Agaricus blazei (Himematsutake) does not alter the development of rat diethylnitrosamine-initiated hepatic preneoplastic foci

Luís Fernando Barbisan,[1] Ana Lúcia Tozzi Spinardi-Barbisan,[2] Eduardo Luís Trindade Moreira,[3] Daisy Maria Favero Salvadori,[2] Lúcia Regina Ribeiro,[2,4] Augusto Ferreira da Eira[5] and João Lauro Viana de Camargo[2,6]

[1]Departamento de Morfologia, Instituto de Biologia and [2]Departamento de Patologia, TOXICAN, Faculdade de Medicina, UNESP, Botucatu, 18618-000, SP, Brazil, [3]Departamento de Patologia e Clínicas, Escola de Medicina Veterinária, UFBA, Salvador, 40170-1110, BA, Brazil, [4]Universidade Luterana do Brasil, ULBRA, Canoas, 92001-970, RS, Brazil and [5]Departamento de Produção Vegetal, Módulo de Cogumelos, Faculdade de Ciências Agronômicas, UNESP, Botucatu 18603-970, SP, Brazil

(Received September 17, 2002/Revised November 11, 2002/Accepted November 14, 2002)

The modifying potential of crude extracts of the mushroom *Agaricus blazei* Murrill (Himematsutake) on the development and growth of glutathione S-transferase placental form (GST-P)-positive liver foci (liver preneoplastic lesion) was investigated in adult male Wistar rats. Six groups of animals were used. Groups 2 to 5 were given a single i.p. injection of 200 mg/kg b.w. of diethylnitrosamine (DEN) and groups 1 and 6 were treated with saline at the beginning of the experiment. After 2 weeks, animals of groups 3 to 6 were orally treated with three dose levels of aqueous extracts of the mushroom *A. blazei* (1.2, 5.6, 11.5, and 11.5 mg/ml of dry weight of solids) for 6 weeks. All animals were subjected to two-thirds partial hepatectomy at week 3 and sacrificed at week 8. Two hours before sacrifice, ten animals of each group were administered a single i.p injection of 100 mg/kg of bromodeoxyuridine (BrdU). Apoptotic bodies and BrdU-positive hepatocyte nuclei were quantified in liver sections stained for hematoxylin and eosin (H&E) (eosinophilic foci) and simultaneously stained for GST-P expression (GST-P-positive foci), respectively. The 6-week treatment with *A. blazei* did not alter the development (number and size) of GST-P-positive foci and did not affect the growth kinetics of liver normal parenchyma or foci in DEN-initiated animals. Our results indicate that the treatment with aqueous extracts of the mushroom *A. blazei* during the post-initiation stage of rat liver carcinogenesis does not exert any protective effect against the development of GST-P-positive foci induced by DEN. (Cancer Sci 2003; 94: 188–192)

Mushrooms have been used as important nutritional and/or therapeutic sources throughout the world since ancient times.[1)] Among the mushroom species of higher *Basidiomicetes*, *Agaricus blazei* Murrill, a species native of Brazil, where it is popularly known as "Cogumelo do Sol," has recently received attention in folk medicine due to its use in the treatment of different ailments. It occurs naturally in the hilly regions of the Atlantic rainforest in the south of São Paulo State, Brazil. Since 1965, this species has been exported to Japan, where it has become popularly known as "Himematsutake." This edible mushroom is also consumed as a food or infusion elsewhere in the world.

In Brazil and Japan, infusion of the dried fruiting bodies of the mushroom *A. blazei* has been popularly consumed both as a stimulant and as an auxiliary treatment for various diseases including diabetes, hyperlipidemia, arteriosclerosis and cancer.[2–5)] Nevertheless, no epidemiological data and few experimental reports exist on the beneficial effects of the crude aqueous extract of this mushroom. Our previous findings demonstrated that the aqueous extract of *A. blazei* provides significant protection against the *in vivo* mutagenicity induced by cyclophosphamide (CPA)[4)] and exerts a hepatoprotective effect on both rat liver toxicity and on the initiation stage of hepatocarcinogenesis (i.e., induction of glutathione S-transferase placental form (GST-P)-positive single cells/mini-foci) induced by a moderately toxic dose of diethylnitrosamine (DEN).[6)]

Considerable efforts have been made to isolate from the *A. blazei* mushroom polysaccharides and protein-bound polysaccharides that have anti-tumoral activity in tumor-bearing mice via activation of the host immune response.[7–9)] However, only the soluble protein-bound polysaccharides ATF have shown direct anti-tumoral activity as seen by selective cell cycle arrest and apoptosis induction in Meth A tumor cells *in vitro*.[2)] In contrast to the well-established antitumoral activity of the mushroom *A. blazei* observed in transplantable tumor models,[7–9)] nothing is known about the modifying potential of this mushroom on chemical carcinogenesis models.

