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Capsicum ethanol extracts and capsaicin enhance interleukin-2 and interferon-gamma production in cultured murine Peyer's patch cells ex vivo

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Abstract

We investigated the effects of red pepper (*Capsicum annuum* Lin.) extracts (capsicum extract) and its main pungent capsaicin on T helper 1 (Th1) and 2 (Th2) cytokine production in cultured murine Peyer's patch (PP) cells in vitro and ex vivo. Direct administration of capsicum extract (1 and 10 μg/ml) and capsaicin (3 and 30 μM) resulted in suppression of interleukin (IL)-2, interferon (IFN)-γ, IL-4 and IL-5 production. In an ex vivo experiment using PP cells removed from the mice after oral administration of capsicum extract (10 mg/kg/day for 4 consecutive days), IL-2, IFN-γ and IL-5 increased in response to concanavalin A (Con A). Oral administration of 3 mg/kg/day capsaicin, one active constituent of the extract, also enhanced IL-2, INF-γ and IL-4 production in response to Con A stimulation but did not influence the production of IL-5. Orally administered capsazepine (3 mg/kg/day), a selective transient receptor potential vanilloid 1 (TRPV1) antagonist, slightly enhanced IL-2 production also irrespective of Con A stimulation. The capsaicin-induced enhancement of both IL-2 and IFN-γ production was not reduced by oral administration of capsazepine (3 mg/kg/day), suggesting a TRPV1 receptor-independent mechanism. Flow cytometric analysis revealed that the population of $CD3^+$ cells in the PP cells was significantly reduced while $CD19^+$ cells increased after oral administration of capsicum extract (1 and 10 mg/kg/day) and capsaicin (0.3 and 3 mg/kg/day). Capsazepine (3 mg/kg/day) weakly but significantly reversed these effects. Orally administered capsicum extract and capsaicin did not change the T cell subset ($CD4^+$ and $CD8^+$), Th1 (IFN-γ^+) and T2 (IL-4^+) ratio. These findings indicate that capsicum extract and capsaicin modulate T cell-immune responses, and their immunomodulatory effects on murine PP cells are partly due to both

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TRPV1-dependent and -independent pathway.

Keywords: Capsicum; capsaicin; Peyer's patch; Th1/Th2; cytokines; TRPV1

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Introduction

The inner surface of the intestinal tract possesses a large area of mucosal membranes, which are continuously exposed to various substances in the intestinal lumen (Mowat et al., 2003). Gut-associated lymphoid tissues exist on the intestinal mucosal site and play an important role in the immune system. Peyer's patches (PP) are considered to be lymphoid tissues where mucosal immune responses such as local IgA production and systemic immunological responses are induced (Mowat et al., 2003). Antigen presentation in PP is important in determining systemic immune responses including T and B cell-dependent immunity (Mowat et al., 2003; Yoshida et al., 2002). Orally administrated hot water extract of cultured mycelia of *Cordyceps sinensis* (Berk.) Sacc was previously shown to increase interleukin (IL)-6 and granulocyte-colony stimulating factor (GM-CSF) production by PP cells in mice (Koh et al., 2002). Juzen-taiho-to, a Kampo prescription, was also shownto enhance production of these cytokines in PP cells from C3H/HeJ mice (Hong et al., 1998). Moreover, we recently reported that culture filtrate of the medicinal entomogenous fungi *Paecilomyces tenuipes* (Peck) Samson (= *Isaria japonica* Yasuda or *Isaria tenuipes*) selectively enhanced T helper 1 cytokine production in cultured murine PP cells from C57BL/6J mice (Takano et al., 2005), and suggested that oral administration of the culture filtrate might increase immune responses (Takano et al., 1996), in part due to enhancement of these cytokines. Therefore, to elucidate the mechanisms of oral immune responses, examination of the cytokine modulating activities in cultured PP cells will be beneficial.

Capsicum annuum (Solanaceae) is used worldwide not only as a food, due to its pungency, but

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also in traditional medicine against gastric ulcers, rheumatism, alopecia and toothache (Szallasi and Blumberg, 1999). Capsaicin is the most well-known and pungent active ingredient of Capsicum. Large numbers of studies have established that capsaicin shows various pharmacological effects and is endowed with a pleiotropic pattern of biological activities, some of which are mediated by the activation of cellular targets different from vanilloid receptor 1 (Szallasi and Blumberg, 1999). Capsaicin has also been shown to have immunomodulatory effects, as indicated by its ability to modulate lymphocyte proliferation and immunoglobulin A, E and G production (Nilsson et al., 1991, Eglezos et al., 1990), regulate the expression of substance P and its receptor in monocytes (Ho et al., 1997), and inhibit cytosolic Ca^{2+} mobilization induced by platelet activating factor in the monocyte cell lines HL-60 (Choi et al., 2000). However, the systemic T-cell-dependent immune response after oral administration of capsaicin remains to be clarified.

In this study, we therefore examine the effects of ethanolic extracts of *C. annum*, capsaicin and a vanilloid receptor-specific antagonist, capsazepine, on the production of T-helper cytokines in cultured PP cells in vitro and ex vivo.

Materials and Methods

Animals

Male C57BL/6J mice, 6 to 10 weeks of age, were purchased from Japan SLC (Shizuoka, Japan). Mice were housed in groups of five in plastic cages with a 12 h light: 12 h dark cycle and free access to water and food ad libitum. Adaptation to these conditions for at least 1 week was allowed

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before commencing the experiment. The experimental procedures complied with the guidelines of the Council for Experimental Animals, the Faculty of Pharmaceutical Sciences, Kanazawa University, Japan. Mice were sacrificed by anesthetization with an overdose of ether.

Materials

RPMI-1640 medium, phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Invitrogen Corp. (Carlsbad, CA USA). Concanavalin A (Con A) (type IV), type I collagenase, capsaicin and capsazepine were obtained from Sigma Chem. Co. (St. Louis, MO). All other reagents were purchased from Wako Pure Chemical Co. (Tokyo, Japan). For analysis of the T, B and T cell subset ($CD4^+$ and $CD8^+$) in PP cells, the following monoclonal antibodies (mAb, Beckman Coulter Inc., Hialeah, FL) were used: anti-CD45RA-fluorescein isothiocyanate (FITC) antibodies (RA3-6B2, IgG2a), anti-CD3-FITC antibodies (KT3, IgG2a), anti-CD4-FITC antibodies (YTS191.1, IgG2b), anti-CD8-phycoerythin (PE) antibodies (KT15, IgG2a), anti-IFN-γ-FITC antibodies (XMG1.2, IgG1) and anti-IL-4-FITC antibodies (BVD-24G2, IgG1). The isotype-matched controls used in this experiment were IgG1 conjugated to FITC, IgG2a conjugated to PE, IgG2a conjugated to FITC and IgG2b conjugated to FITC.

