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The role of Kupffer cells in complement activation in D-Galactosamine/lipopolysaccharide-induced hepatic injury of rats.

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Abstract

To investigate the role of Kupffer cells in complement activation, we used a rat model of acute hepatic injury induced by D-Galactosamine (GalN) and lipopolysaccharide (LPS). In in vivo study, minimal histological changes were observed after i.p. GalN (200 mg/kg) single administration. Complement hemolytic activity (CH 50) decreased to 70% of its initial value 2-3 h after i.p. LPS (1.5 mg/kg) single administration. Massive hepatic necrosis was induced by simultaneous administration of GalN and LPS. After 2-3 h, CH 50 decreased to 70% of its initial value, and deposition of C3 fluorescence (C3) was observed in Kupffer cells. After 4 h, GPT was greatly increased (1286 +/- 240 IU/l), CH 50 was further reduced, and C3 was observed on hepatocyte membranes and in the cytosol. In in vitro study, we used hepatocyte cultures and co-cultures of hepatocytes and Kupffer cells to investigate the participation of GalN, LPS, complement, and Kupffer cells in hepatic cell necrosis. We found no increase of LDH (% leakage) when LPS and complement were added to the medium (22.7 +/- 5.7%). A moderate increase was observed with the addition of GalN (33.2 +/- 2.6%). A remarkable increase was observed only with the addition of GalN, LPS, and complement to the co-culture (50.0 +/- 8.8%). These results suggest that Kupffer cells activated by LPS are very important in promoting acute hepatic injury by complement.

The Role of Kupffer Cells in Complement Activation in D-Galactosamine/Lipopolysaccharide-Induced Hapatic Injury of Rats

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To investigate the role of Kupffer cells in complement activation, we used a rat model of acute hepatic injury induced by D-Galatosamine (GalN) and lipopolysaccharide (LPS). In *in vivo* study, minimal histological changes were observed after i. p. GalN (200mg/kg) single administration. Complement hemolytic activity (CH 50) decreased to 70 % of its initial value 2–3 h after i. p. LPS (1.5mg/kg) single administration. Massive hepatic necrosis was induced by simultaneous administration of GalN and LPS. After 2–3 h, CH 50 decreased to 70 % of its initial value, and deposition of C3 fluorescence (C3) was observed in Kupffer cells. After 4 h, GPT was greatly increased (1286 ± 240 IU/l), CH 50 was further reduced, and C3 was observed on hepatocyte membranes and in the cytosol. In *in vitro* study, we used hepatocyte cultures and co-cultures of hepatocytes and Kupffer cells to investigate the participation of GalN, LPS, complement, and Kupffer cells in hepatic cell necrosis. We found no increase of LDH (% leakage) when LPS and complement were added to the medium (22.7 ± 5.7%). A moderate increase was observed with the addition of GalN (33.2 ± 2.6 %). A remarkable increase was observed only with the addition of GalN, LPS, and complement to the co-culture (50.0 ± 8.8%). These results suggest that Kupffer cells activated by LPS are very important in promoting acute hepatic injury by complement.

Key words : D-Galactosamine, complement, lipopolysaccharide, Kupffer cell, acute hepatic injury

The liver is known to be a complement production site (1); in liver disease it is difficult to determine whether reduced CH 50 is induced by decreased production (2) or by increased consumption of complement (3). In fulminant hepatitis Mackenjee has reported that the most important index correlating with the severity of clinical disease was serum C3 level, and that the proth-

rombin index was less sensitive in differentiating serious from mild illness (4). Kondo *et al.* reported that the decrease in CH 50 appeared before the onset of encephalopathy in fulminant hepatitis. They discussed three possible causes of the decrease in CH 50; decreased production in the liver, activation by immune complex and endotoxin subsequently inducing hepatic injury, and activation by necrotic tissue and cells of the liver (5). The authors of another study classified

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acute viral hepatitis into three forms; a typical classical form, a severe form, and fulminant hepatitis (6). They found that prothrombin time was useful for differentiating a typical from a severe form and fulminant hepatitis, and that CH 50 was useful for differentiating fulminant hapatitis from a typical and a severe form.