Recently developed non-conventional bioassays for carcinogenesis provide a convenient way to evaluate the carcinogenic potential of chemicals.[10–12)] Among these alternative assays, the initiated rat liver bioassay has proved to be a consistent bioassay for detection of hepatocarcinogens.[11, 12)] This 8-week medium-term rat liver assay system—based on the initiation-promotion concept of the carcinogenesis process—uses as an endpoint marker the development of putative preneoplastic altered foci of hepatocytes (AFHs) that express the enzyme GST-P.[11–13)] This is thought to be a practical approach for hazard identification and detection of the potential benefit of chemicals mainly when associated with other preneoplasia-based surrogate endpoints (PSEs).[13)] Among these PSEs, proliferation and apoptosis data for AFHs provide important information to evaluate the modifying potential of chemicals, in particular that of promising chemopreventive compounds.[13)] The purpose of the present study was to evaluate the effects of different concentrations of crude aqueous extracts of the mushroom *A. blazei* on the development and growth of GST-P-positive foci during the post-initiation stage of experimental liver carcinogenesis.

Materials and Methods

Animals. Male 4-week-old Wistar rats were obtained from the CEMIB (UNICAMP, Campinas, SP, Brazil). The animals were kept in polypropylene cages (five animals/cage) covered with metallic grids in a room maintained at 22±2°C, 55±10% humidity and with a 12-h light-dark cycle. They were fed with commercial Purina chow (LABINA, Paulínia, SP, Brazil) and water *ad libitum* during a 2-week acclimation period.

Experimental design. The animals were randomly allocated to six groups: groups 2 to 5 were given a single i.p. injection of 200

[6]To whom correspondence should be addressed. E-mail: decam@fmb.unesp.br

mg/kg b.w. of diethylnitrosamine (DEN, Sigma Chemical Co., St. Louis, MO) and groups 1 and 6 were given a single i.p. injection of saline (solution 0.9% NaCl) only at the beginning of the experiment. After 2 weeks, animals of groups 3 to 6 were treated for 6 weeks with aqueous extracts of mushroom *Agaricus blazei* with mean dry contents of solids of 1.2, 5.6, 11.5 and 11.5 mg/ml, respectively (see below). All animals were subjected to two-thirds partial hepatectomy at week 3 and sacrificed at week 8. Two hours before sacrifice, 10 animals of each group were administered a single i.p. injection of 100 mg/kg b.w. of bromodeoxyuridine (BrdU) (Sigma Chemical Co.) between 8 a.m. and 11 a.m. The protocols used were consistent with the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Bioscience Institute/UNESP Ethical Committee for Animal Research (CEEA) (protocol No. 99/22).

Preparation and administration of *A. blazei*. A sample (lineage AB 99/26) of *Agaricus blazei* Murrill (Agaricaceae) was obtained from the Departamento de Produção Vegetal, Faculdade de Ciências Agronômicas, UNESP, Botucatu-SP, Brazil.

Twenty-five grams of powdered dry fruiting bodies of the mushroom was added to 1000 ml of deionized water (2.5% w/w), and left for 2 h at room temperature. This solution corresponds to the popular form of use of *A. blazei* for beneficial health effects. This solution—referred to subsequently as "the crude aqueous extract"—was centrifuged (800*g* for 10 min) and filtered (commercial non-sterile filter). The final solution was provided as 10% (Ab_1), 50% (Ab_2), and 100% (Ab_3) of the full 2.5% aqueous extract. The mean amounts of solids in Ab_1, Ab_2, and Ab_3 solutions (i.e., mean dry weight of water-extractable material) were 1.2, 5.6, and 11.5 mg/ml, respectively. The solutions were prepared daily and offered *ad libitum* to the rats in aluminum foil-wrapped bottles to avoid light decomposition. It was the sole drinking fluid from 2 weeks after DEN initiation until the end of the experiment (post-initiation phase).

