Mycoscience 39: 351–360, 1998

Modification of cellular immune responses in experimental autoimmune hepatitis in mice by maitake (*Grifola frondosa*)

Keiko Kubo and Hiroaki Nanba

Department of Microbial Chemistry, Kobe Pharmaceutical University, 4–19–1, Motoyamakita-machi, Higashinada-ku, Kobe 658–8558, Japan

Accepted for publication 11 March 1998

Immune response to liver-specific lipoprotein (LSP) is involved in the pathogenesis of chronic active hepatitis. Experimental hepatitis could thus be prepared in C57BL/6 mice by injection of liver-specific protein in a syngeneic liver homogenate with Freund's complete adjuvant. In hepatitic mice treated with maitake (*Grifola frondosa*) fruit bodies, the values of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) values increased temporarily by 2.24–2.79 times and decreased rapidly thereafter. However, in the mice given normal feed, both values increased constantly. Thus, we examined T cell activities both in the exacerbation and remission stages of hepatitis. We suggest that the activation of $CD8^+$ cells is more potentiated than that of $CD4^+$ cells by administration of maitake or the D-Fraction-glucan (β-1,6 glucan having β-1,3 branches), which can enhance immuno-competent cells at the exacerbation stage. However, at the remission stage, marked potentiation of $CD8^+$ cell activity was not observed. These results suggest that depressed suppressor T cell activity is revived by the X-Fraction-glucan (β-1,6 glucan having α-1,4 branched glucan), while the cytotoxic T cell activity, which is activated by the D-Fraction, is restricted, thereby creating a smooth shift from the exacerbation stage to the remission stage.

Key Words——experimental hepatitis; *Grifola frondosa* (maitake); helper T cell; suppressor T cell; X-Fraction-glucan.

A β-1,6-glucan having 1,3 branches, termed the D-Fraction, that was extracted from the fruit body of maitake (*Grifola frondosa*) has been reported to inhibit the growth of solid MM-46 carcinoma (breast tumor) in both oral and intraperitoneal administration by activation of immuno-competent cells (Adachi et al., 1987; Hishida et al., 1988; Nanba et al., 1987). The involvement of immunological functions in the onset and development of hepatitis, in which the cellular immune system plays a particularly important role, has also been reported (Kakumu et al., 1978; Thomson et al., 1974; Vergani et al., 1979; Wands et al., 1975). But we can find no report that mushrooms can cure hepatitis. In this study, the effects of maitake in alleviating liver disorders were examined using hepatic-damaged animal models by active regulation of cytotoxic T (Tc) cells and suppressor T (Ts) cells.

Materials and Methods

Preparation of liver-antigen (Meyer zum Buschenfeld and Miescher, 1972) Fresh livers extirpated from 5–6-wk-old C57BL/6 male mice were homogenized with the same volume of saline at 4°C in a potter homogenizer. The homogenate was centrifuged at $6,000 \times g$ for 10 min at 4°C, and the supernatant was further centrifuged at $150,000 \times g$ for 60 min at below 4°C. The resulting supernatant was used as the crude liver lipoprotein for the liver-specific antigen.

Animals Five-wk-old male C57BL/6 mice (purchased from Clea Japan) were raised on laboratory chow (CE-2, Clea Japan), and water was administered *ad libitum* in a temperature-controlled room, 24 ± 1°C and 55% humidity, under specific pathogen-free conditions for 1 wk before they were used in the experiments.

Immunization with liver-specific antigen (Mori et al., 1985) Six-wk-old C57BL/6 male mice were used. After completely mixing the obtained liver-specific antigen with an equal volume of Freund's complete adjuvant (FCA) at 4°C, immune responses were induced by intramuscularly injecting doses of 10 mg (as protein content) of the antigen into the back of C57BL/6 mice 2–4 times at weekly intervals. Mice immunized with FCA only were used as the control. Variations in glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) values in the blood were used as indicators of liver damage. Blood samples were collected at intervals of mice 7 d after the injections of antigen and assayed to monitor the immune responses.

Preparation of the X-Fraction When the hot-water-extractable fraction was saturated to 50% with EtOH, floating material was obtained that was termed the X-Fraction. The crude X-Fraction was purified by DEAE-Sepharose CL-6B column chromatography by elution with 0.0125 M Tris-HCl buffer (pH 7.25) to eliminate free protein in solution. The eluted material was further purified by Sepharose CL-4B column chromatography by elution with distilled water. The resulting high-

molecular-weight purified X-Fraction (m.w.=787,000 –810,000) was examined using Schlieren ultracentrifugation, and the presence of a single peak indicated that the X-Fraction was purified completely.

Preparation of solid feed of powdered maitake, X-Fraction, or D-Fraction Fruit bodies of maitake were dried at 65°C for 18 h and pulverized to ϕ200 μm. A mixture of 200 g of maitake powder and 800 g of CE-2 chow (Clea Japan) was kneaded thoroughly with 800 ml of distilled water, and the resulting dough was cut into 3 cm cubes and dehydrated at 80°C for 20 h. The solid feed thus obtained was termed used as 20% maitake-feed (20% M-feed). In addition, 100 mg of X-Fraction or 600 mg of D-Fraction (as sugar content) was mixed with 1,000 g of CE-2 feed.

Irradiation with γ-rays C57BL/6 mice were anesthetized with sodium pentobarbital (50 mg/kg weight) and irradiated with γ-rays (3 Gy) from ^{137}Cs at 25°C to specifically inactivate Ts cells.

Preparation and staining of tissue samples Extirpated livers were fixed with 10% neutral formalin solution and embedded in paraffin by the standard method. Liver tissue sections were stained with hematoxylin-eosin solution.

Chemical analysis For blood sampling, the orbital sinus was cut with a heparinized hematocrit tube, and blood was collected in this tube. The capillary tubes were centrifuged at 8,750×g for 4 min, and the separated plasma was frozen until assayed. Values of GOT and GPT were measured by an enzyme method using pyruvic acid oxidase (Transaminase C II-test, Wako).

Cell preparation The spleens of C57BL/6 mice immunized with liver-specific antigen were extirpated, and bacteria-free spleen cells were obtained by passage through a nylon mesh (ϕ150 μm). After destroying erythrocytes with a hemolytic buffer (Tris-NH_4Cl solution), lymphocytes were obtained through a density-gradient separation method using lymphocyte separation medium (Ficoll and sodium metrizoate, specific density of 1.077±0.001 g/ml). These cells were suspended in RPMI-1640 medium (Nissui) containing 20% fetal calf serum (FCS), and non-adherent (T cell-enriched) and adherent (B cell-enriched) cells were separated by use of a nylon wool column (NWC).

