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〔一 般 論 文〕

Pharmacological Activity of Antitumor Drugs in Unphysiologically Conditioned Animals. III.

Preparation of a Model Mouse bearing Immunological Liver Injury in C57BL/6J Mice

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ABSTRACT

An experimental immunological liver injury model was caused by the intravenous injection of an anti-basic liver protein (BLP) antibody in the mice which had been previously immunized with normal rabbit IgG (RGG) and complete Freund's adjuvant. C57BL/6J strain mice indicated the highest susceptability to the immunological liver injury. Typical histopathological changes in the liver were submassive hepatocellular necrosis and infiltration of lymphocytes into the portal tract and sinusoid in necrotic lesion. The liver injury in this model was markedly inhibited by the administration of prednisolone (20 mg/kg, p.o.), cyclophosphamide (15 mg/kg, i.p.), levamisole (10 mg/kg, p.o.), glycyrrhizin (50 mg/kg, i.p.) and cepharanthine (10 mg/kg, i.p.), which act on the immune system. These results suggest that the experimental liver injury model in C57BL/6J mice is useful for unphysiologically conditioned animals model or immunopharmacological research of liver diseases.

INTRODUCTION

The pharmacological effects and toxicity of drugs administered to the human are known to be altered in various unphysiologic or pathologic states. A number of reports have suggested chages in the pharmacokinetis, pharmacological effects, or toxicity of drugs especially in animals and human with liver injury produced by administration of toxic chemicals[1~10].

Since pathologic changes resembling those in human hepatitis are not considered to be reproducible in experimental liver injury models using small animals, there have been no report of evaluation of pharmacological effects or toxicity of drugs in immunological liver injury corresponding to human hepatitis. Moreover, immunological mechanisms are known to be involved in the pathogenesis and progression of hepatitis[11~13].

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In this study, mouse liver injury produced by an immunological mechanism resembling that in human hepatitis was prepared by the use of mouse liver-specific antigen, and whether or not this liver injury can be prevented by drugs currently used for the treatment of hepatitis was examined.

MATERIALS AND METHODS

Animals Inbred male C57BL/6J mice, DBA/2 male mice and ddY male mice, 5 week of age obtained from the Japan SLC in Hamamatsu (Japan) were used for liver injury experiments. The animals had free access to a commercial diet (CE-2, Japan Clea, Tokyo, Japan) and tap water and were kept on a 12 hr light/dark cycle in a temperature controlled room. Male JW rabbits weighing 1.5 to 2.0 kg were used for the preparation of antiserum.

Drugs Prednisolone acetate (Nihon Merk Banyu Pharmaceutical Co. Ltd., Tokyo, Japan), cyclophosphamide (Shionogi pharmaceutical Co. Ltd, Tokyo, Japan), levamisole (Aldrich Chemical Co. Inc., Milwaukee, USA), glycyrrhizin (Minophagen Pharmaceutical Co. Ltd., Tokyo, Japan), cepharanthine (Kaken Shoyaku Pharmaceutical Co. Ltd., Tokyo, Japan) and complete Freund's adjuvant (Nacalai Tesque. Inc., Kyoto, Japan) were purchased. All other chemicals used were analytical grade.

Separation of antigen Basic liver protein (BLP), purified by the method of Mafune et al.[14] and Nagai et al.[15] and previous our report[16], was used as the antigen. The liver of C57BL/6J mice was perfused with physiological saline to remove blood. Physiological saline was added, and 50% homogenates was prepared in a Potter type homogenizer. After centrifugation (4°C, 8,000 rpm, 30 min), the supernate was adjusted to pH 4.8 with acetic acid, resultant insoluble materials were eliminated by centrifugation, and fraction that precipitated by the addition of saturated ammonium sulfate (35—60%) was recovered. This precipitate was dissolved in physiologicl saline and dialyzed against running water for 24 hours and against 0.1 M Tris-HCl buffer (pH 8.0) and 0.005 M Tris-HCl buffer (pH 8.0). It was then chromatographed using a diethylaminoethyl (DEAE) cellulose column equilibrated with 0.005 M Tris- HCl buffer (pH 8.0), and the eluted fraction was collected as BLP.