Tissue processing, histology and immunohistochemical procedures. After sacrifice, samples of the remaining liver lobes were weighed and fixed in 10% phosphate-buffered formalin for hematoxylin and eosin (H&E) staining and immunohistochemical identification of GST-P$^+$ foci and/or BrdU nuclear incorporation, using the avidin-biotin complex (ABC) method.[14] Also, the kidneys were removed, weighed and fixed; a segment of the stomach from each rat was included to confirm nuclear incorporation of BrdU.

For analysis of different types of AFHs, paraffin-embedded liver was cut into 5 μm thick sections and stained with H&E. Based on the cellular appearance under H&E staining, AFHs were classified as clear cell, eosinophilic cell, amphophilic cell, basophilic cell, basophilic tigroid cell foci and mixed types, using previously published criteria.[15] When a single cell type comprised 70% of an AFH, it was classified according to that predominant cell type. For analysis of BrdU nuclear incorporation in GST-P-positive foci, paraffin-embedded liver was cut into 5 μm thick sections and immunohistochemically stained simultaneously with monoclonal antibody anti-BrdU (Sigma Chemical Co.) and polyclonal rabbit anti-rat GST-P (Medical & Biological Laboratories Co., Tokyo). Chromogen color development was done using 3,3′-diaminobenzidine tetrahydrochloride (DAB)-Ni$^+$ and DAB solution for BrdU and GST-P visualization, respectively.[16]

Apoptosis, cell proliferation and GST-P-positive foci quantification. BrdU labeling (BrdU LI%) and apoptotic body (AB%) indices in extra-focal or focal hepatocytes were determined by dividing the number of the BrdU-positive hepatocytes or apoptotic bodies, respectively, by the total number of hepatocytes scored outside or within the foci and multiplying by 100. Criteria for identification and quantification of hepatocytes in apoptosis and of apoptotic bodies were taken from the literature.[17, 18] Approximately 12 000 to 15 000 hepatocytes were scored for each animal in 160 random extra-focal or normal liver microscopic fields under a 50× objective in initiated and non-initiated animals, respectively. The BrdU LI% indices of focal hepatocytes were determined within each GST-P-positive foci (Fig. 1A). The AB% indices of focal hepatocytes were determined in eosinophilic foci (Fig. 1B). A minimum of 100 GST-P-positive or eosinophilic foci larger than 0.15 mm in diameter were analyzed in groups G2 to G5.

GST-P-positive foci larger than 0.15 mm^2 in diameter were measured using a Nikon photomicroscope (Microphot-FXA) connected to a KS-300 apparatus (Kontron Elektronic, Berlin, Germany). Data were expressed as number and area (mm^2) of foci per liver section (cm^2).[11]

Statistical analysis. Body and relative liver weights and food and water consumption data were compared between groups using analysis of variance (ANOVA).[19] The incidence of specific types of AFHs and the number and area of GST-P-positive foci per group were analyzed using the χ^2 test and Kruskal-Wallis test, respectively.[19] Focal, extra-focal and normal liver indices for BrdU nuclear incorporation and apoptosis were compared between groups using the Mann-Whitney or Kruskal-Wallis tests.[19] A significant difference between groups was assumed when $P<0.05$.