Treatment with antibody As shown in Chart 1, a 10 : 1 mixture of 1×10^7 cells/ml and a monoclonal antibody was incubated at 4°C for 60 min. After centrifugation at 300×g for 5 min, the obtained cell pellet was resuspended in 1 ml of RPMI-1640 medium containing 0.1 ml of complement of low-tox guinea pig. After incubation at 37°C for 60 min, cell number was adjusted to 5×10^6 cells/ml with RPMI-1640 medium containing 20% FCS.

Preparation of T cell subsets T cell subsets of helper T cells (CD 4$^+$) or cytotoxic/suppressor T cells (CD 8$^+$) were checked by reaction with anti-Ly 1.2 (CD 4$^+$) monoclonal antibody or anti-Ly 2 (CD 8$^+$) monoclonal antibody, respectively.

Incorporation of ^{3}H-thymidine into cells A mixture of 1×10^6 cells/well with 10 μl of ^{3}H-thymidine (^{3}H-TdR) (370 kBq/ml) was incubated at 37°C for 18 h under 5% CO_2 gas. The cells were collected onto the filter of a cell-harvester (M-24R system, BRANDEL), and then the incorporated radioisotope (RI) activities in the cells were measured with a liquid scintillation counter (LSC-700, Aloka). The amount of ^{3}H-TdR incorporated into T cells was obtained by subtracting RI activities in whole spleen cells treated with Thy 1.2 monoclonal antibody from incorporated RI activities in whole spleen cells. Activities of CD 4$^+$ and CD 8$^+$ cells were expressed as the ratio to whole T cell activities.

Assay for DNA synthesis of suppressor T cell Suppressor T (Ts) cell activity was determined as the ability of cell which was reacted with concanavalin A (Con A). A lymphocyte transformation test was conducted using Con A as a mitogen. Each cell fraction was placed into a microtest plate (FALCON 3072) at 1×10^5 cells/well and incubated at 37°C for 4 d in a 5% CO_2 gas-containing incubator with 1 μg/well Con A. At 18 h before the end of the incubation, 3.7 kBq of ^{3}H-TdR was added. After the incubation, cells were harvested, and the incorporated ^{3}H-TdR was counted with a liquid scintillation counter (LSC-700, Aloka).

Methylation After complete elimination of protein in X-Fraction by treatment with trypsin (specific activity; 3,000 U/mg from pig pancreas, at 37°C, 0.1 M McIlvaine buffer (pH 6.8)) and protease (specific activity; 0.2 U/mg from *Aspergillus oryzae* at 37°C, 0.1 M McIlvaine buffer (pH 8.2)), obtained X-Fraction-glucan was dried. To determine the chemical structure of glucan, methylation was performed according to Hakomori's method (Hakomori, 1964).

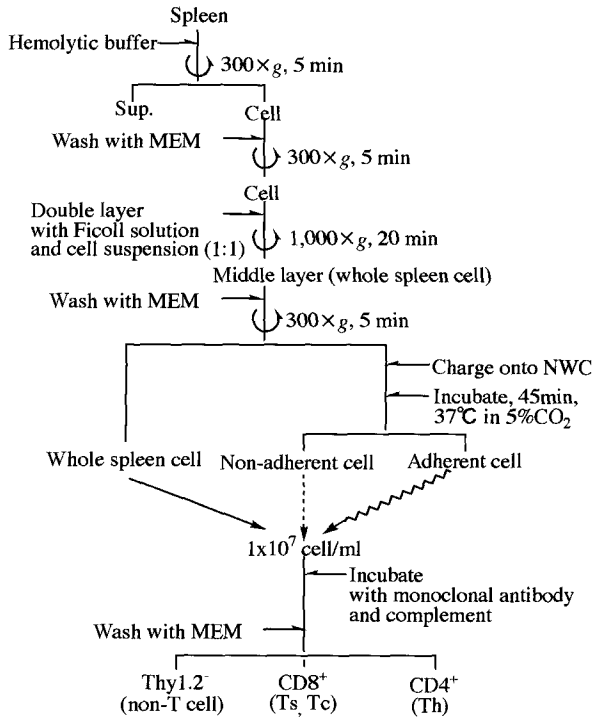

Chart 1. Preparation of T cell subsets.

The X-Fraction-glucan (30 mg) was dissolved in Me_2SO (5 ml) by agitating with a magnetic stirrer at 20–25°C for 5 h. NaH (1.5 g) was washed with 15 ml of petroleum ether, then suspended in Me_2SO (16 ml), stirred in a nitrogen stream at 20–25°C for 0.5 h, and incubated further at 50–60°C for 1.5 h. These two solutions were mixed and incubated at 20–25°C for 4 h under continuous stirring. The temperature was lowered to 4°C, and MeI (8 ml) was added over 1 h period, then stirring was continued at 20–25°C for a further 20–24 h. The reaction mixture was then washed with $CHCl_3$: H_2O (1 : 1) mixture (20 ml), and the methylated product was repeatedly extracted with $CHCl_3$ (5 ml). The combined extracts were evaporated to 1 ml in vacuo. This was dropped into petroleum ether (15 ml), and the pellet produced was collected by centrifugation at 7,000×*g* for 15 min.

The infrared spectrum (Hitachi 270–30) of the methylated material showed no absorption in the 3,200–3,700 cm^{-1} region attributable to the OH- group.

Methanolysis and acid hydrolysis of the methylated β-glucan The methylated compound, was dissolved in 5% MeOH-HCl, and heated at 120°C for 4 h in a sealed tube. After evaporating the solution in vacuo, 10 μl of the methanolyzed compound in 1 ml of MeOH was submitted to gas-liquid chromatography (GLC: Hitachi 663-30) on a glass column (3×100 mm) packed with 60–80 mesh 1.5% neopentyl succinate on Chromosorb. The column temperature was 140°C.

