Immunopharmacology, 26 (1993) 139–146

IMPHAR 00656

Activation of peritoneal macrophages by polysaccharopeptide from the mushroom, *Coriolus versicolor*

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(Received 14 July 1992; accepted 23 October 1992)

Abstract: Polysaccharopeptide (PSP) is a substance produced by an edible mushroom, *Coriolus versicolor* which has been claimed to possess antitumor activity. However, neither tumoricidal activity nor cytotoxicity was observed when five tumor cell lines and mouse peritoneal macrophages were cultured *in vitro* in the presence of 2.5–10 μg/ml PSP. An increase in the production of reactive nitrogen intermediates, reactive oxygen intermediates (superoxide anions) and tumor necrosis factor was measured in peritoneal macrophages collected from inbred C57 mice which had received PSP in the drinking water for 2 weeks. Northern blot analysis also demonstrated that PSP activated the transcription of tumor necrosis factor gene in these cells, indicating that PSP exerted an immunomodulatory effect on the defensive cells.

Key words: Polysaccharopeptide; Macrophage; Tumor necrosis factor; Reactive nitrogen intermediate; Reactive oxygen intermediate

Introduction

Macrophages play an important role in the host defense system by ingestion of infectious microorganisms followed by digestion with lysosomal enzymes (Liu and Ng, 1991). Macrophages can also be activated by lymphokines, endotoxin, and various cell mediators and regulators to kill tumor cells by producing tumor necrosis factor (Keller et al., 1990), oxygen radicals (Ozaki et al., 1986) and reactive nitrogen intermediates (Hibbs et al., 1988, Keller and Keist, 1989). A group of protein-bound polysaccharides have been extracted from an edible mushroom, *Coriolus versicolor*, by scientists in Japan and China, and they include polysaccharide (Ueno et al., 1980; Naruse and Takeda, 1974), Coriolan (Naruse and Takeda, 1974), Krestin (PSK, Hirase et al, 1970) and polysaccharopeptide (PSP, Yang et al., 1987). All these compounds are considered to have antitumor activities by The United States National Cancer Institute (Jong and Donovick, 1989). Most of them are patented and commer-

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Abbreviations: PSP, polysaccharopeptide; CAM, peritoneal macrophages from control mice; PAM, peritoneal macrophages from mice treated with polysaccharopeptide; TNF, tumor necrosis factor; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; mRNA, messenger ribonucleic acids; RNAase, ribonuclease; EDTA, ethylenediaminetetraacetic acid; dCTP, deoxycytidine 5′-triphosphate; 2 × SSC, 2-fold saline-sodium citrate buffer; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; TPA, phorbol 12-myristate 13-acetate 4-O-methylester; LPS, lipopolysaccharide.

cially available. In order to study whether these polysaccharide-bound proteins act by exerting cytotoxicity on tumor cells or by regulating the immune responses of macrophages, an aqueous extract of polysaccharopeptide (PSP) has been administered in the drinking water to inbred mice for two weeks and peritoneal macrophages have been collected to test for the release of tumor necrosis factor, superoxide anions and reactive nitrogen intermediates. The expression of tumor necrosis factor gene in these macrophages in comparison with control cells has been evaluated by Nothern blot hybridization analysis.

Materials and Methods

Preparation of polysaccharopeptide (PSP) solution
Polysaccharopeptide isolated from deep-layer cultivated mycelia of *Coriolus versicolor* was obtained from Landford Co., China. It was dissolved in distilled water and sterilized by passing through a 0.2 μm filter (Millipore, USA). According to the manufacturer, PSP is chemically homogenous as judged by thin layer chromatography, gel filtration and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Only one peak was obtained when subjected to HPLC analysis using GL-C614 column (for carbohydrates) and water as eluent (personal communication from Professor C.F. Lin, Institute for Microbial Resources, Taiwan). PSP has a molecular weight of 100,000 as judged by SDS-PAGE. The percentage carbohydrate composition of PSP is 74.6% glucose, 2.7% galactose, 1.5% mannose, 4.8% xylose and 2.4% fructose. The percentage amino acid composition is 0.4% asparatic acid, 0.23% threonine, 0.32% serine, 0.58% glutamic acid, 0.10% proline, 0.26% glycine, 0.26% alanine, 0.09% cystine, 0.18% valine, 0.04% methionine, 0.22% isoleucine, 0.24% leucine, 0.15% tyrosine, 0.15% phenylalanine, 0.23% lysine, 0.07 histidine, 0.18% arginine and 0.17% tryptophan. The infrared spectrum of PSP revealed three absorption peaks at 3432, 1621 and 1073 cm^{-1} respectively. Its nuclear magnetic resource spectrum showed absorption peaks at 1.0–2.5, 3.0–3.4, 4.5 and 5.4 ppm and a broad peak at 3.0–4.3 ppm. The protein content of the PSP solution was measured by the method of Bradford (1976), and the amount of PSP used for the experiment was calculated as μg protein/ml.