Experimentally, acute injury induced by GalN has commonly been used for determining the various possible mechanisms underlying acute hepatic injury. Decker and Keppler stressed the importance of the metabolic events that comprise the biochemical lesion of the hepatic injury (7). Other aspects of acute hepatic injury induced by GalN have been investigated, *e. g.*, membrane injury (8), endotoxemia (9, 10), the role of Kupffer cells (11, 12), and complement participation (13, 14). Recently further investigations have been made to determine the immunological factors such as cytokines (15) or T lymphocytes (16).

The participation of complement in this hepatic injury has been investigated in several studies; complement deficient mice were shown to develop no GalN hepatitis (10), the time course of CH 50 was reduced after GalN administration, C3 fluorescence was observed on hapatocytes (13), and hepatic injury was reduced by the addition of a complement inhibitor (14). However, to our knowledge, there have been no studies of the relationship between Kupffer cells and complement in acute GalN-induced hepatic injury. In this study, we carried out *in vivo* and *in vitro* investigations of the role of Kupffer cells in relation to complement in acute hepatic injury induced by GalN and LPS.

Materials and Methods

In vivo. Male Sprague-Dawley (SD) rats (Charles River Japan, Kanagawa, Japan), weighing 180 to 220 g, were maintained under standard laboratory conditions, with chow diet and water available *ad libitum*. GalN (Sigma Chemical Company, St Louis, MO, USA) was

diluted with physiological saline to a concentration of 100 mg per ml and neutralized with 1 N sodium hydroxide to pH 7.4. LPS (E. Coli 0111 B 4, Difco, Detroit, MI, USA) was diluted with physiological saline to a concentration of 0.5mg per ml.

The rats were divided into four groups; the first group was given a single intra peritoneal (i. p.) injection of GalN 200 mg/kg body weight (n = 6), the second group received a single i. p. injection of LPS 1.5mg/kg body weight (n = 6). The third group was given i. p. injections of GalN and LPS simultaneously, at the same doses as those in the single injections (GalN/LPS)(n = 12). The fourth group was injected with an equal volume of physiological saline i. p. to serve as histological controls.

Blood samples were obtained from the tail vein or inferior vena cava before, and 1, 2, 3, 4, 6, 8, 10, and 12 h after treatment. GPT levels in serum were measured by the pyruvate oxidase method (Determiner GPT 755^R, Kyowa Medics, Tokyo, Japan). CH 50 was used for evaluating the activity of complement and was measured by a one-point method (17), using sensitized sheep erythrocytes (EA mono-CH 50^R, Ishizu Pharmaceutical Co., Ltd., Osaka, Japan). The grade of hemolysis was read as the percent hemolysis compared to that in the serum before treatment, which was set as 100 %.

Histological examination was performed by light microscopy. The specimens obtained at each time interval were fixed in 10 % buffered formalin. Sections were stained with hematoxylin and eosin (H-E) and periodic acid Schiff reaction with prior diastase digestion (d-PAS). Immunohistological examination of the tissue localization of C3 was according to Tsuji's method (18). The specimens were removed and promptly deep frozen, and they were fixed in saturated ammonium sulfate in 2 % paraformaldehyde solution. They were stained with fluorescein conjugated anti-rat C3 antibody (Organon Teknica Corp., West Chester, PA, USA) by a direct immunofluorescent method.

In vitro. Hepatocytes were prepared from male SD rats (weighing 180 to 220 g) by a collagenase perfusion method described by Seglen (19). The liver was perfused *in situ* via the portal vein with 200 ml of Ca^{2+} free Hanks' balanced salt solution (HBSS), followed by recirculating perfusion with 100 ml of freshly prepared HBSS containing 0.05 % collagenase and 4 mM CaCl$_2$ for 10 min. After perfusion the liver was removed and minced in Eagle's MEM (Gibco Laboratories, NY, USA). The resultant suspension was filtered through four-ply gauze