Extraction and isolation

Capsicum annum L. for medicinal use was purchased from Uchida Wakanyaku Co. Ltd. (Tokyo, Japan). A voucher specimen of this plant (C05205) was deposited in our laboratory at the Faculty of

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Pharmaceutical Sciences, Kanazawa University, Japan.

The dried fruits (1.0 kg) of *C. annum* were macerated in 95% ethanol (EtOH) (1 L, 3 times) at room temperature for 24 h. After filtration, the ethanol solution was evaporated under reduced pressure to give ethanol extract (capsicum extract) (26 g). Capsicum extract (10 g) was then subjected to column chromatography on silica gel (500 g). Elution with a mixture of hexane-ethylacetate (AcOEt) and methanol (MeOH) gave 14 fractions: Fr-1 (5% AcOEt, 1200 ml, 698 mg), Fr-2, (25% AcOEt, 300 ml, 675 mg), Fr-3 (25% AcOEt, 300 ml, 120 mg), Fr-4 (25% AcOEt, 300 ml, 46 mg), Fr-5 (25% AcOEt, 300 ml, 54 mg), Fr-6 (35% AcOEt, 1200 ml, 458 mg), Fr-7 (50% AcOEt, 600 ml, 78 mg), Fr-8 (50% AcOEt, 600 ml, 210 mg), Fr-9 (65% AcOEt, 1200 ml, 1104 mg), Fr-10 (75% AcOEt, 600 ml, 119 mg), Fr-11 (75% AcOEt, 600 ml, 80 mg), Fr-12 (90% AcOEt, 600 ml, 141 mg), Fr-13 (100% AcOEt, 1200 ml, 2844 mg), and Fr-14 (100% MeOH, 1200 ml, 4319 mg). Fr-2 (10 mg) eluted with 25% AcOEt was determined as being composed mainly of carotenoids and was further subjected to high performance liquid chromatography (HPLC; ODS, acetone-H_2O, 90:10) to give the main constituent, β-carotene (3.7 mg, Fig. 1). Fr-9 (10 mg) eluted with 65% AcOEt was determined as being composed mainly of capsaicinoids and was also subjected to HPLC (silica gel, hexane-acetone, 65:35) to give capsaicin (4.1 mg, Fig. 1). The structures of the carotenoids and capsaicinoids were determined by comparing their spectral data with those reported in the literature (Maillard et al., 1997; Mercadante et al., 1999). For ex vivo experiments, capsaicin was purchased from Sigma Chem. Co. (St. Louis, MO). The purity of the capsaicin was checked and further purified by HPLC (acetone-H_2O, 90:10) before use.

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Peyer's patch (PP) cell preparation

C57BL/6J mice were sacrificed with an overdose of ether and their small intestines were placed into a petri dish filled with PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml) on ice (Manhart, 2001). Visible PPs were carefully dissected out from the wall of the small intestines using micro scissors under a microscope (10 Peyer's patches were obtained from each mouse), and were placed in ice-cold complete RPMI-1640 medium containing 5% FBS, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. To obtain a single PP cell suspension, the PPs were digested with type 1 collagenase (70 U/ml) dissolved in the same medium and incubated for 60 min at 37 ^{o}C. After filtration through a 200 μm nylon mesh (Becton Dickinson, Oxnard, CA, USA), the PP cells were washed three times with complete medium. Cell viability was assessed by trypan blue exclusion. Morphological analysis by characteristic non-specific esterase and Giemsa staining revealed that more than 97% of the cells were lymphoids and less than 1% were monocytes. PP cells (3×10^6 cells/ml) suspended in complete medium were seeded in a 24-well tissue culture plate (Becton Dickinson) and cultured with or without 5 μg/ml Concanavalin A (Con A).(Takano et al., 2004)

Test sample treatment

Capsicum extracts, fractions, capsaicin and capsazepine were suspended in medium containing 0.3% EtOH solution. For in vitro assay, various concentrations of these test samples (20 μl/well) were

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added with or without Con A. For ex vivo assay, capsicum extract (1, 3 and 10 mg/kg/day) or fractions (3 mg/kg/day) suspended in 10% EtOH and 50 μl/20g body weight solution was orally injected into mice once a day for four consecutive days. Capsaicin (0.3 and 3 mg/kg/day) or capsazepine (3 mg/kg/day) (Costa et al., 2004) also suspended in 10% EtOH and 50 μl/20g body weight solution was orally injected into mice once a day for four consecutive days. In other case, mice were injected orally with 3 mg/kg/day capsaicin simultaneously with 3 mg/kg/day capsazepine in the same regimen. Control groups received a vehicle (10% EtOH) instead of test samples. In this experiment, capsaicin dosage was estimated by capsaicinoids concentration in the effective doses of both capsicum extract (1 to 2% (w/w) of the extract) (10 mg/kg) and Fr-9 (3 mg/kg) (49 to 51% of the Fr-9) ex vivo.

Cytokine evaluation

To measure cytokine production in cultured PP cells, culture supernatants were collected at 72 h and stored at -80 ^{o}C until use. The levels of IL-2, IL-4, IL-5 and INF-γ in the supernatants were measured by enzyme linked immunosorbent assay (ELISA) using commercial kits (Cytoscreen^{TM}, BIOSOURCE, Camarillo, CA, USA) according to the manufacturer's instructions.

PP cell viability

To assess the correlation between PP cell cytotoxicity and suppression of cytokine production by the test samples, PP cells were seeded on a 24-well tissue culture plate (1.5×10^6 cells/0.5 ml/well)

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and cultured with or without 5 μg/ml Con A for 72 h. The supernatants were then removed and remaining PP cells were added to fresh medium containing MTT solution (50 μl of 5 mg/ml in PBS). The mixture was incubated for 5 h at 37 °C then the reduced formazan was solubilized with 0.5 ml of detergent (10% SDS in 0.05N HCl) for 18 h. Absorbance of the MTT-formazan solution at 590 nm was read using an immunoreader. Cell viability data were recorded as the absorbance ratio (percentage) of the test sample-treated PP cells to non-treated PP cells (Mosmann, 1983). In the ex vivo assay, PP cell viability of treated mice was measured by trypane blue staining (0.4% in PBS).

