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Regulation of Cell Proliferation, Gene Expression, Production of Cytokines, and Cell Cycle Progression in Primary Human T Lymphocytes by Piperlactam S Isolated from *Piper kadsura*

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ABSTRACT

Effects of piperlactam S ($C_{17}H_{13}NO_4$; mol. wt. 295) isolated from *Piper kadsura* on phytohemagglutinin (PHA) stimulated cell proliferation were studied in primary culture of human T cells. The results showed that piperlactam S suppressed T cell proliferation at about 0 to 12 h after stimulation with PHA. Synthesis of total cellular proteins and RNA in activated cell cultures was also suppressed. The inhibitory action of piperlactam S was not through direct cytotoxicity. Cell cycle analysis indicated that piperlactam S arrested the cell cycle progression of activated T cells from the G_1 transition to the S phase. In an attempt to further localize the point in the cell cycle at which arrest occurred, a set of key regulatory events leading to the G_1/S boundary, including gene expression of cytokines and c-Fos protein synthesis, was examined. Piperlactam S suppressed, in activated T lymphocytes, the production and mRNA expression of cytokines such as interleukin-2 (IL-2), IL-4, and interferon-γ in a dose-dependent manner. In addition, Western blot analysis indicated that c-Fos protein expressed in activated T lymphocytes was decreased by piperlactam S. Results of kinetic study indicated that inhibitory effects of piperlactam S on IL-2 mRNA expressed in T cells might be related to blocking c-Fos protein synthesis. Thus, the suppressant effects of piperlactam S on proliferation of T cells activated by PHA seemed to be mediated, at least in part, through inhibition of early transcripts of T cells, especially those of important cytokines, IL-2, IL-4, and arresting cell cycle progression in the cells.

Piper kadsura (Choisy) Ohwi (also named *Piper futokadsura*; Takahashi, 1969) is commonly used in traditional Chinese medicine for the treatment of asthma and rheumatic arthritis (Han et al., 1990). A common feature among these conditions is chronic inflammation (Charles et al., 1997). Many plants belonging to the genus *Piper* have been demonstrated to yield natural products possessing various biological activities (Om et al., 1993; Soren et al., 1993). Several lignans and neolignans have been isolated from the genus and have been demonstrated to possess bioactivities, including antitumor, antiviral, inhibition of cAMP phosphodiesterase, and antimicrobial activities (Chang et al., 1985; MacRae and Towers, 1988; Neerja et al., 1990). A natural lignan-piperenone isolated from the leaves of *P. kadsura* exerts anorexic effects on insects (Kuniaki, 1975; Kuniaki et al., 1976). A well known potent and specific platelet-activating factor receptor antagonist, kadsurenone has been purified from the stem of *P. kadsura* (Shen et al., 1985; Strickler and Stone, 1989). Crotepoxide, a tumor inhibitor, has also been identified from *P. kadsura* (Takahashi, 1969). Although the stem of this plant has been used in Chinese herbal medicinal prescriptions for improvement of asthma for a long time (Takahashi, 1970), there has been a relative scarcity of definitive evidence to establish its immunopharmacological activity. To identify the active ingredients in this plant that are responsible for its possible clinical effects, pure compounds from *P. kadsura* were evaluated in immune response assays.

An intense inflammatory process is a characteristic pathological feature in bronchial asthma (Holgate et al., 1991; Deetz et al., 1997). In asthma patients, there is an accumulation of neutrophils, macrophages, activated mast cells, eosinophils, and T cells in the air spaces after antigen sensitization (Azzawi et al., 1990; Djukanovic et al., 1990; Corrigan and Kay, 1992). There is now convincing evidence that cytokines secreted by T cells or other immune cells, such as interleukin-10 (IL-10), IL-12, and interferon-γ (IFN-γ), in response to antigen stimulation play a role in lung inflammation and asthma (Frew et al., 1990; Dalton and Pitts-Meek, 1993; Goodman et al., 1996). IL-6 serves as chemotactic factor for various leukocyte population and is an important proinflammatory factor (Arai et al., 1990). In pa-

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ABBREVIATIONS: IL, interleukin; IFN-γ, interferon-γ; PHA, phytohemagglutinin; DMSO, dimethyl sulfoxide; EIA, enzyme immunoassay; DEPC, diethyl pyrocarbonate; SSC, standard saline citrate; PCR, polymerase chain reaction.

tients with asthma, the levels of inflammatory cells, T cells, and cytokines have been shown to be significantly elevated in bronchoalveolar lavage fluids, suggesting a possible pathological role for these cells and substances (Bradley et al., 1991; Corrigan and Kay, 1992). One of the therapeutic objectives in asthma and bronchial inflammation is to reduce the local inflammatory response through the reduction of inflammatory cell activation and proliferation and inflammatory cytokine production. Blockade of the T lymphocyte activation and proliferation and cytokine production is one such anti-inflammatory means (Arai et al., 1990).

Passage through T lymphocyte activation and proliferation is a highly regulated process involving ordered expression of a series of control genes such as c-fos, IL-2, and IL-4 (Cantrell, 1996). Growth modulators or other external events that affect the T cell proliferation are ultimately likely to act by controlling the expression or function of the products of these genes (Ajchenbaum et al., 1993). In a previous study, we found that the ethanolic extract of *P. kadsura* inhibited proliferation and IL-2 production in human mononuclear cells activated by phytohemagglutinin (PHA) (Yang et al., 1999). In the present study, piperlactam S was identified from the stem of *P. kadsura*. Primary human T lymphocytes were isolated from peripheral blood and used as target cells. To elucidate the effects of piperlactam S on T cell growth, the tritiated thymidine, tritiated uridine, and tritiated leucine uptake methods were used to detect total cellular DNA, RNA, and protein synthesis in the cultures, respectively. In addition, we determined the effects of piperlactam S on cell cycle progression, production and gene expression of cytokines, and c-Fos protein synthesis in activated T cells and examined their roles in regulation of T cell proliferation.

Materials and Methods

The Source of *P. kadsura*. *P. kadsura* was collected at Pettou, Taiwan and identified by Prof. Cheng-Jen Chou. A voucher specimen has been deposited in the herbarium of the Department of Botany of the National Taiwan University.

Piperlactam S Isolated from *P. kadsura*. The stems of *P. kadsura* (16.3 kg) were air dried and cut into small pieces before grinding. The ground stem was then extracted with acetone three times at room temperature. The solvent was removed under reduced pressure and the residue was partitioned between water and dichloromethane. The organic layers were combined and concentrated to yield the crude extract (240 g). The sample, absorbed on silica gel [sample/adsorbent (v/v) = 1/8], was subjected to dry flash column chromatography. Sufficient hexane was passed through the column to expel all of the air. Extensive gradient elutions were then employed using hexane and ethyl acetate to yield 13 fractions. The like fractions were combined to give eight main fractions with monitoring by thin-layer chromatography and the solvent was removed under reduced pressure. Each combined fraction was further purified by rechromatography and recrystallization. The purity of all pure compounds isolated from *P. kadsura* was analyzed with an HPLC purity program. They were dissolved in dimethyl sulfoxide (DMSO) and then stored at 4°C until for use.

General Methods and Apparatuses for Compound Purification. Melting points were measured on a micromelting point hot-stage apparatus and were uncorrected. ^{1}H-, ^{13}C-, and 2D-NMR spectra were taken on Bruker ACP-300 spectrophotometer with deuterated solvents as internal standard. Dry flash column chromatography was performed on silica gel (230–400 mesh, Merck). Thin-layer chromatography was carried out on precoated kiesel gel 60 F_{254} plates (silica gel plated, 0.25 mm thick, Merck); spots were visualized under UV light (254 and 365 nm) irradiation and by spraying with 10% molybdatophosphoric acid solution followed by heating at 120°C.

