Gastroenterologia Japonica
Vol. 22, No. 4

Printed in Japan

—Original Article—

PROTECTION OF LIVER CELLS AGAINST EXPERIMENTAL DAMAGE BY EXTRACT OF CULTURED LENTINUS EDODES MYCELIA (LEM)

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Summary

Liver cell damage can be induced when isolated liver cells coated with specific antibodies against the liver cell membrane are cultured with peripheral blood mononuclear cells. Although this antibody-dependent cell-mediated cytotoxicity (ADCC) is dependent on the close contact of effector cells with target cells via specific antibodies, a cytotoxic factor or factors causing the inhibition of protein synthesis in liver cells has been detected in the culture supernatant from the ADCC reaction. Similarly, peritoneal exudate macrophages activated by endotoxin lipopolysaccharide also exert cytotoxic effects on isolated liver cells by production of a cytotoxic substance or substances. The liver cell damage caused by either the ADCC or activated macrophage culture supernatants were significantly reduced by pretreating the isolated liver cells with the extract of cultured Lentinus edodes mycelia (LEM). These results suggest that LEM may protect liver cells from immunological damage.

Key Words: *Antibody-dependent cell-mediated cytotoxicity (ADCC), Extract of cultured Lentinus edodes mycelia (LEM), Macrophage-mediated cytotoxicity.*

Introduction

Lentinus edodes, an edible mushroom, is a popular food in several East Asian countries. Recently, some investigators have reported that seroconversion occurs in patients with HBe antigen-positive chronic hepatitis treated with the extract of cultured Lentinus edodes mycelia (LEM)[1]. Furthermore, a multicentered, open study trial has been performed to evaluate the effectiveness and safety of LEM on HBe antigen-positive chronic hepatitis[2]. As a result, it has been concluded that LEM is useful for ameliorating the HBeAg/anti-HBe system, and improvements in the liver function tests, especially the transaminase levels, have been observed in patients with HBe antigen-positive chronic active hepatitis. However, our understanding of the mechanism of LEM is far from complete, although it has been suggested to have an immunomodulatory effect.

Various immunological reactions can be induced against liver specific lipoprotein (LSP). For example, antibody-dependent cell-

Received November 21, 1986. Accepted February 9, 1987.

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mediated cytotoxicity (ADCC) via anti-LSP antibodies is thought to cause liver cell damage. The authors have previously demonstrated that liver cell injury due to ADCC is produced not only by close contact between effector cells and target liver cells via anti-LSP antibodies but also by some chemical mediator(s) released from the K cells[3]. It has also been suggested that liver cell damage may be induced by activated macrophages infiltrating into the liver tissue, as various lymphokines including the macrophage activating factor (MAF), which are produced by stimulating LSP-sensitized T cells by stimulation with LSP, activate macrophages to exert cytotoxic effects on liver cells. In this case, we have demonstrated that liver cell injury is induced not only by cell-to-cell contact between the activated macrophages and target liver cells but also by the production of a cytotoxic factor or factors from the activated macrophages[4,5]. In the present study, the effects of LEM on liver cell damage induced by ADCC or activated macrophages were investigated.

Materials and Methods

1. Materials

Eagle's MEM was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan) and fetal calf serum from M.A. Bioproducts (Maryland, U.S.A.). LSP was prepared from the rat liver according to the method of Meyer zum Büschenfelde and Miesher[6]: Liver homogenate in 0.25 M sucrose was centrifuged at 10,500 g for 1 hr. Approximately 20 ml of the liver supernatant fraction was applied to a Sephadex G-100 column (2.5 × 90 cm) which had been equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl and 1 mM EDTA. The column was eluted with the same buffer, and the first fraction was concentrated to about 20 ml by ultrafiltration using a diafilter (G-05T, Bioengineering Co., Tokyo, Japan) as reported previously[7]. Twenty milliliters of this concentrate was applied to a Sephadex G-200 column (2.5 × 90 cm), and the first fraction was concentrated to about 20 ml. Ten milliliters of this product was in turn applied to a Sepharose 6B column (2.5 × 90 cm) equilibrated and eluted with the same Tris/EDTA buffer. The first fraction was concentrated as above, and the protein content was measured according to the method of Lowry et al.[8]. Anti-LSP antibody was prepared as follows: Male rabbits weighing 3.5 kg were immunized by four weekly injections of LSP (10 mg protein) emulsified in an equal volume of Freund's complete adjuvant. Seven days after the last booster injection, the immunized animals were sacrificed, the sera were separated, and the complement was inactivated by heating at 56°C for 30 minutes. The sera were stored at −70°C until use.