^{13}C-Nuclear Magnetic Resonance (^{13}C-NMR) Spectrum To clarify the chemical bonds of X-Fraction-glucan, 50 mg of the purified materials was dissolved in heavy

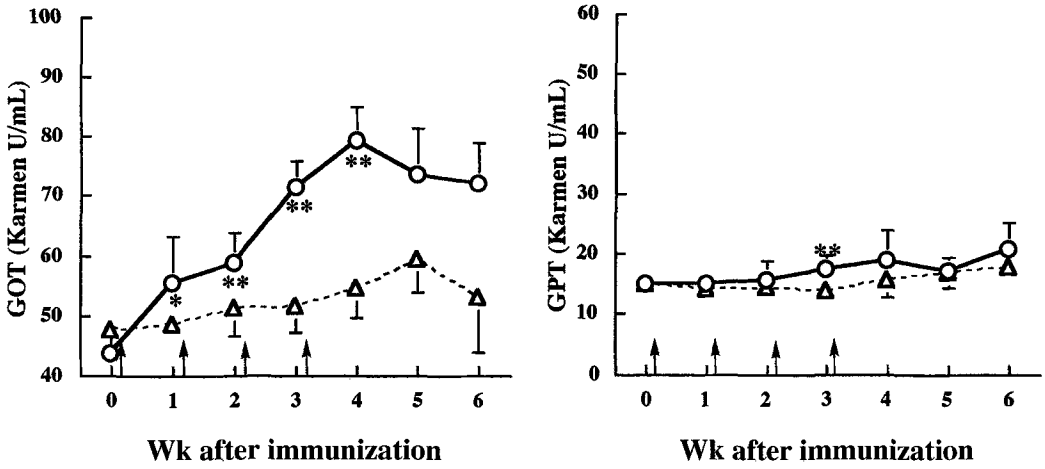

Fig. 1. Profiles of GOT and GPT values in plasma of mice immunized with LSP.
—○—: LSP+FCA. ···△···: FCA. ↑: injection. Significant differences (*t*-test): $^*P<0.05$, $^{**}P<0.01$ (ten mice per group).

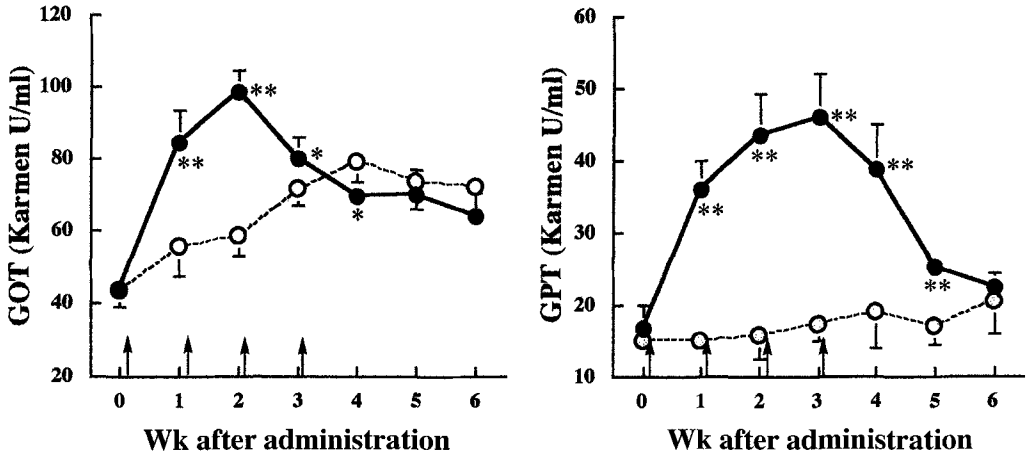

Fig. 2. Effect of maitake (*Grifola frondosa*) fruit bodies on GOT and GPT values of autoimmune hepatitis mice.
—●—: maitake; ···○···: control. ↑: LSP injection. Significant differences (*t*-test): $^*P<0.05$, $^{**}P<0.01$ (10 mice per group).

Abbreviation list: CAH, chronic active hepatitis; Con A, concanavalin A; FCA, Freund's complete adjuvant; FCS, fetal calf serum; GLC, gas-liquid chromatography; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; IL-2, interleukin 2; LSP, liver specific lypoprotein; NWC, nylon wool column; Tc, cytotoxic T cell; TdR, thymidine; Th, helper T cell; Ts, suppressor T cell.

water and analyzed with ^{13}C-NMR (Varian 500) at 25°C.

Statistical examination Student's *t*-test was used to evaluate the significance of differences in the measured values between groups.

Results

Immune response to liver specific lipoprotein (LSP) is involved in the pathogenesis of chronic active hepatitis (Mizoguchi et al., 1982). Therefore, we examined whether liver disorders in experimental model animals are regulated by utilizing the immune response induced by the β-glucan of maitake mushrooms.

Figure 1 shows the variation in GOT and GPT values as a result of the injection of liver antigen. Immunization with the liver antigen increased the GOT values substantially compared with those in the group given adjuvant only. The GOT value was increased about 2-fold after four doses of LSP. GPT values did not increase as markedly as GOT values did, but they did increase slightly with a significant difference as shown in Fig. 1. This result indicates that the injection of LSP can induce the autoimmune hepatitis. These hepatic mice were then fed with 20% M-feed, and the changes in GOT and GPT were examined. As shown in Fig. 2, following the administration of maitake feeds, GOT and GPT values increased temporarily to 2.24 and 2.79 times, respectively, but decreased rapidly thereafter. When the normal feed was administered, both values increased constantly and no reduction was observed. These results indicate that maitake has activity against hepatic liver cells. Based on GOT and GPT values in Fig. 2, we determined the exacerbation stage of hepatitis to be a period of about 1 wk after the second immunization, when liver cell injury seems to occur most actively. The remission stage is thought to begin 1 wk after the 4th immunization, when both the GOT and GPT values start declining. Also, liver tissue sections were prepared and stained with hematoxylin-eosin for histological observation. Figure 3 shows an anatomical pattern of the liver in the exacerba-

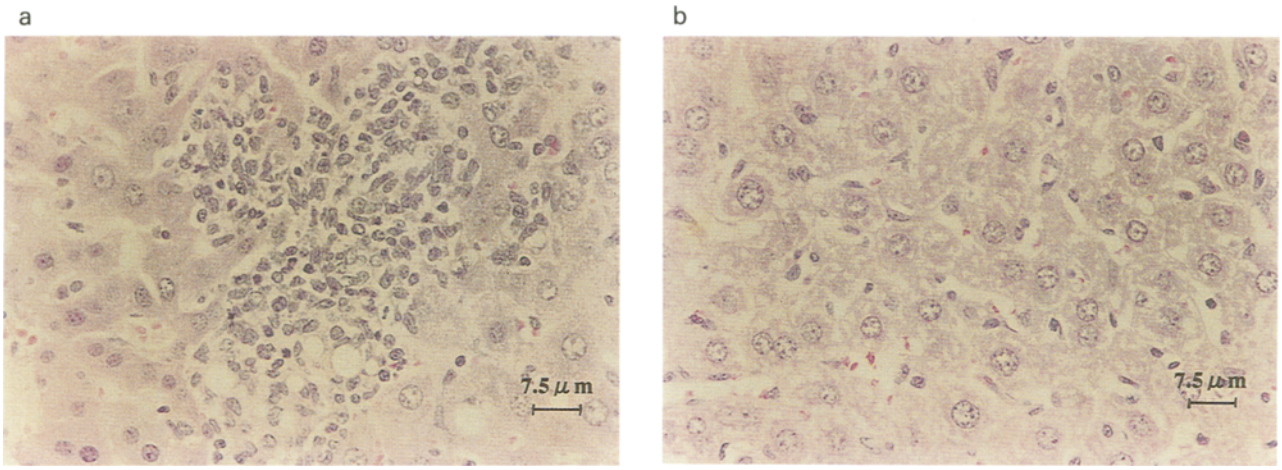

Fig. 3. Anatomical picture of liver tissue on exacerbation stage.
a: maitake treated; b: control (non-treated).