Human Subjects. Fifteen healthy male subjects (25 to 35 years; mean age, 29 years) were chosen for this investigation. The experimental protocol had been reviewed and approved by the institutional human experimentation committee. Written informed consent was obtained from all subjects.

Preparation of Primary Human T Lymphocytes. Heparinized human peripheral blood (80 ml) was obtained from normal healthy volunteers. Human mononuclear cells were isolated by the Ficoll-Hypaque gradient density method as described previously (Yang et al., 1999). After depletion of adherent cells on plastic dishes, T lymphocytes were isolated by erythrocyte rosetting. The erythrocyte rosette positive fraction contained <5% monocytes or B lymphocytes, as assessed by flow cytometric analysis. T cells were resuspended to a concentration of 2×10^6 cells/ml in RPMI-1640 medium (Life Technologies, Gaithersburg, MD), supplemented with 2% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Lymphoproliferation Test. The lymphoproliferation test was modified from that described previously (Yang et al., 1999). The DNA synthesis in proliferating cells was labeled with tritiated thymidine. The density of T lymphocytes was adjusted to 2×10^6 cells/ml before use. Cell suspension (100 μl) was applied to each well of a 96-well, flat-bottomed plate (Nunc 167008, Nunclon, Raskilde, Denmark) with or without 5 μg/ml PHA (Sigma, St. Louis, MO). Cyclosporin A was used as a positive control (Schreiber and Crabtree, 1992). Piperlactam S or cyclosporin A was added to the cells at varying concentrations or at different time. The plates were incubated in 5% CO_2/air humidified atmosphere at 37°C for 3 days. Subsequently, tritiated thymidine (1 μCi/well; NEN, Boston, MA) was added to each well. After a 16-h incubation, the cells were harvested on glass fiber filters by an automatic harvester (Multimash 2000; Dynatech, Billingshurst, UK). Radioactivity in the filters was measured by liquid scintillation counting. The inhibitory activity of piperlactam S on T lymphocyte proliferation was calculated using the following formula:

$$\text{Inhibitory Activity (\%)} = \frac{\text{Control Group (CPM)} - \text{Experiment group (CPM)}}{\text{Control group (CPM)}} \times 100 \quad (1)$$

Assay of Total Cellular RNA Synthesis in T Cells. The assay of total cellular RNA synthesis was modified from the method described previously (Yang et al., 1999). The RNA synthesized in the cells was labeled with tritiated uridine. The density of T lymphocytes was adjusted to 2×10^6 cells/ml before use. Cell suspension (100 μl) was applied to each well of a 96-well, flat-bottomed plate with or without 5 μg/ml PHA. Piperlactam S (25 μM) isolated from *P. kadsura* was added to the cells. The plates were incubated in 5% CO_2/air humidified atmosphere at 37°C for 3 days. Subsequently, tritiated uridine (1 μCi/well; NEN) was added into each well. After a 16-h incubation, the cells were harvested on glass fiber filters by an automatic harvester. Radioactivity in the filters was measured by liquid scintillation counting.

Analysis of Total Cellular Protein Synthesis in T Lymphocytes. Analysis of total cellular RNA synthesis was modified from the method described previously (Yang et al., 1999). The protein synthesized in the cells was labeled with tritiated uridine. The density of T lymphocytes was adjusted to 2×10^6 cells/ml and cultured in leucine-free RPMI-1640 medium before use. Cell suspension (100 μl) was divided into each well of a 96-well, flat-bottomed plate with or without 5 μg/ml PHA. Piperlactam S (25 μM) isolated from *P. kadsura* was added to the cells. The plates were incubated in 5% CO_2/air humidified atmosphere at 37°C for 3 days. Subsequently, tritiated leucine (1 μCi/well; NEN) was added to each well. After a

16-h incubation, the cells were harvested on glass fiber filters by an automatic harvester. Radioactivity in the filters was measured by liquid scintillation counting.

Determination of Cytokines Production in T Lymphocytes. T lymphocytes (2×10^5 cells/well) were cultured with PHA alone or in combination with varying concentrations of piperlactam S for 3 days. The cell supernatants were then collected and assayed for IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-γ concentrations by enzyme immunoassays (EIA; R&D Systems, Minneapolis, MN). No detectable cross-reactivity with other cytokines has been reported for the EIA kits used.

Extraction of Total Cellular RNA. The total cellular RNA was extracted from T lymphocytes by a method described previously (Kuo et al., 1993). The 5×10^6 cells were activated with or without PHA and cocultured with varying concentrations of piperlactam S isolated from *P. kadsura* for different time periods. T cells were collected and washed with cold Tris-saline containing 25 mM Tris, pH 7.4, 130 mM NaCl, 5 mM KCl and then suspended in buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% dextran sulfate. After centrifugation, the supernatants were extracted with a phenol-chloroform mixture. The extracted RNA was precipitated with 100% cold ethanol. The total cellular RNA was pelleted by centrifugation and redissolved in diethyl pyrocarbonate (DEPC)-treated water. The concentration of the extracted RNA was calculated by measuring the absorbance at 260 nm. The ratio of the absorbance at 260 nm to that at 280 nm was always higher than 1.8. The quality of RNA was assessed by the intactness of 28S and 18S bands and lack of degradation on agarose-gel electrophoresis.

Northern Blot Analysis. A 15-μg sample of total cellular RNA was resolved on 6.66% formaldehyde agarose gel and then transferred to nitrocellulose filter (Schleicher & Schuell, Germany) with 20× standard saline citrate (SSC) containing 3 M NaCl and 0.3 M sodium citrate. The filter was prehybridized at 42°C for 4 h (prehybridization solution of 0.1% sodium pyrophosphate, 0.25 mg/ml salmon sperm DNA, and 50% deionized formamide). Deoxycytidine 5′-[α-^{32}P]triphosphate-labeled IL-2 cDNA was then added to the same solution and incubated at 42°C for 12 h. The filter was washed with 0.1× SSC at room temperature for 5 min, and then washed by 0.1× SSC and 0.1% SDS at 50°C for 20 min. Finally, the filter was exposed to X-ray film (Kodak).

Synthesis of First Strand cDNA. Aliquots of 1 μg of RNA were reverse-transcribed using the Advantage RT-for-PCR kit from CLONTECH according to the manufacturer's instructions. Briefly, 1 μg of RNA in 12.5 μl of DEPC-treated water was mixed with 20 μM oligodeoxythymidine $(\text{oligodT})_{18}$, and heated at 70°C for 10 min, then quick-chilled on ice. The following reagents were added to the tube: 6.5 μl of concentrated synthesis buffer (50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTPs, and 0.5 U RNase inhibitor), and 200 U of the Moloney murine leukemia virus reverse transcriptase. The reaction was initially incubated at 42°C for 1 h, and then at 94°C for 5 min to terminate the reaction. DEPC-treated water (80 μl) was added to the tube, which was then stored at −20°C for use in the polymerase chain reaction (PCR).

PCR. PCR was performed in an air thermocycler according to the manufacturer's instructions as described previously (Saiki et al., 1985). Briefly, 10 μl of the first-strand cDNA was mixed with 0.75 μM primers, 4 U of *Taq* polymerase, 10 μl of reaction buffer (2 mM Tris·HCl, pH 8.0, 0.01 mM EDTA, 0.1 mM dithiothreitol, 0.1% Triton X-100, 5% glycerol, and 1.5 mM $MgCl_2$), and 25 μl of water in a total volume of 50 μl. All primer pairs for the cytokines were designed from the published human cDNA sequence data (Gray et al., 1982; Taniguchi et al., 1983; Hirano, 1986; Yokota et al., 1986; Wolf, 1991; Kim et al., 1992). The following c-fos specific primers were used: forward, 5′-CTTCTGCTCTAAAAGCTGCG-3′and reverse 5′-CGACCTGCAGCTCGA-GCACA-3′. The PCR was done at the following setting of the air thermocycler: denaturing temperature of 94°C for 1 min, annealing temperature of 60°C for 1 min, and elongation temperature of 72°C for 80 sec for the first 40 cycles, and finally elongation temperature of 72°C for 10 min. After the reaction, the amplified product was taken out of the tubes and run on 2% agarose gel.

