METHODS

Experimental animals and treatments

Male Wistar rats, weighing 250–300 g, were used in the experiments. All animals received humane care including anesthetic, in compliance with the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine (Tokyo, Japan). To deplete Kupffer cells, gadolinium chloride (Sigma Chemical, St Louis, MO, USA), at 10 mg/kg bodyweight, was injected intravenously from the tail vein twice, 24 h apart.[11] Another group of rats received an equal volume of saline (the non-depleted rats). Twelve hours after the second injection, LPS (from *Escherichia coli* serotype 0111-B4: Sigma Chemical) at 2 mg/kg bodyweight dissolved in saline was injected intravenously into the Kupffer cell-depleted and non-depleted rats. An equal volume of saline without LPS was given to the control group of both the Kupffer cell-depleted and non-depleted rats. In a separate experiment, TNF-α at 5000 U/kg bodyweight was infused continuously for 2 h into the portal vein of both the Kupffer cell-depleted and non-depleted rats.

Detection of superoxide anions released into the hepatic sinusoids

Superoxide anion release into the hepatic sinusoids was measured according to the method by Bautista *et al.* with some modification.[8] After the LPS or saline administration, the portal vein was cannulated with an 18 G Teflon catheter, and the liver was perfused with Hanks' balanced salt solution (HBSS, Gibco BRL, Grand Island, NY, USA) at a rate of 2–3 mL/min per g wet liver. After the blood had been fully removed from the liver, the infusion solution was changed to HBSS containing 50 μmol/L oxidized cytochrome C (Wako Pure Chemical Industries, Tokyo, Japan, solution A). The perfusate was collected from the inferior vena cava every 2 min for 16–20 min, and the absorbance of each sample was measured at 550 nm[12] with a spectrophotometer (MPS-2000, Shimadzu, Kyoto, Japan). In each series, the difference between the absorbance of solution A and the highest absorbance among the samples was expressed as ΔABS. This reflects the formation of the reduced form of cytochrome C converted from the oxidized form in the presence of superoxide anions, and represents the amount of superoxide anions released into the hepatic sinusoids.[9] The superoxide anion level was examined in rats prepared 5 min, 1 h, 3 h, 12 h, and 24 h after the LPS treatment.

Population of Kupffer cells and hepatic macrophages in the liver specimens

Three hours after the LPS challenge, liver samples were obtained from each group and embedded in OCT compound (Tissue-Trek; Sakura Fine Technical Tokyo, Japan). Frozen sections (6 μm) were prepared with a cryostat (Tissue-Trek). Immunohistochemical analysis was performed with a monoclonal antibody to rat macrophages (Mar1; Seikagaku, Tokyo, Japan)[13] or an ED 2 antibody, which specifically recognizes Kupffer cells (Seikagaku).[14] The immune reaction was visualized with a peroxidase-labeled affinity purified antibody to mouse IgG (H + L) adsorbed with rat serum (Kirkegaard & Perry Laboratory, Gaithersburg, MD, USA) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma). The number of positive cells per high-power field (×400) was determined by light microscopy. We prepared five specimens in each group and cells were counted in five high-power fields chosen at random in each specimen.

Neutrophil population in the liver specimens

Liver samples were obtained from each group, fixed with 10% formaldehyde, and embedded in paraffin. Thin sections (3 μm) were prepared and stained with hematoxylin and eosin (H&E). The number of neutrophils per high-power field (×1000) was determined by light microscopy. The cell population was examined as aforementioned in rats prepared 5 min, 1 h, 3 h, 12 h, and 24 h after the LPS treatment.

Levels of TNF-α-and interleukin-8 in the portal vein

A blood sample was collected from the inferior vena cava of each animal 5 min, 1 h, 3 h, 12 h, and 24 h after the LPS or saline treatment. Time-courses of the TNF-α and interleukin (IL)-8 levels were measured in the sera with ELISA kits (Rat TN-Fα immunoassay; Techne Corporation, Minneapolis, MN, USA; and Rat IL-8 measuring kit, Immuno-Biological Laboratories, Gunma, Japan).

Purine nucleoside phosphorylase activity/ glutamic–pyruvic transaminase activity ratio in liver perfusate

To evaluate the endothelial cell damage in the hepatic sinusoids, the purine nucleoside phosphorylase (PNP)/ glutamic–pyruvic transaminase (GPT) ratio[15] was determined in the liver perfusate 3 h after the LPS challenge. The PNP activity was measured by the method described by Hoffee *et al.*[16] Briefly, 100 μL of the liver perfusate was incubated with 100 μL of potassium phosphate buffer (0.05 mol/L, pH 7.4) containing 0.5 nmol/L of inosine (Wako Pure Chemical Industries) and 10 pg/mL of xanthine oxidase (from milk, Sigma) at 37°C. The change in absorbance was measured spectrophotometrically at 293 nm. This represents the PNP activity. The GPT activity in the perfusate was measured with a kit (Unikitrate GPT, Kanto Kagaku, Tokyo, Japan).

Statistical analysis

The data were expressed as means ± SD. Differences between two groups were assessed by the Student's *t*-test. Comparisons among more than three groups were performed by one-way ANOVA with the Newman–Keuls test. The difference was considered significant at $P < 0.05$.

RESULTS

To verify that Kupffer cells were successfully depleted from the liver with gadolinium chloride, ED2-positive cells were immunohistochemically examined in liver specimens. As shown in Fig. 1, ED2-positive cells were observed at very low numbers in the livers of rats given gadolinium chloride, even after the LPS challenge. Gadolinium chloride also significantly reduced the population of cells positive for antimacrophage antibody (Fig. 2) and neutrophils (Fig. 3) in the liver, although they were not totally depleted. The results of the morphometric analysis are summarized in Table 1.

The superoxide anion level in the hepatic sinusoids was examined. Absorbance at 550 nm was measured in the liver perfusate every 2 min and it was found to reach a maximum plateau 8–10 min after the start of HBSS administration containing oxidized cytochrome C (solution A). This finding has already been published elsewhere.[9] The difference between the peak absorbance and the absorbance of solution A was designated as ΔABS. It was significantly higher in the rats given LPS than in those given saline. When superoxide dismutase was simultaneously given in the liver perfusate, it was significantly suppressed and thus, the value of ΔABS was concluded to represent the level of superoxide anion formation in this model.[9]

In the present study, we could reproduce the phenomenon whereby LPS increased the superoxide anion release into the hepatic sinusoids. The time-course of the superoxide anion level in the hepatic sinusoids was studied up to 24 h after the LPS challenge. Superoxide anions were not detectable 5 min after the LPS

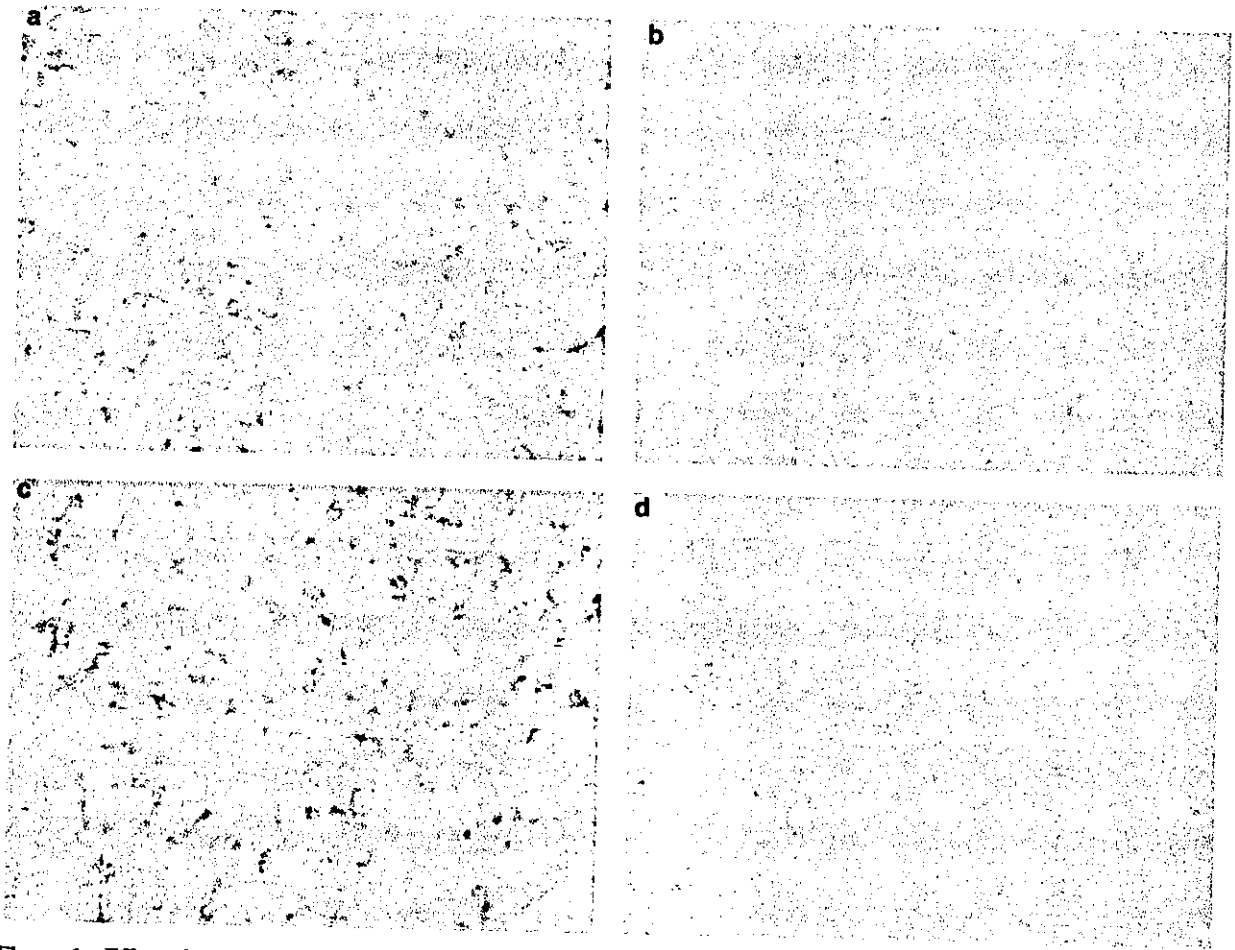

Figure 1 Effect of gadolinium chloride on the population of ED2-positive cells after lipopolysaccharide (LPS) challenge in the liver. The population of ED2-positive cells in the liver was immunohistochemically studied. (a) Rats given saline instead of gadolinium chloride and saline instead of LPS. (b) Those given gadolinium chloride and saline instead of LPS. (c) Those given saline instead of gadolinium chloride and LPS. (d) Those given gadolinium chloride and LPS (immunohistochemistry ×20).

