Jpn. J. Cancer Res. 85, 1298–1303, December 1994

An *in vivo* Study of Hepatic and Splenic Interleukin-1β mRNA Expression Following Oral PSK or LEM Administration

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The effects of orally administered biological response modifiers (BRMs) in preventing postoperative micro liver metastasis of primary colorectal cancer were examined in experimental animals. The two BRMs tested were Krestin (PSK) and *Lentinus edodes* mycelia (LEM). In previous experiments, we found that oral administration of PSK or LEM suppressed liver metastasis and prolonged the survival period. We also found that these agents elevated the liver natural killer (NK) and liver macrophage activities. In the present study *in vivo*, using reverse transcriptase-polymerase chain reaction (RT-PCR), we examined whether or not the liver and spleen have cytokines which would induce NK cells and macrophages, and whether or not the liver and spleen have cytokines induced by NK cells or macrophages. We placed emphasis on the examination of interleukin (IL)-1β expression in the liver and spleen *in vivo*. Two to six hours after oral administration of PSK or LEM (1 g/kg) to mice, IL-1β levels in the liver and spleen rose, and they returned to their baseline levels 24 h later. These findings suggest two possibilities: (1) hepatic IL-1β is potentiated by these agents soon after administration, resulting in activation of liver NK cells or macrophages, or (2) these agents stimulate IL-1β production by liver macrophages, and the produced IL-1β activates liver NK cells or liver macrophages (Kupffer cells). The results of this *in vivo* study suggest that the potentiation of hepatic and splenic IL-1β by PSK and LEM is involved in the early phases of suppression of micro liver metastases of colorectal cancer.

Key words: PSK — LEM (*Lentinus edodes* mycelia) — Liver metastasis — Isogen — IL-1β mRNA

Recent changes (westernization) in the diet of the Japanese people may be related to the fact that the number of patients with colorectal cancer has continued to increase. Methods used to treat this cancer have also changed in recent years. The feasibility of radical treatment, including radical surgery, is greater than ever for this cancer. At the same time, however, postoperative recurrence (especially blood-borne metastasis to the liver) has been highlighted as a serious problem. In some cases, liver metastases can be attributed to the transport of residual cancer cells through blood vessels to the liver after a reduction in immune function due to surgical stress. In other cases, liver metastases are present before surgery, though they are not detected before surgery. Oral drug therapy can be simply and effectively used to cope with these two types of liver metastases in patients with colorectal cancer who undergo radical surgery. In particular, biological response modifiers (BRMs) have recently been attracting much attention because these agents can be useful for such oral drug therapy, while inducing few adverse reactions.

The present study was undertaken to assess the effects of orally administered BRMs in preventing liver metastases and to clarify the mechanisms of their action. The two BRMs tested were Krestin[1-3] (PSK, Sankyo K.K.) and *Lentinus edodes* mycelia[4] (LEM, a hot water extract of mushroom mycelia; Noda Shokukin Kogyo K.K.).

In previous experiments,[5,6] we found that oral administration of PSK or LEM suppressed liver metastasis and prolonged the survival period of rats and mice. We also found that these agents elevated liver natural killer (NK) activity and liver macrophage activity. In the present study *in vivo*, using reverse transcriptase-polymerase chain reaction (RT-PCR), we examined whether or not the liver and spleen have cytokines which would induce NK cells and macrophages, and whether or not the liver and spleen have cytokines induced by NK cells or macrophages. The cytokines examined in this study were interleukin (IL)-1, 2, 4 and 6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ. Taking into account a report that stimulation with PSK *in vitro* elevated IL-1 activity, and that stimulation of macrophages with LEM resulted in IL-1 production, we placed emphasis on the examination of IL-1β expression in the liver and spleen *in vivo*.

MATERIALS AND METHODS

Experimental animals Four-week-old male BDF_1 [$(C57BL/6\times DBA/2)F_1$] mice were used for this study. They were bred under constant temperature and relative humidity, and allowed free access to water.

Dosing schedule Using a stomach tube, PSK or LEM (1 g/kg) was given to the animals. The liver and spleen of these animals were removed 2, 6 or 24 h later. Mice

receiving drug-free physiological saline served as controls. The spleen and liver of the control animals were removed immediately (0 h) after a dose of physiological saline. In this study experiments were done in quadruplicate. Four mice were killed at each time indicated.