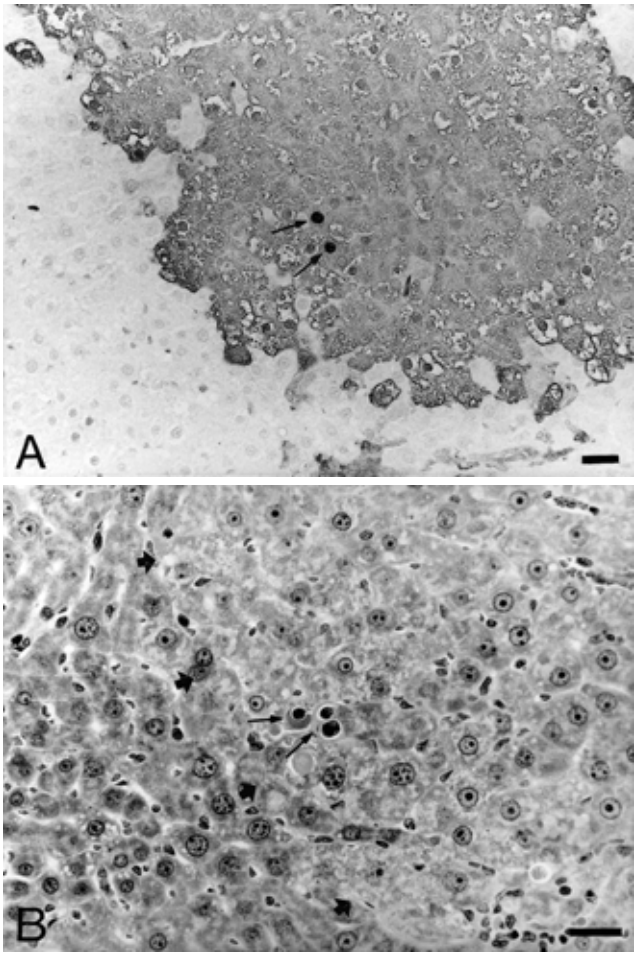

Fig. 1. A) Simultaneous immunohistochemical detection of BrdU nuclear incorporation in GST-P-positive hepatocytes (arrows). Bar=50 μm. B) ABs (apoptotic bodies with condensed chromatin, some of which are phagocytosed, arrows) in eosinophilic hepatocyte foci (arrowheads) (H&E). Bar=20 μm.

Results

Final body and relative liver weights and food and liquid consumption data are presented in Table 1. No differences in mean body and relative liver weights, relative kidney weights (data not shown) and food consumption were observed among the experimental groups at the end of the 8th week, independently of *A. blazei* treatment. During the experiment, drinking fluid consumption was higher in animals treated with 50% and 100% of the full 2.5% aqueous extract of the mushroom *A. blazei* (Ab_2 and Ab_3 solutions, respectively) than in the respective controls (group G1 vs. group G6 and group G2 vs. groups G4 and G5, $P<0.05$). The mean ingestion of solids (water-extractable material) of aqueous extracts of *A. blazei* was 0.18, 0.90 and 1.98 (1.88–2.05) g/kg/day for Ab_1, Ab_2 and Ab_3 groups, respectively (Table 1).

Non-initiated animals (groups G1 and G6) did not develop any preneoplastic liver foci above the cut-off value of the quantitative method, which was 0.15 mm (Table 2). No differences were observed between DEN-initiated animals and DEN-initiated and *A. blazei*-treated animals in relation to the incidences of the types of AFHs identified by H&E staining (Table 2). In DEN-initiated groups, eosinophilic cell foci were more abundant than clear and basophilic cell foci. Although not significantly different, a lower incidence of basophilic cell foci was observed in animals DEN-initiated and treated with Ab_2 and Ab_3 aqueous extract of the mushroom *A. blazei*. No types of AFH other than clear, eosinophilic and basophilic cell foci were found under H&E staining. The 6-week treatment with *A. blazei* aqueous extracts did not alter the development (number and size) of GST-P-positive foci in DEN-initiated animals.

The effects of *A. blazei* treatment on cell proliferation and apoptosis in normal, extra-focal hepatocytes and liver foci are presented in Fig. 2. Overall, the mean BrdU LI% and AB% indices in GST-P-positive and eosinophilic liver foci were approximately 1.5- to 1.9- and 5- to 8-fold higher than in extra-focal or normal hepatocytes of initiated and non-initiated animals, respectively. The *A. blazei* treatment did not interfere with the growth kinetics of liver normal parenchyma or foci.

The administration of 100% of the full 2.5% aqueous extract of *A. blazei* (Ab_3 solution) as the sole drinking fluid for 6 weeks did not induce liver and kidney toxicity or preneoplasia in male Wistar rats at the end of experiment.

