Effect of Kp, an Antitumor Protein-Polysaccharide from Mycelial Culture of *Phellinus Linteus* on the Humoral Immune Response of Tumor-Bearing ICR Mice to Sheep Red Blood Cells

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The immunomodulating activity of Kp, an antitumor protein-polysaccharide preparation from the shake-cultured mycelia of *Phellinus linteus*, was investigated in ICR mice subcutaneously implanted with 1×10^6 cells of sarcoma 180. The mice were intraperitoneally administered with Kp at a dose of 100 mg/kg once daily for five consecutive days starting from 24 hrs after the tumor implantation. Ten days after the last injection, the mice were immunized with 1×10^7 or 4×10^8 sheep red blood cells (SRBC) and five days later, the antibody-forming immune response were assessed by direct hemolytic plaque assay. To an immunization dose of 1×10^7 SRBC, the Kp-treated mice elicited a successful humoral immune response despite the tumor-burden and produced 259×10^3 plaque-forming cells (PFC)/spleen, while the corresponding tumor-bearing control mice showed virtually no response (2.0×10^3 PFC/spleen) (the stimulation index=129.5). However, to an immunization dose of 4×10^8 SRBC, both of the control mice and Kp-treated mice showed almost the same level of strong humoral immune response. From these data it is clear that Kp effectively restores the humoral immune response of the tumor-bearing ICR mice.

Key words: *Phellinus linteus*, Kp, Antitumor, Protein-polysaccharide, Hemolytic plaque assay, Plaque-forming cells (PFC), Humoral immune response, Immunorestoring, Immunomodulator

INTRODUCTION

The antitumor activity of the protein-polysaccharide fraction of *Phellinus linteus*, a basidiomycetous fungus, was first described in 1968 (Ikegawa *et al.*, 1968). However, no investigation on the antitumor activity of the artificially cultured mycelia of *P. linteus* has been carried out until Chung, one of the authors, and his collaborators reported the antitumor activity of Kp, a protein-polysaccharide fraction separated from the shake-cultured mycelia of *P. linteus* (Chung *et al.*, 1991). As described elsewhere (Chung *et al.*, 1993), Kp inhibited the growth of sarcoma 180 in ICR mice when administered prior to the tumor implantation as well as when administered after the tumor implantation. Such a result strongly suggests that Kp exerts the antitumor effect through its immunomodulating activity. In this study, we investigated the immunomodulating activity of Kp on the humoral immune response in tumor-bearing mice by hemolytic plaque assay.

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MATERIALS AND METHODS

Experimental Animals and Tumor Cells

The specific pathogen-free female ICR mice, 4 to 5 weeks of age, were purchased from Genetic Engineering Research Center, KIST, and used as experimental animals. They were stabilized for 7 days before use and kept at 22 ± 2°C with antibiotic-free feed and water *ad libitum* throughout the experiment. Sarcoma 180 cells, maintained by weekly passage in the peritoneum of ICR mice, were used as tumor cells.

Materials

Kp, a water soluble antitumor protein-polysaccharide preparation of *P. linteus*, was prepared from the shake-cultured mycelia as described elsewhere (Chung *et al.*,

1993). Freshly collected sheep blood in Alsever's solution was purchased from Hankook Media Co., Ltd. (Seoul), and used as the source of sheep red blood cells (SRBC). Lyophylized pooled guinea pig serum was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA, USA) and used as complement. Agarose (Low melting point) was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA).

Experimental Schedule

The ICR mice were subcutaneously implanted with 1×10^6 cells of sarcoma 180 in the left groin, and then intraperitoneally administered with Kp at a dose of 100 mg/kg in physiological saline once daily for five days starting from 24 hrs after the tumor implantation. Ten days after the last administration of Kp, the mice were immunized by intraperitoneal injection of 4×10^8 or 1×10^7 SRBC. Five days after the immunization, the mice were sacrificed by cervical dislocation, and the spleen was excised to be subjected to hemolytic plaque assay.

Hemolytic Plaque Assay

Although many modifications have been made since Cunningham published the experimental method (Cuningham, 1965), the hemolytic plaque assay as a whole has generally been accepted as a useful screening method for immunomodulators (Yamada, 1992) as well as a tool to elucidate the action mechanism of the immunomodulators. In this study, the hemolytic plaque assay was performed by localized hemolysis in gel (LHG) method according to Dresser (Dresser, 1986) with some modifications. In brief, the spleen was cut into small pieces and gently teased through a 100 mesh stainless-steel screen. After standing on ice for 5 min to settle down the cell-clumps, the upper layer containing the single cells was taken and washed twice with ice-cold Hank's balanced salt solution (HBSS) and used as effector cells. Throughout the effector cell suspension, special care was taken in order to minimize the possible destruction of the subtle plasma cells.