Cell Cycle Analysis. Procedures for cell cycle analysis have been described previously (Javier et al., 1997). The density of T lymphocytes was adjusted to 2×10^6 cells/ml before use. Cell suspension (1 ml) was divided into each well of a 6-well, flat-bottomed plate (Cellstar 657160, Greiner, Germany) with or without 5 μg/ml PHA. Piperlactam S (25 μM) was added to the cells. The plates were incubated in 5% CO_2/air humidified atmosphere at 37°C for different time periods. The cells were harvested by centrifugation, washed in PBS, pH7.2, and then fixed in 70% ethanol for 30 min at −20°C. After washing the cells once with PBS, the DNA was stained with propidium iodide (4 μg/ml) containing 100 μg/ml of ribonuclease A. Flow cytometry analysis was conducted using a Becton-Dickinson FAS-Can.

Western Blot Analysis. Total cellular protein was extracted from T lymphocytes by a method described previously (Kuo et al., 1993). The density of T lymphocytes was adjusted to 5×10^6 cells/ml before use. Cell suspension (1 ml) was applied into each well of a 6-well, flat-bottomed plate with or without 5 μg/ml PHA. Piperlactam S (25 μM) was added to the cells. The plates were incubated in 5% CO_2/air humidified atmosphere at 37°C for 30 min. Cells were harvested and washed once with PBS containing 0.5 mM EDTA. Then the cells were lysed by a solution containing 20 mM Tris·HCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM EDTA, pH 7.2, 0.5% Triton X-100, 1 mM dithiothreitol, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 10 mM β-glycerophosphate. The lysates were cleared of insoluble material by centrifugation. Proteins (20 μg) were dissolved in the dissociation buffer (2% SDS, 5% β-mercaptoethanol, 0.05 M Tris·HCl, and 20% glycerol, pH 7.6) and boiled for 5 min. Then proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. After blocking the filters with a solution containing 1% BSA, the filters were incubated with mouse monoclonal antibody raised against human c-Fos (Chemicon International Inc., Temecula, CA) Specific reactive proteins were detected by an enhanced chemiluminescence method, employing a rabbit anti-mouse Ig Ab linked to horseradish peroxidase (Amersham, Arlington Heights, IL).

Determination of Cell Viability. Approximately 2×10^5 T cells with or without PHA were cultured with 0.1% DMSO, or 100 μM piperlactam S for 4 days. Total, viable, and nonviable cell numbers were counted under the microscope with the help of a hemocytometer after staining by trypan blue. The percentage of viable cells were calculated:

$$\text{Viability (\%)} = \frac{\text{Viable Cell Number}}{\text{Total Cell Number}} \times 100 \qquad (2)$$

Statistical Analysis. Data are presented as mean ± S.D., and the differences between groups were assessed with Student's t test.

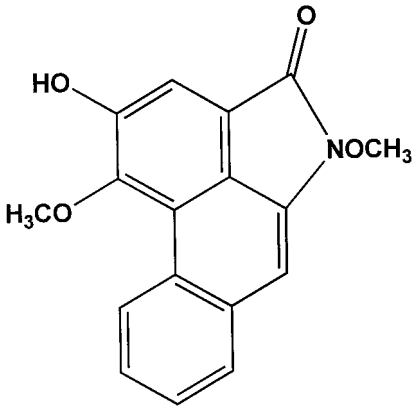
Results

Piperlactam S Identified from *P. kadsura*. To extract pure active compounds from the *P. kadsura*, we used the complete isolation process in each chromatographic cycle and finally HPLC. The compound with the strongest activity (128 mg) was obtained from the third combined fraction as a yellow solid with an m.p. of 242–244°C. NMR and mass spectrometry analyses indicated a structure as shown in Fig. 1. The chemical name of this bioactive component is 10-amino-3-hydroxy-4-methoxy-N-methoxyphenanthrene-1-carboxylic acid lactam ($C_{17}H_{13}NO_4$; mol. wt. 295). The mass and NMR spectra data for the compound were compatible with those previously reported for piperlactam S by Wu et al. (1997). The common name of this component is piperlactam S. The purity of piperlactam S isolated from *P. kadsura* was

assessed by an HPLC purity program (reverse column RP-18). The column was eluted with mixture of acetonitrile and water (3:2, v/v) and analyzed by UV detector at 254 nm. Piperlactam S appeared as a single peak at 6.463 min retention time and its purity was 99.077%.

The Effect of Piperlactam S on T Cell Proliferation. As shown in Fig. 2, treatment with PHA for 3 days stimulated cell proliferation as indicated by about 56-fold increase in tritiated thymidine uptake. Treatment with the vehicle DMSO affected neither the tritiated thymidine uptake in the resting state nor that in the stimulated state. Although similar to cyclosporin A in having little effects on tritiated thymidine uptake in resting T cells, both piperlactam S and cyclosporin A significantly suppressed the enhanced uptake observable in activated cells. Furthermore, the inhibitory effects of piperlactam S on activated T cells were concentration dependent. At 3.125 μM, piperlactam S suppressed PHA-treated T cell proliferation by 46.7 $\pm$ 0.8%. The corresponding degrees of inhibition for 12.5 μM, 25 μM, and 100 μM were 82.0 $\pm$ 3.3%, 92.8 $\pm$ 2.5%, and 98.4 $\pm$ 1.6%, respectively, with an IC_{50} value of 3.5 $\pm$ 1.5 μM. To further delineate the suppressant effects of piperlactam S on total cellular RNA and protein synthesis in PHA-induced T cell cultures, we examined them by the tritiated uridine and tritiated leucine uptake methods, respectively. Results indicated that although piperlactam S had little effect on tritiated uridine uptake (3,500 $\pm$ 900 versus 2,000 $\pm$ 920 cpm) and tritiated leucine uptake (14,300 $\pm$ 1,600 versus 8,000 $\pm$ 5,700 cpm) in resting T cells, the enhanced uptake observable in activated cells was significantly suppressed compared with control cells (106,000 $\pm$ 5,900 versus 30,000 $\pm$ 7,000 cpm, $P < .001$; 170,000 $\pm$ 6,700 versus 25,000 $\pm$ 3,300 cpm, $P < .001$). However, the inhibitory effects of piperlactam S on T cells were not related to direct cytotoxicity because the viability of resting (77.0 $\pm$ 1.2% versus 76.0 $\pm$ 2.1%) or activated T cells (77.4 $\pm$ 1.6% versus 74.3 $\pm$ 3.5%) were not significantly decreased after treatment with 100 μM piperlactam S for 4 days.

Time-Course Analysis of the Effect of Piperlactam S on PHA-Stimulated T Cell Proliferation. Time-course experiments were performed to determine at what point in the activation process piperlactam S inhibited T cell proliferation. Piperlactam S was added to cultures at 0, 2, 4, 6, 8, 12, 16, 24, 36, 48, and 72 h, and the proliferation assay was performed at 88 h. The results indicated that after stimulation, addition of piperlactam S between 0 and 12 h suppressed T cell proliferation (Fig. 3). Addition of piperlactam S at 16, 24, 36, 48, and 72 h of stimulation showed only minimal inhibitory effects on proliferation. The fact that piperlactam S was inhibitory when added between the 0- and 12-h time range suggested that the inhibitory effects of piperlactam S might be related to the blocking biochemical events or



Piperlactam S

Fig. 1. The structure of piperlactam S purified from *P. kadsura*.