Discussion

Recently, our group demonstrated that the aqueous extract of the mushroom *A. blazei* provides significant protection against mutagenicity induced both *in vivo* by CPA and *in vitro* by methyl methanesulfonate (MMS).[4, 5] Two-week treatment with

Table 1. Final body weights, relative liver weights and food and liquid consumption of male Wistar rats at the end of the experiment[1)]

Groups/Treatments[2)]		Effective number of rats	Final body weights (g)	Relative liver weights (%)	Food consumption (g/rat/day)	Liquid consumption (g/rat/day)	Ingestion of solids[3)] (g/rat/day)
Initiated							
G1	Control	15	338.3±34.9	2.6±0.3	25.3±1.7	39.4 ±1.9	—
G6	Ab_3	14	350.6±22.7	2.5±0.2	26.3±1.5	43.8 ±2.5*	1.88±0.37
Non-initiated							
G2	DEN	19	333.3±26.0	2.7±0.3	24.7±1.4	37.67±2.3	—
G3	DEN+Ab_1	18	332.1±26.9	2.6±0.3	24.1±1.3	37.4 ±2.2	0.19±0.04
G4	DEN+Ab_2	18	336.2±28.6	2.7±0.2	24.6±1.8	43.3 ±2.7**	0.98±0.22
G5	DEN+Ab_3	18	332.8±30.8	2.7±0.2	24.3±2.1	44.3 ±1.9**	2.05±0.47

1) Values are means±SD.
2) DEN (diethylnitrosamine 200 mg/kg, i.p., at the beginning of the experiment) and Ab_1, Ab_2 and Ab_3=aqueous extracts of mushroom *Agaricus blazei* with mean dry weight of water-extractable solids of 1.2, 5.6 and 11.5 mg/ml, respectively.
3) Mean ingestion of water-extractable material based on mean dry weight of the aqueous extract of *A. blazei*.
* Statistically significant different from control animals (group G1 vs. group G6, $P<0.05$), ** statistically significant different from DEN-initiated animals (groups G4 and G5 vs. group G2, $P<0.05$).

Table 2. Number and area of hepatic preneoplastic GST-P-positive liver foci in non-initiated and DEN-initiated male Wistar rats at the end of the experiment

Groups/Treatments[2)]		Effective number of rats	Types of altered foci of hepatocytes[3)]			GST-P$^+$ foci[1)]	
			CCF	ECF	BCF	Number (foci/cm^2)	Area (mm^2/cm^2)
Non-initiated							
G1	Control	15	0[4)]	0	0	0	0
G6	Ab_3	14	0	0	0	0	0
Initiated							
G2	DEN	19	10 (53%)	19 (100%)	10 (53%)	9.15±3.73	0.41±0.21
G3	DEN+Ab_1	18	9 (50%)	18 (100%)	6 (33%)	9.33±3.47	0.41±0.20
G4	DEN+Ab_2	18	8 (44%)	16 (89%)	4 (22%)	7.80±3.54	0.32±0.18
G5	DEN+Ab_3	18	10 (56%)	17 (94%)	4 (22%)	7.74±2.61	0.34±0.18

1) Values are means±SD.
2) DEN (diethylnitrosamine 200 mg/kg i.p. at the beginning of the experiment) and Ab_1, Ab_2 and Ab_3= aqueous extracts of mushroom *Agaricus blazei* with mean dry weight of water-extractable solids of 1.2, 5.6 and 11.5 mg/ml, respectively.
3) CCF, clear cell foci; ECF, eosinophilic cell foci; BCF, basophilic cell foci.
4) Non-initiated animals belonging to groups G1 and G6 did not develop preneoplastic foci above the cut-off value of the method for quantitative analysis and therefore are indicated as zero foci.

this mushroom before initiation of rat liver carcinogenesis by DEN exerted a liver-protective effect as seen by serum transaminase levels, regenerative cell proliferation and induction of GST-P-positive single cells/mini-foci 48 h after the initiating procedure.[6] In contrast, the present data indicate that when the *A. blazei* treatment is carried out for 6 weeks after DEN initiation it does not alter the number and/or the aggregate area of GST-positive foci liver of DEN-initiated animals.

Immunological, hypocholesterolemic, antiviral, antibacterial and antiparasitic activities have been associated with substances isolated from higher species of *Basidiomycetes*.[19, 20] However, some species of mushrooms are also common sources of human poisoning (mainly species containing α-amanitin) and have been associated with cancer development in experimental animals (mainly species containing carcinogenic compounds such as hydrazine and diazonium ions).[21–24] The *A. blazei* treatment adopted herein did not induce any toxicity after a 6-week exposure period, as evidenced by unaltered body and relative liver and renal weights, liver and renal morphology, and by the levels of BrdU nuclear incorporation and apoptosis in the liver. This absence of short-term toxicity in a mammal species should be taken into consideration in assessing the public health implications of the increased use of this mushroom by human populations.