and centrifuged at 50 g for 1 min. The pellet was used for the hapatocyte preparation, and the supernatant was used for the Kupffer cell preparation. The pellet was resuspended in fresh medium and centrifuged, after which the cells were washed twice at 25 g for 2 min. In the last washing procedure, Eagle's MEM containing 10 % fetal bovine serum, 0.1μM insulin, streptomycin (50μg/ml), and penicillin (50U/ml)(MEM), was used. Next, Kupffer cells were prepared from the supernatant, according to Knook (20). The supernatant was centrifuged at 60 g, 80 g, and 110 g for 1 min each time, and the last supernatant (non-parenchymal cell suspension) was fractionated in a SCR 20 AB centrifuge fitted with a SRR 6 YA elutriator rotor (Hitachi Koki Co., Ltd., Ibaraki, Japan). The non-parenchymal cell suspension (100ml) was loaded into the mixing chamber at a flow rate of 14 ml/min. The rotor was spun at a constant speed of 2,500 rpm at 4℃ and MEM was added to the chamber. The pump flow rate was adjusted to 14 ml/min for 10 min, 20ml/min for 5 min, and 38ml/min for 2.5 min. The last fraction was selected to provide the Kupffer cell-enriched fraction. The cell suspension was divided into two 50 ml polypropylene centrifuge tubes (Nunc, Roskilde, Denmark), and centrifuged at 450 g for 5 min. The pellet was resuspended in MEM of the same formula as that used for the hepatocyte preparation.

Hepatocytes were adjusted to a density of 1×10^6/ ml, and Kupffer cells to a density of 1×10^5/ml, with MEM. The dish used (Falcon 3001, Becton Dickinson Labware, NJ, USA) was coated with swine-ligament collagen (cellmatrix Type 1-P^R, Nitta Geratin Co., Osaka, Japan). The hepatocyte culture (H group) consisted of 1 ml of the hepatocyte medium and 1 ml MEM in a dish. The co-culture (HK group) consisted of 1 ml each of the hepatocyte and the Kupffer cell medium in a dish. The cultures were incubated at 37 ℃ in a humidified atmosphere containing 5 % CO_2 and air. The Kupffer cell culture, consisting of 1 ml of Kupffer cell medium and 1 ml of MEM in a dish, served as the control for LDH release from all Kupffer cells in a dish. Cell viability was assessed by the trypan blue exclusion test. The purity of Kupffer cells prepared by this method was assessed by their phagocytic action of latex particles ($\phi = 5.4\mu$m Takeda Chemical Ind., Ltd., Tokyo, Japan).

Experimental schedule. The first medium change was made after 3 h to fresh MEM, and non-adherent and dead cells were removed. The next medium change was done after 18 h to fresh MEM (group H 1, HK 1) and to fresh MEM containing 0.4 mM GalN at a neutral pH

(groups H 2, H 3, HK 2, HK 3, and HK 4 described below). The final medium change was 24 h after planting to fresh MEM containing 0.4 m M GalN and/or LPS 10 ug/ml and/or 200 μl/dish of serum which was obtained from the sera of five normal rats for complement containing medium. The same serum was heat-inactivated at 56 ℃ for 30 min to obtain complement-free medium. Each dish was divided into seven groups according to the constituents, namely, H 1; inactivated serum, H 2; GalN and inactivated serum, H 3; GalN, LPS and complement, HK 1; LPS and complement, HK 2; GalN, LPS and inactivated serum, HK 3; GalN and complement, and HK 4; GalN, LPS and complement. Every group was examined with ten dishes. The *in vitro* hepatocytotoxicity experiment was begun 24 h after planting, and a sample (100μl) was obtained for the starting samples from each dish and stored at -20℃ for 24 h.

Assessment of hepatocytotoxicity. Twenty-four hours after the final medium change, a sample (100 μl) was obtained from each dish. Then 1 % Triton X-100 (Ishizu Pharmaceutical Co., Ltd., Osaka, Japan) was added to the medium, and all viable cells were lysed. LDH leakage was assessed as an indicator of cell death. The LDH activity was measured by the formazan method (Sanassey LDH^R, Sankou Pure Chemical Co., Ltd., Tokyo, Japan). Cytotoxicity was expressed as the proportion of LDH leakage into the sample medium compared to the total amount of LDH present in the Triton lysate, subtracting the LDH of starting sample respectively. With regard to the HK groups, the LDH from all Kupffer cells in a co-culture dish was obtained from the Kupffer cell culture medium after the addition of 1 % Triton X-100. The % leakage of LDH was calculated as:

$$\% \text{ leakage} = \frac{\text{Sample LDH} - \text{Starting sample LDH}}{\text{Total LDH} - \text{Starting sample LDH} (- \text{all Kupffer cells LDH})}$$

Data were expressed as mean ± SD, and statistical analysis was performed using Student's *t*-test. A probability of less than 0.05 was considered to be significant.