Animals
One hundred male inbred mice (C57BL/6, 8–12 weeks old) were used in this study. Fifty mice consumed PSP (35 μg/day/mouse) in the drinking water for 2 weeks and another 50 mice receiving only drinking water served as controls. The animals were housed under normal laboratory conditions ($21 \pm 2\,^{\circ}C$, 12/12 h light/dark cycle) and maintained on standard rodent chow.

Tumor cell lines
All tumor cell lines, including P388D1 (mouse monocyte-macrophage, ATCC TIB 63), Sarcoma 180 (mouse sarcoma, ATCC TIB 66), PU5-1.8 (mouse monocyte-macrophage, ATCC TIB 61), B16 (mouse melanoma, NBL 6323) and JAR (human placenta choriocarcinoma, ATCC HTB 144) were maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 mg/l streptomycin and 100 IU/ml penicillin at $37\,^{\circ}C$ in a humidified atmosphere of 5% CO_2.

Cytotoxicity of PSP on mouse peritoneal macrophages
The viability of peritoneal macrophages cocultured with PSP in vitro was measured according to the method of Zhang et al. (1990). Briefly, thioglycolate-elicited peritoneal macrophages were collected from control C57 mice by peritoneal lavage with Hanks balanced salt solution (HBSS) (Liu et al., 1989). Adherent macrophages (1×10^6 cells/ ml RPMI/ well) were incubated with 2.5, 5.0 and 10.0 μg/ml PSP in 24-well culture plates (Nunclone, Nunc, USA). Cells incubated with normal culture medium served as control. After incubation for 24 h, the culture was replaced with fresh HBSS containing 0.005% neutral red (NR) for 1 h, and NR uptake by the cells was determined using a micro ELIZA

autoreader (3550 Bio-Rad microplate reader, USA) at 540 nm.

Production and assays of tumor necrosis factor (TNF)

Adherent peritoneal macrophages (1×10^6 cells/ml RPMI/well) from both PSP-pretreated and control C57 mice were cultured in the presence of 1 μg/ml of lipopolysaccharide (LPS from *E. coli* 0111: B4, Sigma L4391, USA) for 45, 60, 75, 90, 105 and 120 min. The cell-free TNF-containing medium was collected, sterilized by passing through a 0.22 μm millipore filter, and stored at −70 °C before use. For Northern blot analysis, cells (4×10^7 cells/8 ml) were cultured in a 10-cm culture dish (Falcon 1029, Becton Dickenson Labwares, USA) and stimulated with LPS (1 μg/ml) for 30, 60 and 120 min before total cellular RNA was extracted. Cultures without LPS treatment served as controls.

Tumor necrosis factor bioassay

TNF bioactivity was measured using an L929 mouse fibroblast assay (Liu et al., 1991). Briefly, cells (1×10^4 cells/0.1 ml) were cultured in 96-well microtiter plates for 24 h at 37 °C, treated with 2 μg/ml actinomycin D for 2 h and further incubated with 0.1 ml TNF-containing culture medium (1:200 dilution, v/v) for 24 h. The viability of adherent cells was measured by staining with 0.1 ml of crystal violet (0.5% in methanol/water [1:4 v/v]) for 5 min, rinsing 3 times with warm PBS with Ca^{2+} and Mg^{2+}, extracting with acetic alcohol and then reading the absorbance of each well at 595 nm with a microplate ELIZA autoreader (3550 Bio-Rad Microplate reader, USA). The TNF bioactivity was defined as percent inhibition of L929 cell growth according to the following formula:

$$\text{Percentage of tumor cell inhibition} = [(E - n)/E] \times 100\%$$

where n = number of viable cells co-cultured with TNF-containing medium, and E = number of viable cells without treatment.

