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COMMUNICATIONS TO THE EDITOR

Screening of Interleukin-2 Production Inhibitor with Mouse Thymoma EL4 Cells

Sir:

Human interleukin-2 (IL-2) regulates many essential immune functions, but its overexpression is causally related to malignancy, autoimmune disease and renal allograft rejection[1~3]. In order to find immunosuppressants, isotope-labeled enzyme-linked immunosorbent assay (ELISA) and IL-2 reporter gene assay are used to detect IL-2 production, and cyclosporin A (CsA) and FK506 have been found to be powerful IL-2 inhibitors[4~6]. However, these methods are biohazardous, very costly and nonspecific. Moreover, the long-term treatment (over 24 hours) with test samples is cytotoxic to target T cells.

Therefore, we developed a modified method that minimizes this toxic effect by shortening the duration of sample treatment in mouse thymoma EL4, a cytokine-producing T cell line, and also maximizes the amount of test sample applied to the target cells through introduction of a washing step to increase the likelihood of finding a real inhibitor, which might be present at very low concentrations. When culture broth of actinomycetes was applied to EL4 cells for more than 25 minutes, there was a significant decrease in EL4 cell viability. But the cytotoxic effect was negligible if sample volume of up to 40 μl was applied to the cells for 5 minutes (data not shown). Thus, up to 40 μl of test samples can be applied to 200 μl of EL4 cells without any remarkable cell damage if treated for 5 minutes. After extensively examining our system with CsA and FK506, we used the developed method to screen for inhibitors for IL-2 production from the culture broth of actinomycetes and fungi.

For primary screening, the culture broth was extracted with the same volume of acetone and evaporated *in vacuo*. The concentrated residue was dissolved in an equal volume of 50% aqueous methanol. The exponentially growing EL4 cells (ATCC, Bethesda, MD) were divided into 96-well microplates at a density of 4×10^4 cells in 200 μl per well. Fourty μl of culture extract was then added into each well and incubated for 5 minutes in a humidified CO_2 incubator. The supernatants were then removed by inverting the plate on paper towels. The cells were washed with phosphate-buffered saline (PBS), and then re-fed with fresh RPMI1640 (GifcoBRL, Rockville, MD) medium containing 3 ng/ml of phorbol-12-myristate-13-acetate (PMA) (Pharmacia, Peapack, NJ) for 24 hours. The quantity of IL-2 present was determined using CTLL-2 cells, which require IL-2 for proliferation. Thirty μl of culture supernatant from the EL4 cells was added into another well of a 96-well microplate containing 2×10^4 CTLL-2 cells (ATCC, Bethesda, MD) in a total volume of 100 μl. After 24 hours, the level of cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) method[7]. For standardization purposes, human recombinant IL-2 (GifcoBRL, Rockville, MD) was dose-dependently added to CTLL-2 cells in a separate experiment.

Using this improved assay system, we isolated CsA from an unidentified fungus and piericidin A1 (Fig. 1) from a *Streptomyces* sp. as inhibitors of PMA-induced IL-2 production in EL4 cells. Piericidin compounds, isolated from *Streptomyces*, were reported to have antimicrobial, pesticidal, and insecticidal activity[8,9]. However, to the best of our knowledge, the inhibitory potential of piericidin A1 upon IL-2 production in EL4 cells has not been reported until now. In this study, we report for the first time that piericidin A1 inhibits cytokine production in T cells. To isolate piericidin A1 from a *Streptomyces* sp. MT1882-V (KCTC8800P), we used a series of purification techniques,

Fig. 1. Structures of piericidin A1 and glucopiericidin A.