Preparation of antisera Rabbits were immunized by injecting anti-BLP antibody (protein content 300 μg/ml) from C57BL/6J mice and the same volume (2.0 ml) of emulsion of complete Freund's adjuvant (CFA) in the gluteal muscle and subcutaneously in the back and the planta 4 times at 1-week intervals. Elevation in the antibody titer was confired 10 days after the last injection, and antisera were obtained from the blood drawn from the auricular vein. Antisera was absorbed with homologous erythrocytes and kidney homogenate after inactivation of complement at 56°C 30 min, and then precipitated with 30% saturated ammonia sulfate, and precipitated γ-globulin fraction containing IgG and IgM was dissolved in saline and dialyzed against 0.005 M Tris-HCl buffer (pH 8.0). The solution was applied on a DEAE cellulose column equilibrated with 0.005 M Tris-HCl buffer (pH 8.0). The IgG fraction was obtained by collecting passed effluent. It was dialyzed against PBS and stored at −80°C until use.

Preparation of rabbit γ-globulin (RGG) RGG was purified by a routine procedure using the ammonium sulfate fraction and DEAE cellulose column chromatography.

BLP antibody-induced liver injury Lesions were produced according to the method of Nagai et al.[17], which was used to produce RGG-accelerated nephrotoxic serum nephritis. CFA emulsion (0.5 ml) containing RGG at 4 mg/ml was injected intraperitoneally to the mice, and BLP antibody (0.6 ml) was injected into the tail vein after 5 days.

Biochemical analysis and histopathological examination In order to evaluate the severity of the symptoms, blood was collected and the liver was removed and processed for biochemical and histological analysis at 24 hours after injection of anti-BLP antibody and measured mainly for serum glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) using a diagnostic kit from Wako Pure Chemical Co. Ltd., (Tokyo). In some experiments, activities of alkaline phosphatase, γ-glutamyltranspeptidase, leucine aminopeptidase, choline esterase and lactic dehydrogenase and amounts of total bililubin, total protein, total cholesterol, triglyceride, lipophosphatide, blood urea nitrogen, creatinine and uric acid were also measured by an automatic serum analyzer. For the histopathological examination of liver, a portion of the median lobe of the liver was fixed in 10% neutralized formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin-eosin.

Statistics Results were assessed by the Student's t-test.

RESULTS

Liver injuries in C57BL/6J, DBA/2, and ddY mice The RGG-accelerated BLP antibody-induced liver injury was compared among the three mouse strains, namely C57BL/6J, DBA/2, and ddY. BLP antibody collected from C57BL/6J and purified in rabbits was injected to the mice of these three strains, and the blood GOT and GPT activities and the cytochrome P-450 content of liver microsomes were determined after 12 hours and 1, 2, 3, 4, 5, and 6 days (Fig. 1).

The blood levels of GOT and GPT showed peak levels, and the cytochrome P-450 content of liver microsomes was the lowest, 1 day after the antibody administration in mice of all three strains. The increases in the blood GOT and GPT levels and the decrease in the cytochrome P-450 content in liver microsomes were the most remarkable in C57BL/6J mice among the three strains. Histological findings in the liver were consistent with the changes in the blood GOT and GPT levels and the cytochrome P-450 contents of liver microsomes.