Northern blot analysis of TNF mRNA

Total RNA was extracted according to the method of Karlinskey et al. (1989). Briefly, peritoneal macrophages were lysed using a solution of 50 mM Tris at pH 7.5 containing 5 M guanidinium thiocyanate, 10 mM EDTA and 8% 2-mercaptoethanol. Seven volumes of 4 M lithium chloride were added to protect the RNA from RNAase. The lysate was incubated at 4 °C overnight and then spun at 12,000 rpm (Sigma MK2, USA) at 4 °C for 25 min. The RNA was collected, resuspended in 1 ml 3 M lithium chloride, solubilized, and separated on a 1% agarose-6% formaldehyde gel. The RNAs were transferred to a Zeta Probe membrane (Bio-Rad, USA) and hybridized to a 1.7 kb fragment of TNF (kindly supplied by Dr. Beutler (Caput et al., 1983)) at 42 °C overnight. The cDNA probe was labelled with ^{32}P-dCTP (Amersham, UK). Stringent post-hybridization washes were performed in 2 × SSC for 30 min at 37 °C, 2 × SSC-0.2% SDS at 37 °C, followed by 0.2 × SSC-0.2% SDS and 0.1 × SSC-0.1% SDS, both at 42 °C for 30 min. The blots were rinsed with 2 × SSC, air-dried and exposed to Kodak XAR-5 film for 1 day. Equivalent amounts of total RNA per sample were assessed by monitoring with a ^{32}P-labelled actin probe. Autoradiographic exposures were scanned with a densitometer to compare band intensities.

Cytostatic activity of PSP

Tumor cell lines including P388D1, S180, PU5-1.8, B16, and JAR, were cultured at 1×10^4 cells/0.1 ml/well in the presence of a serial dilution of PSP (2.5, 5.0 and 10.0 μg protein/ml RPMI) for 24 h before they were pulsed with 0.5 μCi of [^{3}H]thymidine, [^{3}H]leucine, or [^{3}H]uridine (Amersham, UK) per well for 6 h. The uptake of the radioisotopes by the cells was measured by a liquid scintillation counter (Liu et al., 1989).

Production of reactive nitrogen intermediates (RNI)

Thioglycolate-elicited peritoneal macrophages were collected, rinsed, counted and resuspended

at 1×10^6 cells/ml supplemented DMEM without phenol red. The cells were allowed to adhere onto the surface of the 24-well culture plate for 1 h before they were challenged with LPS (1 μg/ml) for 20 h. Cell-free culture medium (100 μl) was then allowed to react with 50 μl Griess reagent (1% sulfanilamide in 5% H_3PO_4-0.1% naphthalene-ethylenediamine dihydrochloride) and the RNI present in the culture medium was measured by a colorimetric method at 540 nm (Keller et al., 1990).

Production of reactive oxygen intermediates (superoxide anions)

The ability of macrophages to produce superoxide anions was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c (Pick and Mizel, 1981). Briefly, adherent macrophages were incubated in phosphate buffered saline (PBS) containing Ca^{2+} (0.133 g/l) and Mg^{2+} (0.1 g/l), glucose (1 g/l) and 80 μM cytochrome C (Sigma C2506, Type III, USA) and simultaneously stimulated with 1 μM phorbol 12-myristate 13-acetate 4-O-methylester (TPA, Sigma P8139, USA). The cells were incubated at 37 °C for 1 h and the O_2^- released into the culture medium was detected using a microplate ELIZA autoreader at 550 nm. The concentration of O_2^- was expressed as μM/million cells.

Statistics

Data were analyzed using one-way analysis of variance and matched-pair comparisons were further made by Student's *t*-test. The level of significance was taken at $p < 0.05$.