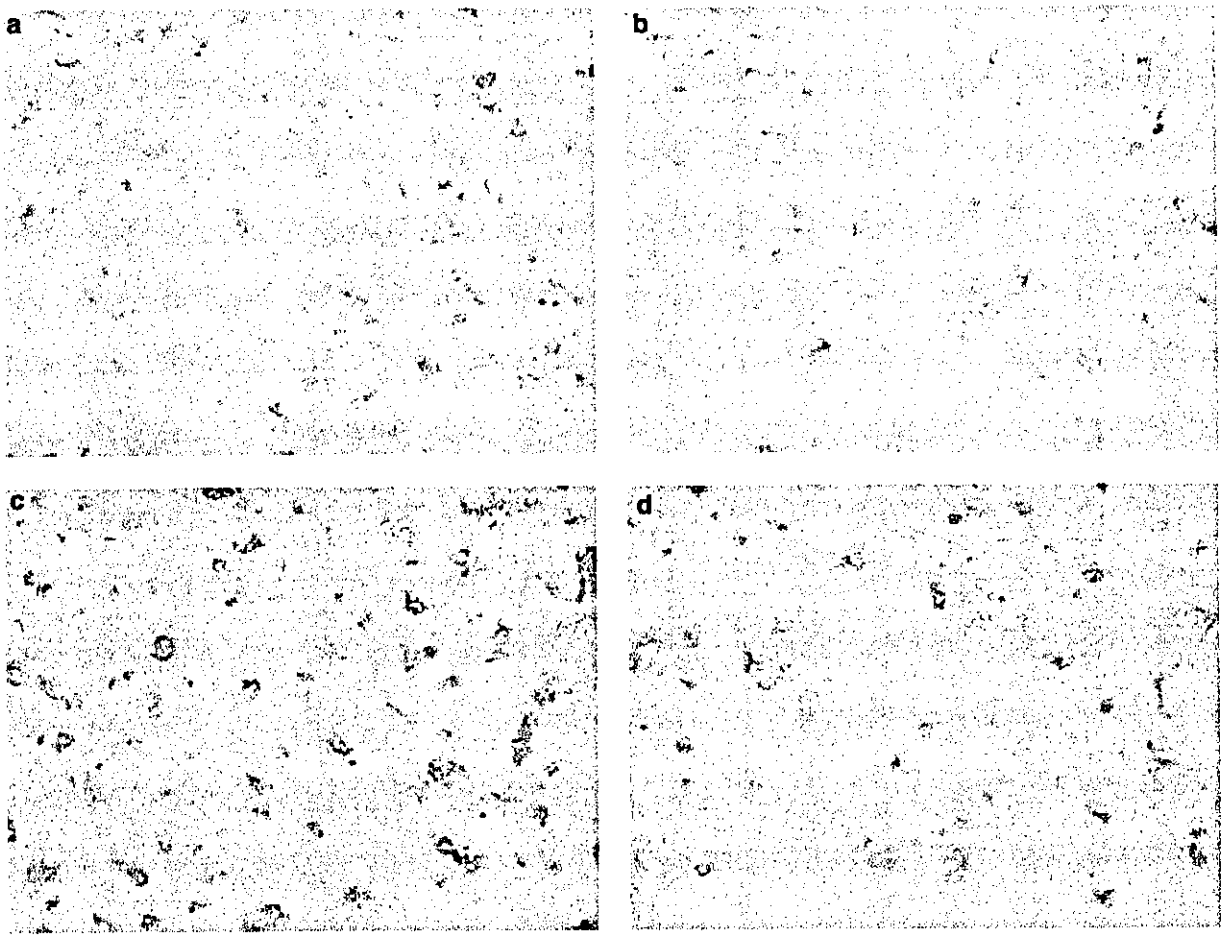

Figure 2 Effect of gadolinium chloride on the population of macrophages after lipopolysaccharide (LPS) challenge in the liver. The population of macrophages in the liver was immunohistochemically studied with an antibody to macrophage. (a) Rats given saline instead of gadolinium chloride and saline instead of LPS. (b) Those given gadolinium chloride and saline instead of LPS. (c) Those given saline instead of gadolinium chloride and LPS. (d) Those given gadolinium chloride and LPS (immunohistochemistry ×20).

Table 1 Effect of gadolinium chloride on the populations of ED2-positive cells, macrophages, and neutrophils in the liver 3 h after lipopolysaccharide (LPS) treatment

	Saline + saline ($n = 5$)	Saline + $GdCl_3$ ($n = 5$)	LPS + saline ($n = 5$)	LPS + $GdCl_3$ ($n = 5$)
No. ED2-positive cells	42 ± 3	ND	65 ± 8*	ND
No. macrophages	73 ± 5	25 ± 4*	127 ± 19**	38 ± 3***
No. neutrophils	0.2 ± 0.4	0.2 ± 0.4	6.6 ± 1.5**	1.4 ± 0.8***,****

*$P < 0.05$ compared with saline + saline; **$P < 0.01$ compared with saline + saline; ***$P < 0.01$ compared with LPS + saline; ****$P < 0.01$ compared with LPS + saline. ND, not detected.

treatment and the level reached its maximum 3 h after the LPS challenge (Fig. 4). Moreover, the level was significantly attenuated in the Kupffer cell-depleted rats compared to the non-depleted rats, although it was still higher than that in rats given saline plus saline (control) 3 h after the LPS challenge (Table 2).

Changes in the superoxide anion level in the hepatic sinusoids after TNF-α infusion for 2 h were also studied. The TNF-α challenge resulted in superoxide anion formation. Similarly to the LPS challenge model, gadolinium chloride significantly attenuated the superoxide anion formation after TNF-α challenge (Table 3), although the level was still higher than in controls.

We also previously reported that LPS treatment increased the PNP/GPT ratio in the liver perfusate, a marker of damage to sinusoidal epithelial cells in the

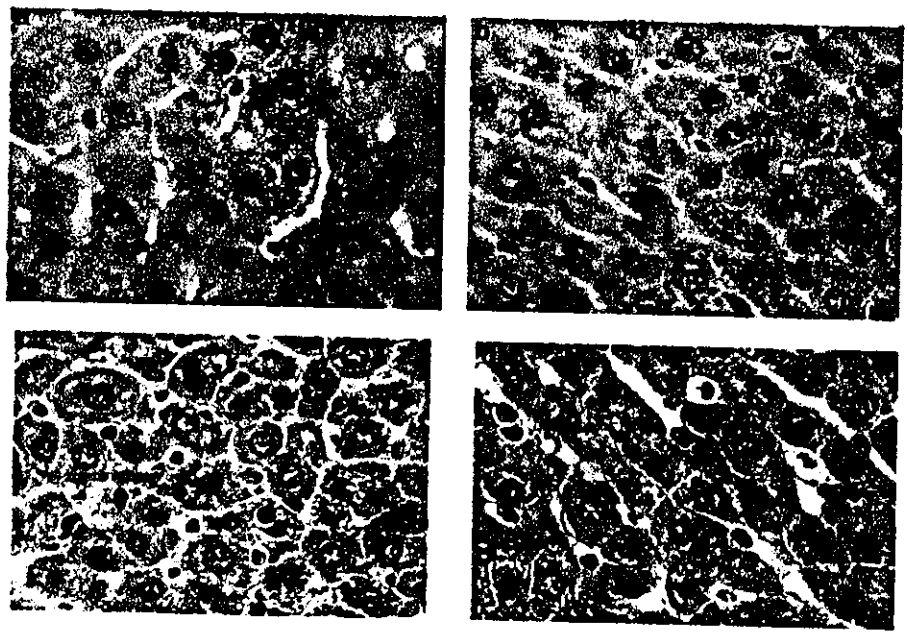

Figure 3 Effect of gadolinium chloride on the population of neutrophils after lipopolysaccharide (LPS) challenge in the liver. The population of neutrophils in the liver was studied. (a) Rats given saline instead of gadolinium chloride and saline instead of LPS. (b) Those given gadolinium chloride and saline instead of LPS. (c) Those given saline instead of gadolinium chloride and LPS. (d) Those given gadolinium chloride and LPS (HE ×40).

Table 2 Effect of gadolinium chloride on the formation of the superoxide anion that is released into the hepatic sinusoids 3 h after lipopolysaccharide (LPS) treatment

	Saline + saline ($n = 3$)	Saline + $GdCl_3$ ($n = 5$)	LPS + saline ($n = 6$)	LPS + $GdCl_3$ ($n = 3$)
Superoxide anion formation (ΔABS)	0.12 ± 0.01	0.12 ± 0.01	0.25 ± 0.01*	0.17 ± 0.01**,†

*$P < 0.01$ compared with saline + saline; **$P < 0.01$ compared with LPS + saline; †$P < 0.05$ compared with saline + saline. ΔABS, difference between the absorbance of the original perfusate and the highest absorbance in the sample perfusate.

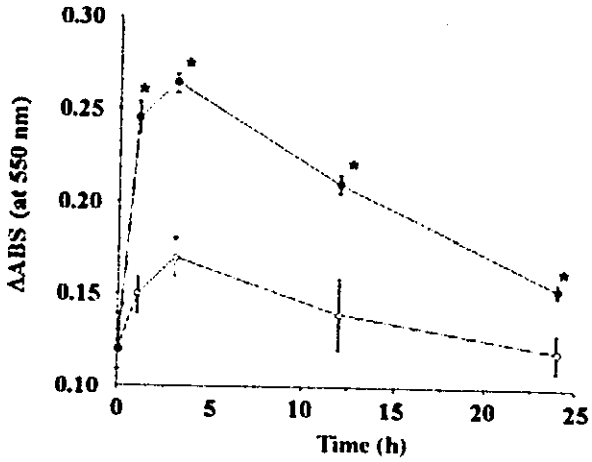

Figure 4 Time-course of the superoxide anion level in the hepatic sinusoids after the lipopolysaccharide (LPS) challenge. The superoxide anion was not detectable 5 min after the LPS treatment and the level was maximal 3 h after the LPS challenge. (○) $GdCl_3$ + LPS; (●) LPS. ΔABS, highest absorbance among the samples. *$P < 0.01$ compared with $GdCl_3$ + LPS.