2. Preparation of LEM

The mushroom spores were germinated and cultured in a liquid medium containing 20 g of malt extract, 2.5 g of yeast extract and 2 g of ammonium tartrate in 100 ml of medium. The resulting mycelial pellet was injected into a solid medium which was composed of bagasse and defatted rice bran (5:1, w/w). The medium was disrupted before the formation of fruit bodies. The disrupted material was incubated in water at 40–50°C for 60 hours to promote autolysis of the mycelia and partial digestion of the culture medium with mycelia enzymes. The digest was then extracted with water at 60°C. The extract was aseptically filtered and lyophilized, and the resulting pale-brownish powder was designated as LEM. The yield of LEM was about 6 g/kg medium (**Fig. 1**).

3. Preparation of culture supernatant from ADCC reaction mixture

Liver cell suspensions were prepared from normal rats according to the method of Bel-

culture medium (bagasse/defatted rice bran 5/1 w/w)
↓
mycelial pellet in suspension culture aseptically inoculated
↓
disrupted before the formation of fruit bodies
↓
incubated in water at 40–50°C for 6 hours
(autolysis and partial digestion of culture medium with mycelial enzymes)
↓
extracted with water at 60°C
↓
filtered and lyophilized
↓
pale brownish powder (LEM)
(6 g/kg medium)
(solubility: 145 g/l)

Fig. 1. Preparation of water-soluble fraction from culture medium of Lentinus edodes mycelia (LEM).

lemann et al.[9]. After the cell concentration was adjusted to 1×10^5 cells/ml in Eagle's MEM containing 10% fetal calf serum, 0.01 ml/ml of antiserum was added to 1.0 ml of the isolated liver cell suspension, and this was incubated at 37°C for 3 hrs. To the antibody-coated hepatocyte culture, 100 ml of lymphocyte-rich mononuclear cells (1×10^7 cells/ml) prepared from the heparinized peripheral blood by Ficoll-Conray density gradient centrifugation were added, and the cell mixture was incubated at 37°C for 2 hrs in a humidified cell-incubator with aeration of 5% CO_2 in air. After incubation, the culture supernatant was separated by centrifugation at 1,000 g for 10 min[10].

4. Preparation of culture supernatant from LPS-activated macrophages

To obtain peripheral exudate cells mainly consisting of macrophages, 20 ml of sterilized Marcol 52 (Esso Oil Co.) was injected into the peritoneal cavity of normal guinea pigs. The peritoneal exudate cells were collected 4 days later by perfusing the peritoneal cavity with 200 ml of Hank's solution. After the oil phase was decanted, the aqueous phase was centrifuged at 800×g for 10 min in the cold. The pellets were washed three times with fresh Hank's solution and suspended in Eagle's MEM supplemented with 10% fetal calf serum to make a cell suspension of 5×10^6 cells/ml. Fifty μg/ml of LPS (Difco Co., Michigan, U.S.A.) was added to the peritoneal exudate cell suspension followed by incubation at 37°C for 48 hrs. The culture supernatant was then separated by centrifugation.

5. Liver cell damage induced by ADCC or activated macrophages and effects of LEM

The culture supernatant (0.5 ml) from the ADCC reaction mixture or activated macrophages prepared as described above was added to 0.5 ml of the liver cell suspension (1×10^6 cells/ml) freshly prepared from the normal rat liver. One μCi of (^{3}H)-L-leucine (specific activity 52 Ci/mmol, Amersham, U.K.) was then added followed by incubation at 37°C for 24 hrs. After incubation, the radioactivity incorporated into the acid-insoluble fractions of the liver cells was measured with a liquid scintillation counter as reported previously[3]. Liver cell damage was evaluated by measuring protein synthesis. To evaluate the effects of LEM on liver cell damage, various concentrations of LEM in Eagle's MEM were added to the liver cell suspension. After incubation at 37°C for 6 hrs, the culture supernatant from the ADCC reaction mixture supernatant from the ADCC reaction mixture or activated macrophages was added, and protein synthesis in the liver cells was determined in the same manner as described above.