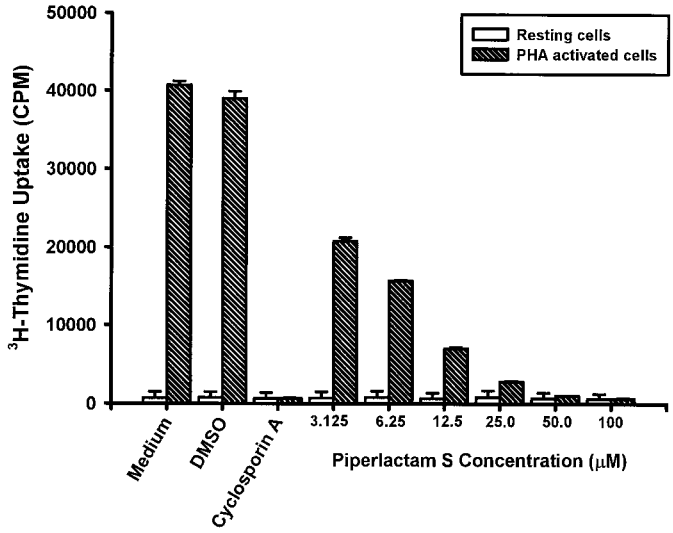


Fig. 2. The inhibitory activity of piperlactam S on T lymphocyte proliferation. T cells (2×10^5/well) were treated by cyclosporin A (6.25 μM) or indicated concentration of piperlactam S with or without PHA (5 μg/ml) for 3 days. The proliferation of cells was detected by tritiated thymidine uptake (1 μCi/well). After a 16-h incubation, the cells were harvested by an automatic harvester, then radioactivity was measured by liquid scintillation counting. Each bar represents the mean of three independent experiments.

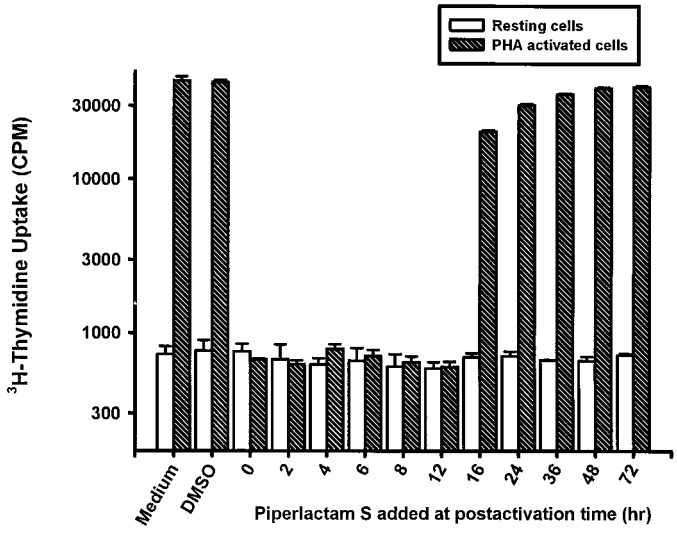


Fig. 3. Kinetics of inhibition of T cell proliferation activated by piperlactam S. T cells (2×10^5/well) were activated with or without PHA (5 μg/ml) and piperlactam S (25 μM) was added at the indicated time. Cells were pulsed on day 3 for 16 h with 1 μCi/well tritiated thymidine. Proliferation assay were performed as described under *Materials and Methods*. Each bar represents the mean of three independent experiments.

genes expression necessary for T cell proliferation activated with PHA during this time.

Effects of Piperlactam S on the Cell Cycle. After stimulation with PHA, T lymphocytes enter the G_1 phase in 2 to 4 h, the S phase after approximately 18 to 24 h, and reach the G_2/M phase by 36 to 48 h (Morice et al., 1993). Because the above data suggested that piperlactam S inhibited T cell proliferation at about 0 to 12 h after stimulation with PHA, we examined further where in the cell cycle of T cells the arrest took place. After incubation with or without piperlactam S for 3 days, cell cycle analyses were performed using a commercially prepared propidium iodide reagent for staining nuclear DNA before flow cytometry analysis. As shown in Fig. 4A, resting T lymphocytes existed almost exclusively in the G_0/G_1 phase, which was not affected by DMSO or piperlactam S treatment. When the cells were stimulated with PHA, then induced into cell cycle, fluorescence intensity increased from that of the G_0/G_1 phase to the S phase and G_2/M phase and DMSO did not affect this fluorescence change. By contrast, after adding piperlactam S to PHA-activated T lymphocytes, almost all cells were still blocked at G_0/G_1 stage compared with the control groups. A computer program was then used to determine the percentage of T cells in the G_0/G_1, S, and G_2/M phases (Fig. 4B). Results indicated that addition of piperlactam S at 2 h after PHA stimulation inhibited 96% of the cells from entering S phase (Table 1). Addition between 16 h and 72 h gradually increased the percentage of cells in S phase and G_2/M phase. These data are in agreement with DNA synthesis data in which addition of piperlactam S at 0 to 12 h after PHA stimulation blocks T cell proliferation.

