Liver Injury Model in Mice for Immunopharmacological Study

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Abstract—Experimental liver injury was produced in mice by the immunological technique. The utility of these models as an immunopharmacological method was investigated. The first model was produced by the injection of anti-basic liver protein (BLP) rabbit antibody into DBA/2 mice that had been previously immunized with rabbit IgG. The second liver injury was caused by injection of anti-liver specific protein (LSP) rabbit antibody into DBA/2 mice. The third model was produced by the injection of bacterial lipopolysaccharide (LPS) into *Corynebacterium parvum* pretreated ddY mice. In all injury models, extensive liver parenchymal cell damage was estimated by elevation of glutamate transaminase (GOT and GPT) activity. These were confirmed by histopathological studies of the liver. Typical histopathological changes in the liver from injured mice were submassive hepatocellular necrosis and infiltration of granulocytes and lymphocytes into the portal tract and sinusoid in the necrotic lesion. Administration of prednisolone and cyclophosphamide for 10 days prior to injection of eliciting antibodies or LPS suppressed the elevation of serum transaminase levels in all experimental liver injury models. Cianidanol and sylbin inhibited the elevation of GOT and GPT in anti-BLP induced liver injured mice. These evidences suggest that the above models are suitable for investigating the remedy for liver diseases.

There is much evidence that an immune reaction plays an important role in certain human liver diseases such as acute viral hepatitis, primary biliary cirrhosis and alcohol or drug induced hepatitis (1–4). For the treatment of the above liver diseases, glucocorticoids and immunosuppressive agents have been used and shown to be effective in controlled trials. However, there are some restrictions to the use of these drugs for the treatment of liver diseases because of their severe adverse effects. At present, other immunological treatments, including immunomodulating agents with low toxicity, are desired. The efficacy of immunomodulating agents is, however, still inconsistent and inconclusive. One main reason for this may be related to the lack of a suitable experimental model for

evaluating the efficacy. The present study, therefore, has been conducted to examine the utility of three kinds of liver injury caused by immunological mechanisms in mice as a model for immunopharmacological research on liver diseases.

Materials and Methods

Animals

DBA/2, C57BL/6, ICR and ddY male mice weighing 18 to 20 g were used for liver injury experiments. Male albino rabbits weighing 2.0 to 2.5 kg were used for the preparation of antiserum.

Drugs

Prednisolone acetate (Nippon Merck Ban-yu, Tokyo) and cyclophosphamide (Shionogi, Osaka) were purchased. Cianidanol was

kindly supplied by Dr. K. Ito (Kanebo, Osaka) and silybin was kindly supplied by Dr. K. Oohira (Taiyo, Gifu).

Drugs were suspended in 0.2% carboxymethyl cellulose saline solution and administered intraperitoneally (0.1 ml/10 g) for 10 days before injection of eliciting antibodies or LPS.

Experimental liver injury

A) Anti-basic liver protein (BLP) antibody induced liver injury: BLP was prepared according to the method of Mafune (5). In brief, DBA/2 mice liver homogenate was centrifuged at 8,000 rpm at 4°C for 30 min. The supernatant was obtained and adjusted to pH 4.8 by addition of acetic acid. After removal of the precipitate by centrifugation, the BLP-rich fraction was precipitated with $(\text{NH}_4)_2\text{SO}_4$ solution at a final saturation between 35–60%. The precipitated proteins were dissolved in distilled water and dialyzed against 0.005 M Tris-HCl buffer (pH 8.0). The solution was applied on a diethylaminoethyl (DEAE) cellulose column equilibrated with 0.005 M Tris-HCl buffer (pH 8.0). The BLP fraction was obtained by collecting passed effluent. The antiserum containing anti-BLP antibody was obtained from rabbits which had been immunized by injection of 1.0 ml of an emulsion containing 300 μg BLP and complete Freund's adjuvant (CFA) intramuscularly 4 times weekly. Antiserum was obtained 7 to 10 days after the last injection and absorbed with homologous erythrocytes and kidney homogenate after inactivation of complement at 56°C for 30 min. Liver injury was induced by a method similar to the previously reported method used for induction of glomerulonephritis (6). DBA/2 mice were immunized by an intraperitoneal (i.p.) injection of 0.5 mg rabbit IgG (RGG) emulsified with 0.25 ml of CFA. Five days later, 0.6 ml of antiserum containing anti-BLP antibody was injected intravenously (i.v.). In order to evaluate the severity of the symptoms, blood samples were collected at 18 hours after injection of anti-BLP antibody and measured mainly for activities of glutamic pyruvic transaminase (GPT) and glutamic oxalacetic transaminase (GOT) in serum. In some experiments, activities of alkaline phosphatase, lactic dehydrogenase, choline