Results

Cytotoxicity of PSP

Table I shows the [^{3}H]thymidine uptake of tumor cells of various tissue origins after PSP treatment for 24 h. No significant difference ($p > 0.05$) from controls was observed in tumor cells after treatment. Results from the neutral red assay also demonstrated that PSP did not affect the viability of mouse peritoneal macrophages (Fig. 1), indicating that PSP at the dose of 2.5 to 10 μg/ml had no direct cytotoxic effect on both normal and tumor cell cultures.

TNF bioassay

In order to study whether PSP could stimulate the production of TNF, macrophages collected from PSP-pretreated mice (PAM) were stimulated *in vitro* with LPS for 45 to 120 min and the level of TNF in the culture medium was measured with a bioassay employing the L929 fibroblast cell line. Fig. 2 shows that the basal level of

TABLE I

Effect of polysaccharopeptide (PSP) on [^{3}H]thymidine uptake (cpm) by timor cell lines

Tumor cell line	PSP (μg protein/ml)			
	0	2.5	5.0	10.0
P388D1	45525 ± 5226	47855 ± 1673	46643 ± 1488	41010 ± 1688
JAR	47792 ± 1327	50367 ± 1178	47412 ± 1905	50352 ± 1671
S180	93652 ± 4471	94846 ± 3430	86239 ± 5233	83412 ± 6332
PU5	51005 ± 7158	58782 ± 2469	54226 ± 1772	43914 ± 10071
B16	6426 ± 664	6137 ± 480	6972 ± 720	6547 ± 1286

Results are presented as mean ± standard deviation ($n = 3$).

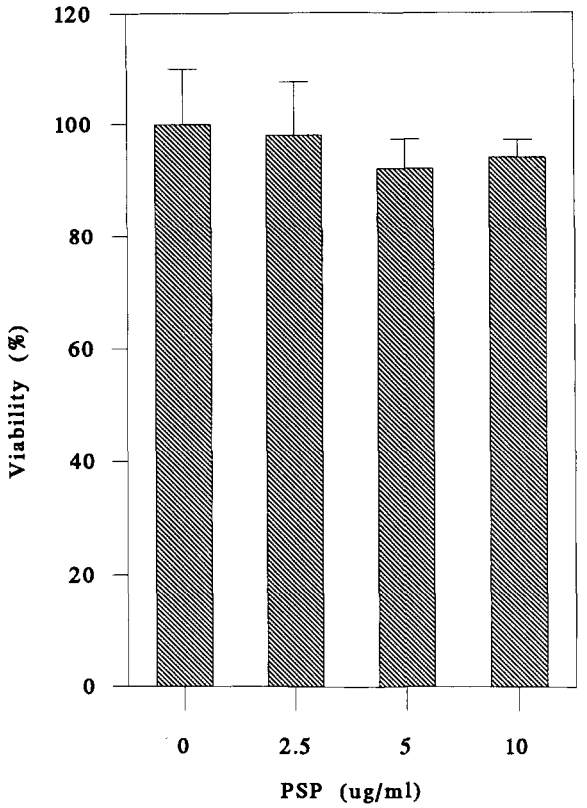

Fig. 1. Lack of significant change in viability of mouse peritoneal macrophages after co-incubation with polysaccharopeptide for 24 h ($n = 12$).