Flow cytometry

PPs (10 /mouse) were collected after 6-days oral injection of extracts, fractions, capsaicin or capsazepine, and dissociated into a single-cell suspension by filtrating through a 200 μm nylon mesh. Erythrocytes in the PP cell suspensions were lysed in 0.75% ammonium chloride buffer (pH 7.6). The resultant cells were counted and resuspended in PBS. Cells (1×10^6) were stained with fluorochrome directly conjugated with mAb (Macey, 1999): anti-CD3-FITC, anti-CD45RA-FITC, anti-CD4-FITC/anti-CD8-PE, anti-IFN-γ-FITC and anti-IL-4-FITC or isotype-matched controls. After 60 min incubation at 4 °C, the cells were washed and resuspended in PBS containing 1 μg/ml of propidium iodide. The lymphocyte population in the PP cells was analyzed using a flow cytometer (FACScan™, Becton Dickinson, San Jose, CA, USA) as described below. Gates were set by forward and side scatter to delineate leukocytes and exclude dead cells; death was confirmed by propidium iodide staining (Takano et al., 2004).

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Data analysis for flow cytometry

Data were acquired using Cell Quest TM software (Becton Dickinson). Between 10,000 and 20,000 events were acquired per sample. All data were representative plots derived from a minimum of three independent experiments in which at least three experimental and three or four control mice were analyzed. Mean FITC- and FTIC-/PE-fluorescence intensities were calculated from fluorescence histograms for the gated population.

Statistical analysis

The mean cytokine production and ratio of lymphocyte or T lymphocyte subpopulations were considered as a single data point for analysis of results from at least three or four independent experiments. All data are expressed as the mean ± SE. Statistical significance was determined by Dunnett's multiple test after one-way analysis of variance (ANOVA) with comparison to a control group, and the differences were considered significant if $p<0.05$.

Results

The murine intestine is a highly regulated immune system producing large amounts of regulatory cytokines, with immune responses dominated by Th2 type cytokines rather than Th1 cytokines (Xu-Amano, 1993). IL-2 and IFN-γ are the major Th1 cytokines involved in cellular immune activation by professional antigen presenting cells such as dendritic cells and monocytes

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(Romagnani, 2004; Sood, 2005).

We previously reported IL-2, IL-4, IL-5 and IFN-γ production in PP cells upon non-specific T-cell stimulation with Con A (Takano et al., 2004); these cytokines were produced 12 h after Con A (5 μg/ml) challenge, increased over 72 h and slightly decreased at 96 h. No increment of these cytokines was observed when the PP cells were cultured without Con A. Based on these data, the present study examined the effects of capsicum extract and capsaicin on PP cell cytokine production 72 h after Con A challenge both in vitro and ex vivo.

PP cells isolated from C57BL/6J mice produced a large amount of IL-2, IL-4, IL-5 and IFN-γ 72 h after Con A stimulation (Figs. 2A, B, C and D). As shown in Fig. 2, capsicum extract and capsaicin strongly inhibited Con A-induced cytokine productions in a concentration-dependent manner. Capsicum extract significantly suppressed four different kinds of T helper cytokines by >90% at a concentration of 10 μg/ml (Fig. 2). Capsaicin, one of the main bitter constituents, completely reduced these cytokines at a concentration of 3 μM (Fig. 2). To examine the PP cell viability, cells were treated with test samples at the same concentrations. A significant cytotoxic effect against the PP cells irrespective of Con A stimulation was observed even at concentrations of 10 μg/ml (for extract) and 3 μM (for capsaicin) (Table 1), which significantly suppressed T helper cytokine production (Fig. 2).

Since capsicum extract and capsaicin suppressed production of T helper cytokines in the cultured PP cells in vitro, we next examined the ex vivo effect of these samples on cytokine production. Capsicum extract was injected into mice once a day for 4 consecutive days then 2 days after the final injection the PP cells were isolated and cultivated for measurement of the T helper

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cytokines. In this experiment, oral injection of capsicum extract and capsaicin showed no toxicity to mice, demonstrated by the fact that the body and liver weights (data not shown) and level of plasma hepatic marker enzyme, alanine aminotransferase (ALT, EC 2.6.1.2), did not change (10 mg/kg/day capsicum extract: 22.1 $\pm$ 1.0 U/ml, n=7, not significant; 3 mg/kg/day capsaicin: 26 $\pm$ 2.9 U/ml, n=6, not significant; vehicle only: 18.3 $\pm$ 1.1 U/ml, n=3). In contrast to the in vitro study (Fig. 2), the production of IL-2 and IFN-γ in PP cells from mice injected with capsicum extract was much more than in those of control mice, especially in response to Con A stimulation (Fig. 3). Oral injection of the extract enhanced Th1 cytokines in response to Con A at doses of 1 to 10 mg/kg in a dose-dependent manner (Figs. 3B and D). Next we examined the influence of the extract on IL-4 and IL-5 production in the PP cells ex vivo. IL-4 and IL-5 are signature cytokines produced by Th2 and are able to direct developing Th0 cells to differentiate into Th2 cells (Xu-Amano, 1993; Romagnani, 2004). EtOH extract did not affect IL-4 production in the PP cells in both the presence and absence of Con A stimulation, whereas it moderately but significantly enhanced IL-5 production by 180.9% (p <0.05) irrespective of Con A (Fig. 4).

In order to isolate the active constituent(s) responsible for Th1 activation (Fig. 2), the capsicum extract was separated into 14 fractions by column chromatography on silica gel. Fig. 5 shows that the stimulatory effect on IL-2 and IFN-γ production in cultured PP cells transferred mainly to Fr-2 and Fr-9, which increased the IL-2 and IFN-γ production co-stimulated with Con A by 187 ± 16% (IL-2 level, Fr-2, p <0.05), 213 ± 20% (IL-2 level, Fr-9, p <0.05), 124 ± 27% (IFN-γ level, Fr-2, not significant) and 134 ± 25% (IFN-γ level, Fr-9, p <0.05). Both fractions were further purified by silica

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gel chromatography and HPLC. Fr-2 was shown to consist mainly of carotenoids (β-carotene; Fig. 1). Fr-9 was mainly composed of capsaicinoids and capsanthin (Fig. 1). Fr-2 showed a toxic action against PP cells atconcentrations over 3 μg/ml, whereas Fr-9 was not toxic at the same concentrations (Table 1). On the other hand, oral injection of Fr-2 at dose of 3 mg/kg/day for 4 consecutive days did not show any significant toxicity to PP cells (Table 2).