esterase and leucine aminopeptidase and amounts of bilirubin, cholesterol, albumin and total protein were also measured by an automatic serum analyzer (Hitachi 705). Pathological changes in the liver were evaluated in a semiquantitative fashion after staining with hematoxylin and eosin.

B) Anti-liver specific protein (LPS) antibody induced liver injury: LSP was purified from DBA/2 mouse liver according to the method of Mcfarlen et al. (7). A 105,000 g supernatant of 50% (W/V) liver homogenate in 0.25 M sucrose was used as the starting material for the preparation of LSP. Supernatant was then applied to a column of Sephadex G-100 (gel bed, 90×2.5 cm) which had been previously equilibrated with 0.1 M Tris-HCl (pH 8.0) containing 0.2 M NaCl and 1 mM disodium EDTA. The column was eluted with the same buffer, and the first peak was collected as a LSP-rich fraction used for liver protein immunogen. Anti-LSP antibody was prepared according to the same method as described above (anti-BLP antibody). Anti-LPS antibody induced liver injury was caused by the injection of 0.6 ml of antiserum into DBA/2 mice. In this case, since preimmunization with RGG did not accelerate the development of disease, the liver injury was caused by the injection of antiserum alone. The animals were sacrificed 48 hours after the injection of antiserum for testing the changes of serum parameters and histopathological changes of the liver. The severity of liver injury was evaluated by the same method as described above.

C) *Corynebacterium parvum* (*C. parvum*) and lipopolysaccharide (LPS) induced liver injury: LPS-induced liver injury in ddY mice that had been pretreated with *C. parvum* was induced by the method of Ferluga et al. (8). In brief, 1 mg of *C. parvum* suspended in 0.5 ml saline was injected i.p. into mice; then 9 days later, LPS at a concentration of 10 $\mu\text{g}/0.5$ ml saline was injected i.v. The animals were killed by exsanguination 2 hours after challenge for testing the severity of the disease. Evaluation of the severity of disease was carried out by the same method as described above.

Statistics

Results were statistically evaluated using

Student's *t*-test. In histopathological studies, the statistical significance was tested by Wilcoxon's *U*-test.

Results

Anti-BLP antibody-induced liver injury:

After an injection of anti-BLP antibody, the elevation of GOT and GPT activities was observed in two strains of mice, C57BL/6 and DBA/2, but was not observed in strains ddY and ICR. In addition, the elevation of serum GOT and GPT activities and histopathological changes in the liver (mainly massive hepatocellular necrosis and infiltration of granulocytes into the necrosis lesion) were accelerated by preimmunization with RGG and CFA. From the results of these preliminary experiments, the following experiments were conducted in DBA/2 mice which were preimmunized with RGG and CFA. Table 1 shows the changes of serum parameters in mice with liver injury. Clear elevation of GOT, GPT and LDH activities and decrease in the amount of bilirubin and albumin were observed. Figure 1 is the histopathological findings of the mouse liver with anti-BLP antibody induced liver injury. Focal hepatocellular necrosis and infiltration of granulocytes into the sinusoid in the necrotic lesion were observed. Histopathological examinations on lung and kidney were also carried out. No significant change was observed in either of the tissues. The effects of prednisolone, cyclophosphamide, silybin and cyanidanol on this experimental liver injury were also studied (Fig. 2). By intraperitoneal administration of

each drug, elevation of both GOT and GPT activities were clearly inhibited. In histopathological studies, prednisolone, cyclophosphamide and silybin showed a tendency to inhibit the pathological changes of the liver.