Liver injury in C57BL/6J mice

1) Effects of RGG immunization

The blood levels of GOT and GPT and the cytochrome P-450 content of liver microsomes were studies in an untreated group, a group administered normal rabbit IgG i.v., a group administered BLP antibody i.v., a group immunized with RGG, a group immunized with RGG and then administered normal rabbit IgG i.v., and a group immunized with RGG and

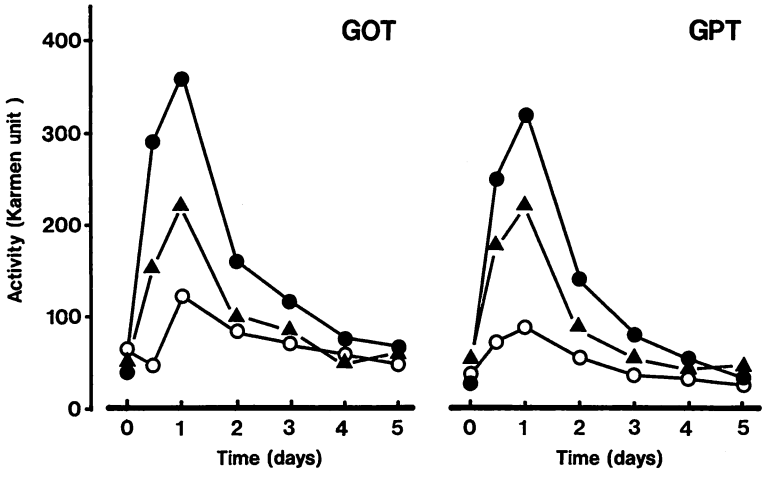

Fig. 1 Strain difference in mice with liver injury induced by RGG-accelerated anti-basic liver protein antibody

Basic liver protein (BLP) from C57BL/6J mice were prepared according to the method of N. Mafune (1982) and Nagai et al. (1988). Anti-BLP antibody was obtained from rabbits which had been immunized by injection of 2.0 ml of an emulsion containing BLP (300 μg protein/ml) and complete Freund's adjuvant intramuscularly or subcutaneously 4 times weekly. C57BL/6J (●), DBA/2 (○), and ddY (▲) mice were immunized by an intraperitoneal injection of 4.0 mg rabbit IgG (RGG) emulsified with 0.5 ml of complete Freund' adjuvant. Five days later, anti-BLP antibody (0.6 ml/animal) was injected intravenously. In order to evaluate the severity of the liver injury, blood sample was collected at 12 hr, 1, 2, 3, 4, 5, and 6 days after the injection of anti-BLP antibody and measured for the activity of GOT and GPT. Values are the means of 6-8 mice ; S.E.s were less than 20% of the respective means.

then administered BLP antibody i.v.

In the normal rabbit IgG group, RGG group, and RGG+normal rabbit IgG group, the blood GOT and GPT levels and the cytochrome P-450 content of liver microsomes were similar to those in the untreated group. An increase in GPT was observed in the BLP antibody group and increases in GOT and GPT and a decrease in the cytochrome P-450 of liver microsomes were noted in the RGG+BLP antibody group. The results of histological examination of the liver were consistent with the increases in GOT and GPT and the decrease in the cytochrome P-450 content of liver microsomes (Fig. 2).

2) Specificity of BLP antibody

Changes in the liver injuries were studied by increasing the dose of BLP antibody injected after RGG immunization. When BLP antibody was administered at 0.1, 0.3, 0.6, and 1.0 ml to mice immunized with RGG, the degrees of increases in the blood GOT and GPT levels and the decrease in the cytochrome P-450 content of liver microsomes 24 hours after antibody injection were dependent on the dose of the antibody (data not shown).

3) Serum biochemical examinations and histological examinations

Various serologic parameters and histological changes were examined 24 hours after

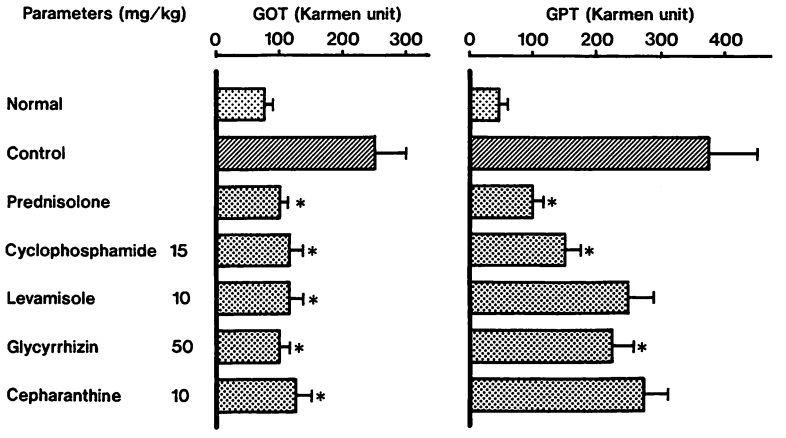

Fig. 2 Effect of various compounds on serum GOT and GPT activities in C57BL/6J mice with liver injury induced by RGG-accelerated anti-basic liver protein antibody

