Effect of Karasurin-A on Nitric Oxide Production by Murine Macrophages and Mitogenic Response of Murine Splenocytes *in Vitro*

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We studied the effect of karasurin-A, a type-I ribosome-inactivating protein (RIP) isolated from fresh root tubers of *Trichosanthes kirilowii* MAX. var. *japonica* KITAMURA, on the mitogenic response of murine splenocytes and nitric oxide (NO) production by murine peritoneal macrophages *in vitro*. Karasurin-A inhibited the lymphocyte proliferation by LPS, ConA and PHA and NO production by LPS at non-cytotoxic concentrations (10–1000 ng/ml for splenocytes and 10–100 ng/ml for macrophages, respectively). These data suggest that karasurin-A has immunosuppressive activity *in vitro*.

Key words karasurin-A; immunosuppressive; mitogen response; NO production

Karasurin-A, which was isolated from fresh root tubers of *Trichosanthes kirilowii* MAX. var. *japonica* KITAMURA (Cucurbitaceae), is a highly basic protein and its complete amino acid sequence has been determined.¹⁾ Karasurin-A shows a high degree of homology with trichosanthin (from the root tubers of *Trichosanthes kirilowii* MAX.,^{2–7)} trichokirin (from the seeds of *Trichosanthes kirilowii* MAX.)⁸⁾ and α - and β -momorcharin (from the seeds of *Momordica charantia*),^{9,10)} which are abortifacient or ribosome-inactivating proteins from other plants of the Cucurbitaceae family.

Leung *et al.* have reported the immunosuppressive activity of α - and β -momorcharin on the mitogenic responses of murine splenocytes to concanavalin A, phytohaemagglutinin and lipopolysaccharide at non-cytotoxic concentrations.¹¹⁾ Yeung *et al.* also isolated an immunosuppressive protein from the root tubers of *Trichosanthes kirilowii* MAX. and reported that the protein was potent inducer of mid-term abortion in mice.¹²⁾ These findings suggested that karasurin-A could have an immunosuppressive activity. In this paper, we studied the immunosuppressive activity of karasurin-A on the mitogenic response of splenocytes and the function of macrophages in mice *in vitro*.

MATERIALS AND METHODS

Chemicals Karasurin-A was isolated and purified from fresh root tubers of *Trichosanthes kirilowii* MAX. var. *japonica* KITAMURA (Cucurbitaceae) as described previously.¹⁾ RPMI-1640 and fetal calf serum (FCS) were purchased from Irvine Scientific Co. (Santa Ana, CA, U.S.A.). Lipopolysaccharide (*Escherichia coli* 0111:B4) was from Difco Laboratories (Detroit, MI, U.S.A.). Concanavalin A and phytohemagglutinin were purchased from Seikagaku Co. (Tokyo, Japan). All other chemicals used were of special grade.

Animals Male ICR mice, 5 weeks old, were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). The animals were housed in standard plastic cages in an air-conditioned room (24 °C) and given commercial diet (CE-2, Kureha Co., Tokyo, Japan) and water *ad*

libitum. The animals were kept under these conditions for at least 7 d after their arrival.

Preparation of Spleen Cell Suspensions Spleen cells were prepared by mincing and tapping the spleen fragments on a stainless 200-mesh in cold RPMI-1640 medium. Erythrocytes were depleted by treatment with 0.83% ammonium chloride-Tris buffer. Cells were washed with RPMI-1640 medium twice, and then suspended in RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, 5% FCS, penicillin 100 U/ml and streptomycin 100 μ g/ml. Cells were cultured at a density of 5×10^6 cells/ml in 0.1 ml culture medium in 96-well cluster plates (Falcon Plastics, NJ, U.S.A.) at 37 °C for 48 h, with or without test samples, under 5% CO₂-95% air.

Mitogenic Activity Proliferation of cells was measured by MTT [3-(4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide, Sigma, MO, U.S.A.)] assay. At 6 h prior to culture termination, 10 μ l MTT solution (10 mg/ml in phosphate buffered-saline) was added to each well and cells were continuously cultured until culture termination. Culture was stopped by addition of 10% SDS (sodium dodecyl sulfate) solution into each well and then the optical density at 577 nm was measured by a Model 450 Micro Plate Reader (Bio-Rad, CA, U.S.A.).