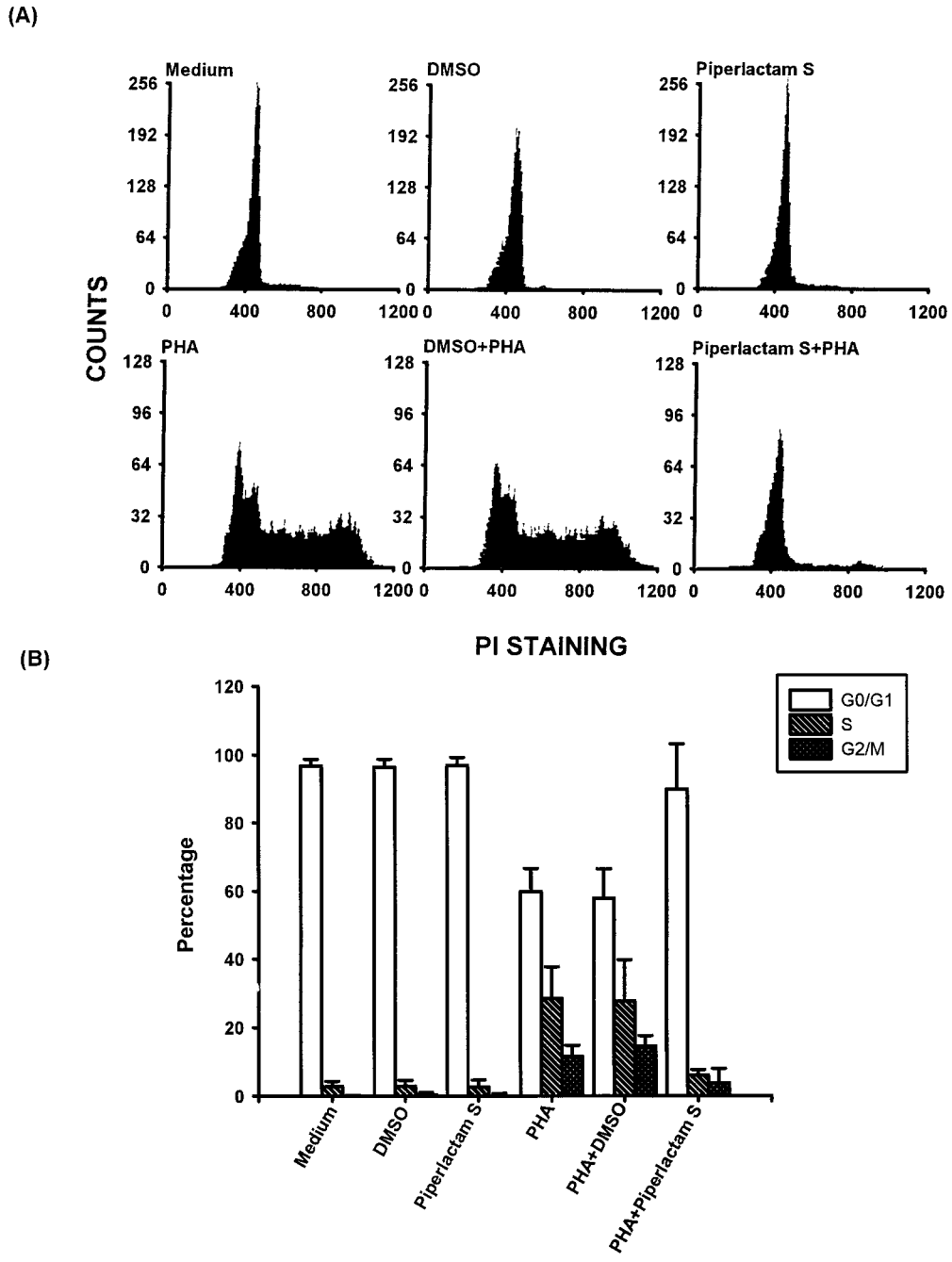


Fig. 4. Ability of piperlactam S to block T cell progression into the S phase of the cell cycle. T cells (2×10^6) were treated by 25 μM piperlactam S with or without PHA (5 μg/ml) for 3 days. For determining the cell counts that entered into the cell cycle, cells from a representative subject were stained with propidium iodide, and the DNA content of the cells was analyzed by flow cytometry as described in *Materials and Methods* (A). A computer program was then used to determine the percentage of T cells in the G_0/G_1, S, and G_2/M phases. Each bar is the mean of three independent experiments (B).

Effects of Piperlactam S on Cytokines Production in T Lymphocytes. To study whether the piperlactam S-suppressed T cell cycle progression and proliferation after stimulation with PHA was related to cytokine production, the cells were incubated with or without varying concentrations of piperlactam S for 3 days. Supernatants were then collected, and the production of IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-γ were assayed by EIA, respectively. As shown in Fig. 5, the stimulated production of IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-γ in activated T cells was significantly suppressed by piperlactam S. Furthermore, the inhibitory activities of piperlactam S were concentration dependent. At 100 μM, the stimulated production of cytokines in activated T cells were completely blocked by piperlactam S, with their concentrations returning to almost the same as those produced in resting cells, which suggests that piperlactam S-suppressed T cell cycle progression and proliferation after stimulation with PHA was related to cytokine production.

Cytokine mRNA Expression in Piperlactam S-Treated T Cells Detected by Reverse Transcription-PCR. Because production of several cytokines in activated T lymphocytes was decreased by piperlactam S, we examined whether cytokine mRNA expression in activated T cells was affected by piperlactam S. Total cellular RNA was extracted from activated T cells in the presence or absence of 25 μM piperlactam S and available for RT-PCR. Initially, we examined the dose-response relationship of the PCR-amplification of cDNA (data not shown). The exponential phase of amplification was determined by performing for 20, 30, 40, and 50 cycles. We found that 40 cycles of PCR were optimal for all the cytokines (data not shown). The results of RT-PCR analyses are shown in Fig. 6A. The mRNA for β-actin was detectable in the samples treated with DMSO (lane 1), PHA (lane 2), DMSO, and PHA (lane 3), and piperlactam S and PHA (lane 4), respectively. The results indicated that neither piperlactam S nor DMSO affected β-actin mRNA expression in T lymphocytes. With the exception of IFN-γ mRNA, resting T cells expressed little of other cytokines' mRNA. After PHA stimulation, the levels of each cytokine mRNA were significantly increased in the cells. By contrast, PCR products for IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-γ amplified from PHA-treated T cells' RNA preparations were attenuated by piperlactam S. As shown in Fig. 6B, laser densitometry analysis demonstrated that the ratios of IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-γ mRNAs to β-actin mRNA in PHA-activated T cells were significantly decreased by piperlactam S. Northern blot analysis confirmed that 0.9-kilobase IL-2 mRNA expression in PHA-activated T cells was blocked by piperlactam S in a dosage-dependent manner (Fig. 7).

c-Fos Protein Expression in Piperlactam S-Treated T Cells Detected by Western Blot Analysis. To elucidate whether piperlactam S-suppressed IL-2 mRNA expression in T cells stimulated with PHA was related to c-Fos protein synthesis, the cells were incubated with or without piperlactam S for 30 min. Then cellular proteins were extracted and c-Fos protein levels were detected by Western blotting. The results indicated that T cells expressed c-Fos proteins after 30-min PHA stimulation (Fig. 8A). The cells incubated with PHA in the presence of 25 μM piperlactam S showed a significant decrease in c-Fos protein levels compared with the control group. The relative absorbance values of the c-Fos protein signal are shown in Fig. 8B. The c-Fos protein signal of piperlactam S-treated cells was significantly lower than that of untreated cells. To prove c-fos-decreased expression preceded a change in cytokine production, the c-fos and IL-2 mRNA expressed in T cells treated with PHA for different time periods were analyzed by RT-PCR. The data showed that c-fos mRNA was expressed in the cells 30 min after activation and decreased 6 h after activation (Fig. 8C). The IL-2 mRNA could be detected in T cells 8 h after activation. When the cells were treated with piperlactam S 2 h after activation, c-fos mRNA was decreased 4 h after activation and IL-2 mRNA expression was blocked 8 h after activation. Thus, we predict that decreasing IL-2 production was related to impairment of c-fos production in T cells treated with piperlactam S.

TABLE 1
Piperlactam S inhibits T cell cycle progression into S phase of the cell cycle

Condition	% Cell		
	G_0/G_1	S	G_2/M
Unstimulated	94.9 ± 3.6	1.1 ± 1.0	4.0 ± 1.8
Time after PHA stimulation			
0 h	97.7 ± 0.7	2.3 ± 0.6	0.0 ± 0.0
2 h	95.8 ± 2.0	2.8 ± 3.0	1.4 ± 0.0
4 h	94.1 ± 1.8	3.9 ± 0.0	2.0 ± 0.0
6 h	93.5 ± 2.9	4.2 ± 0.2	2.3 ± 1.9
8 h	91.3 ± 1.6	5.9 ± 0.4	2.8 ± 3.8
12 h	87.6 ± 1.1	9.1 ± 1.4	3.3 ± 1.9
16 h	79.8 ± 1.5	15.5 ± 0.5	4.7 ± 0.9
24 h	70.6 ± 0.2	19.8 ± 1.0	9.6 ± 1.4
36 h	65.2 ± 2.1	22.9 ± 3.1	11.9 ± 0.9
48 h	63.9 ± 1.5	26.1 ± 1.0	10.0 ± 2.6
72 h	60.9 ± 3.8	28.5 ± 4.2	10.6 ± 3.3
Time of addition of 25 μM piperlactam S after PHA stimulation			
0 h	92.8 ± 1.9	0.4 ± 0.9	6.8 ± 1.7
2 h	92.3 ± 2.2	1.2 ± 1.0	6.5 ± 3.2
4 h	94.0 ± 0.9	0.5 ± 0.8	5.6 ± 2.8
6 h	92.3 ± 1.5	0.7 ± 1.2	7.0 ± 1.9
8 h	92.6 ± 1.1	1.6 ± 0.5	5.7 ± 2.0
12 h	86.7 ± 1.6	0.7 ± 1.1	12.6 ± 3.0
16 h	81.3 ± 2.0	11.3 ± 2.1	7.4 ± 2.2
24 h	72.6 ± 1.9	21.3 ± 1.9	6.1 ± 2.7
36 h	65.0 ± 2.2	20.9 ± 2.5	14.1 ± 3.2
48 h	64.3 ± 1.5	23.1 ± 3.0	12.6 ± 2.3
72 h	61.7 ± 1.0	23.3 ± 2.9	15.0 ± 2.4

In PHA controls, T cells (2×10^6) were stimulated with PHA (5 μg/ml) and collected for cell cycle analysis at the indicated time. In experiment groups, after PHA stimulation, 25 μM of piperlactam S was added at the indicated time, and progression of cell cycle was analyzed on the third day. Analysis of cell cycle was performed as described under Materials and Methods. These data are representative of three separate experiments.

