SELECTIVE SUPPRESSION BY PRODIGIOSIN OF THE MITOGENIC RESPONSE OF MURINE SPLENOCYTES

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In the course of screening for immunomodulating substances among microbial metabolites using a triple mitogen assay system, we detected an immunosuppressive activity which is similar to cyclosporin A. Two active components were isolated as red pigments from the fermentation broth of *Streptomyces hiroshimensis* and they were identified with prodigiosin 25-C and metacycloprodigiosin respectively. These compounds inhibited T lymphocyte proliferation which was induced by plant lectins, concanavalin A (Con A) and phytohemagglutinin (PHA), much more extensively than B lymphocyte proliferation which was induced by lipopolysaccharide (LPS). Prodigiosin 25-C completely inhibited induction of cytotoxic T cells in a mixed lymphocyte reaction (MLR) at 4 ng/ml.

Several low molecular weight immunomodifiers have been discovered by various kinds of screening method from culture broth of microorganisms. Most of them were detected by the screening for enzyme inhibitors,[1~3] antifungal agents[4] or anti-tumor antibiotics.[5] A few groups have searched for immunoactive microbial metabolites with more direct systems.[6] In an accompanied paper,[7] we reported a novel method of screening for immunomodulating substances using three mitogens which are specific for T or B lymphocytes.

In the course of our screening with the system it was found that prodigiosin 25-C and metacycloprodigiosin were potent inhibitors of T lymphocyte proliferation which was induced by Con A and PHA, but less suppressive against B lymphocyte proliferation which was induced by LPS. Furthermore, cytotoxic T lymphocyte induction in MLR was inhibited by prodigiosin 25-C.

Materials and Methods

Mice

Specific pathogen free BALB/c and C57BL/6 mice (male 6~8 weeks old) were obtained from Charles River Japan Co., Ltd., Tokyo.

Mitogens

Concanavalin A (Con A, C2010 highly purified, Sigma Chemical Co., St. Louis, MO, U.S.A.), phytohemagglutinin (PHA, HA16 purified, Wellcome Research Laboratories, Beckenham, England) and lipopolysaccharide (LPS, *Salmonella enteritidis*, Difco Laboratories, Detroit, MI, U.S.A.) were used throughout this work.

Preparation of Murine Splenocytes

Normal male BALB/c mice were sacrificed and the spleen was teased with a plastic syringe in a Petri dish containing 5 ml of Eagle's minimum essential medium (MEM, Nissui Seiyaku, Tokyo). The product was gently strained through a 100-mesh screen to remove clumps and prepare a single cell suspension. These cells were washed three times with MEM and resuspended in RPMI-1640 medium (Nissui Seiyaku) supplemented with 10% fetal calf serum (FCS, Flow Laboratories, Rockville, U.S.A.).

Determination of Lymphocyte Proliferation *In Vitro*

Murine splenocytes were cultured in RPMI-1640 medium supplemented with 10% FCS, at a concentration of 5×10^5 cells/ml (1×10^5 cells/well) with one of the mitogens, Con A, PHA or LPS, and a test sample for 3 days at 37°C in a humidified atmosphere with 5% CO_2. At the end of the culture period they were pulsed with [*methyl*-1′,2′-^{3}H]thymidine (113 Ci/mmol, NEN, Boston, MA, U.S.A.) (0.2 μCi/well) for 5 hours and harvested using a cell harvester (Labo Mash, Labo Science Co., Tokyo). The amount of [^{3}H]thymidine incorporated by the cells was determined by a liquid scintillation counter.

Mixed Lymphocyte Reaction (MLR) and Determination of Killer Activity

Mixed lymphocyte cultures were established by adding 4×10^5 responding lymphocytes in a 0.1 ml-volume to an equal volume of 4×10^5 stimulator cells in flat-bottomed microtiter plates wells. Responding lymphocytes were splenocytes of C57BL/6 mice immunized with spleen cells of BALB/c mice (5×10^6, ip) 2 weeks before. Stimulator cells were splenocytes of BALB/c treated with 50 μg/ml mitomycin C at 37°C for 45 minutes, washed with MEM medium 3 times and resuspended in a culture medium (RPMI-1640 supplemented with 10% FCS). After 5 days of incubation, 1×10^4 ^{51}Cr-labeled target cells, P815, were added to each well and the cultures were further incubated for 4 hours. At the end of the incubation period, 100 μl of the supernatant were removed and the radioactivity was assessed. The spontaneous release was determined by incubating ^{51}Cr labeled P815 cells alone and the maximum release was determined incubating ^{51}Cr-labeled P815 cells with 0.5% sodium dodecyl sulfate. The following formula was used to compute percent lysis.

$$\% \text{ Lysis} = \frac{(\text{Test cpm}) - (\text{Spontaneous cpm})}{(\text{Maximum cpm}) - (\text{Spontaneous cpm})} \times 100$$

Results

Isolation and Identification of Active Substances

In the primary screening system described previously, an methanol extract of the lyophilized culture broth of *Streptomyces hiroshimensis* markedly suppressed T lymphocyte proliferation which was induced by Con A and PHA, leaving B lymphocyte activation by LPS relatively unaffected. The activity was similar to cyclosporin A,[7] an immunosuppressive antibiotic, which is clinically applied in case of tissue or organ transplantation.

