treated rat than in the glucose-fed rat. P, portal vein; C, central vein. Hematoxylin-eosin stain. A, ×200; B, ×350; C, ×350; D, ×150; E, ×350; F, ×350.

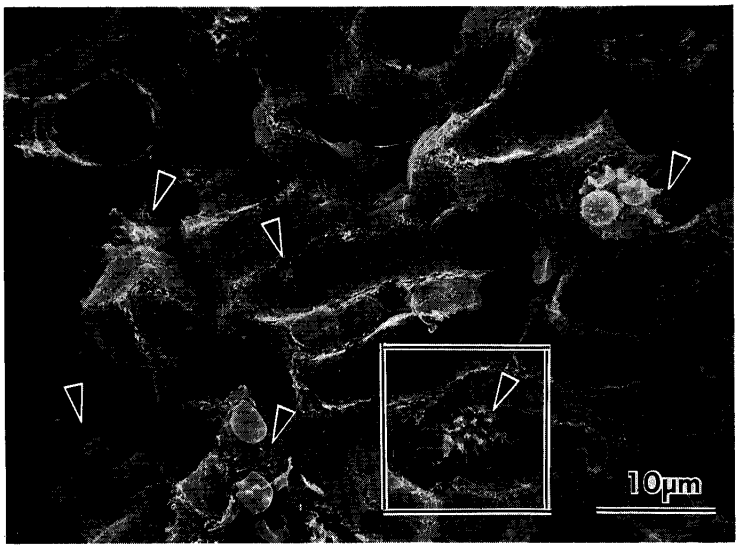


Fig. 3 Scanning electron micrograph of the periportal zone in a glucose-fed rat liver. Many Kupffer cells (arrow heads) are located in the sinusoids.

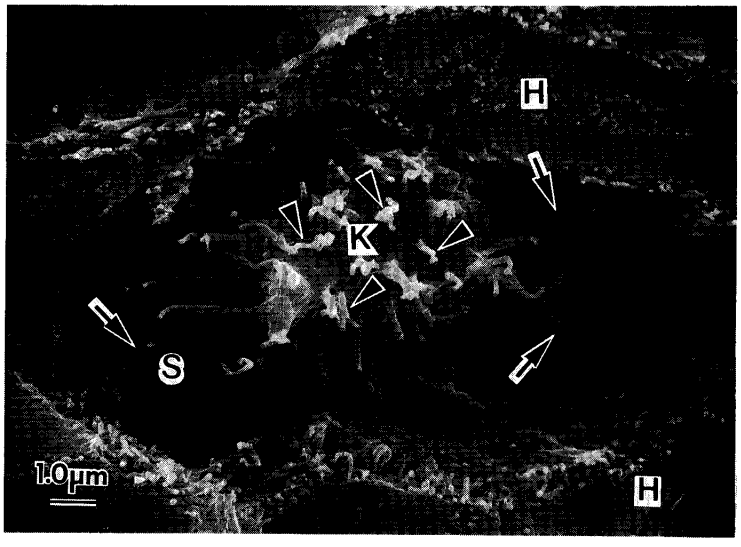


Fig. 4 A closer view of the square in Fig. 3. Note numerous microvilli (arrow heads) and long filopodia (arrows) of the Kupffer cell (K). H, hepatocyte; S, sinusoid.

the E1 and E2, and E1 and S groups. In all groups, Kupffer cells were distributed mainly in the periportal zone and slightly in the middle zone, and few were found in the central zone (Figs. 2A & D). Hepatocytes showed cell atrophy, steatosis and vacuole formation in the central zone (Figs. 2C & F), but not in the periportal zone (Figs. 2B & E). Cellular infiltration and focal necrosis were not observed anywhere in the lobule in any group. In glucose-fed rats, many Kupffer cells were filled by ingested carbon particles (Fig. 2B). In ethanol-treated rats, most Kupffer cells were smaller than those of glucose-fed rats (Fig. 2E).