Table 3 Effect of gadolinium chloride on the formation of the superoxide anion released into the hepatic sinusoids after the tumor necrosis factor (TNF)-α treatment

	TNF-α ± saline ($n = 4$)	TNF-α ± $GdCl_3$ ($n = 4$)
Superoxide anion formation (ΔABS)	0.25 ± 0.01	0.17 ± 0.01*

*$P < 0.05$ compared with TNF-α ± saline. ΔABS, difference between the absorbance of the original perfusate and the highest absorbance in the sample perfusate.

liver, compared with controls.[9] This finding was also reproducible, and the present results showed that the increased PNP/GPT ratio was significantly attenuated by gadolinium chloride treatment (Table 4).

The time-course of the serum TNF-α level in the inferior vena cava was examined in the Kupffer cell-depleted and non-depleted rats up to 24 h after the LPS challenge. The level reached its maximum 1 h after the LPS treatment. There was no significant difference in the TNF-α levels between the two groups (Fig. 5).

Table 4 Effect of gadolinium chloride on the PNP/GPT ratio 3 h after lipopolysaccharide (LPS) treatment

	Saline + saline ($n = 3$)	Saline + $GdCl_3$ ($n = 5$)	LPS + saline ($n = 6$)	LPS + $GdCl_3$ ($n = 3$)
PNP/GPT ratio ($\times 10^4$)	16 ± 5.5	18 ± 2.4	54 ± 2.4*	40 ± 7.0**

*$P < 0.01$ compared with saline + saline; **$P < 0.01$ compared with LPS ± saline. PNP/GPT, purine nucleoside phosphorylase/glutamic–pyruvic transaminase.

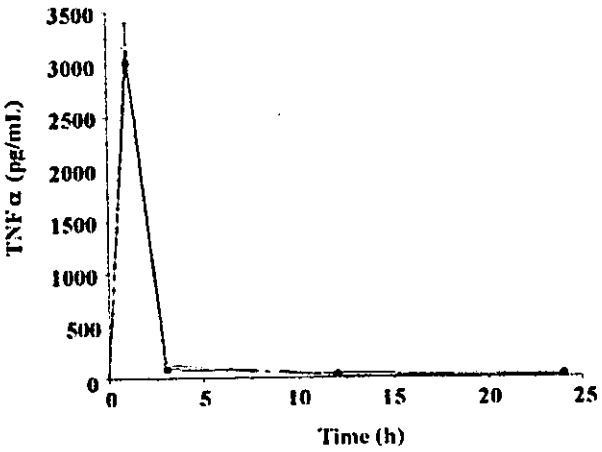

Figure 5 Time-course of the serum tumor necrosis factor (TNF)-α level in the inferior vena cava after the lipopolysaccharide (LPS) challenge. The level was maximal 1 h after the LPS treatment. There was no significant difference in the TNF-α levels between the two groups. (○) $GdCl_3$ + LPS, (●) LPS.

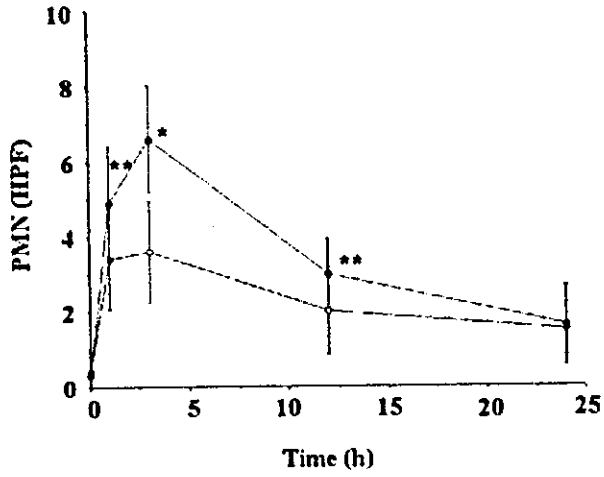

Figure 6 Time-course of the neutrophil population in the liver specimens after the lipopolysaccharide (LPS) challenge. The population was maximal 3 h after LPS challenge in both groups and was significantly lower in the Kupffer cell-depleted rats than in the non-depleted rats. (○) $GdCl_3$ + LPS; (●) LPS. *$P < 0.01$ compared with $GdCl_3$ + LPS; **$P < 0.05$ compared with $GdCl_3$ + LPS.

The time-course of the neutrophil population after the LPS challenge was studied in the Kupffer cell-depleted rats as well as in the non-depleted rats. The population peaked 3 h after the LPS challenge in both groups and was significantly lower in the Kupffer cell-depleted rats than in the non-depleted rats (Fig. 6).

The time-course of the IL-8 level in the inferior vena cava was also studied in the both groups. Concomitantly with the neutrophil population in the liver, the level reached its maximum 3 h after the LPS challenge in both groups and was significantly lower in the Kupffer cell-depleted rats than in the non-depleted rats (Fig. 7).

DISCUSSION

There are several lines of evidence indicating that gadolinium chloride attenuates the progression of endotoxin-related liver damage. The present study aimed to determine the mechanisms behind this. To that end, we studied oxidative stress in the hepatic sinusoids in acute endotoxemia and the effect of gadolinium chloride on it. The oxidative stress was assessed by detection of superoxide anions in the hepatic sinusoids using the methods reported by Bautista *et al.*

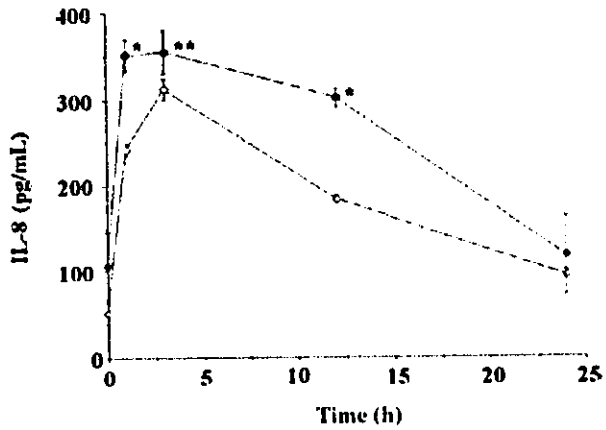

Figure 7 Time-course of the interleukin (IL)-8 level in the inferior vena cava after the lipopolysaccharide (LPS) challenge. The level was maximal 3 h after the LPS challenge in both groups and was significantly lower in the Kupffer cell-depleted rats than in the non-depleted rats. (○) $GdCl_3$ + LPS; (●) LPS. *$P < 0.01$ compared with $GdCl_3$ + LPS; **$P < 0.05$ compared with $GdCl_3$ + LPS.

The present time-course study showed that the superoxide anion level reached its maximum at 3 h after LPS treatment. This observation agrees with the previous report by Bautista and Spitzer.[7] Moreover, the time-course of the serum TNF-α level was examined, because TNF-α has been suggested to be an important factor in stimulating superoxide anion formation in this model.[8] We found that there was a time lag between the peak level of superoxide anions and the maximum serum TNF-α level; the former was 3 h after the LPS challenge whereas the latter was 1 h. However, in view of the time required to prime the Kupffer cells, this time lag is not surprising.

Gadolinium chloride can phagocytically deplete active Kupffer cells from the liver.[11] The present immunohistochemical analysis showed that there were few ED2-positive cells in the hepatic lobules of the gadolinium chloride-treated rats, indicating that Kupffer cell depletion in the present study was successful. The new finding in the present study was that gadolinium chloride treatment significantly reduced superoxide anion release into the hepatic sinusoids after the LPS challenge. This observation is reasonable because gadolinium chloride has been reported to reduce superoxide anion release after the LPS challenge from isolated liver macrophages,[17] which are expected to be the main sources of superoxide anions in this model.[10] We assume that the reduction of superoxide anions in the hepatic sinusoids may be related to a beneficial effect of gadolinium chloride against the progression of LPS-induced liver damage.

In contrast to the level of superoxide anions, there was no significant difference in that of serum TNF-α between the Kupffer cell-depleted rats and non-depleted rats. Because Kupffer cells are one of the sources of TNF-α *in vivo*, this finding was not convincing. Iimuro *et al.* reported that there was no significant difference in levels of serum TNF-α between the Kupffer cell-depleted rats and non-depleted rats,[17] and we could reproduce that observation in the present study. However, because Kupffer cells are one of the sources of TNF-α *in vivo*, this finding was not convincing. We cannot give a reasonable explanation for this. The interpretation for this fact by Iimuro *et al.* was that TNF secretion by liver macrophages would be a small part of the whole and may not affect the TNF level in peripheral blood. Having this view, we could only say that attenuation of the superoxide anion level in the gadolinium chloride-treated rats is at least due to a decrease in the number (or function) of superoxide anion sources. This view is supported by the present observation that the superoxide anion level was attenuated in the gadolinium chloride-treated rats compared to the non-depleted rats after equal doses of TNF were challenged.

It is well known that neutrophils are responsible for the deterioration of various liver injuries including that caused by LPS. Therefore, we examined the time-courses of the neutrophil population and serum IL-8 level, which is an important factor in regulating neutrophil migration *in vivo*, after LPS treatment. We found that the peaks of both factors were at least 3 h (or more) after LPS administration. Moreover, another finding that the time-courses of the two factors were parallel suggests that IL-8 also participates in neutrophil recruitment in this model.

The most interesting finding in the present study was that gadolinium chloride attenuated the serum IL-8 level and neutrophil population in the liver, although it did not affect the level of serum TNF-α, which regulates IL-8 production from various cells[18] after LPS treatment. It is therefore conceivable that factors other than TNF-α are responsible for the reduction of the serum IL-8 level and subsequent neutrophil migration into the liver. Recently, either extracellular or intracellular oxidative stress has been shown to activate IL-8 production and the expression of various adhesion molecules, resulting in an increase in the migration of neutrophils.[19] It is likely that superoxide anions in the hepatic sinusoids promote IL-8 production. In consideration of this view, it is not surprising that gadolinium chloride, which reduces the level of superoxide anions in the hepatic sinusoids, also reduces the serum IL-8 level and subsequent neutrophil migration into the liver.