Each experiment consists of 7 mice. The experimental condition and analysis are the same as in Fig. 1. Blood sample was collected 1 day after the injection of anti-BLP antibody and measured for the activity of GOT and GPT. Control mice were administered with saline (0.1 ml/10 g mouse, i.p.). Prednisolone (20 mg/kg, p.o.), cyclophosphamide (15 mg/kg, i.p.), levamisole (10 mg/kg, p.o.), glycyrrhizin (50 mg/kg, i.p.), or cepharanthine (10 mg/kg, i.p.) was administered for 10 days before the injection of anti-BLP antibody. Significant differences from the control is indicated as * ($p<0.05$).

injection of BLP antibody (0.6 ml) to mice 5 days after immunization with RGG and CFA. After intravenous injection of anti-BLP antibody, GOT, GPT, and LDH showed clear increases, and ALP and T-bil decreased. Biochemical examinations were also performed in the serum obtained 24 hours after administration of acetaminophen (800 mg/kg, i.p.). Increases in T-bil, GOT, GPT, LAP, and LDH and decreases in ALP, T-ch, PL and TG were observed (Table 1).

Although the increases in GOT and GPT were more notable in mice with BLP antibody-induced liver injury than in those with acetaminophen-induced liver injury, there were no changes in T-bil, T-cho, TG or PL in the former mice unlike the latter mice. These results suggest that BLP antibody-induced liver injury is limited to a particular portion of the liver. No changes in the results of biochemical examinations of the blood suggestive of kidney injury were observed in either mice.

Photo 1-a shows the histological profile of the liver of an untreated mouse. However, in the liver of mice administered BLP antibody, punctate necrotic areas were grossly observed in all lobes with occasional adhesion of hepatocytes with the peritoneum. In necrotic areas, inflammatory cells, which were predominantly neutrophils, were found to infiltrate into portal spaces around necrotic areas (photo 1-b). However, no concomitant histological changes were observed in the kidney, lung, or spleen, where histologic profiles were similar to those in untreated animals.

Table 1 Changes of serum biochemical parameters in C57BL/6J mice with liver injury induced by RGG-accelerated anti-basic liver protein antibody.

Parameters		Control	Hepatitis	Acetaminophen (800 mg/kg)
T-bil	(mg/dl)	0.75	0.60	1.40*
GOT	(IU/dl)	181.5	3487.0 *	15156.0 *
GPT	(IU/dl)	46.0	3146.0 *	18663.5 *
ALP	(IU/dl)	110.5	45.5 *	50.0 *
γ-GTP	(IU/L)	5.5	5.0	5.5
LAP	(IU/L)	33.0	79.0 *	162.0 *
CHE	(mg/dl)	1425.5	1368.5	1306.5
TP	(mg/dl)	5.40	4.60	4.35
T-cho	(mg/dl)	87.0	80.5	51.5 *
TG	(mg/dl)	126.0	92.0	69.0 *
LDH	(IU/L)	1111.0	11539.0 *	40755.0 *
PL	(mg/dl)	186.5	141.5	114.0 *
BUN	(mg/dl)	29.9	23.3	27.0
CRE	(mg/dl)	0.50	0.45	0.60
UA	(mg/dl)	2.75	1.70	2.0

The experimental condition for the immunological liver injury is the same as in Fig. 1. Mice were sacrificed 24 hr after intravenous injection of anti-BLP antibody (0.6 ml/mouse), or after intraperitoneal administration of acetaminophen (800 mg/kg), and serum parameters were determined. Values are the means of 6 mice ; S.E.s were less than 20% of the respective means. Significant differences from the control is indicated as* ($p<0.05$). Abbreviations see the "MATERIALS AND METHODS".