Results

In Vivo

GalN administration. Serum GPT level began to increse moderately 6 h after administration of GalN, however, between 6 and 12 h the increase was not so pronounced as at 6 h. Serum
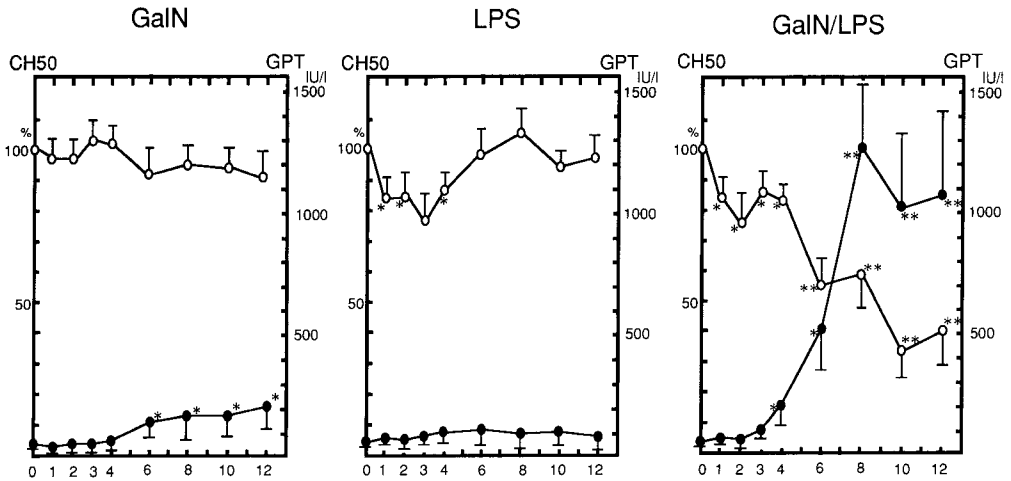
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Fig. 1   Time course of CH 50 and GPT after administration of GalN (D-Galactosamine alone), LPS (Lipopolysaccharide alone), GalN/LPS (GalN and LPS simultaneously). Open circles: CH 50, closed circles: GPT Significantly different from pre treatment. (* p < 0.05, * * p < 0.01).
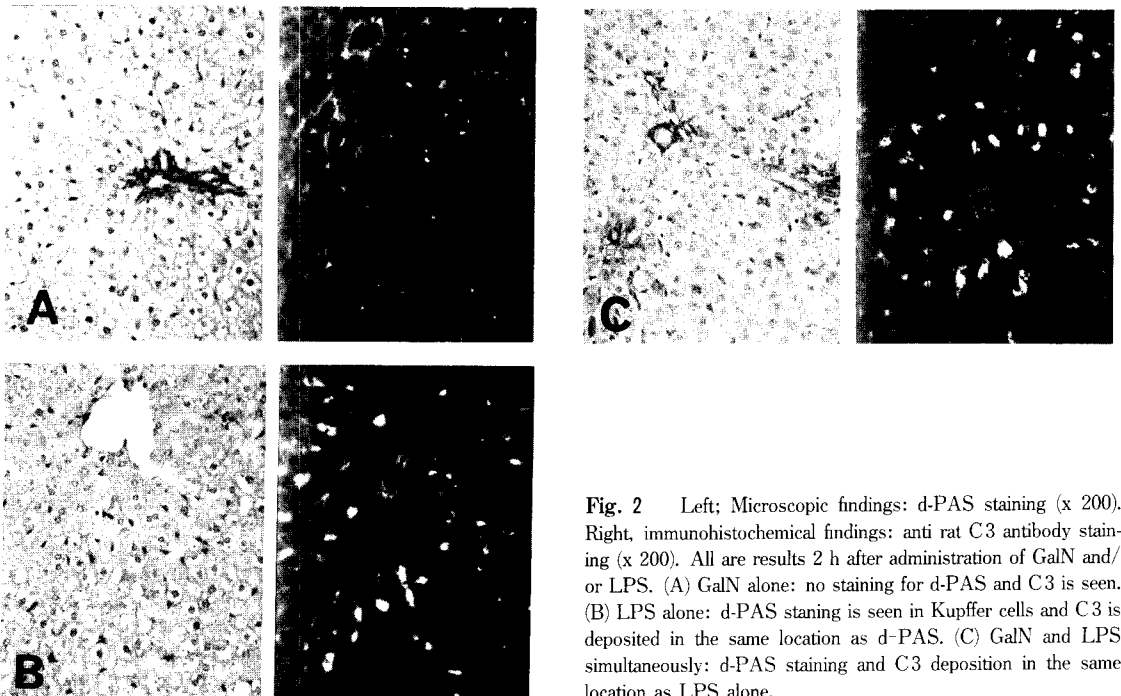