Altered foci of hepatocytes are believed to be an early marker of liver cancer development in rodents, and AFH cell proliferation and apoptosis data provide important information to identify the modifying potential of chemicals.[25] Our data indicate that GST-positive hepatic foci not only showed higher replicative activity, but also enhanced apoptosis when compared to the surrounding normal liver, as already noted by others.[16, 25–27] In rat chemical hepatocarcinogenesis, the apoptosis rate gradually increases in foci and nodules, when compared to normal liver parenchyma, after fasting or promoting stimulus withdrawal.[25–28] The *A. blazei* treatment did not alter the development or growth of the putative liver preneoplastic lesions. The enhanced apoptosis process registered in AFHs was probably an indication of "remodeling" of the foci and was not counterbalancing possibly increased cell replication, since no promoting treatment (i.e., survival factors that favor diminished apoptosis and elevated cell proliferation in AFHs[24, 25]) was used in this study.

Various polysaccharides and protein-bound polysaccharides such as (1→4)-β-D-glucan/(1→6)-β-D-glucan, (1→6)-β-D-glucan-protein and (1→4)-β-D-glucan/(1→6)-β-D-glucan-protein complexes have been isolated from the fruiting bodies of the *A. blazei* mushroom and have shown anti-tumoral activity in tumor-bearing mice, through specific and non-specific immune response activation.[7–9] These compounds appear to stimulate the host immune response by enhancement of macrophage, NK (natural killer cell) and CTL (cytolytic T lymphocyte) activities, which participate in the antitumor response in the sarcoma 180 or Meth A fibrosarcoma-bearing mice systems.

The absence of a beneficial effect by the aqueous extract of *A. blazei* on AFH development could be due to the carcinogenesis step evaluated in the present study. The possibility exists that the extracts could be effective in later stages of rat liver carcinogenesis. Further, it is possible that important water-insoluble components were not extracted in the preparation of the aqueous extract of the mushroom *A. blazei*. Specific components of the lipid fraction of the mushroom *A. blazei* have a modifying influence on chemically induced mutagenesis and carcinogenesis.[3, 7] Linoleic acid diminished benzo(*a*)pyrene mutagenicity in the Ames/*Salmonella*/microsome system[7] and ergosterol inhibited tumor-induced neovascularization in two different Lewis lung carcinoma experimental systems.[3] In the latter work, oral administration of crude (800 mg/kg) and ergosterol-related (400 and 800 mg/kg) lipid fractions of the mushroom *A. blazei* inhibited the growth of sarcoma 180 tumor cells in mice.[3] In our study, a higher dose of *A. blazei* (mean daily ingestion of 2.05 g of solids/kg), provided as an aqueous extract, failed to inhibit the development of preneoplastic foci. It should be noted that the aqueous extracts used in this study mimic more closely than lipid extracts the gross human exposure to this edible mushroom. As indicated in Table 2, the average solid ingestion of mushroom, calculated from the mean ingestion of the aqueous solutions of *A. blazei* (Ab_1, Ab_2 and Ab_3 solutions), represented approximately 1.1, 5.6 and 12.4 times the dose commonly used by human population (0.16 g of extracted solid/kg, estimated for a 70 kg individual who ingests one liter of *A. blazei* infusion).

The present results indicate that the aqueous extract of the mushroom *A. blazei* does not have a modifying influence on the promoting phase of rat chemical hepatocarcinogenesis. Considering the known beneficial effects of this mushroom in other assay systems, it is important to consider that *A. blazei* is not a multipotential and broad-spectrum edible item for cancer prevention. However, the possibility exists that the potential of the aqueous extracts of the mushroom *A. blazei* to block the promotion step of carcinogenesis may be manifested in target organs other than the liver.