Sample (50–100 mg of liver or spleen)
← 1 ml of Isogen
homogenization
store for 5 min at room temperature
← 0.2 ml of chloroform
shake-vortex for 15 s
store for 2–3 min at room temperature
12,000 rpm, 10 min at 4°C
aqueous phase
← same volume of 4 *M* LiCl
store for 2 h at −80°C
12,000 rpm, 10 min at 4°C
precipitate
← 1 ml of 80% ethanol
vortex
8,000 rpm, 6 min at 4°C
precipitate
dry up
← dissolve in water
total RNA solution

Fig. 1. Extraction of total RNA. Method modified from that of Chomczynski and Sacchi.[7]

Total RNA extraction Total RNA was extracted, using a modification of Chomczynski and Sacchi's method[7] with Isogen (Nippon Gene K.K.) (Fig. 1). The first step of this procedure was to add 1 ml of Isogen to each 50–100 mg of the hepatic or splenic tissue and then to homogenize the tissue, using a Polytron homogenizer. The homogenized tissues were left standing at room temperature for 5 min to achieve complete lysis of nuclear protein complexes. Subsequently, chloroform was added to the sample (0.2 ml per 1 ml of Isogen) and the sample was agitated. After standing at room temperature for few minutes, the sample was centrifuged for 10 min at 12,000*g* and 4°C. The aqueous fraction was transferred into a tube and mixed with an equal amount of 4 *M* LiCl. The mixture was the left standing for 2 h at −80°C, after which it was centrifuged for 10 min at 12,000*g* and 4°C to yield RNA pellets. Once the supernatant was discarded, the pellets were centrifuged in 80% ethanol, then air-dried. Total RNA was dissolved in an aqueous solution of sterile diethylpolycarbonate water (DEPC H_2O). Nucleic acid levels were measured in terms of the absorbance at 260 nm. The purity of total RNA was confirmed by using agarose electrophoresis.

RT-PCR assay Oligo-dT (Pharmacia) was added to 200 ng of total RNA. The mixture was left standing for 15

mRNA	5' Sense Primer	3' Antisense Primer	Size of amplified fragment (bp)
HPRT	5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3'	5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3'	138
IL-1β	5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3'	5'-CA GGA CAG GTA TAG ATT CTT TCC TTT-3'	562

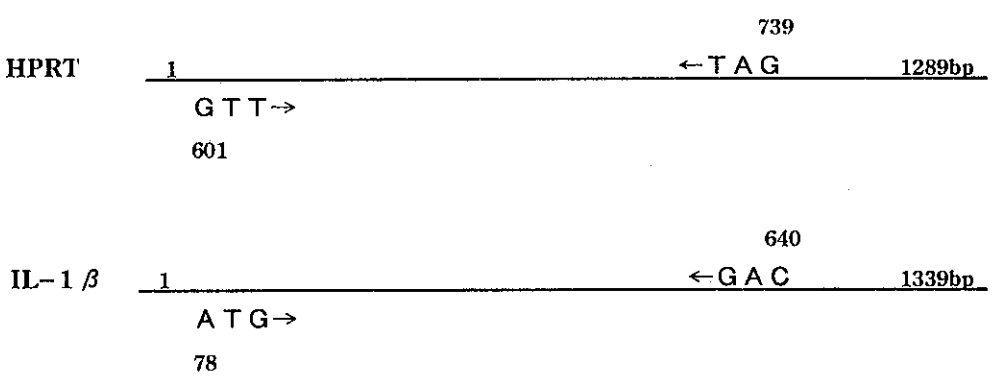

Fig. 2. Primer sequences used for PCR-assisted mRNA amplification.

min at 65°C, then cooled to 20°C in 30 min and annealed. Next, dNTPs and RNase inhibitor (RNAsin, Promega Corp.) were added. The sample was incubated in the presence of Moloney murine leukemia virus-reverse transcriptase (M-MLV RT, Gibco BRL) for 60 min at 37°C and for another 5 min at 99°C, to synthesize complementary DNA (cDNA).[8] Using 1/10 its amount of cDNA as a template, the gene of IL-1β or hypoxanthine phosphoribosyl transferase (HPRT) was amplified by the primer (specific to IL-1β or HPRT) (Clontech Inc.), *Taq* DNA polymerase (Wako K.K.) and dNTPs. One cycle of amplification was composed of 1 min at 94°C, 2 min (annealing) at 60°C and 1 min (synthesis) at 72°C. Forty cycles of amplification were used for IL-1β, and 25 cycles for HPRT. The base sequences of the primers used are shown in Fig. 2.