We have reported that the LPS challenge increased the PNP/GPT ratio in the liver perfusate, a marker of damage to endothelial cells in the hepatic sinusoids, and the superoxide anions in the hepatic sinusoids may, at least in part, have caused this damage.[9] This phenomenon was reproduced in the present study. An important finding in the present study was that the increase in the PNP/GPT ratio, a marker of damage to the hepatic sinusoidal endothelial cells in response to LPS treatments, was significantly attenuated by gadolinium chloride treatment. It can easily be deduced that a direct effect of TNF-α is its involvement in the mechanisms causing damage to hepatic sinusoidal endothelial cells in this model. However, gadolinium chloride reduced the superoxide anion level in the hepatic sinusoids after the LPS challenge and this was associated with a reduction of the damage to the hepatic sinusoidal endothelial cells without any change in the serum TNF-α level. This indicates that superoxide anions released into the hepatic sinusoids per se damage the hepatic sinusoidal endothelial cells after the LPS challenge. As stated here, because gadolinium chloride reduced neutrophil migration into the liver after LPS treatment, the endothelial cell damage is decreased. Inversely, because damage to endothelial cells in the hepatic sinusoids leads to neutrophils sticking to them,[20] the reduction of endothelial cell damage causes a reduction in the liver neutrophil population after LPS treatment in gadolinium chloride-treated rats. In either case, the present finding is in keeping with the fact that Kupffer cell depletion has a protective effect against the progression of LPS-related liver injury.[5,6]

In the present study, the level of superoxide anions after the LPS or TNF-α challenge in the gadolinium chloride-treated rats was significantly higher than in controls, although ED2-positive cells were fully depleted from their livers. This suggests that superoxide anions are also derived from cells other than ED2-positive cells in this model. Because neutrophils and circulating macrophages produce superoxide anions upon various stimuli,[4,18] we assume that these cells could produce superoxide anions in this model. This view is

supported by the present findings that a considerable number of neutrophils and macrophages were still observed in the liver specimens of the gadolinium chloride-treated rats.

Recently, we reported that an ethanol challenge also caused superoxide anion release into the hepatic sinusoids, which was attenuated by Kupffer cell depletion.[21] However, the levels of superoxide anions and subsequent damage to hepatic sinusoidal endothelial cell were much less compared to those in the LPS challenge model. Given that the involvement of neutrophils was minimum in the ethanol challenge model,[21] we assume that superoxide anions only in the hepatic sinusoid cannot activate either IL-8 production or neutrophil migration into the liver, causing further deterioration to the injured liver. Because TNF-α was not detectable in the model,[21] TNF-α could be one of the essential factors to activate IL-8 and neutrophil migration in the liver.

In conclusion, LPS causes the release of TNF-α and superoxide anions into the hepatic sinusoids, which augments IL-8 release as well as endothelial cell damage. These factors contribute to increased neutrophil migration into the liver, resulting in the progression of liver damage. This process may be the reason why alcoholic liver damage progresses when it is concomitant with endotoxemia.

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Journal of Gastroenterology

Editorial

Marked improvement in the resolution of, and survival rates in, spontaneous bacterial peritonitis

Article on page 119

Five days of ceftriaxone to treat spontaneous bacterial peritonitis in cirrhotic patients

França AVC, Giordano HM, Sevá-Pereira T, Soares EC

Spontaneous bacterial peritonitis (SBP) is one of the potentially lethal complications of cirrhosis, and is defined as infected ascitic fluid in the absence of a recognizable secondary cause of peritonitis. It develops in 10%–30% of hospitalized cirrhotic patients, and the mortality rate exceeded 90% when it was first described.[1,2] However, with the early recognition of the disease and prompt and appropriate antibiotic treatment, the in-hospital mortality of an episode of SBP has been reduced to approximately 20%.[3] Nowadays, it appears that practical treatment for SBP has virtually been established, and the remaining problems may be how to achieve a further increase in the survival rate and how to prevent the development of SBP in high-risk patients. Franca and colleagues,[4] in their study reported in this issue of the *Journal of Gastroenterology*, achieved a survival rate as high as 88%, and established a method for managing SBP patients with ceftriaxone, a third-generation cephalosporin.

The infection of ascitic fluid in SBP is considered to be blood-borne and monomicrobial in 90% of patients.[5] The three most common isolates are *Escherichia coli*, *Klebsiella pneumoniae*, and *Pneumococcus*.[6] Although opsonic activity plays an important role as a defense mechanism against bacterial growth, the activity in ascitic fluid is low.[7] Furthermore, the opsonic activity in ascitic fluid is proportional to the total protein concentration in the fluid. Therefore, SBP is most likely to occur in cirrhotic ascites.[8]

The infection of ascitic fluid leads to an elevation of the polymorphonuclear leukocyte (PMN) count in the ascites, which represents evidence of the failure of the first line of defense, i.e., the peritoneal macrophages, to kill invading bacteria. Therefore, elevation of the PMN count to more than 250/mm^3 in ascitic fluid has been adopted as a diagnostic criterion of SBP, without consideration of the detection of bacteria in ascites cultures. Furthermore, bacterial and PMN counts in ascites increase rapidly after infection unless antibiotic treatment is started.[9] Therefore, the prompt initiation of antibiotic administration is crucial for saving patients.

At the start of therapy, a broadspectrum antibiotic is warranted, because the species of causative bacteria range from gram-negative intestinal flora to pneumococcus. Cefotaxime or a similar third-generation cephalosporin is recommended as the initial treatment for SBP in many guidelines[3] and reviews,[6] because the effectiveness of this antibiotic has been well established in several studies.[10,11] After the identification of the causative bacteria and determination of their sensitivity to antibiotics, a narrower spectrum of coverage can be determined. Apart from the resolution of infection, the administration of albumin appears to be effective for preventing renal failure, and this has resulted in a reduced mortality rate.[12]

The antibacterial treatment should be continued for approximately 5 days, on the basis of a report that the PMN count in ascites decreases below 250/mm^3 in a mean period of 5 days; the antibiotic treatment can then be safely discontinued.[13] Moreover, it has been shown that 5-day treatment with cefotaxime is as effective as 10-day treatment.[14] Franca et al.[4] considered these reports, and evaluated the effectiveness of the antibacterial treatment on day 5 from the date of administration.

Several risk factors for the development of SBP have been demonstrated, such as low total protein concentrations (less than 1.0g/dl) in ascitic fluid, gastrointestinal hemorrhage, and a prior occurrence of SBP.[3,6] Therefore, several therapies, such as the oral administration of norfloxacin, have been proposed as primary or secondary prophylactic therapies for high-risk patients, and the effectiveness of norfloxacin has been confirmed in several studies.[3,6]

Regarding the prediction of prognosis of SBP, there are few reports to date. Toledo et al.[11] analyzed 51 clini-

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cal and laboratory variables in 213 episodes of SBP treated with cefotaxime; using stepwise logistic regression, they identified four independent factors associated with the resolution of infection: band neutrophils in the white blood cell count, community-acquired versus hospital-acquired peritonitis, blood urea nitrogen level, and serum aspartate aminotransferase level. Using a similar method, Franca et al.[4] examined 21 factors in 33 SBP patients treated with ceftriaxone, although no factor was selected as a significant predictor of prognosis on Cox regression analysis after a follow-up of fixed duration (5 days). The correlation between renal function and survival in SBP patients has been demonstrated, and the effectiveness of albumin administration for preserving renal function has been suggested, as mentioned above. In addition to these previous findings, Franca et al.[4] found that renal dysfunction (elevation of serum urea or serum creatinine) may have been associated with the resolution of SBP in 5 days.

In the reports to date that have described the treatment of SBP, 10% has been the lowest mortality rate.[12] This excellent result was achieved by a combination treatment using cefotaxime and albumin. In contrast, Franca et al.[4] reported an almost comparable mortality rate, of 12%, achieved with ceftriaxone treatment alone; furthermore, in most of their patients (73%), the infection had resolved within 5 days from the start of the treatment.

From these results, the outstanding effectiveness of ceftriaxone treatment for SBP was clearly confirmed, and a practical method for managing SBP patients was well established.

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Brain glutamine and glutamate levels in patients with liver cirrhosis: assessed by 3.0-T MRS

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Abstract

Background/Objectives: Magnetic resonance spectroscopic (MRS) studies have revealed abnormal metabolism in the brain of patients with liver cirrhosis, including an increase in total brain glutamine (Gln) and glutamate (Glu) levels (Glx). However, with conventional MRS techniques, it was difficult to separate the Glx signals. Using a high-magnetic field MR equipment and a newly developed data processing method, we attempted to separate the Glx signals on an MRS. *Subjects and Methods:* Twenty-three patients with liver cirrhosis and 11 healthy adults were enrolled in this study. After designating a region of interest in the occipital lobe gray matter of each subject, ^{1}H(proton)-MRS was performed using 3.0-T MR equipment. *Results:* MRS conducted using the 3.0-T MR equipment allowed Gln signals in the brain to be distinguished from the Glx signals. The brain signal intensity of Gln was found to be significantly higher in the liver cirrhosis group (0.658 ± 0.23) than in the control group (0.473 ± 0.08) ($P < 0.05$). Neither the Glu nor the Gln signal intensity showed any correlation with the blood ammonia level. *Conclusion:* High-magnetic field MRS allowed us to separate the Glx signals in the brain and revealed that the increase in the total brain Glu and Gln levels in patients is solely attributable to an increase in the level of Gln.

Keywords: Liver cirrhosis; Magnetic resonance spectroscopy; Astrocyte; Glutamate; Glutamine

1. Introduction

Hepatic encephalopathy is a complication of liver cirrhosis, which describes a spectrum of neuropsychiatric abnormalities seen in these patients, including disturbances of consciousness. In cases of chronic liver failure, as prevails in cases of uncompensated-liver cirrhosis, the nervous system is exposed to certain toxic substances such as ammonia for prolonged periods of time, resulting in abnormalities of neurotransmitters, receptors, as well as in the blood–brain barrier. As a result, the sensitivity of these patients to toxic substances is increased, which hastens the onset of hepatic encephalopathy. The brain under this condition is called a "hypersensitive brain" [1].

In recent years, close attention has been paid to the dysfunction of astrocytes associated with elevated brain levels of toxic substances because it has been suggested that this may be involved in the pathogenesis of hepatic encephalopathy [2]. According to one theory, the degradation of toxic substances such as ammonia in the brain results in elevation of the glutamine (Gln) level in astrocytes, which causes both swelling and dysfunction of these cells [2]. In relation to hypotheses explaining the pathogenesis of hepatic encephalopathy, the following sequence of metabolic events were postulated from the results of spectroscopic studies. As results of the increase in ammonia level, the synthesis of glutamine (Gln) from glutamate (Glu) intensifies and Gln accumulates in the astrocytes. Astrocytes are the only cells in the CNS having Gln synthesis pathway, which is the main path of ammonia transformation. A previous magnetic resonance spectroscopic (MRS) study revealed that the level of Gln in the brain was high even in cases of liver cirrhosis not complicated by apparent encephalopathy [3]. However, in MRS using the conventional 1.5-T equipment, it was difficult to distinguish between glutamine (Gln) and glutamate (Glu) signals, and the total combined Gln and Glu level in the brain was used as an indicator of the increased Gln levels [3]. Thus, it was difficult to determine by conventional

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MRS if the elevation of the total Gln and Glu levels was attributable to elevation of the Gln level alone, or rather an elevation of both the Gln and the Glu levels. Therefore, using high-magnetic field MR equipment with high resolution, we attempted to distinguish the Gln from the Glu signals and analyzed the relationship between the brain Gln and Glu levels to various blood biochemical data in patients with liver cirrhosis.