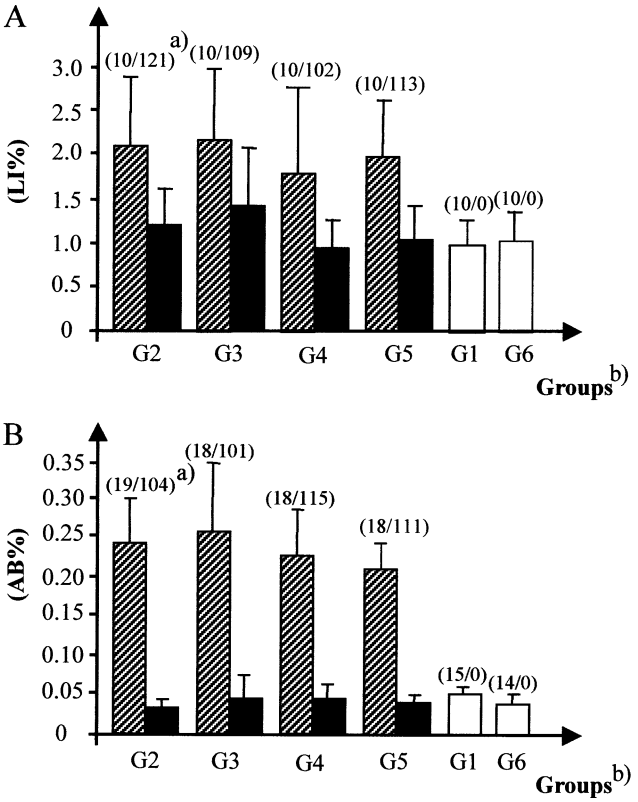

Fig. 2. A) BrdU labeling indices (LI%) in normal, extra-foci and GST-P-positive foci in the liver of non-initiated and DEN-initiated animals (values are means±SD). B) Apoptotic bodies indices (AB%) in normal, extra-foci and eosinophilic foci tissues in the liver of non-initiated and DEN-initiated animals (values are means±SEM).[a] Number of animals analyzed/number of AFHs analyzed.[b] Groups: G1, non-treated; G2, DEN; G3, DEN+Ab_1 solution; G4, DEN+Ab_2 solution; G5, DEN+Ab_3 solution; G6, Ab_3 solution. ▨ foci tissue, ■ extra-foci tissue, □ normal tissue. See "Materials and Methods."

The authors gratefully acknowledge the technical support provided by Paulo Roberto Cardoso and Maria Luíza Falaguera Ardanaz. This study was supported by FAPESP (98/07726-5) and FUNDUNESP (No. 119/99-DFP).