6. Statistical analysis

All results are indicated as mean ± SE. Significant differences were calculated by analysis of the variance test ($p<0.05$) and further by student's t-test.

Results

1. Effects of LEM on liver cell damage induced by culture supernatant from ADCC reaction mixture

When liver cell damage induced by ADCC was determined by measuring protein synthesis, it was found that the incorporation of (^{3}H)-L-leucine into the protein was reduced to 68.2 ± 4.8% of the control in which the culture supernatant from the ADCC reaction mixture was not added (**Fig. 2**). However, the impaired protein synthesis in the liver cells was found to improve, when the cells were pretreated with LEM. When the liver cells were pretreated with 10, 50, 100, 200, 300, and 400 μg/ml of LEM, protein synthesis was 121.8 ± 8.5%, 139.1 ± 10.5%, 169.5 ± 10.1%, 181.2 ± 11.2%, 153.1 ± 20.2%, and 121.0 ± 12.4%, respectively, compared with when not pretreated with LEM (**Fig. 3**).

2. Effects of LEM on liver cell damage induced by culture supernatant from activated macrophages

The cytotoxic activity of the activated macrophages was evaluated, and the uptake of (^{3}H)-L-leucine into the protein was found to be reduced to 68.4 ± 4.6% of the control as shown in **Fig. 2**. However, when the culture supernatant was added to the liver cells pretreated with various doses of LEM, the im-

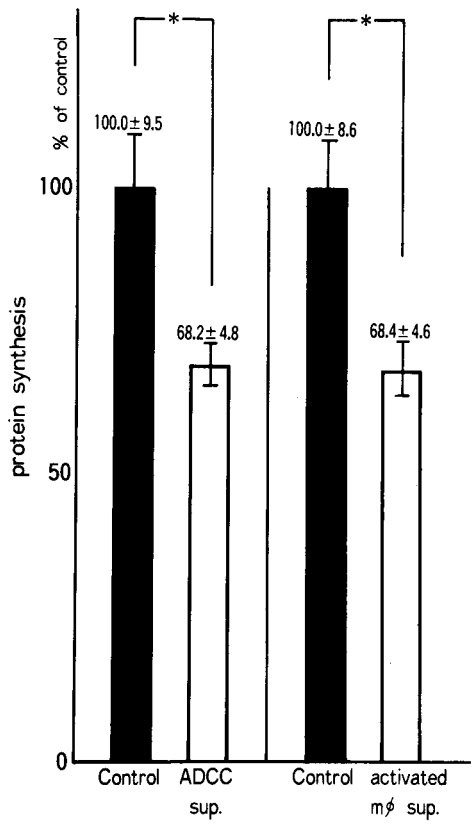

Fig. 2. Liver cell damage induced by culture supernatant from antibody-dependent cell-mediated cytotoxic reaction mixture and activated macrophages (n=6, *p<0.01). Liver cell damage was evaluated by the uptake of (^{3}H)-L-leucine in hepatocytes. Values are given as means ± SD. Control (left): 1784 ± 169 cpm, Control (right): 1898 ± 163 cpm.

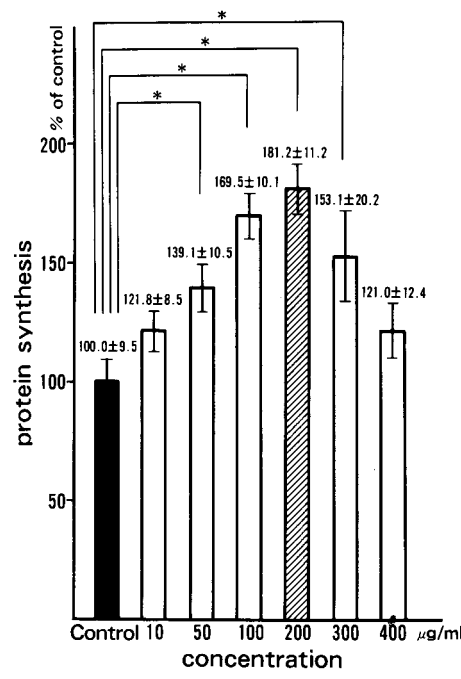

Fig. 3. Effect of LEM on liver cell damage induced by culture supernatant from antibody-dependent cell-mediated cytotoxic reaction mixture (n=10, *p<0.01). Liver cell damage was evaluated by the uptake of (^{3}H)-L-leucine. Values are given as mean ± SD. Control: 1640 ± 156 cpm.