The amplified PCR products were separated by agarose electrophoresis and transferred onto nylon membrane filters (Dupont). They were labeled with ^{32}P by means of 5′-phosphorylation, using a 20-mer oligonucleotide specific to IL-1β (5′-CTTTGAAGTTGACGGACCCC-3′ (nucleotides 143′–162′)) or HPRT (5′-GTTTGTTGT-TGGATATGCCC-3′ (nucleotides 645–664)) as a T4 kinase. Using them as probes, Southern hybridization was performed. The filter was washed, dried and autoradiographed, while simultaneously, the radioactivity of each positive band was measured, using an AMBIS image analyzer. The increase in IL-1β was measured using the following equation: Increase in IL-1β = (IL-1β radioactivity at each point of measurement/HPRT radioactivity at each point)/(control IL-1β radioactivity/control HPRT radioactivity).

Thus, the increase in IL-1β was expressed as an increase in the ratio of IL-1β to HPRT when compared to the control group.

The radioactivity of PCR products showed a concentration-dependent increase when total RNA was diluted serially and IL-1β and HPRT were used as probes. We confirmed that this change is quantitative in nature (Fig. 3).

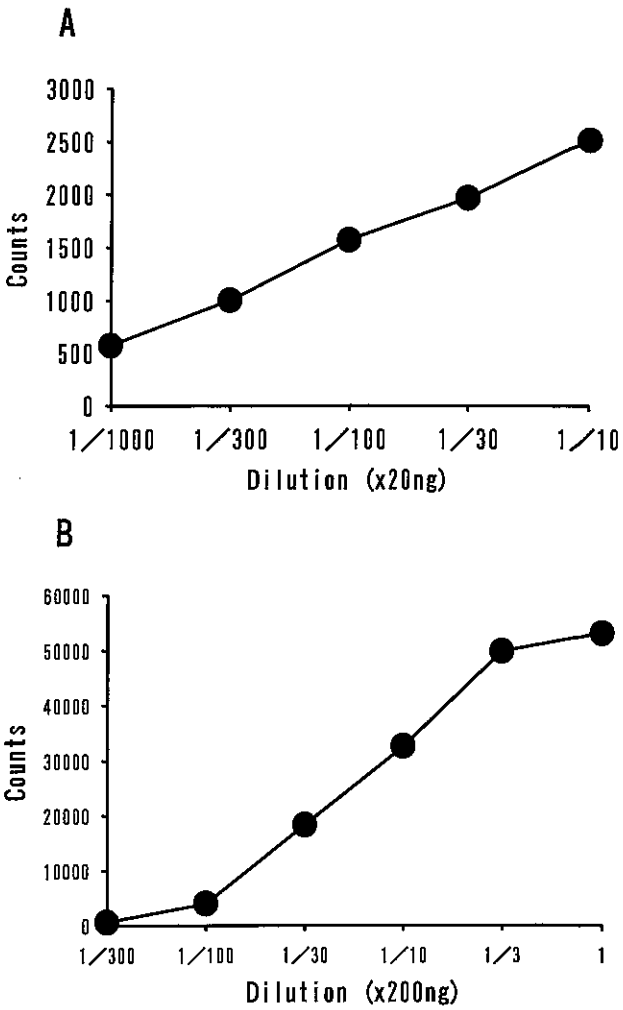

Fig. 3. Radioactivity of PCR-amplified mRNA of HPRT (A) and IL-1β (B). ^{32}P-Labeled specific 20-mer synthetic oligonucleotides for HPRT and IL-1β were used as the probes for Southern hybridization. Radioactivity of each positive band was measured using an AMBIS image analyzer.

RESULTS

When PCR products were subjected to autoradiography, the band due to IL-1β of the liver and spleen was most intense 2 h after oral administration of PSK or LEM, when compared to the control group. It decreased in intensity thereafter. The intensity of HPRT, which was used as an internal control, did not differ between the animals treated with PSK or LEM and the control group. Representative results are shown in Fig. 4.

When the amount of IL-1β relative to the internal control was analyzed, the radioactivity of the IL-1β positive bands of liver tissues showed an approximately 4.4-fold increase ($P<0.002$) at 2 h after PSK administration (n=4) when compared with the radioactivity in the control group (n=4). The radioactivity of IL-1β-positive bands of liver tissues decreased thereafter and returned to the control level 24 h after PSK administration (n=4). After LEM administration, the radioactivity of the IL-1β-positive bands of liver tissues showed an approximately 5.2-fold increase at 2 h (n=4) when compared to the control group. At 6 h (n=4), a 6-fold increase ($P<0.001$) was evident, but the level was equal to the control level 24 h after the LEM dose (n=4), as shown in Fig. 5.