Capsaicin has also been shown to have modulatory effects to B cell immune responses (Nilsson et al., 1991; Eglezos et al., 1990). In the present experiment, we therefore noted the richness of Fr-9 in capsaicinoids, which potentiated IL-2 and IFN-γ production similar to Fr-2, and evaluated the effect of capsaicin, a major pungent component of Capsicum, on cytokine production in PP cells ex vivo.

PP cells from mice treated with capsaicin at doses of 0.3 and 3 mg/kg/day produced IL-2 and IFN-γ unlike those from control mice (Figs. 6A-D), whereas orally injected capsaicin at the same doses did not affect IL-5 production (Figs. 7A-D). The levels of IL-4 co-stimulated with Con A in the PP cells from capsaicin-treated mice rose approximately 1.4 times more than those from the control (Fig. 7B).

It is well known that capsaicin and other pungent vanilloids activate a cell surface receptor called transient receptor potential vanilloid 1 (TRPV1), which is expressed mainly in nociceptive neurons. TRPV1 is a cation channel activated by pungent vanilloid compounds, extracellular protons or noxious heat and plays a central role in neurogenic inflammation (Szallasi, 2001; Szallasi and Blumberg, 1999). To clarify the relationship between the capsaicin-induced enhancement of cytokine production in PP cells and TRPV1, the effect of oral administration of the specific TRPV1 receptor

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antagonist capsazepine (Fig. 1) was examined in capsaicin-treated mice. As shown in Figs. 6 and 7, IL-2 and IFN-γ production in PP cells co-stimulated with Con A were significantly potentiated by oral administration of capsazepine alone at a dose of 3 mg/kg/day (Figs. 6B and D). Oral administration of capsazepine alone also accelerated Con A-stimulated IL-4 production, but did not accelerate IL-5 production at the same dose (Figs. 7B and D). Administration of capsazepine (3 mg/kg/day) simultaneously with capsaicin (3 mg/kg/day) resulted in enhanced IL-2 production in response to Con A (Fig. 6B), while the secretion of IFN-γ, IL-4 and IL-5 in PP cells was not affected (Figs. 6 and 7).

We determined the number of PP cells from mice orally treated with tested samples. The number of PP cells from normal mice was 26.1 ± 1.0 ($\times 10^6$) cells/mouse. Orally administered capsicum extract significantly increased PP cell number, whereas capsaicin (3 mg/kg/day) decreased the number of PP cells (Table 1). Administration of capsazepine alone or capsazepine + capsaicin had no effect on the number of PP cells (Table 1). Oral administration of capsicum extract, capsaicin and capsazepine, respectively, caused no cytotoxicities toward PP cells in contrast to the in vitro experiment (Table 2).

To clarify what kinds of T lymphocytes when treated with capsicum extract, capsaicin, capsazepine and capsaicin + capsazepine increase or decrease the PP cells, PP cells were stained with FITC- or PE-conjugated specific T, B and T helper-subset lineage markers and subjected to flow cytometric analysis. PP cells are mainly composed of T and B cells, and it is well understood that T cells are a source of immunomodulatory cytokine production (Bhide et al., 2001). Flow cytometric analysis revealed that the percentages of T ($CD3^+$) and B ($CD19^+$) lymphocytes in normal mice were

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27.1% and 69.4%, and that of the T cell subpopulation, $CD4^+$ and $CD8^+$, were 25.2% and 5.9%, respectively (Table 3). After administration of higher doses (3 mg/kg/day) of both capsicum extract or capsaicin, the percentage of $CD3^+$ cells decreased from 7 to 10% of the total T cell population, whereas they increased $CD19^+$ B lymphocytes by 78.1% (capsicum extract, $p<0.05$) and 77.0% (capsaicin, $p<0.05$) at 10 and 3 mg/kg/day. On the other hand, capsazepine did not change the percentages of T and B cells, the T cell subset and T helper subset in PP cells (Table 3). The populations of IFN-γ and IL-4 positive cells in the T lymphocytes significantly decreased with capsaicin treatment (3 mg/kg/day) but the ratio of the T helper subset was unchanged (control group, IFN-γ^+:IL-4^+ = 1:1.76; capsaicin IFN-γ^+:IL-4 = 1:1.88). Administration of the TRPV1 antagonist capsazepine inhibited the capsaicin-induced decrease in $CD3^+$ cells and increase in $CD19^+$ cells (Table 3).

Discussion

The present study demonstrated that ethanol extract of red pepper (*C. annum*) and its main piquant component capsaicin directly inhibit T helper cytokine production in cultured PP cells in vitro, whereas oral injection enhances the levels of IL-2 and IFN-γ in response to Con A. The capsicum extracts and capsaicin had an opposite effect on immune responses in vitro and ex vivo, suggesting an indirect effect in vivo. Capsicum has shown a wide range of pharmacological properties including pain relief, antigenotoxic, antimutagenic, and anticarcinogenic effects (Surh and Lee, 1995). To our knowledge, the present results provide the first evidence that oral administration of capsicum extract

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and capsaicin regulate the production of T helper 1 cytokines such as IL-2 and IFN-γ in PP cells.

Capsaicin reportedly causes cell death of neuronal cells (Szallasi and Blumberg, 1999) as well as non-neuronal cells and cell lines such as Vero monkey kidney cells (Creppy et al., 2000), SHSY-5Y human neuroblastoma cells (Richeux et al., 1999), ECV340 human endothelial cells (Richeux et al., 2000), A172 human glioblastoma cells (Lee et al., 2000), BEAS-2B human bronchiolar epithelial cells and A549 human lung adenocarcinoma cells (Reilly et al., 2003), and a variety of other cell lines. Further, functional vanilloid receptors have been found on rat $CD3^{high}$ as well as $CD3^{negative}$ thymocytes and are associated with the selective ability of capsaicin to trigger DNA fragmentation in thymocytes (Amantini et al., 2004). In the present study, direct treatment of PP cells with capsaicin reduced cell viability at concentrations of 3 and 30 μM (Table 1). Furthermore, oral administration of capsaicin at a dose of 3 mg/kg (for 4 consecutive days) selectively reduced the $CD3^+$ T cell population without changing the ratio of Th1/Th2 cells in the PP, and capsazepine, a prototype of the TRPV1 antagonist, ameliorated the capsaicin-induced reduction of $CD3^+$ T cells (Table 3). Thus, it is reasonable to assume that TRPV1 is also expressed on T lymphocytes in PP and that the capsaicin-induced T cell reduction was mediated by its interaction with TRPV1 since it was partly inhibited by capsazepine.