SEM evaluation. In the control (G, S) groups, especially in the G group, many Kupffer cells were observed to have a large and round cell body, numerous microvilli and long filopodia (Figs. 3, 4). In contrast, many Kupffer cells had a flat cell body and no microvilli of filopodia in the ethanol-

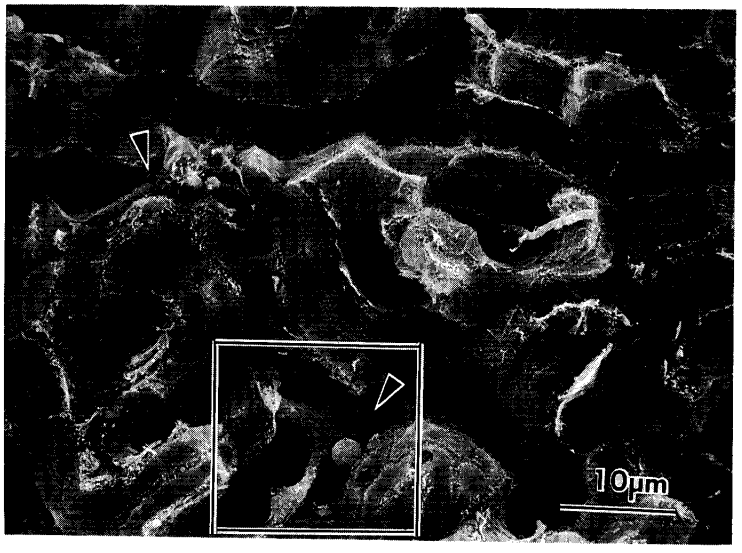


Fig. 5 Scanning electron micrograph of the periportal zone in an ethanol-treated rat. A few Kupffer cells (arrow heads) are located in the sinusoids.

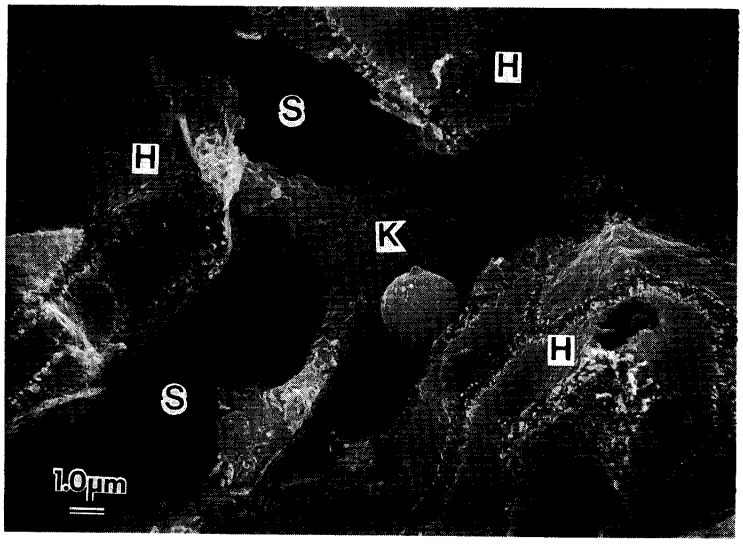


Fig. 6 A closer view of the square in Fig. 5. Note disappearance of microvilli and filopodia of the Kupffer cell (K). H, hepatocytes; S, sinusoid.

treated (E1, E2) groups (Figs. 5, 6). The ratio of injured Kupffer cells with a flat cell body and without microvilli or filopodia was significantly higher in the ethanol-treated groups (E1, E2) than in the control groups (G, S; Fig. 1). Injured Kupffer cells were more frequent in the S group than in the G group, but there was no significant difference between the E1 and E2 groups.

Relationships among carbon clearance, Kupffer cell number, liver weight and, morphological changes. Correlation between carbon clearance and Kupffer cell number per 0.01 mm^2 liver lobule is shown in Fig. 7. By multiple regression analysis, carbon clearance was significantly explained by Kupffer cell number and liver weight, which were selected commonly by the forward selection method, backward elimination method and stepwise method. The linear multiple regression model was as follows: Carbon clearance $=17.0\times$Kupffer cell number per 0.01

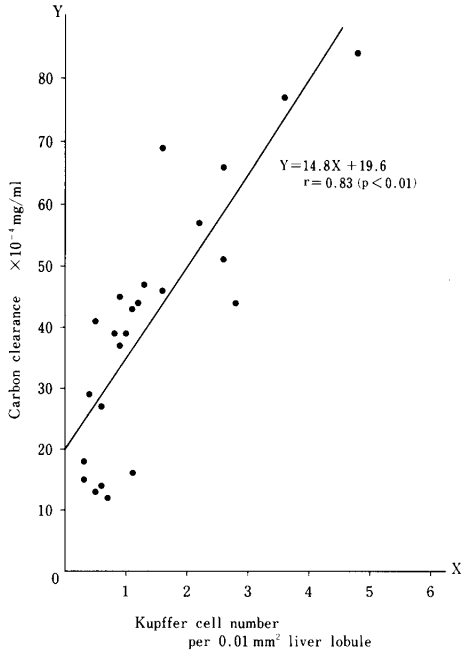


Fig. 7 Relationship between carbon clearance and Kupffer cell number per 0.01 mm^2 liver lobule.

mm^2 liver lobule $-4.5 \times$ liver weight $+48.9$. The multiple correlation coefficient was 0.87 ($p < 0.01$), and multicolinearity was excluded by making the variance inflation factor less than 10. The number of injured Kupffer cells was the third parameter in importance, but there was no significant additive importance to the formula. However, when the Kupffer cell number was excluded in the analysis, surface changes of Kupffer cells became the most important parameter for the explanation of carbon clearance. Body weight and spleen weight were not so important as parameters.