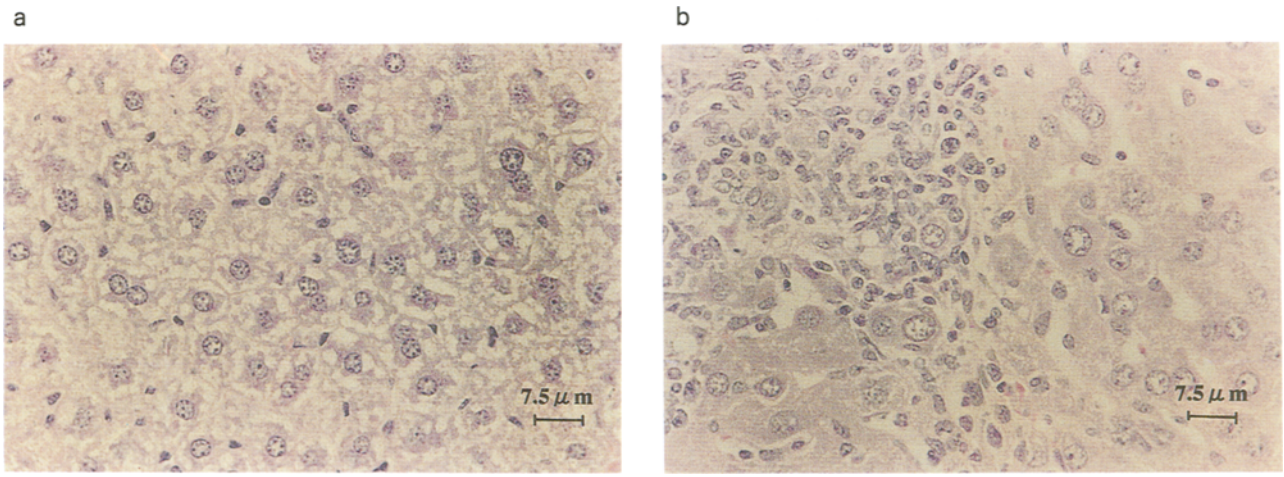

Fig. 4. Anatomical picture of liver tissue on remission stage.
a: maitake treated; b: control (non-treated).

tion stage. After administration of maitake, inflammatory cells such as lymphocytes, polymorphonuclear cells and plasmocytes were observed (Fig. 3a). On the other hand, in the control group mice (the group without maitake treatment), vacuolation of the liver cells was found, but infiltration and necrosis were not observed (Fig. 3b). These results indicate that the cytotoxic T (Tc) cell activity was not enhanced sufficiently when maitake was not administered. Figure 4 shows a micrograph of the remission stage. Although the values of GOT and GPT were observed to decrease in the maitake-treated mice during this stage, necrosis of liver cells and infiltration of inflammatory cells were visible. However, binuclear cells were also observed, suggesting that the regeneration of liver cells began after the elimination of necrotic cells (Fig. 4a). On the other hand, severe infiltration of inflammatory cells and also inflammatory cells in liver lobules were observed in the control group in the remission stage (Fig. 4b). In addition, in the control group, regenerating cells like those in the maitake-treated mice were not observed.

These facts suggest that autoimmue chronic hepatitis occurs more severely in the control mice than in the maitake-treated mice, because serious symptoms like those of hepatitis were observed during the remission stage. To clarify the suppression of the autoimmune hepatitis by maitake, adherent and non-adherent spleen cells were fractionated using a nylon wool column (NWC). Monoclonal antibodies (Ly 1.2 or Ly 2) which bind specifically to each T cell subset were used for separation of CD 4^+ and CD 8^+ cells. In the exacerbation stage, the incorporated ^{3}H-thymidine doubled on immunization with the liver antigen. As shown in Fig. 5, the activation of CD 8^+ cells was potentiated 1.25-fold by maitake administration. This result indicates that either Ts cells or Tc cells are activated by maitake. The results in the remission stage are shown in Fig. 6. In both the maitake-treated and non-treated (control) groups, CD 4^+ cell activities were enhanced compared to CD 8^+ cell activities. Even on maitake administration, potentiation of CD 8^+ cell activity was not observed as it was in the exacerbation stage. When the activation tests of T cells were repeated 8 times, the CD 4^+/CD 8^+ ratio of the M-feed group was 0.79–0.97-fold lower than that of the control group in the exacerbation and remission stages, as shown in Fig. 7. In addition, the ratio for T cells was

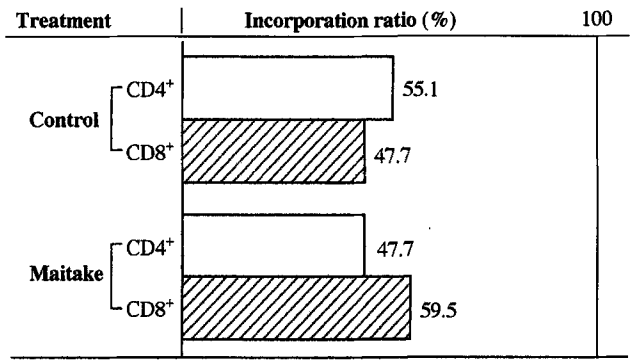

Fig. 5. Incorporation ratio of ^{3}H-thymidine into T-cell subsets obtained from C57BL/6 mice immunized with LSP in the exacerbation stage.
Immunization was performed 2 times at weekly intervals.
Eight mice per group.

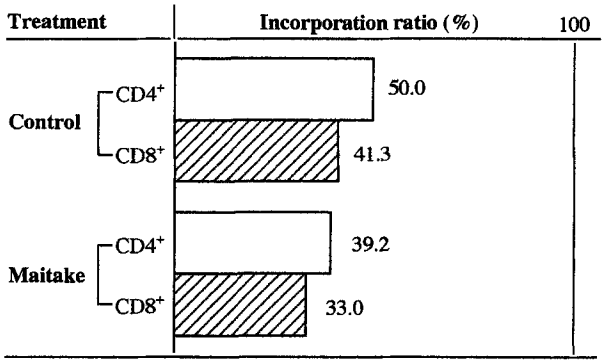

Fig. 6. Incorporation ratio of ^{3}H-thymidine into T-cell subsets obtained from C57BL/6 mice immunized with LSP in the remission stage.
Immunization was performed 4 times at weekly intervals.
Eight mice per group.