2. Subjects and methods

2.1. Subjects

The subjects of the study were 23 liver cirrhosis patients with no history of brain disease, who were inpatients at the First Department of Internal Medicine, Iwate Medical University School of Medicine (Table 1). There were 15 males and 8 females, with a mean age of 61 years (mean ± S.D. = 60.7 ± 10.8 years). The diagnosis of liver cirrhosis was confirmed by a general evaluation of the findings on diagnostic imaging (abdominal ultrasonography, CT, etc.), histological examination, and blood biochemical data. The liver cirrhosis was etiologically related to excessive alcohol consumption in one case, viral infection in 20 cases (HCV in 14, HBV in 2, and HCV + alcohol in 4 cases), and was of unknown etiology in 2 cases. The condition was judged to be child-pugh class A in 2 cases, class B in 18 cases, and class C in 3 cases. Eleven healthy adults without a history of liver disease and free of organic brain disease served as controls (nine males and two females; mean age ± S.D., 38.5 ± 11 years).

2.2. Methods

The equipment used for the MRS was a Signa Horizon LX-VH/i 3.0-T (GE Medical Systems). A cubic region of interest (ROI) with the dimensions of 20 mm × 20 mm × 20 mm was designated in the occipital lobe gray matter of each subject and the ^{1}H(proton)-MR spectrum of this region was measured by the point-resolved spectroscopy (PRESS) sequence method (Fig. 1). Measurements of MRS were performed under overnight fasting condition. The measured parameters were as follows: repetition time (TR), 2000 ms; echo time (TE), 60 ms; and number of summations (NS), 192. To quantify the levels of *N*-acetylaspartic acid (*N*-AA), glutamate (Glu), glutamine (Gln), and creatine (Crn), phantom solutions of each of these substances at a known level of concentration were prepared in advance and used for measuring spectral signal intensities. The concentration of each phantom solution was adjusted with a phosphate buffer (pH 7.4) to a final concentration of 5.0 mm.

Table 1
Characteristics of the cirrhotic patients and a control group

Characteristic	Controls	Liver cirrhosis
Number	11	23
Gender male	9	15
Height (cm)	169.7 ± 4.1	161.1 ± 8.9
Weight (kg)	66.4 ± 9.2	60.6 ± 10.6
BMI	23.2 ± 3.1	23.2 ± 2.7
Female	2	8
Height (cm)	160.0 ± 0.0	147.3 ± 6.0
Weight (kg)	55.5 ± 4.5	50.8 ± 8.5
BMI	21.5 ± 1.5	23.4 ± 3.3
Age (yr)	38.5 ± 11.0	60.7 ± 10.8
Child-Pugh classification		
Grade A	–	2
Grade B	–	18
Grade C	–	3

Age, height, weight, BMI represent: mean ± S.D.

Since it is difficult to measure absolute levels of metabolites by non-invasive means in MRS, the levels of Gln and Glu were determined as molar ratios relative to the internal standard (*N*-AA level) [2].

Previous reports on measurements using MRS have suggested that Glu can be quantified on the basis of the spectra yielded at two different echo times [4,5]. Therefore, in the present study, we attempted to measure the Glu intensity at various TEs, using a 3.0-T MR device. Separation of the Glu from the Gln signals was the best when the TE was 60 ms. Fig. 2 shows the brain MR spectra of individual subjects, as well as the spectra of the reference materials used for the quantification of *N*-AA, Gln, and Glu. In this figure, (A) represents the spectrum of the brain in a case of liver cirrhosis, (B) represents the *N*-AA and Crn spectra of the reference solution, (C) shows the Glu and Crn spectra of the reference solution, and (D) indicates the Gln and Crn spectra of the reference solution. Each substance showed its own unique spectrum. The main signals of Glu and Gln were recorded at between 2.0 and 2.5 ppm.

Separate quantifications of brain Glu and Gln were performed by the computerized processing of the data (transferred from the MR equipment to the computer) using the GRAMS/32 (Galactic Industries, Corp. Salem, NH, USA) program and Excel (Microsoft Corp., Tokyo, Japan). The procedure for this newly developed method of separate quantification of Glu and Gln is shown below (Fig. 3). First, the *N*-AA spectrum (b) was subtracted from the subject's spectrum (a) to yield the amount of *N*-AA. On the differential spectrum (c), the amount of *N*-AA can be determined based on the point at which the signal of the methyl group of *N*-AA (2 ppm) disappears. In the second step, the Glu spectrum (d) was subtracted from the differential spectrum (c) obtained in the first step to yield the amount of Glu. The Glu signal at a point near 2.3 ppm was used as an indicator for this step (e). In the third step, the Gln spectrum (f) was subtracted from the differential spectrum obtained in the second step (e) to yield the amount of Gln. The Gln signal in the vicinity of 2.4 ppm served as an indicator for this step (g).

In each subject, the blood biochemical parameters, including the venous blood level of ammonia (μg/dl:enzyme assay) and serum albumin (g/dl), the serum prothrombin

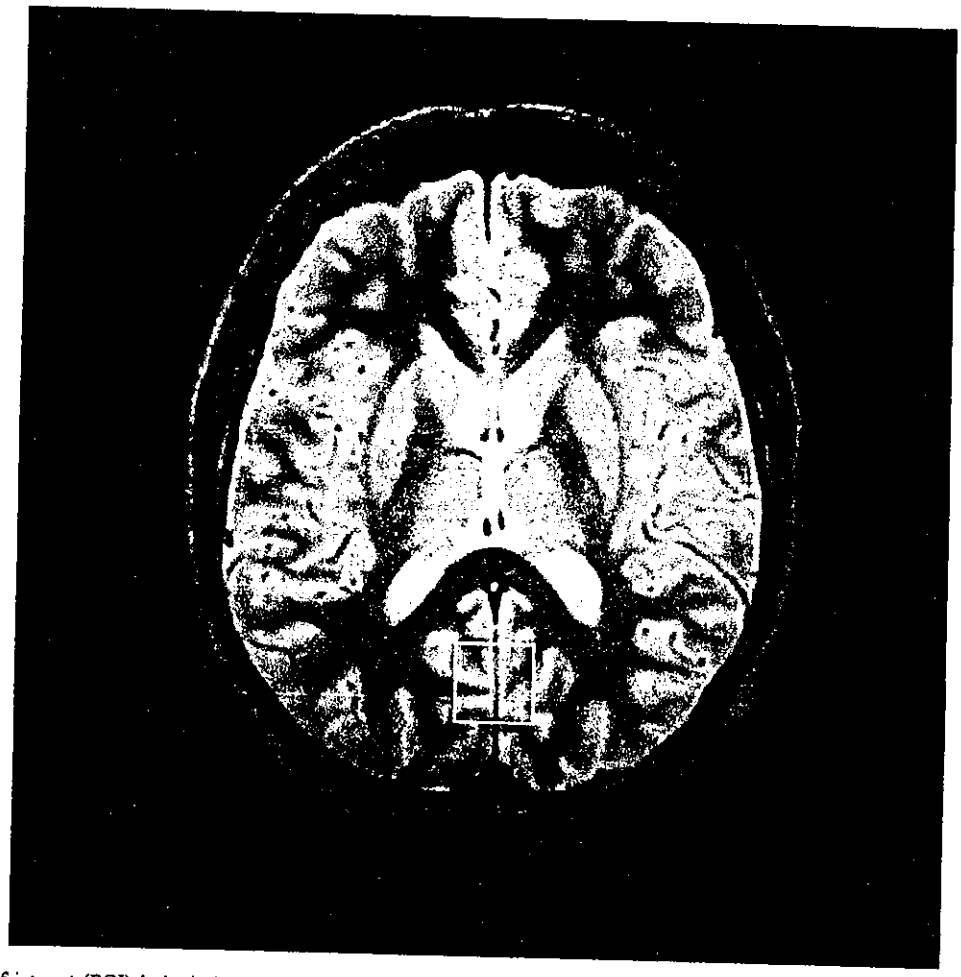

Fig. 1. The region of interest (ROI) in brain MRS. A cubic region of interest (ROI) with the dimension of 20 mm × 20 mm × 20 mm was designated in the occipital lobe grey matter of each subject in brain MRS.

time (%), the serum total bilirubin level (mg/dl), and the plasma free amino acids including Gln (μmol/l:HPLC) and Glu (μmol/l:HPLC), were also measured.

2.3. Statistical analysis

Values were expressed in mean ± S.D. Student's *t*-test and Mann–Whitney's *U*-test were used for the comparison of parameters between the two groups. Fisher's PSLD was used for testing correlations. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Brain glutamine and glutamate levels compared with healthy controls

The molar ratio of the total amount of Glu and Gln to *N*-AA, i.e., (Glu + Gln)/*N*-AA, was significantly higher in the liver cirrhosis group (1.305 ± 0.33) than in the control group (1.046 ± 0.11) ($P < 0.05$) (Table 2). The molar ratio of brain Gln to *N*-AA (Gln/*N*-AA) was also significantly higher in the cirrhosis group (0.658 ± 0.23) than in the control group (0.473 ± 0.08) ($P < 0.05$). On the other hand, the molar ratio of brain Glu to *N*-AA (Glu/*N*-AA) did not differ significantly between the control group (0.573 ± 0.06) and the liver cirrhosis group (0.648 ± 0.15). None of these molar ratios showed any significant correlation with the level of severity of liver disease.

3.2. Relationship to the blood biochemical data

The venous blood ammonia level showed no significant correlation with the brain Gln or Glu signal intensity (Table 3). No significant correlation was observed between these two parameters even when the cirrhosis group was subdivided into the high-ammonia level group (over 60 μg/dl; Gln/*N*-AA 0.656 ± 0.27) and the normal-ammonia level group (less than 60 μg/dl; Gln/*N*-AA 0.659 ± 0.19).