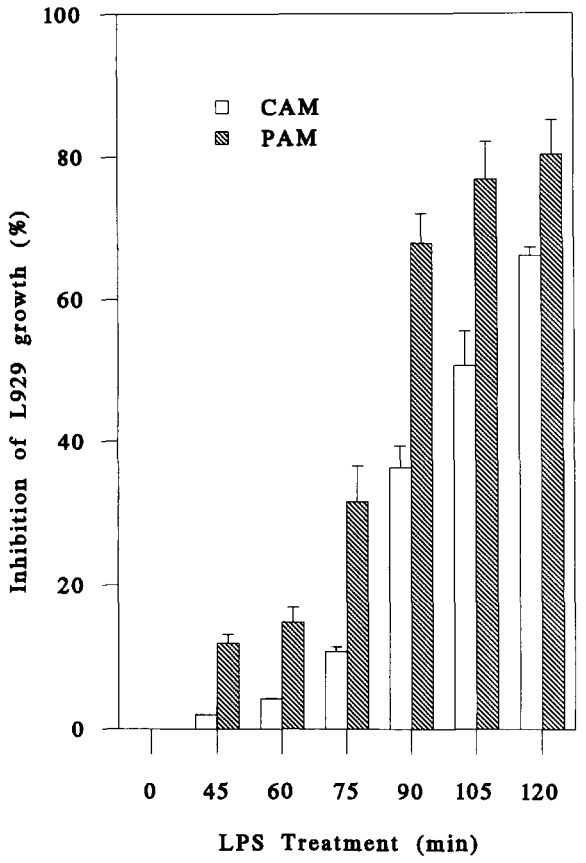

Fig. 2. Percentage of tumor cell growth inhibited by TNF-containing media produced by peritoneal macrophages from control and PSP-treated mice. Cultures ($n = 6$) were stimulated with lipopolysaccharide for 0, 45, 60, 75, 90, 105 and 120 min.

TNF in culture media with PAM was similar to that in macrophages from control mice (CAM, at 0 min). However, a significant ($P < 0.05$) increase of TNF was produced by PAM starting from 45 to 120 min of LPS stimulation, indicating that PSP enhanced TNF production.

Northern blot analysis of TNF gene expression by PAM and CAM also showed that the levels of TNF mRNA in both groups increased with the duration of LPS stimulation (Fig. 3), PAM with an earlier TNF transcription. Normal macrophages had a lower level of TNF mRNA after 1 h of LPS stimulation, but reached a level similar to that of PAM after 2 h. The level of TNF mRNA in PAM at 1 h after correlation with an equal amount of actin was increased more than 3-fold (Fig. 4) when compared with the respective control. The augmented of TNF pro-

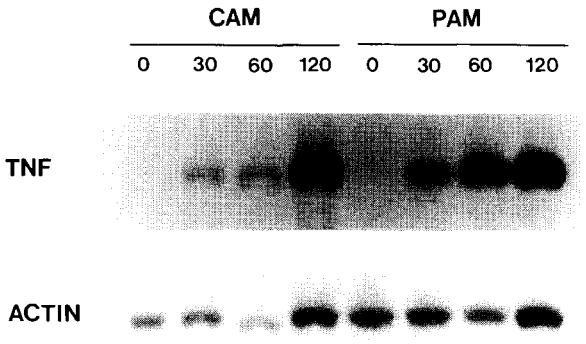

Fig. 3. Northern blot analysis of TNF transcripts in macrophages from control (CAM) and PSP-treated (PAM) mice. Cells were stimulated with lipopolysaccharide for 0, 30, 60 and 120 min.

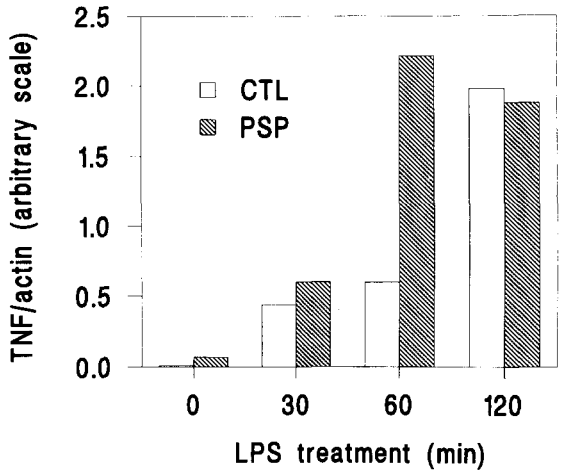

Fig. 4. Kinetics of TNF expression in macrophages from control and PSP-treated mice in response to LPS stimulation for 0, 30, 60 and 120 min. The hybridization intensities of TNF were normalized to an equal amount of actin.

duced by PAM as revealed by the L929 fibroblast bioassay was a result of an earlier and higher transcription rate of TNF gene.

Production of reactive nitrogen and oxyen intermediates

After treatment with PSP the capacity of peritoneal macrophages to generate RNI and ROI which were released into the culture media was significantly ($p < 0.05$) increased (Table II). About 266% and 275% increase of RNI and ROI production by macrophages collected from mice with prior PSP treatment were measured when compared with the respective controls.