Discussion

In the present study, the active mechanisms of piperlactam S from *P. kadsura* on human T lymphocyte proliferation activated with PHA were defined. Results demonstrated that increase in total cellular DNA, RNA, and protein synthesis induced by PHA was inhibited by piperlactam S. The growth-suppressive actions of piperlactam S were not explained by a drug-induced reduction in cell viability. Delayed addition of piperlactam S reduced its antiproliferative activity. Cell cycle analysis revealed that piperlactam S inhibited the entry of PHA-stimulated T cells into the S phase of the cell cycle, observations that were consistent with data that piperlactam S suppressed PHA-driven T cell proliferation. In addition, we observed that piperlactam S decreased production and mRNA expression of cytokines and c-Fos protein levels in activated T cells. We suggest that piperlactam S interferes

with some regulatory events required for entry of PHA-activated T cells into the S phase, and then the cell proliferation is suppressed.

Piperlactam S is an alkaloid and has been isolated from *Piper puberullum* (Wu et al., 1997). This is the first report of a naturally occurring *N*-oxygenated methoxy aristolactam identified from *P. kadsura*. So far, no immunopharmacological functions have been described for this compound. The present results show that piperlactam S suppressed activated T cell proliferation and cytokines production. The possible inhibitory effect of DMSO on T cells was studied in these experiments. DMSO did not change T cell proliferation and viability. Therefore, the inhibitory function of piperlactam S was probably not related to DMSO. The morphology and characteristics of T cells treated with or without piperlactam S were similar, suggesting that the inhibitory effects of piperlactam S were not related to the pH, osmolarity, or other physiologic variables in different preparations (data not shown). The effects of piperlactam S on T lymphocytes were determined at the third day or early after PHA activation and in almost all experiments, the concentration of piperlactam S used was 25 μM. Results of cell viability indicated that there was no significant cell death in T cell cultures after treatment with 100 μM piperlactam for 4 days. We suggest that under 100 μM and during this time, the inhibitory effects of piperlactam S on T cells were not cytotoxic, although we cannot exclude out the possibility that piperlactam S may have toxic effects on T cells after chronic treatment or at higher concentrations. However, we have used piperlactam S above 100 μM to treat several tumor cell lines for 5 days. The preliminary results indicated that piperlactam S could not block tumor cell growth.

Tritiated uridine and tritiated leucine uptake indicated that piperlactam S decreased the total cellular RNA and protein synthesis in PHA-activated T cell cultures. Piperlactam S did not affect total cellular RNA and protein synthesis in the resting cells and could not block β-actin mRNA expression in resting and activated T cells. The results of RT-PCR indicated that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in resting and activated T cells were not decreased by piperlactam S (data not shown). However, the values of tritiated uridine and tritiated leucine uptake in each T cell (CPM/cell) were calculated. The preliminary results showed piperlactam S did not change the values of tritiated uridine and tritiated leucine uptake in each activated T cell although RNA and protein synthesis increased in each activated T cell compared with each resting cell. We predict that the impairments of total cellular RNA and protein synthesis in activated T cells treated with piperlactam S may be related to decrease in the proliferating cell number.

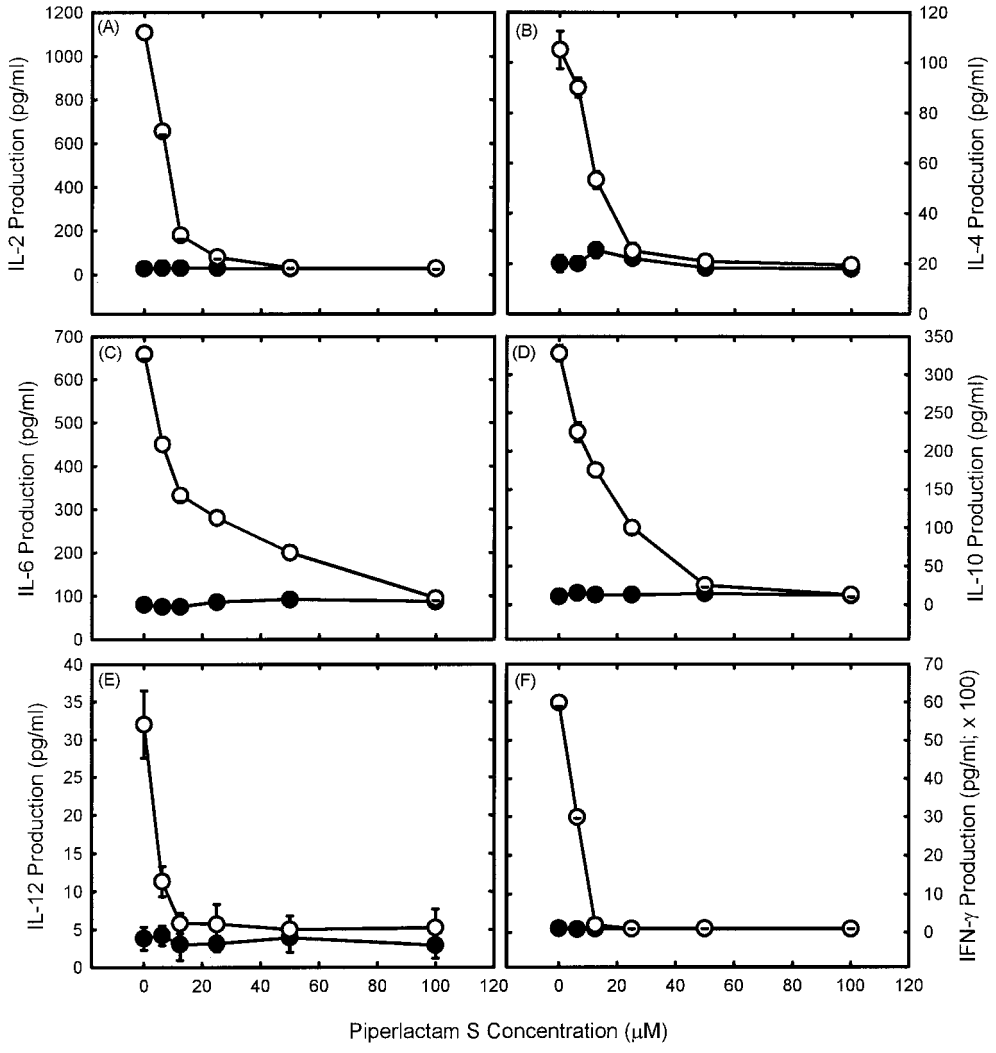


Fig. 5. Cytokines production in T lymphocytes treated with piperlactam S. T cells (2×10^5/well) were treated by 0, 6.25, 12.5, 25, 50, and 100 μM piperlactam S with or without PHA (5 μg/ml) for 3 days. Then the cell supernatants were collected and IL-2 (A), IL-4 (B), IL-6 (C), IL-10 (D), IL-12 (E), and IFN-γ (F) concentration was determined by EIA, respectively. Each point is the mean of three independent experiments. ●, resting cells; ○, PHA activated cells.

Although the attenuation of cytokines and c-Fos production was part of protein decrease in total T cell population, the inhibitory effects of piperlactam S on cytokines and c-Fos production in activated T cells were rather specific.