Determination of Nitric Oxide (NO) Production by Murine Peritoneal Macrophages Murine peritoneal macrophages were obtained from ICR mice by lavage with 8.0 ml cold RPMI-1640, four days after i.p. injection of 2.0 ml 3.0% thioglycollate broth (Wako Junyaku, Osaka, Japan). Cells were seeded in 96-well cluster plates (Falcon Plastics, NJ, U.S.A.) at a density of 2×10^6 cells/ml in RPMI-1640 supplemented with 10% FCS. After 2 h incubation at 37 °C under 5% CO₂-95% air, nonadherent cells were removed by washing with the medium and remaining cells were incubated at 37 °C with medium containing various concentrations of sample or LPS (10 μ g/ml). After 20 h incubation, 100 μ l cell-culture supernatant was added to 100 μ l Griess reagent (0.05% N-1-naphthyl-ethylene diamine-dihydrochloride/0.5% sulfanilamide/2.5% phosphoric acid), and incubated at room temperature for 10 min in a 96-well microplate. Absor-

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bance was read at 577 nm in a Model 450 Microplate Reader (Bio-Rad, CA, U.S.A.). Sodium nitrite was diluted in the medium and used as a standard.

Statistical Analysis Results are given as mean $\pm$ S.E.M.; mean values were compared by the two-tailed Student's *t*-test.

RESULTS AND DISCUSSION

The *in vitro* effect of karasurin-A on proliferation of murine splenocytes to various mitogens is shown in Fig. 1. Karasurin-A reduced the mitogenic responses to LPS, ConA and PHA in a dose dependent manner. Karasurin-A showed cytotoxicity to murine splenocytes only at doses of 10000 ng/ml. Comparing the degree of inhibition of lymphocyte proliferation, it was likely that T lymphocytes were more susceptible to karasurin-A. With respect to NO production by murine intraperitoneal macrophages, karasurin-A reduced NO production in a dose-dependent

manner from 100—10000 ng/ml (Fig. 2). Karasurin-A showed cytotoxicity at much lower doses (over 1000 ng/ml) than in the case of splenic lymphocytes. It has been reported that karasurin-A strongly inhibits the growth of BeWo cells (human choriocarcinoma) and the IC_{50} was about 300 ng/ml.^{1c)} Thus, the immunosuppressive activity of karasurin-A may be due to direct immunocytotoxicity. The mechanism of inhibition of lymphocyte proliferation or reduction of NO production remains obscure. Since karasurin-A is a ribosome-inactivating protein (RIP), inhibition of protein synthesis could be involved in the reduction of lymphoproliferation and NO production. Recently, RIP has been linked to antibodies to form immunotoxins specifically toxic to target cells and the immunotoxins are thought to be potentially useful in the therapy of malignant, parasitic and autoimmune diseases. However, some RIPs are inactivated by linkers used in the preparation of conjugates and, consequently, not all RIPs are equally suitable for the preparation of immu-

Fig. 1. The Effect of Karasurin-A on the Proliferation of Murine Spleen Cells

Each column represents the mean $\pm$ S.E.M. of triplicate determinations from a representative experiment that was replicated three times with similar results. ***p* < 0.01, **p* < 0.05 vs. the control (open columns).

Fig. 2. The Effect of Karasurin-A on NO Production by Murine Peritoneal Macrophages

(A) NO production, (B) cytotoxicity (MTT method). Each column represents the mean $\pm$ S.E.M. of triplicate determinations from a representative experiment that was replicated three times with similar results. ***p* < 0.01; **p* < 0.05 vs. the control.

notoxins. Alternatively, administration of immunotoxins elicits an immune response against both the antibody and the toxic moiety of the immunotoxins. Since many RIPs are immunologically distinct, we need to characterize the immunological properties of karasurin-A.

In this study, we demonstrated that karasurin-A has immunosuppressive activity through the reduction in macrophage function and the inhibition of lymphocyte proliferation which are important steps in the host-defence system. Further immunological studies on karasurin-A *in vivo* are needed.

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