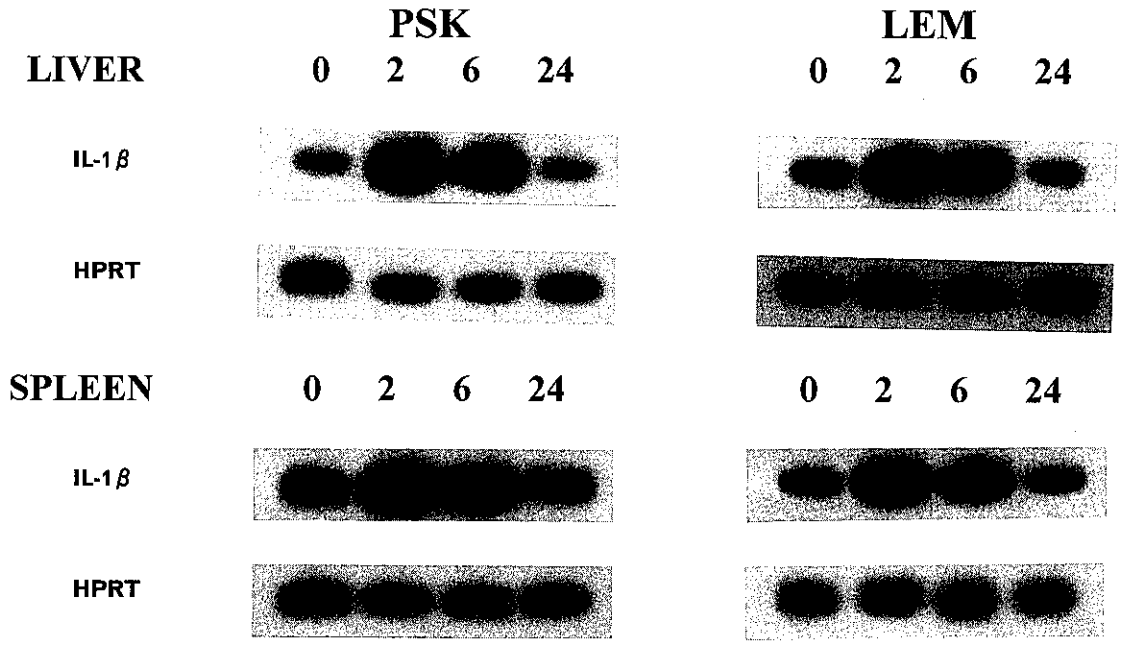

Fig. 4. PCR-assisted mRNA amplification of IL-1β and HPRT.