1. Chang R. Functional properties of edible mushrooms. *Nutr Rev* 1996; **54**: S91–3.
2. Fujimiya Y, Suzuki Y, Takakura R, Ebina T. Tumor-specific cytocidal and immunopotentiating effects of relatively low molecular weight products derived from the basidiomycetes, *Agaricus blazei* Murrill. *Anticancer Res* 1999; **19**: 113–8.
3. Takaku T, Kimura Y, Okuda H. Isolation of an antitumor compound from *Agaricus blazei* Murrill and its mechanism of action. *J Nutr* 2001; **131**: 1409–13.
4. Delmanto RD, de Lima PLA, Sugui MM, da Eira AF, Salvadori DMF, Speit G, Ribeiro LR. Antimutagenic effects of *Agaricus blazei* Murrill mushroom on the genotoxicity induced by cyclophosphamide. *Mutat Res* 2001; **496**: 15–21.
5. Menoli RCR, Mantovani MS, Ribeiro RL, Speit G, Jordão BQ. Antimutagenic effects of the mushroom *Agaricus blazei* Murrill extracts on V79 cells. *Mutat Res* 2001; **496**: 5–13.
6. Barbisan LF, Miyamoto M, Scolastici C, Salvadori DMF, Ribeiro LR, da Eira AF, de Camargo JLV. Influence of aqueous extract of *Agaricus blazei* mushroom on rat liver toxicity induced by different doses of diethylnitrosamine. *J Ethnopharmacol* 2002; **83**: 25–32.
7. Kawagishi H, Inagaki R, Kanao T, Mizuno T, Shimura K, Ito H, Hagiwara T, Nakamura T. Fractionation and antitumor activity of the water-insoluble residue of *Agaricus blazei* fruiting bodies. *Carbohydr Res* 1989; **186**: 267–73.
8. Itoh H, Ito H, Amano H, Noda H. Inhibitory action of a (1→6)-β-D-glucan-protein complex (FIII-2-b) isolated from *Agaricus blazei* Murrill ("Himematsutake") on Meth A fibrosarcoma-bearing mice and its antitumor mechanism. *Jpn J Pharmacol* 1994; **66**: 265–71.
9. Ito H, Shimura K, Itoh H, Kawade M. Antitumor effects of a new polysaccharide-protein complex (ATOM) prepared from *Agaricus blazei* (Iwade Strain 101) "Himematsutake" and its mechanisms in tumor-bearing mice. *Anticancer Res* 1997; **17**: 277–84.
10. Dragan YP, Rizvi T, Xu Y-H, Hully JR, Bawa N, Campbell HA, Maronpot RR, Pitot HC. An initiation-promotion assay in rat liver as a potential complement to the 2-year carcinogenesis bioassay. *Fundam Appl Toxicol* 1991; **16**: 525–47.
11. Ito N, Tsuda H, Tatematsu M, Inoue T, Tagawa Y, Aoki T, Uwagawa S, Kagawa M, Ogiso T, Masui T, Imaida K, Asamoto M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats; an approach for a new medium-term bioassay system. *Carcinogenesis* 1988; **9**: 387–94.
12. Ito N, Hasegawa R, Imaida K, Hirose M, Shirai T. Medium-term liver and multi-organ carcinogenesis bioassay for carcinogens and chemopreventive agents. *Exp Toxicol Pathol* 1996; **48**: 113–9.
13. Moore M, Tsuda H, Tamano S, Hagiwara A, Imaida K, Shirai T, Ito N. Marriage of a medium-term liver model to surrogate markers—a practical approach for risks and benefit assessment. *Toxicol Pathol* 1999; **27**: 237–42.
14. Hsu SM, Raine L, Fanger N. Use of avidin-biotin-peroxidase complex (ABC) and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981; **29**: 557–80.
15. Goodman DG, Maronpot RR, Newberne PM, Popp JA, Squire RA. Proliferative and selected other lesions in the liver of rats. In: Guides for toxicologic pathology. Washington: STP/ARP/AFIP; 1994. p. 1–22.
16. Dragan YP, Hully J, Mass M, Pitot HC. Incorporation of bromodeoxyuridine in glutathione S-transferase-positive hepatocytes during rat multistage hepatocarcinogenesis. *Carcinogenesis* 1994; **15**: 1939–47.
17. Goldsworthy TL, Fransson-Steen R, Maronpot RR. Importance of and approaches to quantification of hepatocyte apoptosis. *Toxicol Pathol* 1996; **24**: 24–35.
18. Levin S, Bucci TJ, Cohen SM, Fix AS, Hardisty JF, LeGrand EK, Maronpot RR, Trump BF. The nomenclature of cell death: recommendations of an ad hoc committee of the Society of Toxicology pathologists. *Toxicol Pathol* 1999; **27**: 484–90.
19. Snedecor GW, Cochran WG. Statistical methods. 7th ed. Ames: Iowa State University Press; 1980. p. 1–507.
20. Wasser SP, Weis AL. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. *Crit Rev Immunol* 1999; **19**: 65–96.
21. Ooi VEC, Liu F. A review of pharmacological activities of mushroom polysaccharides. *Int J Med Mushrooms* 1999; **1**: 195–206.
22. McPartland JM, Vilgalys RJ, Cubeta MA. Mushroom poisoning. *Am Fam Physician* 1997; **55**: 1797–809.
23. Larry D, Pageaux GP. Hepatotoxicity of herbal remedies and mushrooms. *Semin Liver Dis* 1995; **15**: 183–8.
24. Toth B. Mushroom toxins and cancer. *Int J Oncol* 1995; **6**: 137–45.
25. Schulte-Hermann R, Timmermann-Trosiener I, Barthel G, Bursch W. DNA synthesis, apoptosis, and phenotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion. *Cancer Res* 1990; **50**: 5127–35.
26. Stinchocombe S, Buchmann A, Bock KM, Schwarz M. Inhibition of apoptosis during 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated tumor promotion in rat liver. *Carcinogenesis* 1995; **16**: 1271–5.
27. Schulte-Hermann R, Bursch W, Grasl-Kraupp B, Müllauer L, Ruttkay-Nedecky B. Apoptosis and multistage carcinogenesis in rat liver. *Mutat Res* 1995; **333**: 81–7.
28. Grasl-Kraupp B, Ruttkay-Nedecky B, Müllauer L, Taper H, Huber W, Bursch W, Schulte-Hermann, R. Inherent increase of apoptosis in liver tumors: implications for carcinogenesis and tumor regression. *Hepatology* 1997; **25**: 906–12.