Fig. 2   Left; Microscopic findings: d-PAS staining (x 200). Right, immunohistochemical findings: anti rat C3 antibody staining (x 200). All are results 2 h after administration of GalN and/or LPS. (A) GalN alone: no staining for d-PAS and C3 is seen. (B) LPS alone: d-PAS staning is seen in Kupffer cells and C3 is deposited in the same location as d-PAS. (C) GalN and LPS simultaneously: d-PAS staining and C3 deposition in the same location as LPS alone.

CH 50 level was not found to change significantly (Fig. 1). C3 fluorescence was not observed at any location in the lobule for 12 h. With H-E stainig, inflammatory mononuclear cells slightly infiltrated the lobule after 6 h, and scattered focal necrosis was observed after 10-12 h. With d-PAS staining, no positive cells were found until 10 h, but after 10 h slight staining was
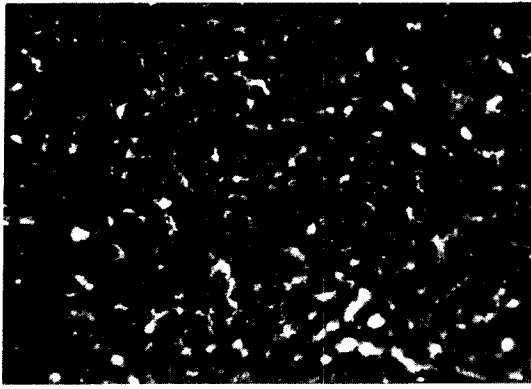

Fig. 3 Four hours after administration of GalN and LPS immunohistochemical findings showing C3 deposition in Kupffer cells and on hepatocyte membrane (x 200).

detected in the focal necrosis (Fig. 2 A).

LPS administration. Serum GPT level was not elevated for 12 h. After 1 to 4 h serum CH 50 level decreased to 70 % - 80 % of the initial value, from 6 h returning to the initial value (Fig. 1). C3 fluorescence was observed after 1 h in sinusoidal lining cells, particularly in Kupffer cells, and continued to be observed in the same location, but not in any other location up to 12 h. With H-E staining, no hepatocellular necrosis was observed for 12 h. With d-PAS staining, positive cells were observed after 2 or 3 h in sinusoidal lining cells, and a few positive cells in the same location continued to be observed up to 12 h (Fig. 2 B).

Simultaneous GalN and LPS administration. Serum GPT level began to increase after 4 h, increasing markedly after 6 to 8 h. Some rats died after 8 h; at the end of the experiment mortality was 7/12. After 1 to 4 h serum CH 50 level decreased to 70 % -80 % of the initial value, this change being similar to that after LPS administration. However, after 6 h there was a remarkable decrease of CH 50 with a further subsequent decrease up to 12 h (Fig. 1). C3
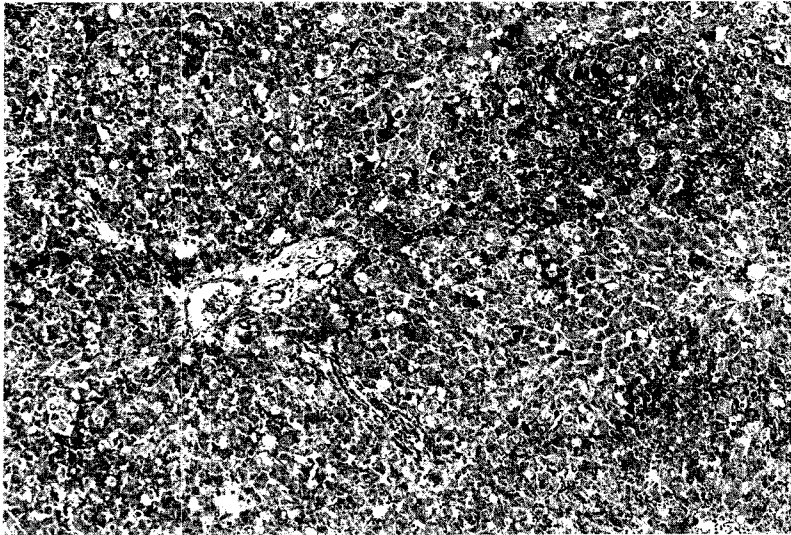