TABLE II

Production of reactive nitrogen intermediates (RNI) and superoxide anions (O_2^-) by macrphages of C57 mice pretreated with polysaccharopeptide (PSP) for 2 weeks ($n = 6$)

Radicals	Control	PSP
RNI ($\mu M/10^6$ cells)	2.72 ± 0.25	7.24 ± 0.50***
O_2^- ($\mu M/10^6$ cells)	2.95 ± 0.33	8.14 ± 0.95***

*** Level of significance at $p < 0.01$.

Discussion

A number of polysaccharide-bound proteins have been extracted from fungi, including *Coriolus, Corticium* and *Microsporus* species by Japanese investigators (Naruse and Takeda, 1974; Hirase et al., 1970). Several species of *Coriolus* were reported to produce antitumor polysaccharide-protein complexes (Jong and Donovick, 1989). Since these substances were plant extracts, they might contain several active ingredients. However, so far unfractionated preparations have been widely used in almost all experiments (Hori and Ryoyama, 1990; Sakagami et al., 1991). Inhibition of in vivo solid tumor growth was demonstrated but undesirable side effects were not observed (Sakagami et al., 1991). These special features might be one of the major rationales why the United States National Cancer Institute selects natural products as a source of new drugs.

The PSP from *Coriolus versicolor* has been characterized by Yang (1987). It has a molecular weight of about 100,000. The polysaccharide moiety consists of glucose, galactose, mannose, xylose and fructose. Its polypeptide moiety is rich in aspartic and glutamic acids. It is soluble in hot water, heat stable and light stable. The PSP is classified as an antitumor chemical by the United States National Cancer Institute (Jong and Donovick, 1989), but its biological activity is mostly unknown.

Our results show that polysaccharopeptide (PSP) did not exert any cytotoxicity on cultured mouse peritoneal macrophages nor five tumor cell lines, including two macrophage-like cells (PU5 and P338D1); and one each of human choriocarcinoma cell line (JAR); mouse melanoma cell line (B16) and mouse sarcoma cell line (S180). It could hardly stimulate the production of reactive nitrogen and oxygen (superoxide anions) intermediates and TNF by macrophages in the culture (unpublished data). However, when mice were pretreated with PSP, the production of these molecules by macrophages was greatly enhanced, indicating that PSP is an immuno-

modulatory rather than a direct cytotoxic substance on the tumor cells tested in this study. Northern blot analysis of TNF mRNA provided further support that macrophages upon exposure to PSP had an earlier TNF transcription, consequently leading to a higher level of TNF mRNA within the first hour of LPS stimulation. More than 3.5-fold increase of TNF mRNA over the respective control was detected and this difference was minimized after 2 h. Our assays show that PSP enhanced TNF gene expression at both the transcriptional and translational levels.

The molecular basis for the tumoricidal activity of activated macrophages is not clearly known, but their secretory products, such as TNF, and reactive nitrogen and oxygen intermediates, might play an important role in this process (Ding et al., 1988; Kilbourn et al., 1984; Nathan and Hibbs, 1991). Reactive nitrogen intermediates suppress mitochondrial respiration of tumor cells, hence exhibiting their cytotoxicity against target cells (Kilbourn et al., 1984; Mauel et al., 1991; Takema et al., 1991), while ROI appear to be important components of the oxygen-dependent killing of ingested microbes by phagocytes (Fridovich, 1976). It is therefore possible that enhancement of production of reactive intermediates and TNF underlies the tumoricidal activity of polysaccharopeptide. In addition to its anti-tumor activity, TNF acts as a pluripotent mediator in activating immune cells (Andrews et al., 1990). However, it cannot induce RNI production because they arise by two independently regulated pathways (Ding et al., 1988). We have also observed increases of signal proto-oncogenes, such as c-fos and c-myc in PAM (unpublished data). Hence it is not known whether PSP directly modulates cytokine action or participates in signal transduction in the macrophages. Experiments are currently in progress to study the effect of polysaccharopeptide on the activation of macrophages.

Acknowledgements

The authors would like to thank Mr. E. Wong, Mr. S. Wong and Ms. K. Lau for their technical assistance. This study was supported by the endowment fund of the Hong Kong University and Polytechnic Grant Committee.

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