There was no significant relationship between the serum albumin level, prothrombin time, or the serum total bilirubin level and the brain Gln or Glu signal intensity. In regard to the relationship between plasma free amino acid levels

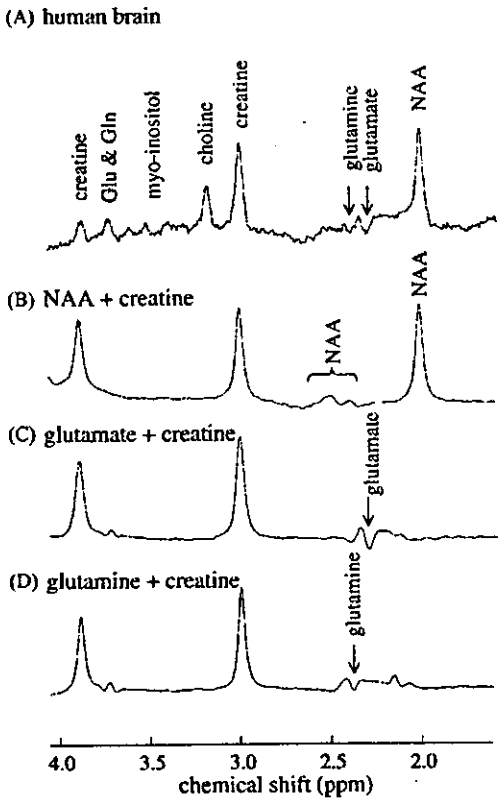

Fig. 2. ^{1}H-MR spectra of the subjects' brains and reference solutions. A 10-Hz filter was used to obtain the spectra of the solutions, so as to compare these with the spectra in vivo. Measurements were conducted by the PRESS method (TR 2000 ms, TE 60 ms, NS 192). Phantom solutions were measured by the PRESS method at ROI with dimensions of 20 mm × 20 mm × 20 mm, TR of 3000 ms, TE of 60 ms and NS of 64. (A) ^{1}H-MR spectra of the brains of patients with liver cirrhosis. An ROI with the dimensions of 20 mm × 20 mm × 20 mm was designated in the occipital lobe gray matter. (B) Reference spectra for quantification of NAA. NAA and creatine were dissolved in phosphate buffer (pH 7.4), to obtain a 5.0 mm solution of each. (C) Reference spectra for quantification of glutamate. Glutamate and creatine were dissolved in phosphate buffer (pH 7.4), to obtain a 5.0 mm solution of each. (D) Reference spectra for quantification of glutamine. Glutamine and creatine were dissolved in phosphate buffer (pH 7.4), to obtain a 5.0 mm solution of each.

and the Gln and Glu signal intensities, it was found that the latter exhibited no significant correlation with the Fischer's ratio, aromatic amino acid (AAA) levels, plasma Glu levels, or plasma Gln levels.

Table 2
MRS findings in liver cirrhosis and controls

	Gln + Glu/*N*-AA (molar ratio)	Gln/*N*-AA (molar ratio)	Glu/*N*-AA (molar ratio)
Contorols (11)	1.046 ± 0.11	0.473 ± 0.08	0.573 ± 0.06
Liver cirrhosis (23)	1.305 ± 0.33[a]	0.658 ± 0.23[a]	0.648 ± 0.15
Child-Pugh classification			
Grade A (2)	1.566 ± 0.40	0.937 ± 0.26	0.629 ± 0.15
Grade B (18)	1.272 ± 0.35	0.620 ± 0.23	0.652 ± 0.17
Grade C (3)	1.330 ± 0.07	0.697 ± 0.12	0.634 ± 0.08

[a] Control vs. liver cirrhosis ($P < 0.05$).

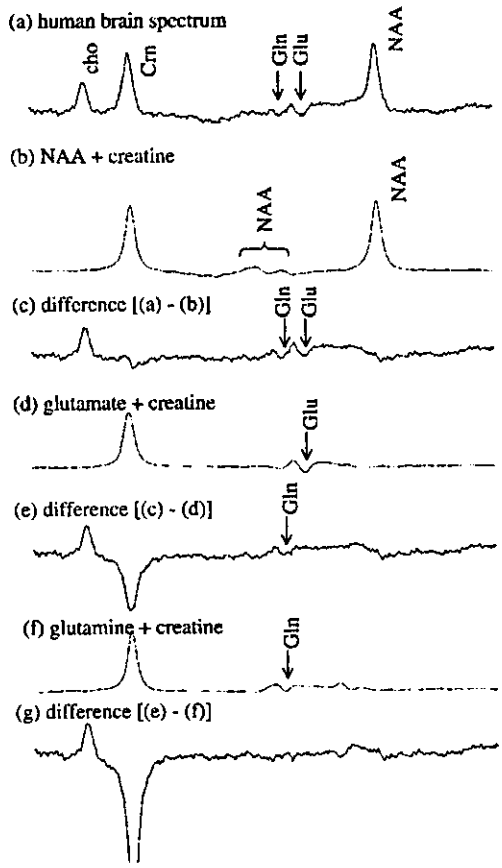

Fig. 3. Method used for quantifying glutamine and glutamate. (a) ^{1}H-MR spectra of the brains of patients with liver cirrhosis; (b) reference spectrum for quantification of NAA; (c) differential spectrum (a)–(b); (d) reference spectrum for quantification of glutamate; (e) differential spectrum (c)–(d); (f) reference spectrum for quantification of glutamine; (g) differential spectrum (e)–(f).

Table 3
Correlation matrix of MR findings and biochemical data

	Gln/*N*-AA		Glu/naa	
	r	P	r	P
B-NH3	0.063	0.778	0.079	0.721
T-Bil	0.276	0.290	0.172	0.516
Albumin	0.191	0.388	0.010	0.971
PT(%)	0.023	0.918	0.097	0.715
Fischer's ratio	0.028	0.941	0.171	0.463
AAA	0.293	0.200	0.005	0.983
Glutamate (blood plasma)	0.300	0.146	0.187	0.423
Glutamine (blood plasma)	−0.120	0.609	0.183	0.432

B-NH3: blood ammonia; PT: prothrombin time; AAA: aromatic amino acids; Fischer's ratio: branched chain amino acids/AAA.

4. Discussion

In a previous study, an increase in the brain Glx signal intensity was demonstrated in patients with liver cirrhosis [3]. The present study shows that this increase is attributable to an increase in the signal intensity of Gln alone.

Hepatic encephalopathy refers to a spectrum of neuropsychiatric abnormalities, including disturbances of consciousness, and occurs in association with severe liver disease, including fulminant hepatic failure and liver cirrhosis. The severity of this condition ranges widely, from mild cases detectable only by quantitative neuropsychiatric functional testing [6], to deep coma with absent response to stimulation.

Recently, close attention has been paid to swelling of astrocytes as a possible cause of hepatic encephalopathy. Astrocytes constitute the only compartment of the brain in which ammonia is detoxified by biosynthesis of Gln. A recent ^{1}H-MRS study revealed that the balance of astrocyte volume is lost in patients with chronic hepatic encephalopathy [2]. When the cells swell up due to elevation in the osmotic pressure, myo-inositol, which is a component of astrocytes and serves as an organic electrolyte [7], is released from the cells. In cases of hepatic encephalopathy, the brain Glx signal intensity, as measured by ^{1}H-MRS, is increased, while that of myo-inositol is markedly decreased. These changes have been reported to be correlated with the severity of coma seen in patients with hepatic encephalopathy [8]. It has been proposed that in cases of hepatic encephalopathy, the Glx levels in the astrocytes rise to degrade the excessive ammonia, resulting in swelling of the astrocytes.

Substances or factors known to cause swelling of astrocytes, besides ammonia, include hyponatremia, several neurotransmitters, TNF-α, and benzodiazepines [9]. It is thought that as the Glx-level rises, myo-inositol is released, and the resultant swelling of astrocytes induces gliopathy (compromised glial cell dysfunction), which is associated with disturbed communication between the astrocytes and neurons.

Previous studies in vitro have shown that the swelling of astrocytes is associated with effects such as activation of MAP-kinase [9], up-regulation of peripheral-type benzodiazepine receptors [10], changes in the protein phosphorylation-Ca^{2+} equilibrium [11], and changes of cellular pH [12]. It has also been shown experimentally that swelling of astrocytes alkalizes intracellular granules and thus affects the functions of cell receptors [3]. In our previous ^{1}H-MRS study of patients of liver cirrhosis who did not have any apparent hepatic encephalopathy, we found that the myo-inositol signal intensity was low and the Glx level was high, similar to the findings in cases with hepatic encephalopathy, and that these parameters were correlated with the severity of the liver disease. This suggests that brain metabolism is already affected in cases of liver cirrhosis even before the appearance of signs of hepatic encephalopathy [13]. The concentrations of brain GLN and GLU were evaluated using biopsied human brain tissue. Rothman et al. [4] reported that the gray matter concentration of GLU was 7.8 ± 4.0 μmol/g and that of GLN was 4.1 ± 2.0 μmol/g. They reported that the gray matter concentrations of GLU were larger than that of GLN in healthy controls. We also clarified that the intensity of GLU is larger than that of GLN in healthy controls. In the cirrhotic group, however, the intensity of GLU is smaller than that of GLN and it is estimated that there were abnormalities in the metabolic pathway of GLU and GLN in cirrhotic patients.

With the conventional-technique of MRS, for which a 1.5-T MR device is used, while an increase in the Glx signal intensity in the brain could be detected, increased Gln levels in situ could not be discerned (a hypothesis proposed by Haussinger et al. [12]). In the present study, we conducted MRS using a high-magnetic field and high-resolution MR equipment, and analyzed the data for known concentration levels of *N*-AA, Crn, Gln, and Glu by a new data processing method, and adequate echo time. This study demonstrated that the increased Glx signal intensity in the brain previously observed in cases of liver cirrhosis is attributable to an increase in the Gln alone. We have thus endorsed the validity of the theory of abnormal brain metabolism in situ.

In the present study, the Gln signal intensity did not exhibit any correlation with the venous blood ammonia level or indeed any of the other blood biochemical parameters tested. The blood samples for quantification of ammonia were collected in the early morning before breakfast on the day on which MRS was performed, and there was no significant time lag between the blood sampling and MRS. However, it remains unknown whether or not the blood ammonia levels have any immediate effects on the Gln level in the glial cells. It also seems likely that chronic exposure of cells to blood ammonia may be involved in the increased brain Gln level. This is, however, a still untested hypothesis. In the present study, venous blood was used for measuring the blood ammonia level. However, the venous blood ammonia level may not always be correlated with the brain Gln level, when one considers the removal of ammonia in the peripheral muscle. Therefore, it may be necessary to study the relationship between the arterial blood ammonia level and the brain Gln level. In the present study, the mean age differed between the control group and the liver cirrhosis group. However, in the cirrhosis group, no effects of aging on the Gln or Glu signal intensity were noted (data not shown). There were several reports about the effect of age on brain metabolite concentrations. Chang et al. [14] reported that concentrations of creatine, choline and myo-inositol increased with age. In contrast, there was a relatively stable concentration of Glx measured by a 1.5-T MRS in 36 normal healthy volunteers (19–78 years).