Effects of drugs

Effects of prednisolone, cyclophosphamide and levamisole, which are used clinically for the treatment of hepatitis, were examined in this liver injury model. Prednisolone (20 mg/kg) and levamisole (10 mg/kg) were administered orally, and cyclophosphamide (15 mg/kg) was administered intraperitoneally for 10 days before BLP antibody injection. All drugs inhibited increases in the GOT and GPT activities, particularly GOT, in this liver injury model (Fig. 2). Prevention of liver injury by the drugs was evident also by histological examinations.

DISCUSSION

Antigens localized in specific tissues are called tissue-specific antigens and have long been studied in connection with the problems of tissue differentiation, tumor-specific antigens, and gene expression. In addition, as these antigens appear in the blood in particular diseases, they have been regarded as clinical markers. Recently, there have been a number of reports that antibodies to tissue-specific antigens are involved in injuries of particular tissues in autoimmune diseases.

There have also been many reports concerning tissue-specific antigens of the liver, such as F-antigen, LSP(LP-1), LP II, and LM-Ag, γ-EST, and BLP [14,18~22]. F-antigen is an

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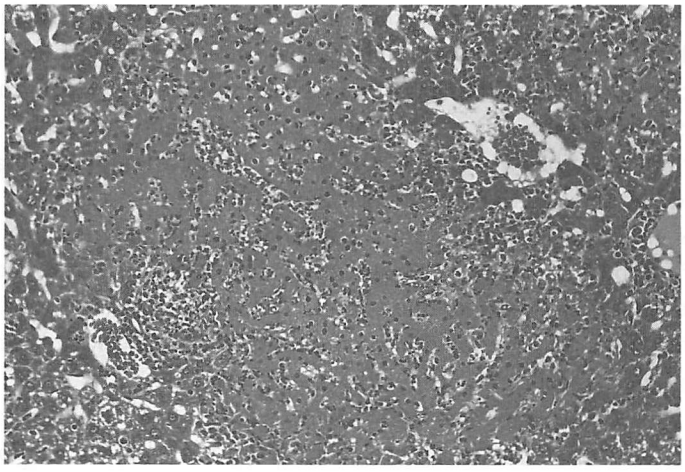

(B)

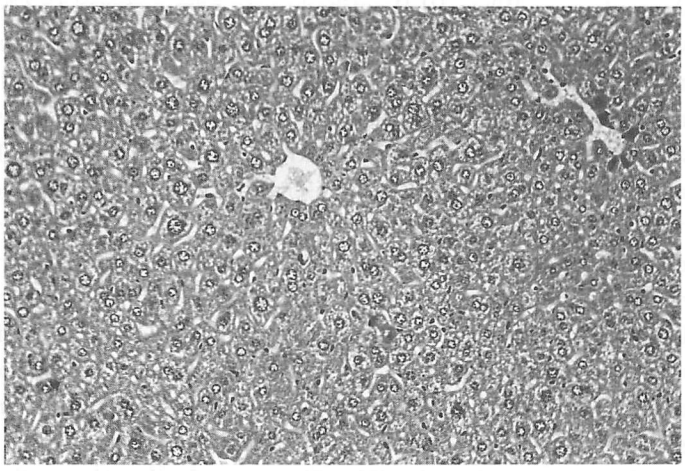

Photo. 1 Histopathological picture of injurious liver induced by RGG-accelerated anti-basic liver protein antibody in C57BL/6J mice

Mice were sacrificed 24 hr after intravenous injection of anti-BLP antibody (A) or intravenous injection of saline (B) (H.E.-stain, ×100). Submassive hepato-cellular necrosis and infiltration of granulocytes into necrotic lesion were found compared to normal mice (saline-treated mice).

alloantigen produced by immunizing mice with liver homogenate of inbred mice and, along with H-2 antigen, is of great interest in the field of immunogenetics. LSP and LM-Ag are antigens present in the hepatocellular membrane and have attracted paritcular attention as antigens related to autoimmune hepatocyte injury in liver diseases such as chronic active hepatitis. γ-EST is a basic protein separated from the rat yolk sac tumor and is present widely in hepatoma, carcinoembryonic tissues such as the embryo and placenta, and the serum and ascites of cancer-bearing rats.