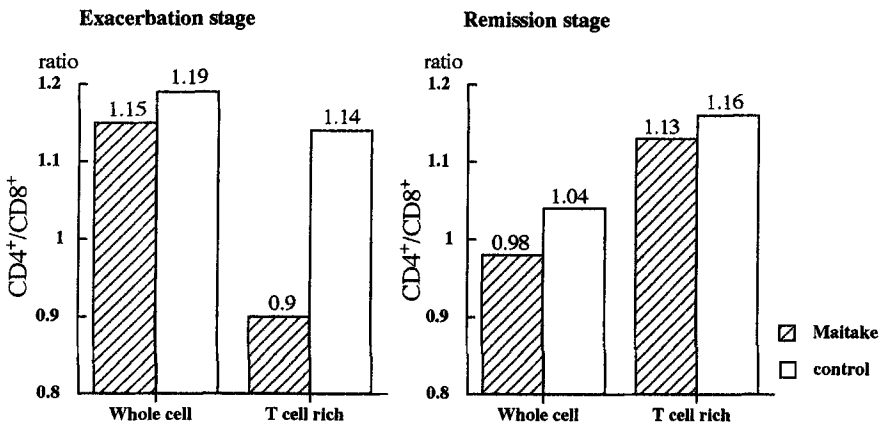

Fig. 7. Effect of maitake (*Grifola frondosa*) fruit bodies on CD4$^+$/CD8$^+$ cell ratio in the exacerbation and remission stages of hepatitis.
Eight mice per group.

Table 1. Incorporation of ^{3}H-thymidine into concanavalin A-reacted T cells in the remission stage.

Treatment	Whole spleen cell ($\times 10^3$ dpm)	NWC-non-adherent cell ($\times 10^3$ dpm)
Maitake	77.7±3.1 (1.47) **	84.3±0.8 (2.07) **
Control	52.8±2.7 (1.00)	40.8±1.2 (1.00)
FCA	115.5±3.1	107.3±3.3
Normal	75.2±0.7	73.8±3.0

Significance differences (*t*-test): $^{**}P<0.01$. Control: hepatitic mice (normal feed). Normal: normal mice (normal feed). Eight mice per group.

Table 2. Incorporation of ^{3}H-thymidine into concanavalin A-reacted T cells in the exacerbation stage.

Treatment	Whole spleen cell ($\times 10^3$ dpm)	NWC-non-adherent cell ($\times 10^3$ dpm)
Maitake	12.1±0.9 (0.69) **	34.5±2.8 (0.56) **
Control	17.6±1.2 (1.00)	61.3±2.1 (1.00)
FCA	22.5±1.9	118.2±4.4
Normal	14.3±1.6	79.1±1.0

Significant differences (*t*-test): $^{**}P<0.01$. Control: hepatitic mice (normal feed). Normal: normal mice (normal feed). Eight mice per group.

0.80-fold lower in the exacerbation stage than in the remission stage. These results suggest that activation of CD 8$^+$ cells, i.e., Ts cells or Tc cells, occurs strongly during the exacerbation stage, and that the activated T cells gradually decline during the remission stage.

Thus, to examine which T cells are activated, Con A was used as a mitogen for T cells in the remission stage in order to measure the activation of Ts cells. The results are shown in Table 1. On treatment with maitake, the activities of whole spleen cells or the T cell-enriched fraction were observed to be potentiated by 1.47 and 2.07 times, respectively, compared to those in the control group. This result suggests that maitake induced the activation of Ts cells during the remission stage. Table 2 shows the activation of cells treated with Con A in the exacerbation stage. Incorporation of ^{3}H-TdR into Con A-reacted T cells in both the whole spleen cells and the T cell-enriched fraction decreased by 0.69 and 0.56 times respectively, but the decreases were not prominent in the control group. These results indicate the possibility that maitake revives the depressed Ts cell activity while restricting the Tc cell activity, thus promoting a smooth shift from the exacerbation stage to the remission stage. As shown in Table 3, the ratio of Thy 1.2 positive cells in the NWC-adherent cell fraction of the maitake-treated group increased during the exacerbation stage to 1.66 times that of the control group. As shown in Fig. 8, the CD 4$^+$/CD 8$^+$ cell ratio was depressed by the maitake treatment. Histomorphology and cell level examination suggest that Ts cells are activated by administration of maitake and restrict the chronic development of hepatitis. To confirm this suggestion, irradiation with a low amount of ^{137}Cs γ-ray was performed to

Table 3. Percentage of Thy 1.2 monoclonal antibody-positive cells in NWC-adherent cells in spleen from LSP immunized C57BL/6 mice.

Treatment	Thy 1.2 monoclonal antibody-positive cells	
	Exacerbation stage	Remission stage
Maitake	54.4% (1.66) (1.00)	25.9 (1.13) (0.48)
Control	32.8 (1.00) (1.00)	22.9 (1.00) (0.70)

Eight mice per group.

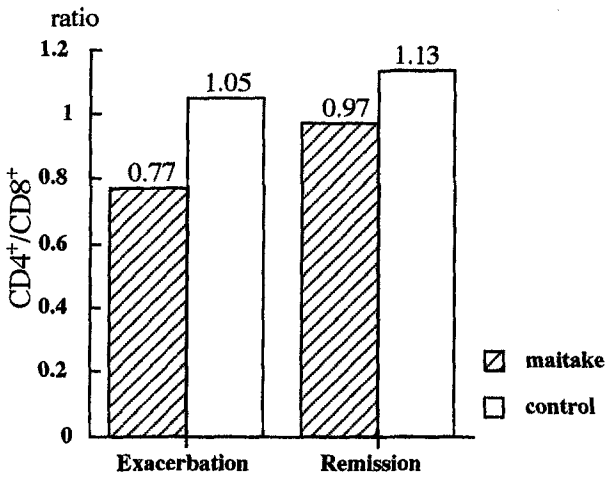

Fig. 8. Effect of maitake (*Grifola frondosa*) fruit bodies on NWC-adherent cell at each stage of hepatitis. Eight mice per group.