A stock culture of *S. hiroshimensis* was inoculated into 100 ml of the seed medium in a 500-ml flask. The medium consisted of glucose 20 g, glycerol 20 g, soybean meal 20 g, L-methionine 1 g, KH_2PO_4 1 g, $MgSO_4\cdot7H_2O$ 0.5 g and $CaCO_3$ 10 g per liter of water (pH 6.5). The seed culture was incubated at 30°C for 2 days on a reciprocal shaker. Twenty ml of the seed culture was added to 1 liter of the same medium in a 5-liters flask. They were incubated at 30°C for 6 days on a rotary shaker.

A flow diagram for isolation of active components is illustrated in Fig. 1. The fermentation broth was filtered and the activity was found in the filter cake. The mycelium was extracted with methanol and ethyl acetate. The extracts were pooled and concentrated *in vacuo*. The concentrate was extracted with chloroform and the chloroform layer was concentrated to a small volume *in vacuo*. The resulting oily residue was chromatographed on silica gel. The active fraction was eluted with hexane - acetone (85: 15) and was concentrated *in vacuo*. The residue was a mixture of active substances and it was further fractionated by a silica gel column chromatography using solvent mixtures of chloroform - acetone containing NH_4OH. Two reddish pigments, compounds **A** and **B**, were isolated. Both of them were pH indicators.

Fig. 1. A flow diagram for the isolation of active compounds from culture broth of *S. hiroshimensis*.

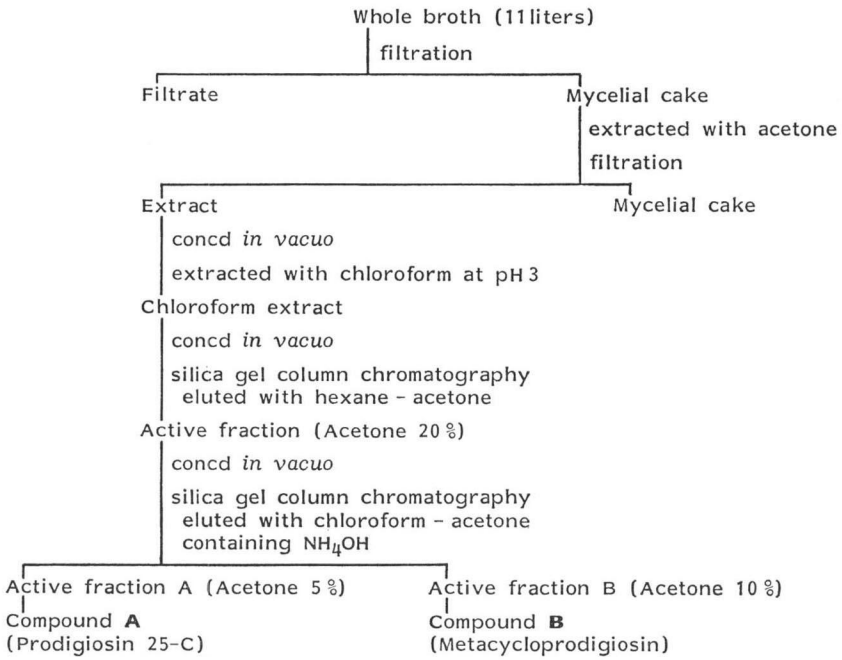
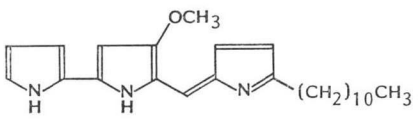
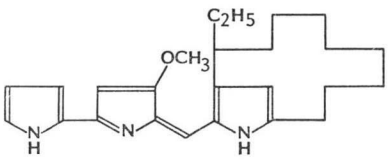


Fig. 2. Structure of prodigiosin 25-C (**A**) and metacycloprodigiosin (**B**).



Prodigiosin 25-C (**A**)

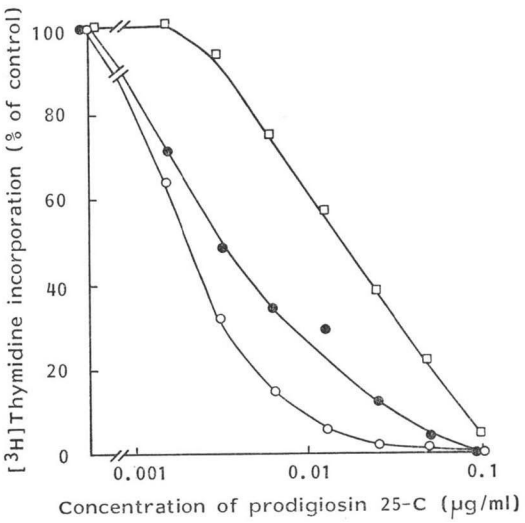


Metacycloprodigiosin (**B**)

Fig. 3. Differential suppression of mitogenic responses of mouse spleen cells by prodigiosin 25-C.