Anti-LSP antibody induced liver injury:

From the preliminary experiments, the preimmunization with RGG did not affect the onset of liver disease. This injury model was, therefore, caused by antibody alone. Table 2 shows the changes of serum parameters in anti-LSP antiserum (1.0 ml) treated mice. Clear elevation of GOT, LDH and LAP activities and the amount of bilirubin and triglyceride was observed by the injection of anti-LSP antibody. Significant decrease of ALP activity was also observed.

Fig. 1. Liver of mouse that was treated with anti-BLP antibody after preimmunization with RGG. Mouse was sacrificed 18 hours after the injection of antiserum. Hematoxylin and eosin. $\times 80$.

Table 1. Change of serum biochemical parameters on RGG-pretreated anti-BLP antibody-induced liver injury in mice

Item	Non-treatment	RGG+a-BLP
GOT (mU/ml)	81.3 $\pm$ 3.41	163.2 $\pm$ 12.03*
GPT (mU/ml)	16.3 $\pm$ 1.74	311.2 $\pm$ 25.37*
ALP (mU/ml)	668.0 $\pm$ 6.93	442.0 $\pm$ 31.43*
LDH (mU/ml)	360.0 $\pm$ 33.3	4889 $\pm$ 343*
BIL (mg/dl)	0.74 $\pm$ 0.22	0.42 $\pm$ 0.06*
CHO (mg/dl)	114.3 $\pm$ 4.45	102.0 $\pm$ 2.45
CHE (mU/ml)	3817.0 $\pm$ 253.0	3671.2 $\pm$ 169.93
ALB (g/dl)	1.38 $\pm$ 0.04	1.02 $\pm$ 0.03*
TP (g/dl)	5.49 $\pm$ 0.11	5.33 $\pm$ 0.19
LAP (mU/ml)	62.3 $\pm$ 6.52	65.2 $\pm$ 3.20

Each value represents the mean $\pm$ S.E. of 5 to 6 animals. *: $P < 0.05$

Fig. 2. Effect of prednisolone (10 and 20 mg/kg/day), cyclophosphamide (5 and 10 mg/kg/day), silybin (100 and 300 mg/kg/day) and cianidanol (500 and 1000 mg/kg/day) on the elevation of serum GOT and GPT activities induced by anti-BLP antibody after the preimmunization with RGG. Each drug was administered for 10 days before the injection of anti-BLP antibody. Nor, Normal; Cont, Control. *: $P < 0.05$, t: $P < 0.01$.

Table 2. Change of serum biochemical parameters on anti-LSP antibody-induced liver injury in mice

Item	Non-treatment	anti-LSP
GOT (mU/ml)	53.5 $\pm$ 3.05	706.4 $\pm$ 64.0**
GPT (mU/ml)	12.6 $\pm$ 0.94	252.6 $\pm$ 23.6**
ALP (mU/ml)	826.7 $\pm$ 19.0	508.2 $\pm$ 22.4**
LDH (mU/ml)	365.6 $\pm$ 32.1	3148.6 $\pm$ 378.0**
BIL (mg/ml)	0.684 $\pm$ 0.02	0.822 $\pm$ 0.054**
TG (mg/dl)	102.2 $\pm$ 6.4	150.2 $\pm$ 10.3*
CHO (mg/dl)	102.9 $\pm$ 2.5	101.4 $\pm$ 3.2
ALB (g/dl)	1.94 $\pm$ 0.03	1.93 $\pm$ 0.01
TP (g/dl)	5.01 $\pm$ 0.07	5.70 $\pm$ 0.05**
LAP (mU/ml)	59.6 $\pm$ 0.65	64.4 $\pm$ 0.98**

Each value represents the mean $\pm$ S.E. of 10 or 11 animals. *: $P < 0.05$, **: $P < 0.01$.