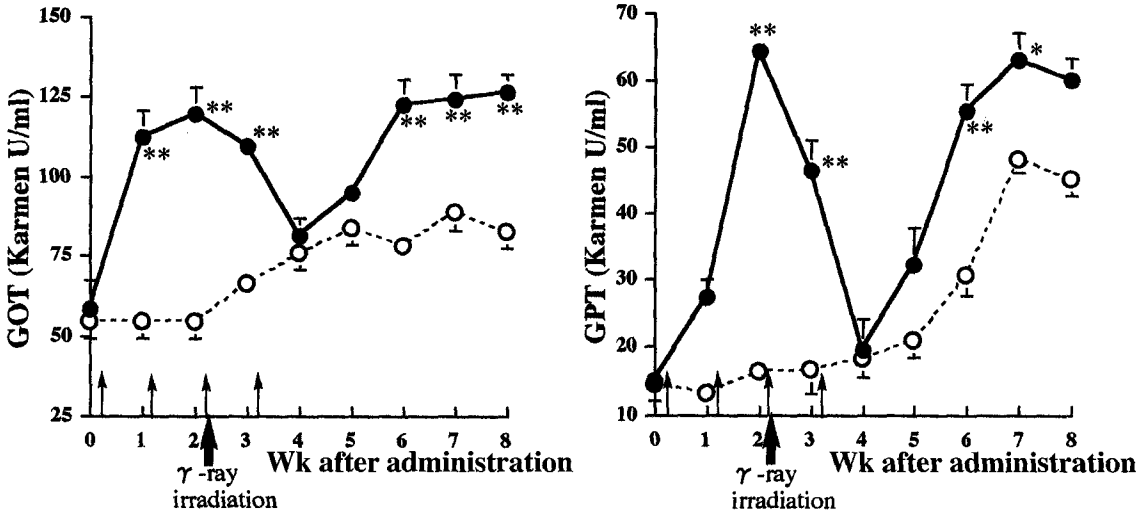

Fig. 9. Profiles of GOT and GPT values after γ-ray irradiation.
—●—: maitake; ···○···: control. ↑ : injection. Significant differences (*t*-test): $^{*}P<0.05$, $^{**}P<0.01$ (eight mice per group).

injure mainly suppressor T cells. As shown in Fig. 9, following the temporary increase and subsequent decline towards the remission stage, both GOT and GPT values began increasing again. This result indicates that the Ts cells which were damaged by γ-ray irradiation need to be activated for the inhibition of Tc cell activity. Figure 10 shows the results of the CD 4^+ and CD 8^+ cell activation ratio 4 wk after γ-ray irradiation of the maitake-administered hepatic mice. The activation of CD 8^+ cells apparently decreased due to the γ-ray irradiation, but CD 4^+ cell activities increased. These results suggest that the Ts cells in the T cell subsets are destroyed by the γ-rays. To confirm which components in maitake exhibit these anti-hepatitic activities, two kinds of glucans, named the D-Fraction and the X-Fraction, were prepared by 50–80% saturation with EtOH. Beta-1,6 glucan having β-1,3 branched chains, termed as the D-Fraction, has

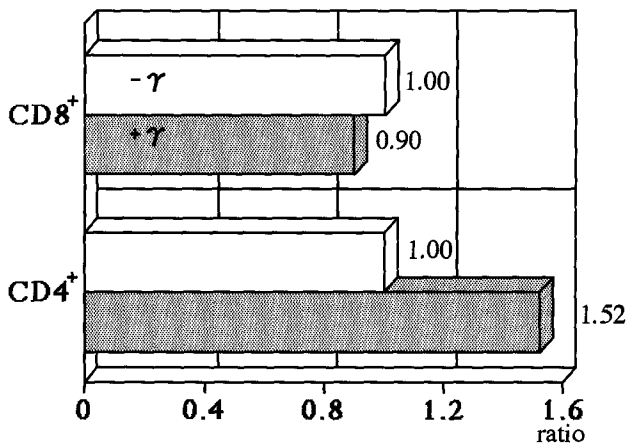

Fig. 10. Activity-ratio of $CD4^+$ and $CD8^+$ cells before and after treatment with γ-ray irradiation.
Eight mice per group. 1.00 indicates the activity of cell which have no γ-ray irradiation.

been reported to enhance the activities against cellular immune-competent cells such as macrophages, NK, LAK, killer T cells and Tc cells (Adachi et al., 1987). Here, we report that, when the glucans in floating material from a 50% EtOH solution (m.w. 550,000-558,000: X-Fraction-glucan) was methylated by Hakomori's method, as shown in Table 4a, 3 mol of 2,3,4,6-tetra-, 3 mol of 2,3,4-tri-, and 2 mol of 2,3-di-*O*-methyl-D-glucose were detected. Moreover, as shown in Table 4b, on ^{13}C-NMR analysis, signals at 78.8 (78.0) ppm, corresponding to a 1,4-bond, and signals at 71.0, (70.1) ppm, corresponding to a 1,6-bond, were observed.

When this fraction was treated with β-glucosidase, a small amount of glucose was detected, and on treatment with α-glucosidase, glucose was liberated (data not shown). These results indicate that the chemical structure of the X-Fraction-glucan is a β-1,6 main chain having α-1,4 side chains as shown in Fig. 11. However, the position of the side chains is not yet determined. The X-Fraction also contains about 35% protein. When the D-Fraction was administered to hepatitic mice, as shown in Fig. 12, the GOT and GPT values increases but when the X-Fraction was administered, they decreased. These results suggest that the D-Fraction activates the Tc cells which can injure the injured hepatic cells, and the X-Fraction suppresses the Tc cell activity. As shown in

Table 4. Analyses of glucan in X-Fraction by methylation (a) and ^{13}C-NMR (b).
(a) Methylated sugar.

	molar ratio
2,3-di-*O*-methyl-D-glucose	2.1
2,3,4-tri-*O*-methyl-D-glucose	3.0
2,3,4,6-tetra-*O*-methyl-D-glucose	3.1

(b) Peaks of ^{13}C-NMR: 100.5, 99.2, 78.8 (78.0), 73.9, 72.1, 71.0, 70.1, 61.1 (60.1) ppm.

Table 5, during the remission stage, Con A-reacted T cell activity was enhanced by 2.2-fold, but during the exacerbation stage, the activity was decreased by 0.50 by the X-Fraction. These results suggest that Con A-reacted T cell activity is enhanced by the X-Fraction-glucan in the remission stage.

To clarify which components of the X-Fraction induce the enhanced activity against Ts cells, the 35% protein in the X-Fraction was completely eliminated by the protease. The obtained X-Fraction-glucan indicates the activation of T cells as shown in Fig. 13.