Previous research has demonstrated that capsaicin inhibits IL-2 production (Gertsch et al., 2002) by capacitative calcium entry in Jurkat cells (Fischer et al., 2002). Capsaicin has also been reported to suppress cell surface expression of CD69, CD25 and ICAM-1 on Jurkat cells in vitro (Sancho et al., 2002). In our experiment, treatment of PP cells with capsicum extract or capsaicin

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showed concentration-dependent suppression of IL-2, IFN-γ, IL-4 and IL-5 (Fig. 2) secretions in cultured PP cells, dramatically reducing their viability (Table 2). Our in vitro data were in agreement with previous reports in which capsaicin reduced T cell function and cytokine production (Gertsch et al., 2002; Bruce et al., 2002; Sancho et al., 2002).

Unexpectedly, PP cells from mice injected orally with capsicum extract or capsaicin significantly secreted Th1 cytokines, IL-2 and IFN-γ, in response to Con A (Fig. 3). Further, the increment of IL-2 and IFN-γ production with capsaicin treatment could not be abolished when the TRPV1 antagonist capsazepine was simultaneously administered (Figs. 6 and 7). Interestingly, the same dosage (3 mg/kg/day) and regimen (4 consecutive days) of capsazepine significantly increased Con A-induced IL-2 and IFN-γ production in cultured PP cells. These results indicated that TRPV1 agonist capsaicin and its antagonist capsazepine showed stimulatory effects on Th1 cytokine production ex vivo through a TRPV1-independent pathway.

Capsaicin-sensitive primary afferents are well known to be involved in nociception and neurogenic inflammation. In contrast to its acute excitatory activity, capsaicin also exhibited analgesic properties, when used to treat pain associated with diabetic neuropathies or rheumatoid arthritis (Szallasi and Blumberg, 1999; Tominaga and Tominaga, 2005). This paradoxical effect correlated to the ability of capsaicin to desensitize nociceptive terminals following long-term exposure (Tominaga and Tominaga, 2005). In our experiment, a short-term administration (once a day for 4 consecutive days) of capsaicin showed stimulatory effect on Th1 cytokine production in PP cells. Therefore, further studies (including long-term administration of capsaicin) were necessary to

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clarify relationship between capsaicin-induced T helper 1 cytokines production in PP cells and function of TRPV1 ex vivo. We are now planning to investigate the effects of short-term and long-term administration of capsaicinoids on the production of T helper cytokines in PP cells by using TRP V1 knockout mice in the next step.

Recently, it was reported that an intraplantar injection of capsaicin into the dorsal aspect of the rat hind paw produced early upregulation of proinflammatory cytokines such as IL-1β, TNF-α, IL-6, and nerve growth factor (Saade et al., 2002). Basu and Srivastava (2005) more recently showed that bone marrow-derived mouse dendritic cells express TRPV1, and that ligand capsaicin transmits the immunological inflammatory signals. Thus, it is assumed that in vivo administration of capsaicin might up-regulate T cell immune responses and that the mechanisms of the immunopotent effect of capsaicin might be TRPV1 independent. More specifically, capsaicin is known to mediate neuroactive ligands such as Substance P, bradykinins and enkephalins (Wang, 2005). It is also noteworthy that Substance P and bradykinins have been shown to mediate NF-κB, a transcriptional factor central to innate immune responses (Pan et al., 1996; Lieb et al., 1997). Endogenously produced Substance P has been shown to contribute to lymphocyte proliferation by activation of dendritic cells and T cell antigen receptor ligation (Lambrecht et al., 1999). Indeed, PP cells from mice intravenously injected with Substance P at dose of 27 ng/kg strongly secreted IL-2 and IFN-γ irrespective of Con A (unpublished data). Moreover, we also found that an intravenous injection (0.1 mg/kg) of neurokinin-1 receptor antagonist, sendide (Sakurada et al., 1989), completely abolished the capsaicin-induced Th1 cytokine secretion co-stimulated with Co A (unpublished data). It might

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therefore be discussed that the mechanisms by which capsaicin potentiated Th1 cytokine production ex vivo was in part due to endogenous neuropeptide Substance P. Further investigation in line with this idea is underway.

Most pharmacological studies performed so far have focused on capsaicinoids; however, the medicinal properties attributed to capsicum might be related to different compounds. In this experiment, the active fractions in capsicum extract, which enhanced T helper cytokine production, were shown to be not only a fraction rich in capsaicinoids (Fr-9) but also one rich in carotenoids (Fr-2) (Fig. 5). HPLC analysis of Fr-2 revealed that it contained mainly β-carotene (Fig. 1), β-cryptoxanthin, and lycopene. Satoh et al., (2004) recently reported that splenocytes from mice fed β-carotene (Fig. 1) produced more IL-2 and IFN-γ than those from control mice. Thus, it is possible to suggest that the immunopotent activity of capsicum extract might also be due to carotenoids in the extract. Further purification and chemical characterization of Fr-2 as well as evaluation of their inductive effects on T helper cytokines in PP cells are necessary.

In summary, the present study demonstrated that orally administered capsicum extract and its main pungent capsaicin selectively modulate the production of T helper 1 cytokines such as IL-2 as well as IFN–γ, but have no significant effect on the levels of IL-4 and IL-5, via a TRPV1-independent pathway. These findings suggest that capsicum extract and capsaicin do not cause non-specific immune responses like those induced by Con A. Further, the balance between Th1 and Th2 is of central importance for immune responses of mucosal T cells (Neurath et al., 2002). It is also indicated that overexpression of Th2 and/or Th1-depolarization predispose for allergic diseases such as allergic

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asthma (Neurath et al., 2002). Taken together, a moderate intake of capsicum or capsaicin has potentialities as therapeutic or preventive agents for treatment of immune diseases such as allergies and parasitic diseases.

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Figure legends

Fig. 1. Chemical structures of β-carotene, capsaicin and capsazepine.

Fig. 2. Suppressive effects of capsicum extract and capsaicin on IL-2, IFN-γ, IL-4 and IL-5 production in cultured PP cells in vitro. PP cells were directly treated with capsicum extract or capsaicin + 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. Panel A: IL-2 production, panel B: IFN-γ production, panel C: IL-4 production and panel D: IL-5 production. All data are expressed as the mean ± SD of quadruplicate cultures, [a] $p<0.05$ and [b] $p<0.01$ compared with control cultures. Results of one experiment repeated twice with similar results. CE: capsicum extract.