It, therefore, appears unlikely that the difference in age between the two groups served as a significant confounding factor in the study. According to the previous reports [2,15,16], abnormal metabolism of Glx and myo-inositol were detected in various brain regions (basal ganglia, temporal lobe, occipital lobe, etc.) in patients with liver cirrhosis complicated by hepatic encephalopathy. In the present study, only one ROI was designated in the occipital lobe when performing MRS. It would therefore be desirable to examine other areas of the brain also for the presence or absence of similar changes.

It has recently been reported that abnormal metabolism of Glx and myo-inositol in the brain can be reversed by treatment. Haseler et al. [17] examined the changes in the Glx and myo-inositol levels before and after administration of a synthetic bisaccharide (lactulose, 60 ml/day for 7 days), in comparison with those in an untreated group. Their study revealed a 15% decrease in the Glx and a 29% increase in the myo-inositol levels in the lactulose-treated group, while no such change was observed in the untreated group. It has also been reported that these abnormalities could be reduced by liver transplantation [18]. These findings suggest that measurements using MRS may also be useful for evaluating the responses to treatment. Measurements using MRS may also be useful for the early detection of the subclinical hepatic encephalopathy, which can only be detected by quantitative neuropsychiatric function tests. From our data, the main reason for the increase of Glx intensity was the increase of GLN in the brain. However, the level of GLU also increased following the increase of the GLN intensity in patients with liver cirrhosis. Thus, the clinical significance of the separation of Glx is to be able to evaluate the effect of the treatment or the clinical condition for cirrhotics from the balance of GLU and GLN intensities.

In brief, high-magnetic-field MRS revealed that the increase in the total brain Glu and Gln levels, previously observed as a sign of abnormal brain metabolism associated with liver cirrhosis, is attributable to an increase in the level of Gln alone. The study results also suggested that the brain Gln level shows no significant correlation with the venous blood ammonia level. In the future, it would be desirable to conduct a chronological study of the changes in the Gln levels in the brain in cases of liver cirrhosis under follow-up, with or without active treatment.

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Development of quantitative neuropsychological tests for diagnosis of subclinical hepatic encephalopathy in liver cirrhosis patients and establishment of diagnostic criteria—multicenter collaborative study in Japanese

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Abstract

At present, there are no generally accepted diagnostic criteria or methods for subclinical hepatic encephalopathy (SHE) associated with liver cirrhosis. We therefore developed an easily conducted computer-aided quantitative neuropsychiatric function test system for use in routine medical practice. We established normal values in healthy Japanese subjects and determined differences between healthy persons and liver cirrhosis patients without clinical encephalopathy in a multi-center clinical trial. The test system consists of eight tests: number connection tests A and B, a figure position test, a digit symbol test, a block design test, and reaction time tests A, B and C. The test results were affected by age, but not by gender or facility. No learning effect was noted. The results were therefore reported by 5-year quartile ranges and differences were evaluated between 542 healthy subjects and 292 cirrhotic patients. When the cut-off value was set at the 10th/90th percentile of the results in healthy subjects, the results of each of the 8 tests were abnormal in about 25% of cirrhotic patients, and at least 1 of the 8 tests gave values greater than the 10th/90th percentile cut-off value in 58.2% of the 292 liver cirrhosis patients. SHE patients were thought to be

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included in these 58.2% of patients. The developed test makes it possible to quantitatively assess neuropsychiatric function, and the results obtained can be used as a basis for the diagnosis of SHE.

Keywords: Liver cirrhosis; Subclinical encephalopathy; Neuropsychological tests; Computer-aided test system

1. Introduction

Subclinical hepatic encephalopathy (SHE), which gives abnormal results to sensitive quantitative neuropsychiatric function tests without showing any abnormal physical findings, is seen in 30–84% of liver cirrhosis patients. The importance of SHE is being increasingly recognized as it may interfere with the activities of daily living [1–14]. Recently, Ferenci et al. [15] presented new proposals regarding the definition and classification of hepatic encephalopathy and the degree of associated coma. They named SHE "minimal hepatic encephalopathy (mHE)" as low-grade hepatic encephalopathy and emphasized its importance.

SHE includes pre-clinical hepatic encephalopathy and is associated with no clinical symptoms or signs of the degree of coma being grade I or 0 according to the conventional grading system. There are no standard diagnostic criteria, and it is diagnosed at individual medical institutions by their own diagnostic methods based on the paper–pencil test and electrophysiological tests, including EEG [3]. However, none of these tests is satisfactory in terms of ease in operation or reproducibility, and the need for development of a simpler quantitative test has been pointed out.

Since SHE is associated with a reduction in performance cognition, a combination of multiple neuropsychiatric function tests designed to assess performance cognition is used for its diagnosis. Recently, Kircheis et al. reported that the critical flicker-frequency (CFF) test alone makes it possible to easily diagnose SHE in liver cirrhosis [16]. However, because results of the CFF are normal in about 40% of SHE patients diagnosed by conventional neuropsychiatric function tests, the CFF has limitations as a diagnostic method although it is a simple, easy-to-use method [17].

We developed a computer-aided simple neuropsychiatric test system consisting of eight tests that can be easily conducted on outpatients using a touch panel. In order to evaluate the utility of this test system, we first conducted a pilot study in healthy subjects and identified factors that influence the results of the test. This was followed by a multiple cooperative study designed to establish standards for neuropsychiatric functions in healthy subjects and liver cirrhosis patients. The criteria for the SHE diagnosis were established based on the findings obtained.

2. Materials and methods

The test system consisted of the following eight tests and was designed to assess psychomotor, attention, memory and special functions: number connection tests A and B (NCT-A and B), figure position test (FPT), digit symbol test (DST), block design test (BDT), and reaction time tests A, B and C (RTT-A, B and C) [10–13,18,19]. The system was simplified so that two-dimensional operations using a computer were possible. All tests can be completed in about 20 min, including the time needed for practice and operation guide.

2.1. Neuropsychological (NP) test system

Software was developed by Otsuka Pharmaceutical Co., Ltd., Kokuyo Co., Ltd., and ISB Co., Ltd. Hardware consisted of a personal computer (OS: window3.1, ThinkPad 365X, IBM) and a 33 cm size touch panel connected to the PC (GUNZE Access Vision AV4833FT, Gunze Ltd., Tokyo, Japan).

2.2. Number connection tests (NCTs)

Two tests were conducted, NCT-A, in which the time needed to serially connect figures from 1 to 20 on the touch panel was determined (time limit: 60 s), and NCT-B, in which the time needed to connect figures from 1 to 10 and 10 Japanese characters was determined (time limit: 180 s).

2.3. Figure position test (FPT)

Subjects were asked to remember the shape and position of 2–4 figures displayed on the panel for 15 s. They were asked to return each figure to its original position after randomly moving them on the panel, and the time needed to complete this task was determined (time limit: 90 s). This test was developed after the tactual performance test.

2.4. Digit symbol test (DST)

Nine different symbols were displayed on the panel in 60 s, and subjects were asked to select a digit corresponding to each symbol on the panel. The number of correct answers was determined (maximum number of questions: 40).

2.5. Block design test (BDT)

Six different cards were displayed on the panel, and the time needed to complete the same design as that displayed on the panel was determined (time limit: 60 s).

2.6. Reaction time tests (RTTs)

Three types of RTT were conducted: RTT-A, in which the reaction time needed by subjects to press an enter key after the color of a circle on the panel changed from white to red; RTT-B, in which subjects were asked to press the enter key when the color changed from white, blue or yellow to red; and RTT-C, in which subjects were asked to press the enter key when a combination of white, blue and yellow changed to that of yellow and red.

2.7. Construction of the NP test system (pilot study)

A pilot study was conducted at three university hospitals in Japan between June 1996 and March 1997. One hundred and twelve healthy subjects under no medical treatment, who were almost evenly divided into 3 age brackets (40–49, 50–59, and 60–69), were enrolled. Informed consent was obtained from all volunteers after providing thorough information on the objectives and method of the study. One of the objectives of this pilot study was to determine if the test system could be operated without any problem. In addition, the correlation between the test results and factors that were thought to influence them (age, gender and facility) was evaluated. The tests were conducted three times by repeating the tests 1 and 7 days after the first tests in order to determine the presence or absence of learning effects (effects of familiarization with the tests). The tests were always conducted in an independent test room between 10 a.m. and 4 p.m. in order to minimize the environmental effects.

2.8. Establishment of normal ranges and estimation of cut-off values

Cut-off values were estimated in order to establish the normal ranges and extract a population showing abnormal neuropsychological function test results. The following number of subjects, aged between 40 and 69, were enrolled at 14 university hospitals between June 1996 and March 1997: 328 patients with hepatic cirrhosis and 550 healthy subjects (not included in the 112 enrolled in the pilot study). Healthy subjects under no medical treatment and without hepatic disease were enrolled. Hepatic cirrhosis was diagnosed based on liver biopsy, imaging diagnosis, or clinical diagnosis combining objective findings and hematological tests. Those with any of the following were excluded: previous neurological focal episode or other neurologic illness, history of psychiatric illness, history of consumption of psychotropic drugs, and clinical hepatic encephalopathy (≤grade II). Those under treatment with lactulose, poorly absorbed antibiotics or branched-chain amino acid to deal with hyperammonemia were enrolled if they could tolerate the tests.

Informed consent was obtained from all subjects after thoroughly explaining the objectives, methods and other relevant details of the study.

3. Statistical analysis

In data analyses, subjects were grouped by age at 5-year intervals since findings obtained in the pilot study showed that age affects analytical results when they are grouped at 10-year intervals. Subjects in each age bracket were classified into the healthy subject group and the cirrhotic patient group. Differences between the two groups were shown by the quartile range. The upper and lower 10, 20 and 30 percentiles were estimated for each test item in healthy subjects in order to evaluate cut-off values as percentages of upper and lower test values obtained in cirrhotic patients.

Test values were handled as mentioned below. Namely, the actual time needed to complete the task was reported in NCT-A and NCT-B. The number of correct answers were reported in the DST. The mean effective test time, i.e. the test time spent for correct operations divided by the number of correct operations, was reported for the FPT, BDT, RTT-A, -B and -C.