The central event in generation of immune responses is the activation and clonal expansion of T cells (Charles et al., 1997). During the process of differentiation, T cells spontaneously arrest in G_0 and may remain quiescent for long periods of time until exposed to specific antigen or mitogens. Interaction of T cells with antigens or PHA initiates a cascade of biochemical events that induces the resting T cells to enter the cell cycle then proliferate and differentiate. In our studies, the results indicated that almost all unstimulated primary T lymphocytes existed at the G_0/G_1 phase and after stimulation with PHA, entry into cell cycle was induced. It has been demonstrated in many previous studies with T cells that a series of genes such as IL-2 and c-fos are included in a carefully controlled order as the cells pass through G_0, G_1, and S phases (Ajchenbaum et al., 1993). For example, transition from G_0 to G_1 is marked by transcriptional activation of the IL-2 receptor and IL-2 genes (and in some cases IL-4). Subsequent G_1 events and initiation of DNA synthesis are dependent on induction of IL-2 receptor and on a supply of IL-2 from autocrine or external supply. Although, the molecular mechanisms involved in regulating passage through cell cycle in T cells stimulated with PHA remain largely unknown, growth modulators or other external events that affect T cell proliferation are ultimately likely to act by controlling the expression or function of the products of these genes (Ajchenbaum et al., 1993). The present results indicated that piperlactam S suppressed PHA-activated T cells proliferation and blocked PHA-induced progression of T cell

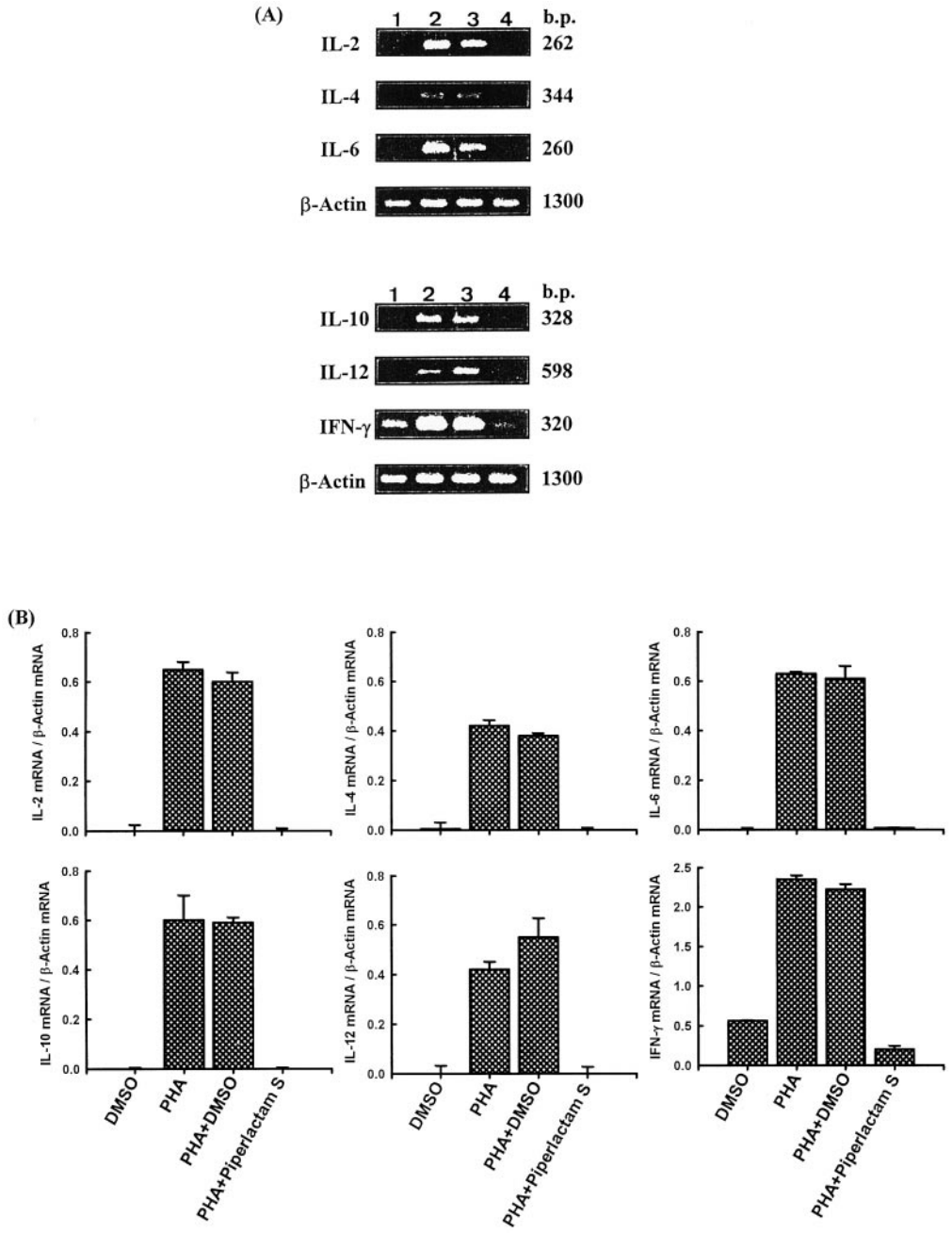


Fig. 6. Effects of piperlactam S on cytokines mRNA expression in T cells detected by RT-PCR analysis. T cells (5×10^6) activated with or without PHA (5 μg/ml) in the presence or absence of 25 μM piperlactam S for 3 days. The total cellular RNA was isolated from T cells treated with DMSO (lane 1), PHA (lane 2), PHA and DMSO (lane 3), or PHA and piperlactam S (lane 4), respectively. Aliquots of 1 μg of RNA were reverse-transcribed for synthesis of first-strand cDNA. Briefly, 10 μl of the first-strand cDNA was applied for the PCR test. The PCR was done as described under *Materials and Methods*. After the reaction, the amplified product was taken out of the tubes and run on 2% agarose gel (A). Graphical representation of laser densitometry of various cytokines expression in resting or PHA-stimulated T cells in the presence or absence of piperlactam S. Each band was quantified using laser-scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA). The ratio of each cytokines mRNAs to β-actin mRNA was calculated. Each bar is the mean of three independent experiments (B).

cycle from G_1 transition to S phase. These results demonstrated that piperlactam S acted similarly to the immunosuppressive agent cyclosporin A, retaining T cells predominantly in either the G_0/G_1 phase or the early S phase of the cell cycle (Schreiber and Crabtree, 1992). In preliminary experiments, we also found that the levels of cyclin E mRNA in PHA-activated T cells were decreased by piperlactam S (data not shown). Data from primary human T lymphocytes indicate that cyclin E is likely to play a regulatory role in the cell cycle (Charles, 1993). We suggest that inhibitory effect of piperlactam S on T cells proliferation may be related to arresting of cell cycle progression in the cells.