Fig. 3. Effects of capsicum extract on IL-2 and IFN−γ production in cultured PP cells ex vivo. Mice were treated orally with capsicum extract or vehicle once a day for 4 consecutive days, and the PP cells were collected 6 days after the first day of treatment and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-2 and IFN-γ production in the cultured supernatants were measured using an ELISA kit. Panel A: IL-2 production in PP cells without Con A stimulation, panel B: IL-2 production in PP cells with Con A stimulation, panel C: IFN-γ production in PP cells without Con A stimulation and panel D: IFN-γ production in PP cells with Con A stimulation. All data are expressed as the mean ± SD of quadruplicate cultures, [a] $p<0.05$ compared with control cultures. Results of one experiment

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repeated twice with similar results. CE: capsicum extract.

Fig. 4. Effects of capsicum extract on IL-4 and IL-5 production in cultured PP cells ex vivo. Mice were treated orally with capsicum extract or vehicle once a day for 4 consecutive days, and the PP cells were collected 6 days after the first day of treatment and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-4 and IL-5 production in the cultured supernatants were measured using an ELISA kit. Panel A: IL-4 production in PP cells without Con A stimulation, panel B: IL-4 production in PP cells with Con A stimulation, panel C: IL-5 production in PP cells without Con A stimulation and panel D: IL-5 production in PP cells with Con A stimulation. All data are expressed as the mean ± SD of quadruplicate cultures, [a] $p<0.05$ compared with control cultures. Results of one experiment repeated twice with similar results. CE: capsicum extract.

Fig. 5. Effects of fractions separated from the capsicum extract on IL-2 and IFN−γ production in cultured PP cells ex vivo. Mice were treated orally with a fraction or vehicle once a day for 4 consecutive days, and the PP cells were collected 6 days after the first day of treatment and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-2 and IFN-γ production in the cultured supernatants were measured using an ELISA kit. Panel A: IL-2 production in PP cells without Con A stimulation, panel B: IL-2 production in PP cells with Con A stimulation, panel C: IFN-γ production in PP cells without Con A stimulation and panel D: IFN-γ production in PP cells with Con A stimulation. All data are expressed as the mean ± SD of quadruplicate cultures, [a] $p<0.05$ compared

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with control cultures. Results of one experiment repeated twice with similar results. CE: capsicum extract.

Fig. 6. Effects of capsaicin and capsazepine on IL-2 and IFN–γ production in cultured PP cells ex vivo. Mice were treated orally with capsaicin (Cap), capsazepine (CZ), capsaicin + capsazepine (Cap/CZ) or vehicle once a day for 4 consecutive days, and the PP cells were collected 6 days after the first day of treatment and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-2 and IFN-γ production in the cultured supernatants were measured using an ELISA kit. Panel A: IL-2 production in PP cells without Con A stimulation, panel B: IL-2 production in PP cells with Con A stimulation, panel C: IFN-γ production in PP cells without Con A stimulation and panel D: IFN-γ production in PP cells with Con A stimulation. All data are expressed as the mean ± SD of quadruplicate cultures, [a] $p<0.05$ compared with control cultures. Results of one experiment repeated twice with similar results.

Fig. 7. Effects of capsaicin and capsazepine on IL-4 and IL-5 production in cultured PP cells ex vivo. Mice were treated orally with capsaicin (Cap), capsazepine (CZ), capsaicin + capsazepine (Cap/CZ) or vehicle once a day for 4 consecutive days, and the PP cells were collected 6 days after the first day of treatment and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-4 and IL-5 production in the cultured supernatants were measured using an ELISA kit. Panel A: IL-4 production in PP cells without Con A stimulation, panel B: IL-4 production in PP cells with Con A stimulation,

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panel C: IL-5 production in PP cells without Con A stimulation and panel D: IL-5 production in PP cells with Con A stimulation. All data are expressed as the mean ± SD of quadruplicate cultures, [a] $p<0.05$ compared with control cultures. Results of one experiment repeated twice with similar results.

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Table 1

Effects of capsicum extract and capsaicin on the cell viability of cultured PP cells*

Treatment	Concentration	Cell viability (%) Con A(-)	Cell viability (%) Con A (+)	*n*
-	-	96.8 + 2.2	90.2 + 1.9	4
Capsicum extract	1 μg/ml	83.1 ± 1.7 [a]	76.1 ± 1.1 [a]	3
	10 μg/ml	67.2 ± 1.0 [b]	64.4 ± 2.2 [b]	4
Fr-2	3	87.1 ± 1.4 [a]	74.4 ± 2.6 [b]	4
	10	70.4 ± 1.0 [b]	63.0 ± 1.0 [b]	4
Fr-9	3	93.3 ± 1.1	92.1 ± 1.3	3
	10	94.6 ± 0.4	87.0 ± 1.8	4
Capsaicin	3 μM	73.3 ± 2.9 [b]	73.9 ± 1.9 [a]	3
	30 μM	60.2 ± 2.8 [b]	66.0 ± 3.8 [b]	4

*PP cells were cultured with capsicum extract or capsaicin at the indicated concentrations together with 5 μg/ml Con A for 72 h. Cytokine production (Fig. 2) and cell viability were then measured by MTT methods (see the materials and methods). Cell viability data represent the absorbance ratio (percentage) of sample-treated PP cells to non-treated PP cells. [a] $p<0.05$ and [b] $p<0.01$ compared with Con A-treated control cultures.

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Table 2

PP cell number and cell viability in mice orally treated with capsicum extract or capsaicin*

Treatment	Dose (mg/kg/day)	PP cell number ($\times 10^6$ cells/mouse)	Cell viability (%)	*n*
Control	–	26.1 ± 1.0	95.5 ± 0.1	7
Capsicum extract	1	23.9 ± 1.5	94.0 ± 0.2	6
	10	30.1 ± 1.8 *	93.4 ± 0.2	6
Fr-2	3	24.1 ± 1.5	96.1 ± 1.7	5
Fr-9	3	28.5 ± 1.0	93.8 ± 1.6	5
Capsaicin	0.3	20.8 ± 0.3	95.1 ± 0.1	6
	3	18.9 ± 0.6 *	94.5 ± 0.3	6
Capsazepine	3	28.0 ± 1.5	96.1 ± 1.0	5
Capsaicin/capsazepine	3 + 3	24.1 ± 2.7	96.6 ± 1.0	5

*Capsicum extract and capsaicin were injected orally once a day for 4 consecutive days; the final injection was conducted 2 days before collecting the PP cells. The PP were then collected (10 Peyer's patches/mouse) and counted using a hemocytometer with trypan blue staining. Data are expressed as the mean + S.E. of 5 to 7 mice. *$p<0.05$ compared with the control mice.