A subcommittee was created to establish a data handling policy. It excluded all "outliers due to apparent errors in operation" (data from subjects who did not completely understand the operation procedures and those who undertook the tests under apparently abnormal physical conditions due to night duties or a lack of sleep) from analysis. All data from subjects younger than 40 and those aged 70 or older were excluded from analysis as a deviation from the protocol.

4. Results

4.1. Construction of the NP test system

Results obtained from the 112 healthy subjects showed no significant differences in the test results by gender or facility or any significant learning effects on the test results due to repetition of the tests by each subject. However, the test results were found to be affected by age as is typically shown by the results of NCT-B shown in Fig. 1. As is evident from this figure, the test time clearly increased with age in healthy subjects when they were divided into groups at 10-year intervals.

4.2. Establishment of normal ranges and estimation of cut-off values

The NP test system was conducted in a total of 834 subjects, including 542 healthy subjects and 292 liver cirrhosis patients. However, 8 healthy subjects and 37 liver cirrhosis patients were excluded due to violations of the protocol in terms of the age. The backgrounds of healthy subjects and cirrhotic patients are shown in Table 1. Relatively old males were predominant among cirrhotic patients ($P < 0.0001$). The severity of liver cirrhosis was mild in most patients according to the child classification system, and the cause was viral in

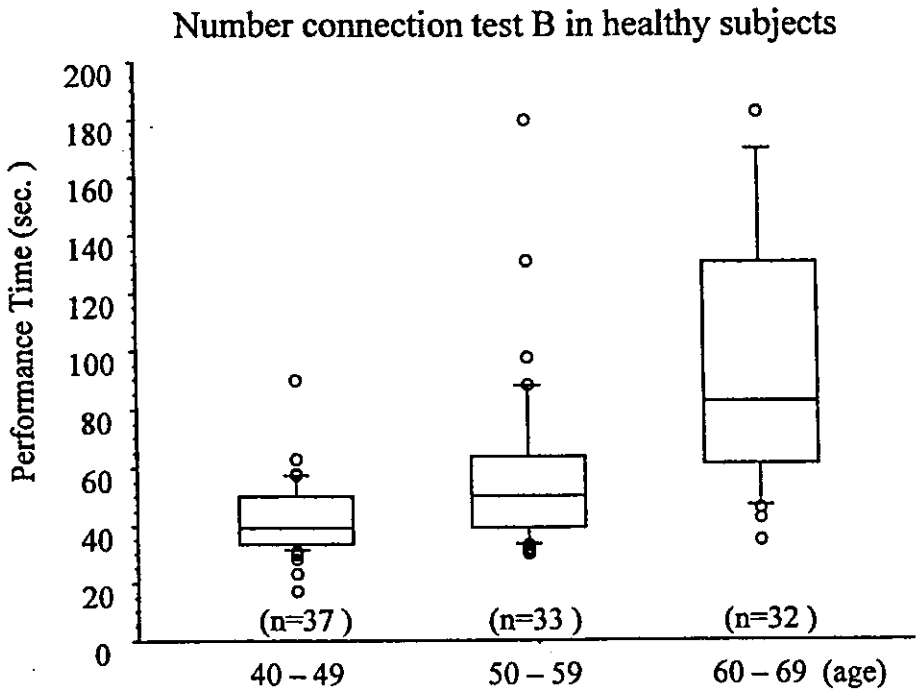

Fig. 1. Results of number connection test B in healthy subjects are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of boxes represent the first and third quartiles.

the great majority of patients (81%). Overall, 131 cirrhotic patients (45.0%) were receiving at least 1 drug for hyperammonemia: 74 (25.3%) lactulose, 107 (36.6%) BCAA, and 9 (3.1%) poorly absorbed antibiotics.

The number of subjects analyzed with respect to each test item ranged between 517 and 542 healthy subjects (95.4–100%) and between 273 and 290 cirrhotic patients (93.5–99.3%). Because the pilot study showed that the test re-

Table 1
Clinical and laboratory characteristics of cirrhotic patients and healthy subjects

Items	Cirrhotic patients, *n* (%)	Healthy subjects, *n* (%)	*P*-value
Total	292	542	
Sex			
Male	191 (65.4)	275 (50.7)	
Female	101 (34.6)	267 (49.3)	<0.0001[a]
Age (years)			
40–44	14 (4.8)	98 (18.1)	
45–49	27 (9.2)	112 (20.7)	
50–54	33 (11.3)	109 (20.1)	
55–59	54 (18.5)	99 (18.3)	
60–64	82 (28.1)	79 (14.6)	
65–69	82 (28.1)	45 (8.3)	
Mean ± S.D.	59.0 ± 7.3	52.6 ± 7.9	<0.0001[b]
T-bilirubin (mg/dL) (mean ± S.D.)	1.6 ± 1.9		
ALT (IU/L) (mean ± S.D.)	70.1 ± 50.0		
Child classification			
A	130 (44.5)		
B	134 (45.9)		
C	28 (9.6)		
Etiology			
Virus	237 (82.2)		
Alcohol	39 (13.4)		
Others	16 (5.5)		

[a] Chi-square test.
[b] Student's *t*-test.

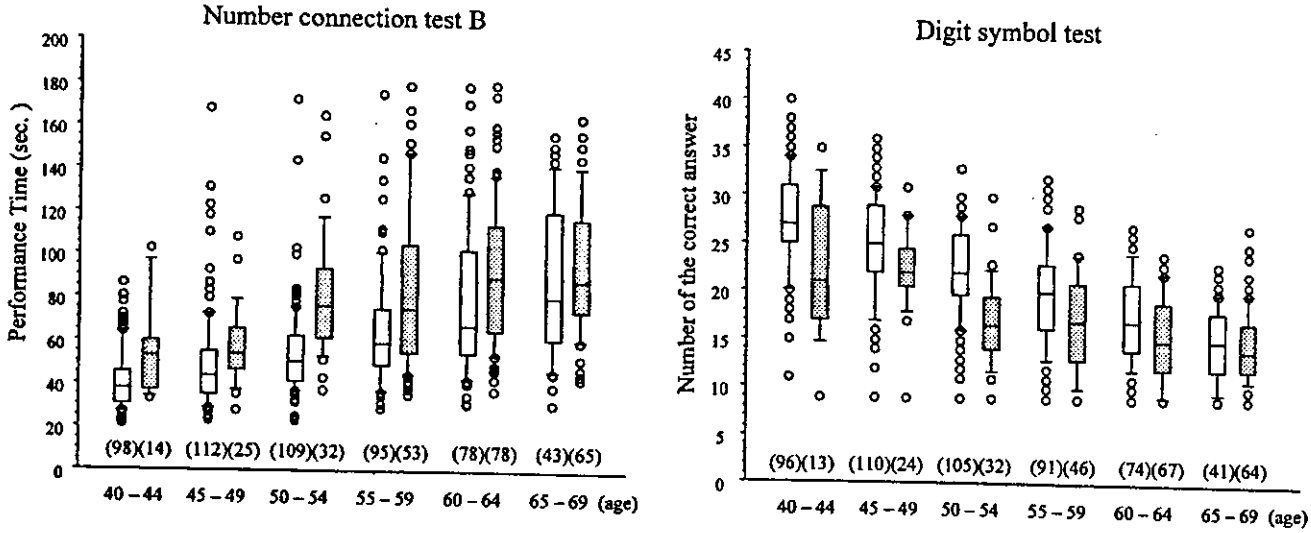

Fig. 2. Results of number connection test B and digit symbol test in healthy subjects and cirrhotic patients are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of boxes represent the first and third quartiles: (□) healthy subjects; (▨) cirrhotic patients (*n*).

sults and their standard deviations become increasingly variable with age (data not shown), test data were compiled at 5-year age intervals and shown by the quartile range (box and whisker plots). Results of NCT-B and DST are shown in Fig. 2 as typical results obtained. Test results obtained from both healthy subjects and cirrhotic patients showed clear age-related changes, with the test time increasing and the number of correct answers decreasing with age. The test time increased and the number of correct answers decreased in cirrhotic patients compared to healthy subjects regardless of age up to 65. Among those aged 65 or older, however, there were no significant differences between healthy subjects and cirrhotic patients. Results of all tests showed similar effects of aging as well as the differences between healthy subjects and cirrhotic patients.

Data were missing from 11 healthy subjects (2%) for DST and 1 healthy volunteer (0.2%) for all of the 7 other tests, while among hepatic cirrhosis patients, 7 (2.4%) gave no data for NCT-A, 25 (8.6%) for NCT-B, 19 (6.5%) for FPT, 46 (15.8%) for DST, 6 (2%) for BDT, and 11 (3.8%) for RT-A, B and C. DST data were missing from the largest number of subjects in both the healthy volunteer and hepatic cirrhosis patient groups.

Cut-off values were determined based on the upper and lower 10th, 20th and 30th percentiles, which are regarded as outliers in healthy subjects, and were set at the upper and lower 10th percentile due to great variability because of the effects of aging on standard deviations. The percentage of liver cirrhosis patients who were regarded as giving abnormal values based on the 10th/90th percentile cut-off value in healthy subjects and the test results obtained at 5-year intervals ranged between 9 and 47% for NCT-B and 21.0% overall and between 2 and 44% for DST and 18.7% overall. The percentage of liver cirrhosis patients who gave abnormal values in the eight tests varied according to the age bracket and ranged between 10 and 25% (Table 2). Of the 292 cirrhotic patients, 170 (58.2%) showed deviations from the 10th percentile cut-off value with respect to at least 1 test item.

5. Discussion

We developed a computer-aided quantitative neuropsychological test system in order to facilitate the diagnosis of SHE associated with liver cirrhosis.

Results obtained with this test system showed apparent differences between healthy subjects and liver cirrhosis patients without clinical hepatic encephalopathy (grade 0 or 1). Both healthy subjects and liver cirrhosis patients showed increases in the test time and decreases in the number of correct answers with age, and it was evident that the results of the neuropsychological tests need to be analyzed at 5-year age intervals. The results of the present study thus indicated that major shortfalls of the this test method are the great variability of the test results in both healthy subjects and liver cirrhosis patients and increases in the test time with age even in healthy subjects. The test method seemed to be able to differentiate healthy subjects from liver cirrhosis patients and attribute the difference to subclinical encephalopathy if they are younger than 65. In older patients, however, discrimination seemed to be impossible and the test method seemed to have its own limitation.

Although the tests were not repeated in the same patients, it was thought that they could be properly conducted in liver cirrhosis patients as well as in healthy subjects because data were missing from only a few patients.

Among various factors with possible effects on the test results, blood ammonia levels affected the test results, but the severity of hepatic dysfunction did not. Test results obtained in the present study were not significantly different