The SRBC in Alsever's solution were washed three times with ice-cold HBSS and resuspended in Dulbeco's modified Eagle's medium (DME/High glucose, Hyclone, Logan, Utah, USA) into 20% just before use. After revival according to the manufacturer's direction, the pooled guinea pig serum was adsorbed using packed SRBC for 30 min on ice and then 1 : 10 diluted with DMEM just before use. The hemolytic reaction was performed in agarose gel in 6-well tissue culture plates. Each well received 2 ml of 1.5% agarose-DMEM as a bottom layer and then 500 μl of the reaction mixture, which consisted of 250 μl of 0.75% agarose-DMEM, 125 μl of the appropriately diluted spleen cell suspension and 125 μl of 20% SRBC, was added onto the solidified bottom layer. After an incubation at 37℃ for 90 min, 100 μl of 1 : 10 diluted complement was added and further incubated for 40 min to develop the hemolytic plaques. The experiment was carried out in triplicates and statistical significance was evaluated by student's t-test.

RERSULTS AND DISCUSSION

The tumor-bearing control mice, immunized with 1×10^7 SRBC, showed virtually no humoral immune response in that they produced only 2.0×10^3 PFC/spleen which is almost the same minimal response shown by the nonimmunized nontumor-bearing normal mice (1.6×10^3 PFC/spleen) (Table 1) Since 1×10^7 SRBC

Table I. Hemolytic Plaque-forming Cells (PFC) in the Spleen of ICR Mice

	Sarcoma −180	SRBC	No. of mice (M± SD)	spleen weight (mg) ($\times10^7$)	No. of splenic leukocyte (M± SD)	PFC/spleen ($\times10^3$)	PFC/ 10^6 cells	Stimulation Index[d]
control	1×10^6	4×10^8	4	315± 44	30.8± 6.89	447± 81	1450	−
Kp (100mg/kg)	1×10^6	4×10^8	4	283± 58[a]	28.1± 5.96[a]	414± 157[a]	1473	0.93
control	1×10^6	1×10^7	10	240± 54	21.4± 3.67	2.0± 0.5	9.5	−
Kp (100mg/kg)	1×10^6	1×10^7	10	317± 69[b]	29.1± 3.94[a]	259± 53.5[c]	889	129.5
normal	−	−	2	190± 28	18.9± 6.30	1.6± 0.5	8.6	−

[a]not significant at $p=0.05$.
[b]significant ($p<0.05$).
[c]significant ($p<0.01$).
[d]the stimulation index (SI) was calculated as the following.

$$SI=\frac{\text{PFC/spleen of the Kp-treated mice}}{\text{PFC/spleen of the control mice}}$$

is a high enough dose to elicite a strong humoral immune response in normal mice (Hudson and Hay, 1989), the result is a good evidence that subcutaneous implantation of 1×10^6 sarcoma 180 cells almost completely suppressed the humoral immune response of the ICR mice to 1×10^7 SRBC.

Despite the tumor burden, however, the Kp-treated mice showed a strong humoral immune response to 1×10^7 SRBC in that they produced 259×10^3 PFC/spleen. The stimulation index (PFC count of treated mice/PFC count of control mice) calculated was as high as 1295. From this result, it is clear that Kp exerts a remarkable immunorestoring activity in the immunosuppressed mice due to the tumor-burden.

However, to 4×10^8 of SRBC the tumor-bearing mice elicited almost the same level of antibody producing immune responce irrespectively of Kp-treatmemt. (the number of PFC/spleen of the control and the Kp-treated mice, respectively, were 447×10^3 and 414×10^3) (Table 1).

From these results, it is obvious that Kp effectively restores the suppressed humoral immune response of the tumor-bearing mice to the submaximal dose (1×10^7 SRBC) of an antigen while not enhancing the response to a maximal antigen dose (4×10^8 SRBC) which is high enough even for the the tumor-bearing control mice to respond to.

Similar experiments were carried out on protein-polysaccharides of *Coriolus versicolor*, using the tumor-bearing (Kim, 1992) as well as the nontumor-bearing mice (Ohno *et al.*, 1973). In Kim's study using the tumor-bearing ICR mice, the stimulation index was only 4.8. According to Ohno and his collaborators, PS-K, a protein-bound polysaccharide of the cultured mycelia of *C. versicolor*, increased the PFC count in the C57BL/6 mice immunized with 1×10^7 SRBC, the stimulation index being 52.25. Although it is difficult to evaluate the effectiveness of these protein-polysaccharides based on the the results of different studies, it seems likely that Kp has stronger immunomodulating activity than the protein-polysaccharides of *C. versicolor*.

In our recent study, Kp was found to exert mitogenic effect on the splenic lymphocytes of BALB/c mice (unpublished data). These findings and the results of present study strongly suggest that the antitumor activity of Kp might be at least partly from its immunorestoring activity on the humoral immune system.

CONCLUSION

In a hemolytic-plaque assay, Kp, a protein-polysaccharide preparation isolated from the mycelial culture of *P. linteus*, showed a strong immunorestoring activity in sarcoma 180-bearing ICR mice.

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