Figure 3 shows the histopathological findings of the mouse liver with anti-LSP antibody induced liver injury. Focal hepatocellular necrosis and infiltration of granulocytes into the portal tract and sinusoid in the necrotic lesion were observed. No significant histo-

pathological change was observed in the lung and kidney. The effects of prednisolone, cyclophosphamide, silybin and cianidanol on this experimental liver injury were studied (Fig. 4). Prednisolone and cyclophosphamide inhibited the elevation of both GOT and GPT

activities. Contrary to the above two agents, silybin and cianidanol did not affect the eleva-

Fig. 3. Liver of mouse that was treated with anti-LSP antibody. The mouse was sacrificed 48 hours after the injection of antiserum. Hematoxylin and eosin. $\times 95$.

tion of serum transaminases. In the histopathological studies, no significant difference between the control and drug treated groups was observed.

LPS-induced liver injury in *C. parvum* pre-treated mice: The symptoms of liver injury in terms of the elevation of serum transaminase and histopathological changes of the liver were exacerbated by increasing each amount of *C. parvum* and LPS. From the results of preliminary experiments, the most suitable condition for the onset of disease was the i.v. injection of LPS at a dose of 10 μ g after the i.p. injection of *C. parvum* at a dose of 0.5 mg. The changes of serum parameters are indicated in Table 3. Significant elevation of GOT, GPT, LDH and LAP activities and bilirubin, triglyceride and total protein levels were observed. Figure 5 shows the histopathological findings of mouse liver with LPS induced liver injury. Focal necrobiosis and increased in-

Fig. 4. Effect of prednisolone (10 and 20 mg/kg/day), cyclophosphamide (5 and 10 mg/kg/day), silybin (100 and 300 mg/kg/day) and cianidanol (500 and 1000 mg/kg/day) on the elevation of serum GOT and GPT activities induced by anti-LSP antibody. Each drug was administered for 10 days before the injection of anti-LSP antibody. Nor, Normal; Cont, Control. *: $P < 0.05$, †: $P < 0.01$.

Table 3. Change of serum biochemical parameters in *Corynebacterium parvum* and LPS-induced liver injury in mice

Item	Non-treatment	Coryne+LPS
GOT (mU/ml)	38.8±1.16	1832.1±304**
GPT (mU/ml)	15.8±0.76	298.3±50.17**
ALP (mU/ml)	444.6±30.4	510.2±48.3
LDH (mU/ml)	413.4±23.3	23625.2±4995.9**
BIL (mg/ml)	0.950±0.04	1.640±0.128**
TG (mg/dl)	152.2±13.2	204.8±17.1*
CHO (mg/dl)	133.0±4.3	152.1±13.9
ALB (g/dl)	2.14±0.04	2.02±0.076
TP (g/dl)	5.59±0.11	6.07±0.21
LAP (mU/ml)	48.0±1.71	166.6±22.03**

Each value represents the mean±S.E. of 10 or 11 animals. *: $P<0.05$, **: $P<0.01$.

Fig. 5. Liver of mouse that was treated with bacterial LPS after the injection with *Corynebacterium parvum*. The mouse was sacrificed 2 hours after the injection of LPS. Hematoxylin and eosin. $\times 80$.

flammatory cell filtration in the periportal tissue were observed. Similar changes were observed in the spleen, but not in the lung and kidney. The effect of drugs on the elevation of GOT and GPT in LPS induced liver injury mice is indicated in Fig. 6. Prednisolone, cyclophosphamide and cianidanol inhibited the elevation of transaminase activities, but silybin did not affect the elevation.