BLP discovered by Mafune et al.[14] is a basic protein specific to and rich in the liver compared with other organs. This BLP is reported to be differenct from known liver-specific antigens in the physical properties and immunological responsiveness. However, it is

considered to be most suitable as a material for liver-specific antigen, because it is abundant in the liver and has previously reported characteristics of liver-specific antigens such as the commonness within species and the appearance of autoantibodies. The fact that rat BLP antiserum obtained by immunizing a rabbit with rat BLP reacts with the liver of the rabbit suggests production of antibody to BLP of the self. Proliferation of rat fetal hepatocytes in culture is inhibited by the addition of rat BLP antibody. In this process, a large number of dead hepatocytes are suspended in the culture medium, presumably because of the cytotoxic effect of rat BLP antibody on rat fetal hepatocytes. Therefore, BLP anti-body appears likely to be involved as an autoantibody in immunological liver injury.

In the present study, we attempted to produce a model mouse with liver injury resulting from an immunological mechanism using BLP as the antigen.

Liver injury was produced in mice by intravenous injection of BLP antibody derived from C57BL/6J mice 5 days after intraperitoneal administration of RGG obtained from normal rabbits with CFA emulsion. When BLP antibody was administered to C57BL/6J, DBA/2, and ddY mice, liver injury was severest in C57BL/6J mice. In this strain, the severity of liver injury was dependent on the dose of BLP antibody. Serum biochemical studies showed abnormalities only in parameters of liver functions, and histological lesions were demonstrated only in the liver with no abnormalities in the kidney, spleen, or lung. The liver injury in this model was markedly inhibited by the administration of prednisolone, cyclophosphamide, and levamisole, which act on the immune system. From these results, liver injury in this model was considered to be caused by immunological mechanism unlike liver injuries induced by the administration of chemicals such as carbon tetrachloride and acetaminophen.

The total bilirubin level including direct-reacting and indirect-reacting bilirubin is known to increase in humam hepatitis, but the serum total bilirubin level tended to decrease in this mouse liver injury model induced by BLP antibody. Furthermore, in an early stage of humans hepatitis, the structure of hepatic cords appears normal, a few hepatocytes are swollen, degenerated, or necrosed, and mild infiltration of migrating cells such as macrophages, lymphocytes, and neutrophils is observed. In advanced stages, marked hepatocellular necrosis is oberved in the entire liver, and a very small number of hepatocytes are left, exhibiting features of acute hepatic atrophy, and the limiting lamina that demarcates a hepatic cord from Glisson's capsule is destroyed due to disintegration of hepatic cords and infiltration of inflammatory cells. In the present liver injury model, however, the histopathologic profile of the liver differed slightly from that in human hepatitis. Pathologic changes in human hepatitis are generally considered not to be reproducible in small animals, and this point needs improvements.

No drug specifically effective for hepatitis has been developed, and the disease is usually treated with anti-inflammatory agents and immunomodulators. In the present experimental model, the anti-inflammatory steroid prednisolone and cyclophosphamide with an immunosuppressive effect markedly reduced the liver injury. The development of liver injury was also inhibited by levamisole with immunomodulator effects.

These findings suggest that this mouse liver injury model is useful for clarification of the immunological mechanism of liver injury or as a model of a particular pathologic state. Moreover, considering the fact that drugs acting on the immune system have little effect on

hepatitis induced by chemicals such as carbon tetrachloride and galactosamine, the present model developed by an immunological process is considered to be useful also for screening of drugs for the treatment of immunoreactive liver injuries.

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