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Table 3

Effects of capsicum extract, capsaicin and capsazepine on the PP lymphocyte population in mice*

Treatment	Dose (mg/kg/day)	$CD3^+$ (%)	$CD19^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)	IFN-γ^+ (%)	IL-4^+ (%)	*n*
Control	-	27.1 ± 1.6	69.4 ± 3.5	25.2 ± 2.1	5.9 ± 0.7	9.2 ± 0.3	16.2 ± 0.1	7
Capsicum extract	1	25.8 ± 1.9	69.6± 1.9	19.0 ± 0.9	5.3 ± 1.1	8.1 ± 1.7	14.9 ± 0.2	6
	10	20.4 ± 1.0 [a]	78.1 ± 1.6 [a]	17.4 ± 1.0 [a]	5.1 ± 1.2	8.5 ± 1.2	15.0 ± 1.8	6
Capsaicin	0.3	22.1 ±1.4	74.1 ± 1.3	21.3 ± 1.5	5.2 ± 0.4	8.7 ± 0.9	14.8 ± 0.3	6
	3	17.6 ± 0.3 [a]	77.0 ± 2.1 [a]	13.3 ± 1.2 [a]	4.8 ± 1.0	5.0 ± 0.3[a]	9.4 ± 2.2 [a]	6
Capsazepine	3	29.9 ± 2.1	68.4 ± 0.7	20.6 ± 0.8	7.2 ± 0.3	8.3 ± 0.4	14.0 ± 1.0	5
Capsaicin/capsazepine	3 + 3	28.7 ± 0.4	66.3 ± 0.2	24.2 ± 0.3	4.2 ± 0.1	8.8 ± 0.4	13.5 ± 0.6	5

*Capsicum extract, capsaicin, capsazepine or capsaicin + capsazepine were injected orally once a day for 4 consecutive days. PP cells (1×10^6 cells) were collected 2 days after the final injection and stained with lineage specific FITC- or PE-conjugated mAb. Data are expressed as the mean ± S.E. of 5-6 mice.

[a] $p < 0.05$ compared with the control mice.

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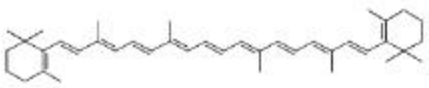
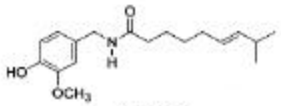
Takano et al., Figure 1



β-Carotene



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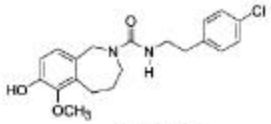

Capsazepine

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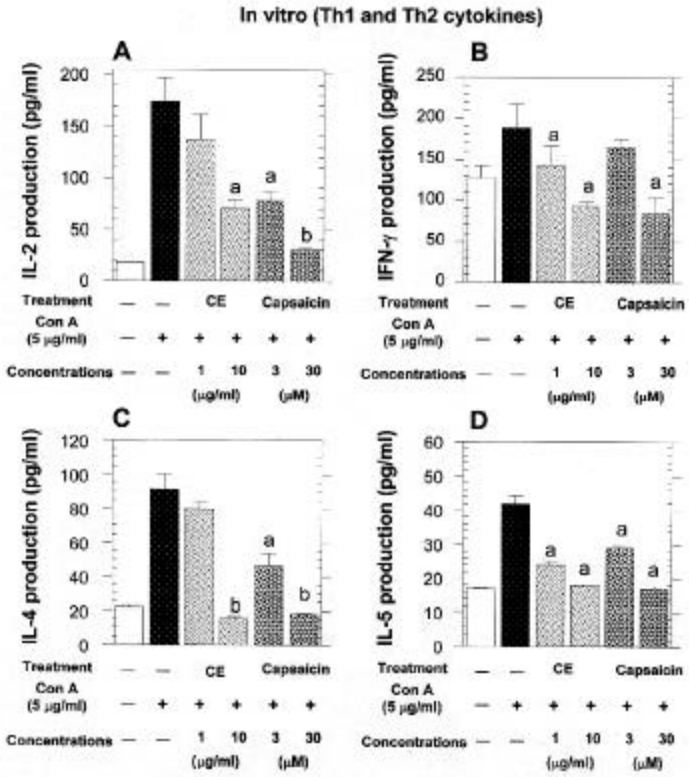

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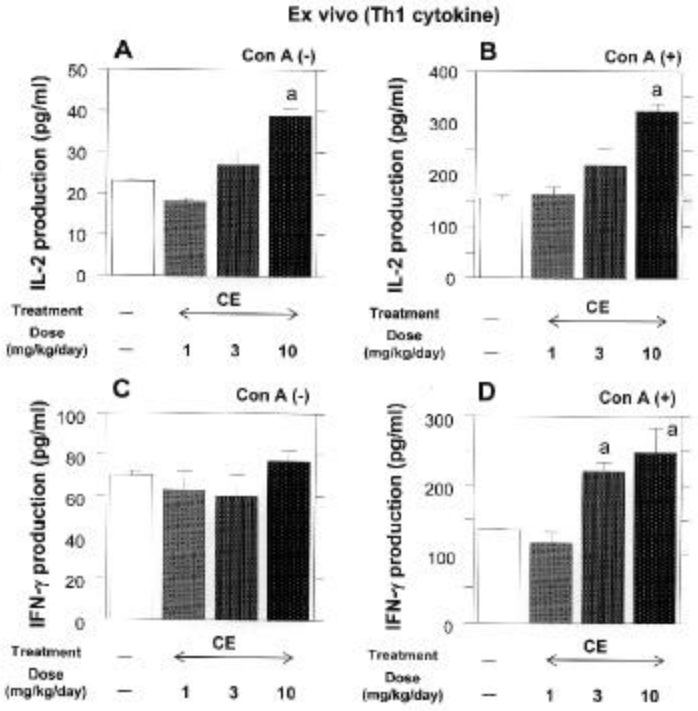