Fig. 4 Twelve hours after administration of GalN and LPS microscopic findings showing massive hepatocellular necrosis (H-E staining x 100).

fluorescence was observed after 1 h in Kupffer cells, as after LPS administration alone (Fig. 2 c), and after 3-4 h on hepatocyte membranes (Fig. 3). From 6 h to the end of the experiment C3 fluorescence was observed in necrotic area. With H-E staining, hepatocytes exhibited ballooning and acidophilic degeneration after 4 h. After 6 h, focal necrosis and infiltrating mononuclear cells were observed at various locations throughout the lobule. After 8 h, the necrotic area was extended and at 10-12 h massive necrosis was observed (Fig. 4). With d-PAS staining, positive cells were observed after 2 h in sinusoidal lining cells, similarly to the pattern after LPS administration alone, and there was an increase until 6 h.

In Vitro

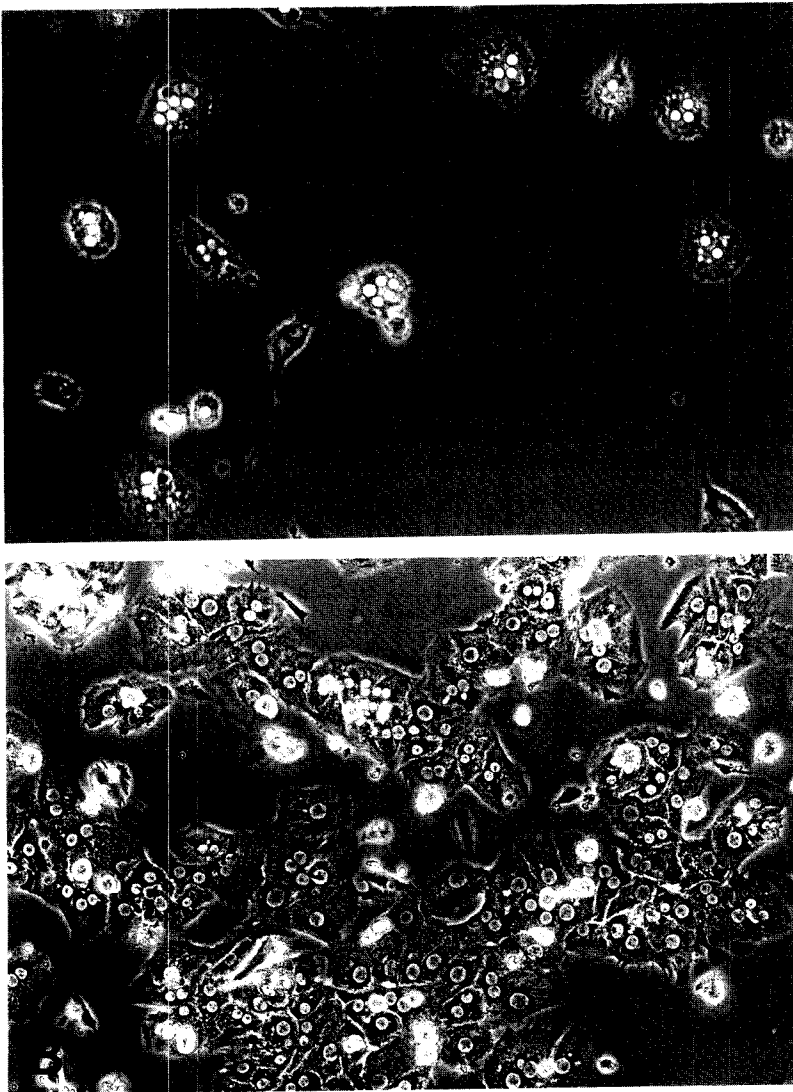For this method of hepatocyte and Kupffer

Fig. 5   (A) Phase contrast microphotogaph showing Kupffer cells ingesting several numbers of latex particles ($\phi = 5.4\mu$m) (x 400). (B) Phase contrast microphotograph showing hepatocytes co-cultured with Kupffer cells (x 200).