Discussion

The results reported here indicate that three immunological liver injuries caused by anti-BLP antibody, anti-LSP antibody and bacterial LPS are suitable models for immunopharmacological research of liver diseases. The primary basis for the validity of these models is the effectiveness of agents which are used for the clinical treatment of

liver disease in these pharmacological models. Moreover, another reason is that these models are produced by immunological procedures. In previous pharmacological studies, chemical compounds such as CCl_4 , galactosamin and α -naphthyl isothiocyanate induced liver injury models were mainly used (9–12). However, some reports indicated a few problems utilizing these chemically induced experimental liver injuries as a model for pharmacological research (13, 14). The major problem seems to be related to the reproducibility and quantitative analysis of experiments. Moreover, as is well-known, immunologic mechanisms play a very important role in the onset and development of certain liver diseases such as acute viral hepatitis, primary biliary cirrhosis and alcohol or drug induced hepatitis, so a suitable experimental liver disease model caused by immunologic methods is highly desirable (1–4). Regarding the immunologically induced model, experimental hepatitis has previously been produced by immunization with homologous liver antigen or injection of heterologous anti-liver antibody (15–19). However, most of these are models for research in pathology or biochemistry, and no attempt has been made to use these models as a pharmacological tool. Moreover, the above studies were carried out by using an unpurified antigen. In the present study, therefore, we tried to evaluate immunologically induced liver disease by employing antibody against specific liver proteins in mice as a model for pharmacological study.

Among the present models, anti-BLP anti-

Fig. 6. Effect of prednisolone (10 and 20 mg/kg/day), cyclophosphamide (5 and 10 mg/kg/day), silybin (100 and 300 mg/kg/day) and cianidanol (500 and 1000 mg/kg/day) on the elevation of serum GOT and GPT activities induced by LPS after the injection of *Corynebacterium parvum*. Each drug was administered for 10 days before the injection of LPS. Nor, Normal; Cont, Control. *: $P < 0.05$, †: $P < 0.01$.

body induced liver injury is a useful tool for evaluating the efficacy of an immunomodulator. This model was produced by using heterologous anti-liver protein antibody after the immunization with heterologous immunoglobulin (RGG). By the preimmunization with RGG, the onset of disease is accelerated, so that drug efficacy can then be evaluated. Contrary to the anti-BLP antibody induced liver injury model, the anti-LSP antibody induced model was produced by the injection of an antibody without the preimmunization with RGG. Whereas the clinical signs and histopathological features were similar in these two models, the drug efficacy, especially that of an immunomodulator, is different between the two models. This may be due to the presence or lack of an antibody production stage. Regarding the liver injury caused by LPS and *C. parvum*, Ferluga et al. (8) reported that this model is suitable for

studying the pathogenesis of hepatitis and the remedy for hepatitis. The present study, however, indicated that this model is also insufficient for investigating agents with an immunomodulating property.

At present, specific drugs are not yet available for the treatment of liver disease. Nonspecific anti-inflammatory drugs and occasionally immunosuppressive drugs such as glucocorticoids and cyclophosphamide significantly suppressed the development of liver injury. In addition, cianidanol and silybin which are reported to have an immunomodulating activity, show similar suppression on RGG accelerated anti-BLP antibody induced liver disease. Cianidanol is reported to be effective in viral hepatitis by modulating the immune response against the hepatitis virus (20, 21). Further study will elucidate the utility of immunomodulators for the therapy of liver disease. More detailed experiments

are being planned to develop new agents for the treatment of liver disease by utilizing these experimental models.