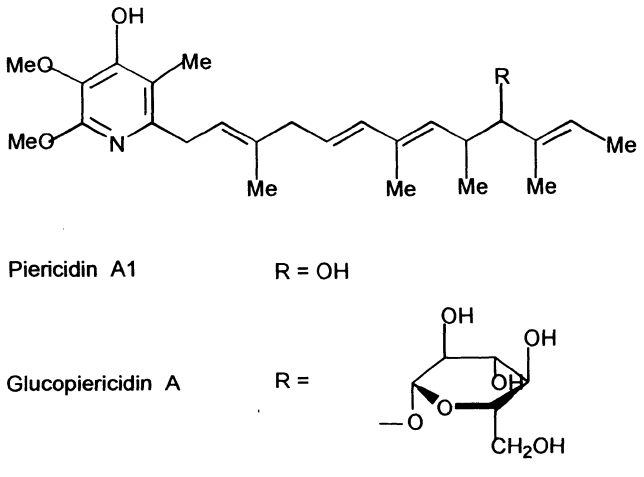
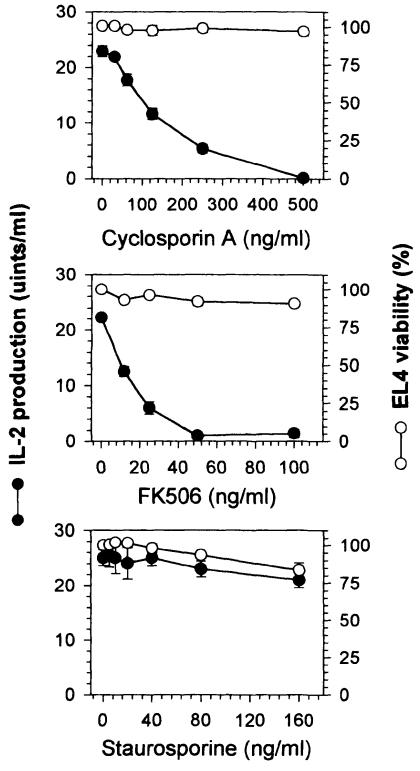
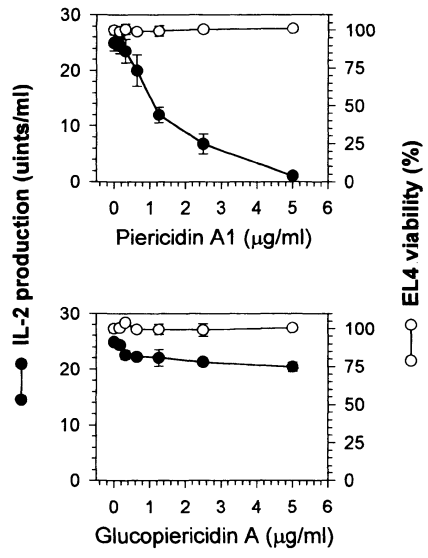


Fig. 2. Effects of cyclosporin A, FK506 and staurosporine on PMA-induced IL-2 production in EL4 cells.



1 unit of IL-2 defines the amount that promotes the growth of CTL-L2 cells of $O.D_{540}$ 1.0 and points show means±S.D. from a triplicate experiment.

Fig. 3. Effects of piericidin A1 and glucopiericidin A on PMA-induced IL-2 production in EL4 cells.



1 unit of IL-2 defines the amount that promotes the growth of CTLL-2 cells of $O.D_{540}$ 1.0 and points show means±S.D. from a triplicate experiment.

which included Amberlite XAD-2, EtOAc extraction, silica gel, Sephadex LH-20 and Lobar RP-18 as described previously[8~10]. Glucopiericidin A, a derivative of piericidin A1, was also purified through similar chromatographic techniques. In each purification step, inhibiting fractions were collected for further purification by TLC on silica gel TLC plates (0.25 mm thickness, Merck Co., Germany) developed with a chloroform : MeOH (9 : 1), and inhibitory activity was tested against PMA-induced IL-2 production in EL4 cells.

To determine the inhibitory pattern of IL-2 production, piericidin A1, glucopiericidin A, staurosporine, CsA, or FK506 was applied to EL4 cells at different concentrations. Fig. 2 shows that FK506 has an IC_{50} of 15 ng/ml for the inhibition of IL-2 production whereas CsA needs a ten times higher concentration (about 150 ng/ml) to produce the same level of inhibition, which is consistent with a report that FK506 is 10 to 100 times more potent than cyclosporin A at inhibiting IL-2 mRNA synthesis in Jurkat cells[11]. However, when EL4 cells were treated with staurosporine, a protein kinase C inhibitor, only cell toxicity was observed without any inhibition of IL-2 production. In Fig. 3, piericidin A1, which was isolated as an IL-2 inhibitor, dose-dependently reduced IL-2 production without causing EL4 cell damage, similar to CsA and FK506 (Fig. 2). Moreover, glucopiericidin A, piericidins derivative, showed no inhibitory activity on IL-2 production in EL4 cells, similar to staurosporine.

The present study shows that even as little as 5 mininutes treatment with test samples could be as efficient as longer treatment in finding inhibitors of IL-2 production from many screening sources, and by using this improved method, it is possible to distinguish between a real inhibitor of IL-2 production and cytotoxic compounds, like staurosporine. Thus, other specific compounds affecting IL-2 production can also be selected using this method and subsequently isolated with ease. Additionally, the introduction of a sample washing step after 5 minutes greatly increases the amount of sample applied to target cells, and thus increases the likelihood of selecting an IL-2 inhibitor. IL-2 gene expression is induced *via* Ca^{2+}-

calmodulin-dependent serine/threonine phosphatase, calcineurin, or ras-mediated pathways. Moreover, CsA or FK506 blocks IL-2 production through the inhibition of calcineurin activity[12,13]. Therefore, whether the modified method we described here can be applied to ras-raf1-1-mediated IL-2 gene expression requires more study. In addition, because EL4 cells are known to express other cytokine genes in addition to IL-2, our assay system may be very useful for the selection of inhibitors of these cytokines.

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