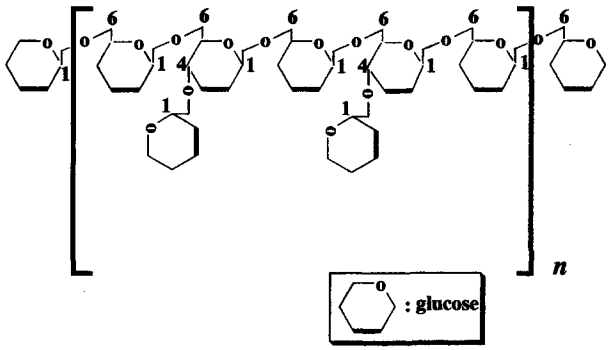

Fig. 11. Chemical structure of glucan in X-Fraction of maitake (*Grifola frondosa*) fruit bodies.

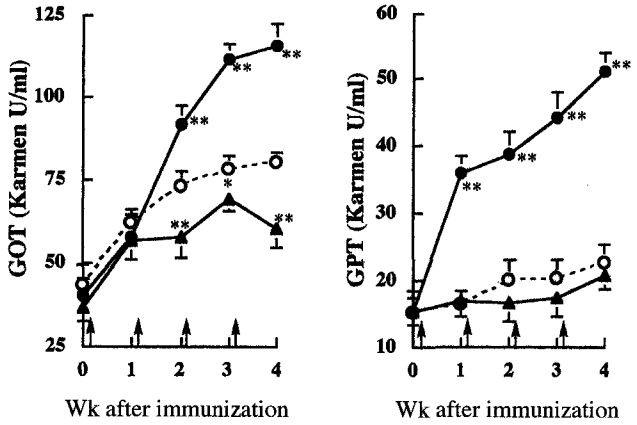

Fig. 12. Effects of D-Fraction and X-Fraction in fruit bodies of maitake (*Grifola frondosa*) on GOT and GPT values of autoimmune hepatitis mice.
—●—; D-Fraction, —▲—; X-Fraction, ···○···; control. ↑; LSP injection. Significant differences (*t*-test): $^{*}P<0.05$, $^{**}P<0.01$ (eight mice per group).

Discussion

Cellular immune regulation is said to be closely involved in the onset, development, and recovery of hepatitis. The β-glucan in maitake termed the D-Fraction can enhance the cellular immune system (Hishida et al., 1988). Therefore, we examined the influence of maitake on the mechanism of recovery from hepatic disorders. An experimental allergic hepatitis model was created by administering the liver cell membrane-specific lipoprotein (LSP) to C57BL/6 mice. The experimental hepatic models were prepared according to the report that chronic active hepatitis (CAH) occurs on immunizing a rabbit for a long period with human LSP with Freund's complete adjuvant (Meyer zum Buschenfelde et al., 1972). In a state of hepatitis, the damaged hepatocytes must be eliminated for recovery. These hepatocytes are destroyed by Tc cells, which are activated by interleukin-2 (IL-2) released by helper T (Th) cells. The temporary increase observed in GOT and GPT values is thought to be due to the destruction of hepatocytes. If the destruction of hepatocytes occurs excessively, serious tissue injury results and the immune responses are not sufficient for recovery, so that continuous infection would be established. However, these actions controlled by the immunocompetent cells, and the activated Tc cells would destroy and eliminate only the inflammatory hepatocytes

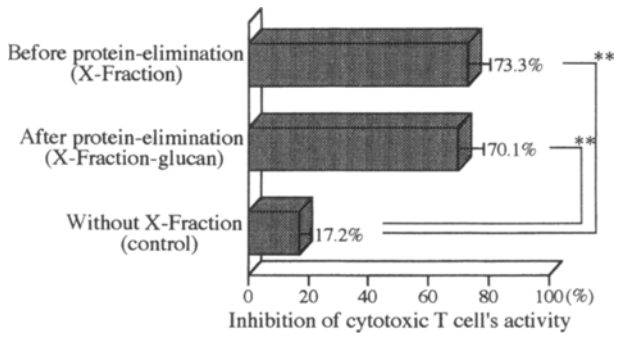

Fig. 13. Activation effects of X-Fraction or X-Fraction-glucan against concanavalin A-reacted $CD8^{+}$ (Ts) cell.
Significant differences: $^{**}P<0.01$ (eight mice per group).
Inhibition (%) =
$$\left(1-\frac{\text{specific release (dpm)}-\text{spontaneous release (dpm)}}{\text{maximal release (dpm)}-\text{spontaneous release (dpm)}}\right)\times 100$$

Table 5. Effect of X-Fraction of maitake (*Grifola frondosa*) fruit bodies on the activity of concanavalin A-reacted T cells.

	Treatment ($\times 10^3$ dpm)	
	Control	X-Fraction
Exacerbation stage	61.3±2.1 (1.00)	30.6±1.9 (0.50)**
Remission stage	40.8±1.2 (1.00)	90.1±0.6 (2.21)**

Control: hepatitic mice (normal feed). Significant differences (t-test): $^{**}P<0.01$ (eight mice per group).

in order to terminate the exacerbation stage. That is, the activated Tc cells appear to function so as to cause a prompt shift to the remission stage modulated by the activated Ts cells. However, if the function of Ts cells deteriorates, the injury to hepatocytes by Tc cells is maintained. The exacerbation stage is prolonged and does not shift to the remission stage. As a result, chronic hepatitis is observed.

The reaction of various mitogens with the lymphocytes from hepatitis patients was studied. In case of CAH patients, B cell activities are not altered, but the responses to PHA and Con A, which act as the mitogens for T cells, decrease significantly (Hodgson et al., 1978; Peavy and Pierce, 1974). This indicates that the activity of Ts cells is suppressed in CAH patients. These reports correspond with results of this study. As shown in Fig. 2, GOT and GPT values temporarily increased on administration of maitake, and then decreased rapidly. This fact suggests that a prompt shift from the exacerbation stage to the remission stage is induced. But the hepatic mice without maitake treatment (the control group) did not show these responses.

Our study of histopathology, shown in Fig. 3a, number of infiltrated cells were observed in the exacerbation stage in the maitake-treated group, and the destruction and elimination of damaged hepatocytes were confirmed to accompany the temporary increase in GOT and GPT values at this stage. In maitake-fed mice, in spite of the continuous immunity with hepatic antigen, a rapid decrease in GOT and GPT values was observed in the remission stage, and necrosis and infiltrated cells were still seen in the hepatic tissues of this stage. However, the revival of damaged cells was already completed, and the enzymes in the blood had no influence (Fig. 4a).