On the other hand, cytokine production, such as IL-2 and IL-4, in PHA-activated T cell cultures was decreased by piperlactam S. Recently, external IL-2 was added into PHA-activated T cell cultures in the presence of piperlactam S and the cell proliferation was determined in a preliminary study. Although 50% proliferation of piperlactam S-treated T cells could be restored by IL-2 at 10 U/ml, other cytokines such as IL-4 and IFN-γ might help to restore it (data not shown). Another possibility is that higher concentration of IL-2 (above 10 U/ml) might be required to enhance the recovery of piperlactam S-treated T cell proliferation. We predict that one of factors contributing to the arrest of cell cycle progression is deficiency in IL-2 or other cytokine production in T cells. The impairment of cytokine production was related to piperlactam S suppressing the mRNA transcription of these

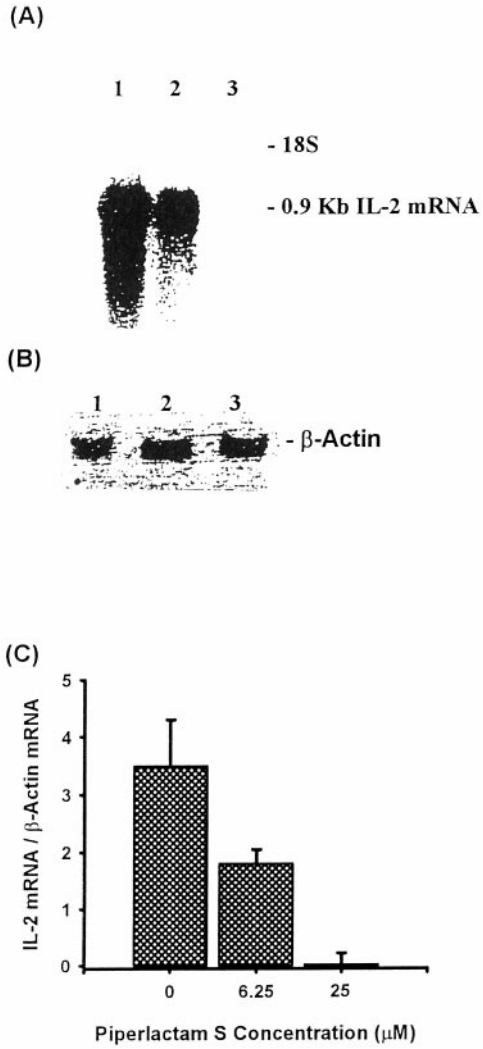


Fig. 7. IL-2 mRNA expressed in piperlactam S treated T cells detected by Northern blot analysis. T cells (5×10^6) were activated with PHA (5 μg/ml) in the presence or absence of piperlactam S for 3 days. Then total cellular RNA was isolated from the cells and analyzed in 6.66% formaldehyde agarose gel and hybridized with ^{32}P-labeled IL-2 (A) or β-actin cDNA (B). Lane 1 indicates the RNA extracted from PHA-activated T cells. Lanes 2 and 3 indicate the RNA extracted from activated T cells treated with 6.25 μM and 25 μM piperlactam S, respectively. Each IL-2-exposed band was quantified using laser-scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA). The ratio of IL-2 mRNAs to β-actin mRNA was calculated. Each bar is the mean of three independent experiments (C).

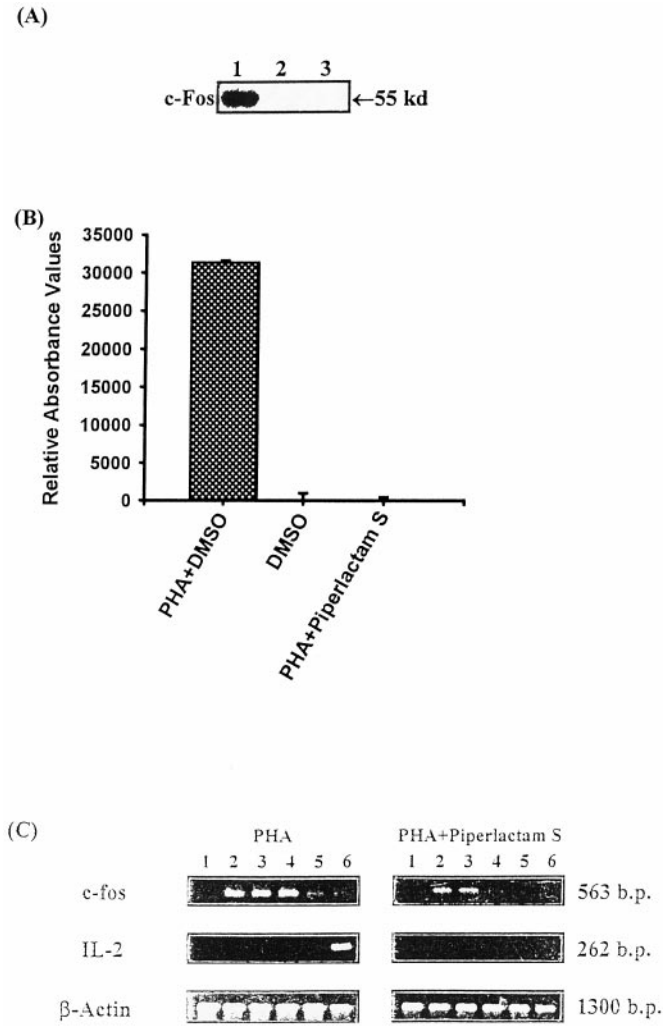


Fig. 8. c-Fos protein levels are decreased by piperlactam S after a 30-min stimulation with PHA and kinetic studies of c-fos and IL-2 mRNA expression in activated T cells. T cells (5×10^6) were stimulated with PHA (5 μg/ml) in the presence or absence of 25 μM piperlactam S for 30 min. Lysates (20 μg of protein) were run on a 10% SDS-polyacrylamide gel and analyzed by immunoblotting with anti-c-Fos antibody. Lane 1, stimulated cells; lane 2, unstimulated cells; lane 3, stimulated cells in the presence of piperlactam S (25 μM) (A). Bar graph represents the relative absorbance values of the c-Fos protein signal. These results are representative of three separate experiments (B). In kinetic studies of c-fos mRNA and IL-2 mRNA expression, T cells were activated with PHA (5 μg/ml) or 25 μM piperlactam S were added 2 h after activation. Then total cellular RNA was isolated from T cells at 2 min (lane 1), 30 min (lane 2), 2 h (lane 3), 4 h (lane 4), 6 h (lane 5), and 8 h (lane 6) after activation. The RT-PCR was done as described under *Materials and Methods*. After the reaction, the amplified product was taken out of the tubes and run on 2% agarose gel (C).

cytokines. However, these actions are similar to those of cyclosporin A, which induces arrest early in G_1 of the T cell cycle by inhibiting IL-2 transcription (Schreiber and Crabtree, 1992). In addition, many previous studies showed that c-Fos combined with c-Jun proteins and the activator protein-1 complex is an important regulatory factor for IL-2 mRNA transcription in T lymphocytes (Charles et al., 1997). Western blot analysis indicated that piperlactam S decreased c-Fos protein levels in T cells activated with PHA. The kinetic studies indicated that decreased expression of c-fos mRNA preceded a change in IL-2 mRNA. We suggest that impairment of IL-2 mRNA transcription in activated T cells is related to the inhibitory effect of piperlactam S on c-Fos protein synthesis.

From the present results, we hypothesize that inhibitory mechanisms of piperlactam S on PHA-activated T cell proliferation, at least in part, are related to: 1) piperlactam S changing the c-Fos protein levels and affecting cytokine mRNA levels in the cells; 2) cytokine production decrease as the entry into S phase of the cell cycle induced by PHA was blocked; and 3) inhibition of entry into the S phase of the cell cycle causing the antiproliferative effect of piperlactam S on T cells. It is believed that arthritis, asthma, cough, and rheumatism are related to overexpression of T cell-mediated inflammatory responses (Corrigan and Kay, 1992; Goodman et al., 1996). Thus, results of the present study indicate that piperlactam S included in *P. kadsura* may also have acted to reduce tissue inflammation, in part by inhibiting T lymphocyte proliferation and cytokine gene expression. Piperlactam S may be an immunosuppressive agent and a growth modulator for T cells. Our observation correlated with *P. kadsura* putative pharmacological activities. Although in vitro studies do not necessarily predict in vivo outcomes, such studies have provided insights into molecular targets, as illustrated by the effects of piperlactam S on cytokine genes and c-Fos proteins. The relative contributions of these activities to the potent immunosuppressive by piperlactam S in vivo remain to be elucidated.

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