2, H 3, and HK 3. This group was represented by H 2, and GalN was added to each of their media. H 2 had only added GalN (33.2 ± 2.6 %). HK 2 had added LPS as well as GalN (35.9 ± 5.8 %). H 3 had added LPS and complement as well as GalN (32.4 ± 8.7 %), while HK 3 had added complement as well as GalN (36.2 ± 4.7 %). The % leakage of LDH was statistically not significant in any one of the subgroups in the second group. In the third group was only HK 4, which had added GalN, LPS, and complement. It developed the most remarkable leakage (50.0 ± 8.8 %), significantly different from the other two groups ($p < 0.01$).

Discussion

In vivo examination showed only a moderate increase of GPT and minimal histological changes were induced by the administration of GalN alone. These results are similar to those described by Decker (7), who found acidophilic bodies and focal necrosis were induced by the same dose. These results are explained by inducing biochemical mechanisms by which GalN inhibits RNA and protein synthesis and induces hepatocyte membrane damage. These effects have been shown to be dose-dependent and to lead from reversible to irreversible liver injury over time (8, 21). It appeared that the dose and time course used in this experiment would not have led to the development of massive hepatic necrosis.

Following LPS administration, there was no hepatic injury, but activation of complement was shown in the serum CH 50 level and the deposition of C3 in Kupffer cells. GalN-induced endotoxemia is due to GalN-induced degranulation of mast cells, subsequent histaminemia induces edema of the colonic wall, and this may allow increased endotoxin absorption (10). Regarding the time course of cellular distribution of endotoxin in the liver, LPS detection by peroxidase-staining has been reported in a few sinusoidal cells 2 h after injection (22). In our experiment, C3

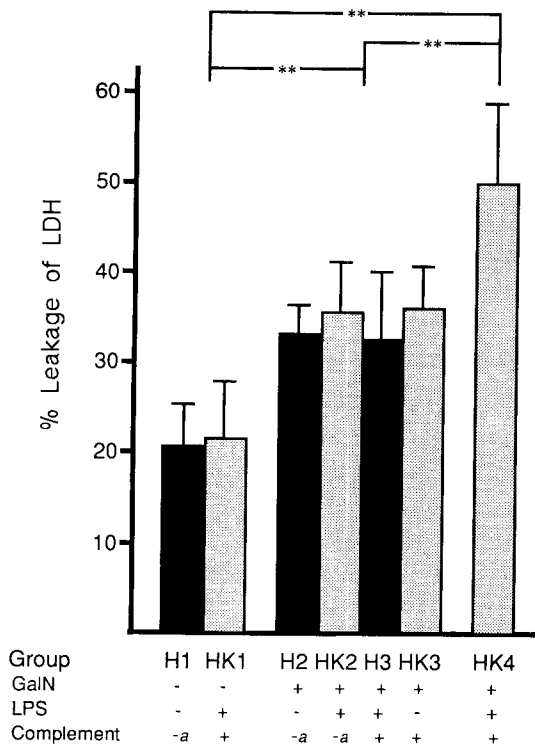| Group	H1	HK1	H2	HK2	H3	HK3	HK4
GalN	-	-	+	+	+	+	+
LPS	-	+	-	+	+	-	+
Complement	-a	+	-a	-a	+	+	+

Fig. 6   Percent leakage of LDH 24h after administration. The seven experimental groups were divided into 3 groups according to % leakage of LDH. One group consisted of H 1 and HK 1, one of H 2, H 3, HK 2, and HK 3, and the third of HK 4.
Significantly different from each of the three groups (* * p < 0.01). − a: Heat-inactivated normal rat serum.

cell isolation, cell viability was 90 % –95 %, as assessed by the trypan blue exclusion test. The purity of Kupffer cells was 80 % –90 %, as assessed by their phagocytic action of latex particles (Fig 5). As indicated in Fig. 6, the seven groups were divided into 3 groups according to the statistical significance of the % leakage of LDH. The first group consisted of H 1 and HK 1. H 1 had no added factor to the medium, i. e., control (21.7 ± 4.3 %). HK 1 had added LPS and complement (22.7 ± 5.7 %). The % leakage of LDH was statistically not significant in this group. The second group consisted of H 2, HK

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was observed in sinusoidal cells, particularly in Kupffer cells where d-PAS staining was observed at the same time. These results suggest that, primarily, LPS activates an alternative complement pathway, and is subsequently phagocytosed by Kupffer cells.