These histopathological findings were also studied at the cellular level, and it was confirmed that the activity of CD 8^+ cells was increased by maitake administration in both the exacerbation and remission stages. However, both Tc cells and Ts cells are classified as CD 8^+ cells, and Con A was used as a mitogen specifically for Ts cells. As a result, it was observed that the Con A-reacted T cell activity of the maitake-treatment group substantially increased by 2.07 times in the remission stage compared to that of the control group. On the contrary, this activity was confirmed to be inhibited in the exacerbation stage by 0.56 times compared to the control group. It has been reported that the Tc cells which destroy the injured hepatocytes are contained in NWC-adherent cells (B cell-enriched fraction) (Mori et al., 1986). As shown in Table 3, 54.4% of the T cells in the maitake group were positive to the Thy 1.2 monoclonal antibody, especially in the exacerbation stage. The percentage of the Thy 1.2 monoclonal antibody positive cells of NWC-adherent cells which was obtained from the treatment groups, decreased in the remission stage.

Further, a low dose of γ-rays, sufficient to damage the function of Ts cells was irradiated during the shift from the exacerbation to the remission stage. The shift to the remission stage was not observed even with the maitake treatment after γ-irradiation. This result suggests that the activity of the γ-ray responsive T cells (probably Ts cells) is needed for the shift to the remission stage.

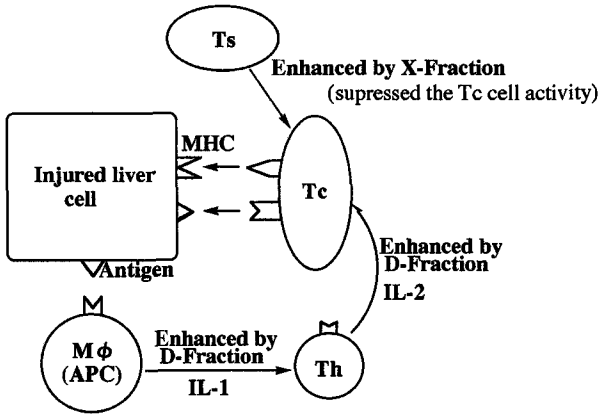

Fig. 14. Possible regulation mechanisms for recovery of autoimmune hepatitis by cellular-immune system with D- or X-Fraction of maitake (*Grifola frondosa*) fruit bodies.

To clarify which materials in maitake enhance Tc and Ts cells, two glucans were purified from the fruit bodies in maitake. We propose that, in the exacerbation stage, the D-Fraction (β-1,6 glucan having 1,3 branches) activates Tc cells which damage and destroy the injured hepatocytes, and in the remission stage, the X-Fraction (β-1,6 glucan having α-1,4 branches) enhances the activity of Ts cells which suppresses the activity of Tc cells.

Furthermore, it is necessary to collect only Ts cells in CD 8^+ cells which were activated by the X-Fraction in maitake. We speculate that maitake has the ability to regulate and improve these response mechanisms appropriately as shown in Fig. 14.

Acknowledgements——The authors thank Ms. Sumiko Takii for her assistance and Mr. Yasuo Ohdaira, Yukiguni Maitake Ltd., for supplying the maitake.

Literature cited

Adachi, K., Nanba, H. and Kuroda, H. 1987. Potentiation of host-mediated antitumor activity in mice by β-glucan obtained from *Grifola frondosa* (Maitake). Chem. Pharm. Bull. **35**: 262–270.

Hakomori, S. 1964. A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. **55**: 205–208.

Hishida, I., Nanaba, H. and Kuroda, H. 1988. Antitumor activity exhibited by orally administered extract from fruit body of *Grifola frondosa* (Maitake). Chem. Pharm. Bull. **36**: 1819–1827.

Hodgson, H. J. F., Wands, J. R. and Isselbacher, K. J. 1978. Alteration in suppressor cell activity in chronic active hepatitis. Proc. Natl. Acad. Sci. USA **75**: 1549–1553.

Kakumu, S., Hara, T., Goji, H. and Sakamoto, N. 1978. Lymphocyte cytotoxicity against chang liver cells in chronic active hepatitis. Cell Immunol. **36**: 46–53.

Meyer zum Buschenfelde, K. H. and Miescher, P. A. 1972. Liver specific antigens purification and characterization. Clin. Exp. Immunol. **10**: 89–102.

Meyer zum Buschenfelde, K. H., Kossling, F. K. and Miescher, P. A. 1972. Experimental chronic active hepatitis in rabbits following immunization with human liver proteins. Clin. Exp. Immunol. **11**: 99–108.

Mizoguchi, Y., Monna, T., Yamamoto, S. and Morisawa, S. 1982. Studies on the mechanism of liver injury by antibody-dependent cell-mediated cytotoxicity (ADCC): partial purification of cytotoxic factor detected in the culture supernatant of ADCC reaction. Gastroenterogia Japonica **17**: 360–367.

Mori, T., Mori, Y., Ogawa, M., Ueda, S., Yoshida, H., Iesato, K., Kato, I., Wakashin, Y. and Wakashin, M. 1986. Cytotoxic activities of sensitized spleen cells from hepatitis mice against hepatocyte: characterization of immune mechanisms responsible for hepatocellular damages in microcytotoxicity assay. Kanzo **27**: 425–430.

Mori, Y., Mori, T., Ueda, S., Yoshida, H., Iesato, K., Wakashin, Y., Wakashin, M. and Okuda, K. 1985. Study of cellular immunity in experimental autoimmune hepatitis in mice: transfer of spleen cells sensitized with liver proteins. Clin. Exp. Immunol. **61**: 577–584.

Nanba, H., Hamaguchi, A. and Kuroda, H. 1987. The chemical structure of an antitumor polysaccharide in fruit bodies of *Grifola frondosa* (Maitake). Chem. Pharm. Bull. **35**: 1162–1168.

Peavy, D. and Pierce, C. W. 1974. Cell-mediated immune responses in vitro. I. Suppression of the generation of cytotoxic lymphocytes by concanavalin A and concanavalin A-activated spleen cells. J. Exp. Med. **140**: 356–369.

Thomson, A. D., Cochran, A. M. G., McFarlane, I. G., Eddleston, A. L. W. F. and Williams, R. 1974. Lymphocyte cytotoxicity to isolated hepatocyte in chronic active hepatitis. Nature **252**: 721–722.

Vergani, G. M., Vergani, D., Jenkins, P. J., Portmann, B., Mowat, A. P., Eddleston, A. L. W. F. and Williams, R. 1979. Lymphocyte cytotoxicity to autologous hepatocytes in HBsAg-negative chronic active hepatitis. Clin. Exp. Immunol. **38**: 16–21.

Wands, J. R., Perroto, J. L., Alpert, E. and Isselbacher, K. J. 1975. Cell-mediated immunity in acute and chronic hepatitis. J. Clin. Invest. **55**: 921–929.