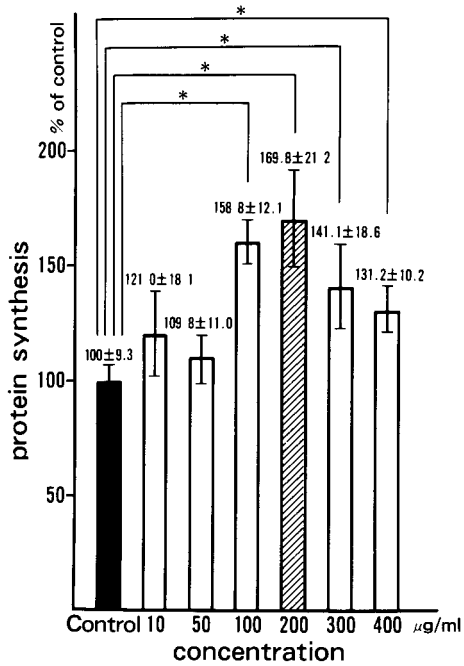

Fig. 4. Effect of LEM on liver cell damage induced by culture supernatant from activated macrophages (n=10, *p<0.01). Liver cell damage was evaluated by the uptake of (^{3}H)-L-leucine. Values are given as means ± SD. Control: 1724 ± 160 cpm.

paired protein synthesis improved significantly. Thus, when the liver cells were pretreated with 10, 50, 100, 200, 300, and 400 μg/ml of LEM, protein synthesis was 121.0 ± 18.1%, 109.8 ± 11.0%, 158.8 ± 12.1%, 169.8 ± 21.2%, 141.1 ± 18.6%, and 131.2 ± 10.2%, respectively (**Fig. 4**).

These results indicated that pretreatment of the liver cells with LEM protected them from damage by the activated macrophages as well as ADCC reaction. The most effective concentration of LEM was found to be 200 μg/ml in both cases.

Discussion

Autoantibodies which react with hepatocyte membrane lipoprotein complex (LSP) have been detected in the sera obtained from patients with untreated chronic active hepatitis, and it has been suggested that these autoantibodies may be involved in causing peripheral hepatocyte necrosis which is a characteristic of this condition[11]. Various immunological reactions can be induced against LSP, and it is thought that ADCC via anti-LSP antibodies is related to the induction of liver cell damage[12–14]. The authors have previously demonstrated that liver cell injury due to ADCC is produced not only by close contact between effector cells and target liver cells via anti-LSP but also by some chemical mediators produced by the effector cells[3]. Furthermore, it has been suggested that liver cell damage may be induced by activated macrophages infiltrating into the liver tissue, because various lymphokines including MAF are produced from LSP-sensitized T cells by stimulation with LSP and because the MAF-activated macrophages are presumed to participate in the induction of liver cell injury. We have demonstrated that liver cell injury is induced not only by cell-to-cell contact between activated macrophages and target liver cells but also by the production of a cytotoxic factor or factors due to the activated macrophages[4,5].

Recently, it has been shown that LEM is beneficial in the therapy of HBe antigen-positive chronic hepatitis[1,2]. That is, LEM is a drug which is useful for ameliorating the HBeAg/anti-HBe system, and improvements in the liver function tests especially the transaminase levels have been observed. Furthermore, LEM is known to have an immunomodulatory effect[15–17] and anti-tumor effect[18–25]. However, the detailed functional mechanism of LEM has yet to be clarified.

In this experiment, we confirmed the protective effects of LEM on experimentally-induced liver cell damage. Pretreatment of liver cells with LEM protected them from cytotoxic effects. Moreover, we found that the protective effects of LEM were dose-limited. Thus the most effective concentration of the drug

was 200 μg/ml, and larger amounts were not necessarily beneficial. Arichi et al.[26] have reported that saikosaponin strongly suppresses hepatic injury induced by D-galactosamine using serum transaminase as the marker, and that when saikosaponin was administered in to the peritoneum 2 hours before D-galactosamine administration, 2 mg/kg was more effective than 20 mg/kg. Since the localization and metabolism of LEM in the liver cells are not clear, further investigations are undoubtedly necessary for the complete understanding of the mechanism of its effects.

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