The mouse spleen cells were cultured with 1 μg/ml Con A or PHA or 4 μg/ml LPS in the presence of indicated amount of prodigiosin 25-C for 3 days. The cells were allowed to incorporate [^{3}H]thymidine for 5 hours before harvest.

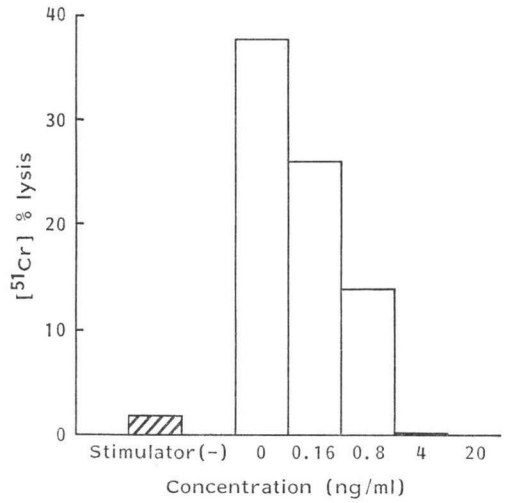
□ LPS, ● PHA, ○ Con A.



Maximum absorptions in the visible spectrum of compound A were at 529 nm in acid methanol and 460 nm in alkaline methanol, respectively. In the mass spectrum of compound A the strong peaks were shown at *m/z* 91, 252 and 393. These data determined for compound A corresponded to those reported for prodigiosin 25-C.[8,9] NMR and IR spectra of compound A were identical with those of prodigiosin 25-C. Com-

Fig. 4. Suppression of cytotoxic T cell induction in MLR by prodigiosin 25-C.

C57BL/6 splenocytes and mitomycin C-treated BALB/c splenocytes were co-cultured in the presence of indicated amount of prodigiosin 25-C for 5 days. Four hours before the end of the culture, ^{51}Cr-labeled target cells (P815) were added and the radioactivity released in the culture medium was counted.



pound **B** which was similar to compound **A** in most physico-chemical properties was identified with metacycloprodigiosin.[10]

Suppression of T Lymphocyte Proliferation by Prodigiosin

Fig. 3 shows the relationship between the dose of prodigiosin 25-C and the proliferative responses of splenocytes induced by mitogens. The addition of prodigiosin 25-C resulted in a dose dependent inhibition of the responses. Con A induced incorporation of [^{3}H]thymidine was most sensitive to the antibiotic. But the results were similar to that shown in the case of PHA. The cell proliferation induced by LPS was significantly less sensitive than the other two. LPS is a B lymphocyte specific mitogen in the murine system and two plant lectins, Con A and PHA, stimulate only T lymphocytes.[11] Therefore the present results suggest that prodigiosin 25-C differentiates T from B lymphocyte proliferation.

The effects of metacycloprodigiosin were similar to those of prodigiosin 25-C but its suppressive activity was a little less potent.

Suppression of MLR Killer Induction by Prodigiosin 25-C

The target cell, P815, expresses H-2^d antigen on its surface. Therefore C57BL/6 cytotoxic T cells stimulated by the presense of mitomycin C treated BALB/c (H-2^d) splenocytes kill the P815 cells and make them release ^{51}Cr into the culture medium. In the absence of prodigiosin 25-C in the culture, lysis of the target cells was 38%. However, the killer induction was partially inhibited by very low concentration of prodigiosin 25-C (0.16 ng/ml), and it was completely suppressed at 4 ng/ml of the compound (Fig. 4). Since viability of the splenocytes was comparable when assayed by the trypan blue dye exclusion test after 5 days treatment with or without 4 ng/ml of prodigiosin 25-C, the suppression was not resulted from general cytotoxicity of the antibiotic. Thus, prodigiosin 25-C is a potent inhibitor of cytotoxic T cell induction.

Discussion

The inhibitory profile of prodigiosin 25-C was similar to that of cyclosporin A.[7] Cyclosporin A has been reported to be preferentially active against T lymphocytes[12] and to suppress Con A and PHA induced cell activation more extensively than LPS induced cell activation.[7] However, these two compounds seem to have different mechanisms of action on T cell proliferation. Because, suppression by prodigiosin 25-C of Con A induced T cell proliferation was more significant at optimal concentration of Con A (2~3 μg/ml) than at sub-optimal concentration (0.3~1 μg/ml) (data not shown), whereas cyclosporin A inhibited Con A response more extensively at the lower Con A concentration. This

tendency is specific for Con A and is not detectable in the case of PHA and LPS responses.

Prodigiosin 25-C completely inhibited cytotoxic T cell induction in MLR at low concentration (4 ng/ml). When added in the assay period, prodigiosin 25-C did not inhibit the expression of killer activity at this concentration (data not shown). Therefore, prodigiosin did not affect killer function directly. The result suggest that prodigiosin 25-C inhibits some step of metabolic pathway or cell to cell interactions required for the induction of cytotoxic T cells. The effect of metacycloprodigiosin was similar to prodigiosin 25-C.

Thus, these two pigments, prodigiosin 25-C and metacycloprodigiosin, are expected to be applied for the research of the immune responses including T cell activation.

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