Discussion

Carbon clearance in the present experiment was decreased to less than 13% of that of the normal rats ($48 \pm 18 \times 10^{-3}$ mg/ml) (15). RES function is known to decrease in starved animals (16). Rats of the E1 and G groups were given 28 Cal per kg body weight per day, which is about 10% of the calorie intake of well-fed rats. Carbon clearance of the E1 group (4 g ethanol per kg body weight per day) was lower than in the G group, even though they were given the same number of calories. Carbon clearance of the E2 group (8 g ethanol, 56 Cal per kg body weight per day) was decreased to only 3% of the normal rat, and was lower than even that of the E1 and S groups. These results demonstrated that ethanol aggravated phagocytic activity of RES.

Multiple regression analysis demonstrated that carbon clearance was mostly explained by Kupffer cell number per unit area and liver weight. That is, carbon clearance depended mostly on the total Kupffer cell number in this experiment, though intravenously injected carbon particles are taken up mainly by the liver, and moderately by the spleen in normal animals (17, 18). Phagocytic activity of the liver and spleen is known to be influenced by their weight (10). As the liver was 18 to 33 times heavier than the spleen, the spleen did not play an important role in this experiment. It is well known that intravenous infusion of ethanol and starvation do not effect hepatic blood flow (16, 19). Therefore, clearance of carbon particles used in this experiment reflected predominantly phagocytic activity in the liver, that is, of Kupffer cells.

Kupffer cell number counted by LM was less than 58 % of that of normal rats (15) in the present groups. Kupffer cell number of the E2 group decreased to 11% of normal rats, and was less than in the G and S groups. However, the number of the E1 group did not differ from that of the S group. That is, Kupffer cells decreased in starvation, and ethanol reduced Kupffer cell number more.

Most Kupffer cells lost microvilli and filopodia in the E2 (74%) and E1 (67%) groups, whereas 54-75% of the cells retain-

ed them in the G and S groups. Surface morphology of Kupffer cells reflects their phagocytic activity; Kupffer cells with numerous microvilli and filopodia indicates vigorous phagocytic activity (15, 20-22). The number of injured Kupffer cells was the third most important parameter of carbon clearance. In the analysis without Kupffer cell number, surface changes of Kupffer cells became the most important parameter of carbon clearance. Therefore, the number of injured Kupffer cells is important for carbon clearance, as is the total Kupffer cell number. Ethanol induced impairment of carbon clearance in the E2 group by decrease of Kupffer cell number and also by decrease of the activity of each Kupffer cell as shown by disappearance of microvilli and filopodia.

Cytoskeletal changes of hepatocytes have been reported in alcoholic liver injury; ethanol injures microtubles of hepatocytes (23), and the Mallory body is known to be proliferated with intermediate filaments (24, 25). Therefore, disappearance of microvilli and filopodia of Kupffer cells demonstrated in the present study may be due to the disturbance of their cytoskeletal filaments induced by ethanol.

Hepatocytes showed marked changes such as cell atrophy, steatosis and vacuole formation in the central zone where Kupffer cells were scanty, whereas hepatocytes were well preserved in the periportal zone where Kupffer cells were present. These changes of hepatocytes in the central zone might be due to starvation (26), because they were observed in all groups. These findings also suggested the possibility that the decrease in Kupffer cell number induced hepatocyte changes. Kupffer cells have several functions to help hepatocytes such as phagocytosis, detoxification of endotoxin, secretion of mediators, regulation of microcirculation and metabolic breakdown of lipids (27). It can be expected that some disturbance may occur on hepatocytes when Kupffer cells are injured and/or their number decreases. In this study, it was shown that ethanol induced a decrease in the activity of each Kupffer cell and decrease in the number of Kupffer cells. Therefore, Kupffer cells may play an important role in the development of alcoholic liver injury.

Acknowlegements. The authors wish to thank Mr. N. Hayashi and Mr. N. Kishimoto, Electron Microscope Division of the Research Labolatory, Okayama Medical School, for their technical assistance. They also thank Mr. H. Takata for preparing the animals.

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Received: March 25, 1986
Accepted: October 1, 1986

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