Taking these results of separate GalN or LPS administration into account, we examined the participation of complement in hepatic injury when GalN and LPS were administered simultaneously. In the time course up to 4 h after simultaneous administration, the changes of CH 50 and C 3 deposition were similar to these with LPS adminstration alone. However, after 4 h the deposition of C 3 on the hepatocyte membrane differed from that with LPS administration. Thereafter, a remarkable increase of GPT and massive hapatic necrosis developed. Liehr *et al.* reported C 3 deposition on hepatocyte membranes and subsequent accumulation in necrotic areas in a similar experimental model (13). They concluded that the decrease in complement hemolytic activity was due to increased consumption, rather than due to disorders in factors for complement synthesis. These investigators found that it was necessary to prepare membrane damages in hepatocytes by pretreatment with GalN, before the hepatocytes became targets for complement-mediated cytolysis. They discussed events similar to the later events in our experiments, but they did not refer to early events, *i. e.*, up to 4 h C 3 deposition in Kupffer cells.

In our *in vitro* examination, we investigated the participation of each of four factors, that is GalN, LPS, complement, and Kupffer cells in conditions excluding disturbance of the microcirculation and injury by polymorphonuclear and lymphocytic cellular infiltration. With regard to LDH leakage, the first group HK 1, showed similar levels to the control; this indicates that LPS and complement develop no cytotoxicity despite the presence of Kupffer cells. This supports the results of an *in vivo* examination which showed that LPS administration alone did not lead to hepatic injury (23). The second group,

comprising the four groups H 2, H 3, HK 2, and HK 3, showed moderate leakage which was not significant in each group. The leakage appeared to be due to GalN addition, since GalN was the common factor in these four groups. The mechanism of LDH leakage from GalN-treated hepatocytes in a monolayer culture has been investigated by examining metabolic consequences (24). The third group, HK 4 alone, consisting of all four factors, had the most leakage of LDH. The mechanism of hepatic cell injury in this group is considered in the participation of four factors. That is, preincubation with GalN for 6 h induced membrane damage as a result of GalN metabolism. Additional LPS activated complement which was added simultaneously. Subsequently Kupffer cells phagocytosed LPS, and were activated. Then Kupffer cells promoted complement to induce hepatic cell injury by the formation of membrane attack complex. This supports the results of an *in vivo* examination following GalN and LPS administration which showed massive hepatic necrosis.

With regard to these promotive processes, it is well known that Kupffer cells produce various kinds of cytokines, such as tumor necrosis factor (TNF), interferon, interleukin, and others (15). Previous studies have indicated that the complement system had an essential role in the development of bowel injury after TNF and LPS administration (25). On the other hand, complement regulatory membrane proteins appear to fulfill the function of protecting host cells from accidental attack by complement (26). If such a protective mechanism was disturbed or deficient, it would be easy to predict complement injury to host cells, such as occurs in paroxysmal nocturnal hemoglobinuria (27). As mentioned above in this experimental model of acute hepatic injury, we hypothesize that Kupffer cells phagocytose LPS and complement which was activated by LPS, and that Kupffer cells subsequently secrete some chemical mediator (s) which activate complement for phagocytosis much more LPS. And by such secretion complements acquire to react to the

hepatocyte membrane which has been damaged by GalN.

In additinal experiments, we found high leakage of LDH, such as occurred in HK 4, in hepatocyte culture medium consisting of GalN and complement to which had added the supernatant of Kupffer cell culture medium consisting of LPS and complement (date not shown). This result shows that some promoting soluble factor (s) exist (s) in the Kupffer cell culture medium consisting of LPS and complement. Iwaki *et al.* reported soluble factor (s) including a hepatocytotoxic factor in serum obtained from rats after adminstration of LPS (14). Yamashita reported a hepatocytotoxic factor participating with complement in rats after administration of TNF (28). Further studies must be done to elucidate the factor (s) that promote complement activation.

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