References

- 1 Almeida, J.D. and Waterson, A.P.: Immune complex in hepatitis. *Lancet* **2**, 983–984 (1969)
- 2 Duddely, F.J., Fox, R.A. and Sherlock, S.: Cellular immunity and hepatitis associated. Australian antigen liver disease. *Lancet* **1**, 723 (1972)
- 3 Eddelstone, A.L.W.F. and Williams, R.: Inadequate antibody response to HBsAg or suppressor T-cell defect in development of active chronic hepatitis. *Lancet* **2**, 1543 (1974)
- 4 Edgington, T.G. and Chisari, F.V.: Immunological aspects of hepatitis B virus infection. *Am. J. Med. Sci.* **270**, 213–216 (1975)
- 5 Mafune, N.: Purification, characterization and clinical aspects of liver specific antigen. *Hokkaido Med. J.* **57**, 491–501 (1982)
- 6 Nagai, H., Takizawa, T., Nishiyori, T. and Koda, A.: Experimental glomerulonephritis in mice as a model for immunopharmacological studies. *Japan. J. Pharmacol.* **32**, 1117–1124 (1982)
- 7 Mcfarene, I.G., Wojcicka, B.M., Zucker, G.M., Eddleston, A.L.W.F. and Williams, R.: Purification and characterization of human liver specific membrane lipoprotein (LSP). *Clin. Exp. Immunol.* **27**, 381–390 (1977)
- 8 Ferluga, J., Kaplun, A. and Allison, A.C.: Protection of mice against endotoxin-induced liver damage by anti-inflammatory drugs. *Agents Actions* **9**, 566–574 (1979)
- 9 Lidstrom, T.D. and Andres, M.W.: Effect of cycloheximide and actinomycin D on carbon tetrachloride hepatotoxicity. *Toxicol. Appl. Pharmacol.* **42**, 167–173 (1977)
- 10 Imaizumi, Y., Sugimoto, T. and Kasai, T.: Effect of diisopropyl 1,3-dithiol-2-ylindenemalonate (NKK-105) on fatty liver induced by carbon tetrachloride. *Japan. J. Pharmacol.* **31**, 15–21 (1981)
- 11 Williams, D.E. and Priestly, G.: Evaluation of prophylactic efficacy of silymarin in CCl₄-induced hepatotoxicity. *Res. Commun. Chem. Pathol. Pharmacol.* **6**, 185–194 (1973)
- 12 Tajima, S., Takebe, H., Sato, I., Ikeda, Y., Imai, E., Ito, K. and Nose, T.: Protective effect of cianidanol (KB-53) on experimental liver injury in mice. *Folia Pharmacol. Japon.* **81**, 519–528 (1983) (Abs. in English)
- 13 Rechnagel, R.O.: Carbon tetrachloride hepatotoxicity. *Pharmacol. Rev.* **19**, 145–208 (1967)
- 14 Paronetto, F. and Popper, H.: Hetero-, iso- and autoimmune phenomena in the liver. In *Textbook of Immunopathology*, Edited by Miescher, P.A. and Müller-Eberhard, H.J., p. 789–818, Grune and Stratton, Inc., New York (1976)
- 15 Bartholomaeus, W.N., O'Donoghue, H. and Reed, W.D.: Thymus dependence of autoantibody responses to liver specific lipoprotein in the mouse. *Clin. Exp. Immunol.* **55**, 541–545 (1984)
- 16 Bartholomaeus, W.N., Reed, W.D., Joske, R.A. and Silkin, K.B.: Autoantibody responses to liver-specific lipoprotein in mice. *Immunology* **43**, 219–226 (1981)
- 17 Buschenfelde, K.H., Kossling, F.K. and Miescher, P.A.: Experimental chronic active hepatitis in rabbits following immunization with human liver proteins. *Clin. Exp. Immunol.* **10**, 99–108 (1972)
- 18 Kuriki, J., Murakami, H., Kakumu, S., Sakamoto, N., Yokochi, T., Nakashima, I. and Kato, N.: Experimental autoimmune hepatitis in mice after immunization with synergistic liver proteins together with the polysaccharide of *Klebsiella pneumoniae*. *Gastroenterology* **84**, 596–603 (1983)
- 19 Goldgraber, M. and Kirsner, J.: The hypersensitive state and liver: a critical review. *Am. J. Med. Sci.* **241**, 109–114 (1961)
- 20 Kakumu, S., Murakami, H. and Kuriki, A.: Activation of suppressor function of human peripheral blood T cell by (+)cyanidanol: its application to chronic active liver diseases. *Clin. Exp. Immunol.* **52**, 430–436 (1983)
- 21 Ikeda, Y., Kawasaki, K., Sato, I., Tajima, S., Ito, K. and Nose, T.: Effect of cianidanol (KB-53) on the activity of mouse natural killer cells. *J. Pharmacobiodyn.* **7**, 21–23 (1984)