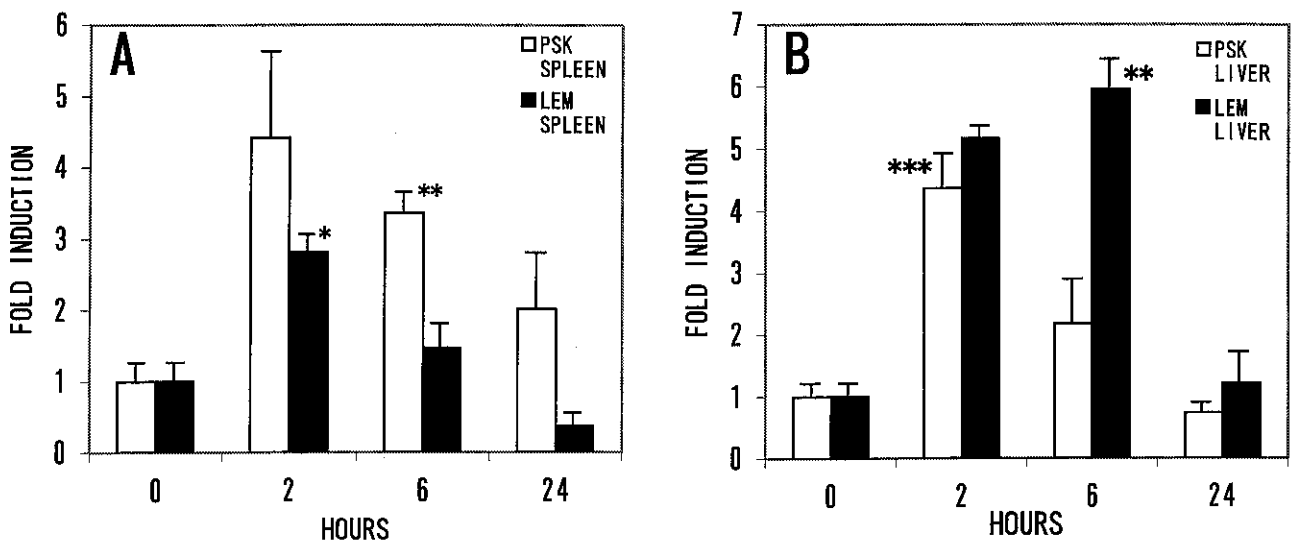

Fig. 5. Quantitative analysis of IL-1β. Mice (BDF_1, 4-week-old) were given p.o. one shot (1 g/kg) of PSK or LEM. Total RNA was collected according to the method of Chomczynski and Sacchi[7] 2, 6, 24 h after administration, from spleen (A) and liver (B). Data are presented the mean±SD of standardized mRNA levels. * $P<0.0001$, ** $P<0.001$, *** $P<0.002$.

When splenic tissues were examined, the radioactivity of IL-1β-positive bands showed an approximately 4.4-fold increase at 2 h after the PSK dose (n=4) when compared to the control group (n=4). This difference was significant ($P<0.001$). The radioactivity of the PSK treatment group then decreased but it was still about twice the control level at 24 h after the PSK dose (n=4). After the LEM dose, the radioactivity showed an approx-

imately 2.8-fold increase ($P<0.0001$) at 2 h (n=4) when compared to the control group (n=4). The activity then decreased and returned to the control level at 24 h (n=4), as shown in Fig. 5.

DISCUSSION

The incidence of colorectal cancer has been increasing in Japan, accompanied with an increase in patients with liver metastases of this cancer. Liver metastasis after surgical resection of the primary colorectal tumor has become a serious problem which greatly affects the prognosis for recovery from colorectal cancer. Bearing this in mind, we conducted the present study to assess the effects of oral BRM therapy in preventing micro liver metastasis after resection of primary colorectal cancer, and to clarify the mechanism of such effects.

Fujita[9] carried out an experiment to analyze the fate of orally administered BRM, and found that blood radioactivity after oral administration of ^{14}C-PSK to mice reached a peak in 30–60 min and decreased thereafter. This suggests that ^{14}C-PSK is absorbed rapidly from the intestines and that the absorbed ^{14}C-PSK is transferred into organs and then eliminated from the body. We estimate that PSK is transferred into the liver and spleen at least 1 h after its administration, and that PSK begins to induce cytokines 1 h or more after its administration. In the present study, therefore, we removed organs 2 h or more after oral administration of BRM.

With regard to whether PCR amplification in this study is really quantitative or not, we have shown in Fig. 3 that there is a linear relationship between the dose of RNA (0.02 ng to 2 ng for HPRT, or 2 ng to 60 ng for IL-1β) and the counts of radioactivity (500 cpm to 2,500 cpm for HPRT, or 1,000 cpm to 5,000 cpm for IL-1β) that were obtained from the predicted autoradiography bands. In this study, we used a dose of RNA within these ranges. Therefore, it is conceivable that PCR amplification as well as RT-PCR assay for the levels of mRNA is quantitative as long as the obtained radioactivity exists within the ranges shown in Fig. 3. Hepatic and splenic IL-1β levels of mice showed an increase 2–6 h after oral administration of PSK or LEM, and returned to their baseline levels 24 h after administration, as shown in the Fig. 5. A preliminary experiment had revealed no significant change in any other cytokines examined (IL-2, 4, and 6, TNF-α and IFN-γ) after oral administration of the same BRM (data not shown).

These results suggest two possibilities. First, it seems possible that IL-1β in the liver is potentiated soon after administration of BRM, resulting in activation of liver NK cells or macrophages. In view of the previous findings that *in vitro* stimulation of mouse spleen cells with PSK induced the elevation of IL-1 activity[10] and that *in vitro* stimulation of macrophages with LEM induced IL-1 production,[11–13] it is also possible that the BRM first stimulates IL-1β production by liver macrophages and IL-1β thus formed activates liver NK cells or macrophages. With regard to the effect of IL-1β on the activation of NK cells or macrophages, the *in vitro* experiments showed that IL-1 influences the activation of NK cells and induces macrophage cytotoxic activity.[14]

The present results suggest for the first time that the potentiation of hepatic and/or splenic IL-1β by PSK or LEM, as observed in this study *in vivo*, is probably involved in early phases of the suppression of micro liver metastases. We are planning to identify hepatic cells which produce IL-1β, using an RT-PCR assay of hepatic cell fractions. Furthermore, we intend to explore IL-1β-positive cells in various tissues, using *in situ* hybridization.

(Received July 5, 1994/Accepted September 9, 1994)

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