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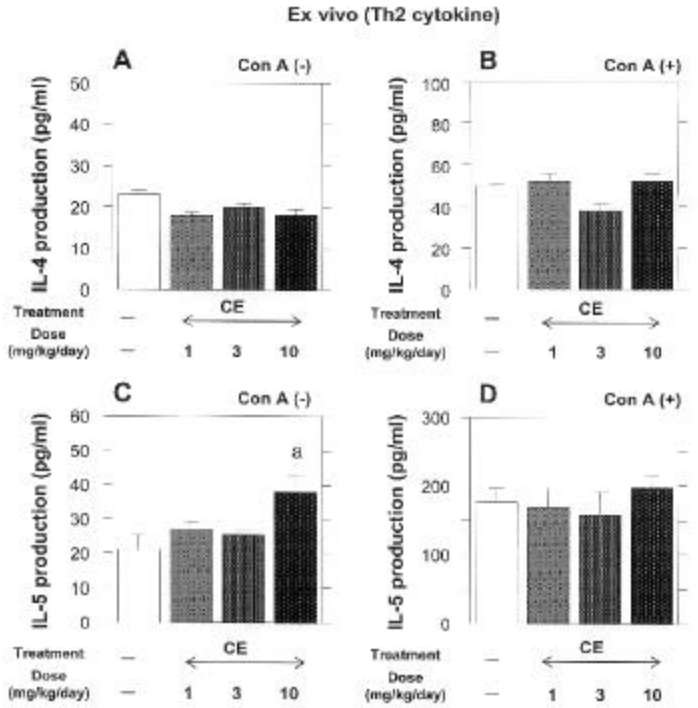

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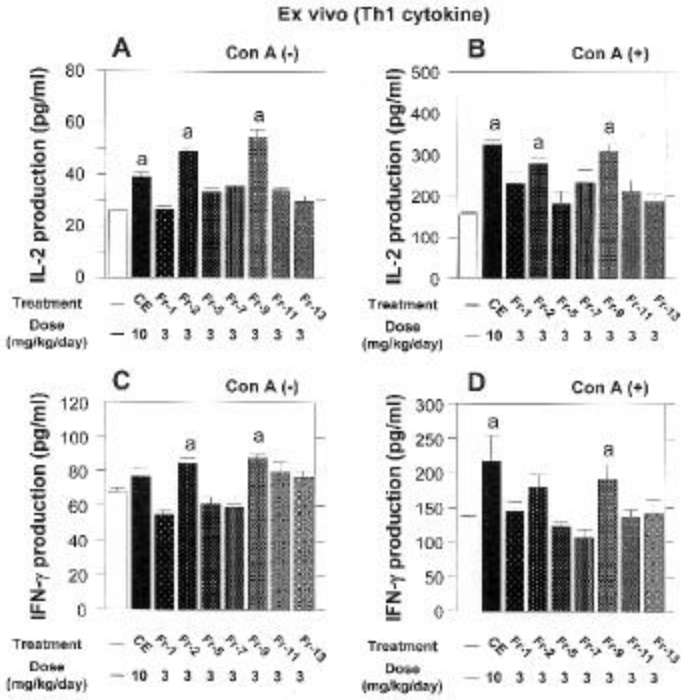

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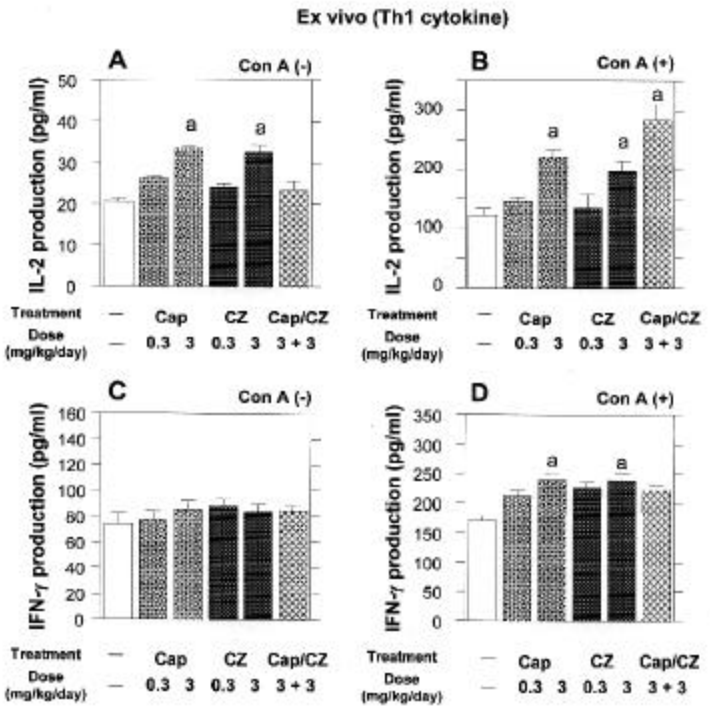

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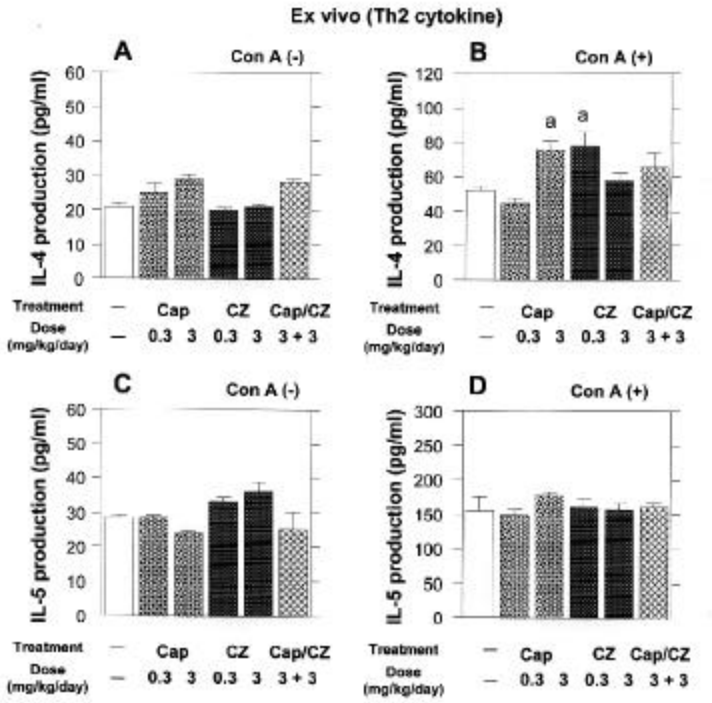

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