Dietary *Curcuma xanthorrhiza* Roxb. Increases Mitogenic Responses of Splenic Lymphocytes in Rats, and Alters Populations of the Lymphocytes in Mice

Sedarnawati YASNI, Kiyotaka YOSHIE, Hiroshi ODA, Michihiro SUGANO, and Katsumi IMAIZUMI

Department of Food Science and Technology, School of Agriculture, Kyushu University, Fukuoka 812, Japan

Department of Bacteriology, School of Medicine, Kagoshima University, Kagoshima 890, Japan

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Summary Rhizomes of *Curcuma xanthorrhiza* Roxb. (*C. xanthorrhiza*), a medicinal plant in Indonesia, has been shown to exert diverse physiological functions. Hitherto, a little attention has been paid to its effect on immune functions. This study was carried out to determine the effect of this medicinal plant on mitogenic response of splenic lymphocytes in rats and population of splenic lymphocytes and macrophages and peripheral blood macrophages in mice. Mitogenic responses of splenocytes to phytohemagglutinin, concanavalin A, and pokeweed mitogens were examined in rat fed *C. xanthorrhiza* for 3 weeks. The medicinal plant increased the blastogenesis to these mitogens. Flow cytometric analysis was carried out for mice fed the medicinal plant for 3 to 5 weeks. *C. xanthorrhiza* increased the proportion of the splenic T cells throughout the experimental period, but exerted a variable effect on B cells and T cell subsets, that is, elevations of B cells at 3 weeks and of Th cells at 4 weeks without any elevation of Ts cells. The effect of this medicinal plant on a proportion of macrophages from the spleen and peripheral blood was not consistent. Thus, the present study suggests that *C. xanthorrhiza* contains some principle(s) activating T and B cell-mediated immune functions.

**Key Words:** *Curcuma xanthorrhiza*, mitogenic response, lymphocytes, macrophages, flow cytometry

*Curcuma xanthorrhiza* Roxb. (*C. xanthorrhiza*, Zingiberaceae family, commonly known as Temu lawak or Javanese Turmeric in Indonesia) which is found both wild and cultivated in Indonesia has been traditionally used as vegetables and traditional medicines (1). Either fresh rhizomes or decoction of dried sliced rhizomes have been used to treat various stomach diseases and liver disorders (2). *C. xanthorrhiza* is also utilized as a health tonic in Indonesia (3). In the previous
paper we have shown a beneficial effect of *C. xanthorrhiza* on streptozotocin-induced diabetic rats, that is, lowering serum glucose and triacylglycerols (4). In addition, this plant increased the survival rate under the condition of suppressed immuno potency induced by streptozotocin (unpublished observation).

Rhizomes of *Curcuma longa* L. (*C. longa*), commonly known as turmeric which belongs to the same genus of *C. xanthorrhiza*, contain strong coloring pigment used widely in Asian countries as a condiment, particularly as an ingredient of curry powder. Current traditional Indian medicine claims the use of this powder against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorders, rheumatism, and sinusitis (2). Chandra and Gupta showed an anti-inflammatory and anti-arthritic activity of volatile oil of *C. longa* (5). More recently, Gonda *et al.* have shown that polysaccharides of *C. longa* exhibited enhancing phagocytic activity (6). Since little attention has been focused on the role of *C. xanthorrhiza* on the immune system, we have examined the effects of dietary supplementation of this medicinal plant on lymphocyte blastogenesis in rats, and populations of splenic lymphocytes and macrophages in mice by using flow cytometric analysis. Changes in macrophage populations of peripheral blood cells were also examined in mice.

**MATERIALS AND METHODS**

**Diets.** Six-week-old Sprague-Dawley rats (Seiwa Experimental Animals Co., Fukuoka) and 6-week-old female mice (Kuroda Laboratory Animal Center, Inc., Kumamoto) were housed in stainless steel cages with wire mesh bottoms and in plastic cages, respectively. These animals were maintained in a light-(12 h/day) and temperature-controlled room (23°C). They were freely given a diet and water. The composition of the control diet is as follows (wt%): 20 casein (vitamin-free, Wako Pure Chemicals Co., Osaka), 5 safflower oil (local market), 15α-cornstarch (Nippon Shokuhin Kakoh Ltd., Aichi), 5 cellulose (Toyo Roshi Co., Tokyo), 1 vitamin mixture (AIN-76 mixture, Oriental Yeast Co., Tokyo), 3.5 mineral mixture (AIN-76 mixture, Oriental Yeast Co., Tokyo), 0.2 choline bitartrate (Katayama Chemicals Co., Osaka), 0.3 DL-methionine (Nacalai Tesque Inc., Kyoto) and sucrose to 100 (7). Powdered rhizomes of *C. xanthorrhiza* (P.T. Mustika Ratu Jakarta, Indonesia), which were freeze-dried, milled, and sifted through a 100-mesh sieve, were added to the control diet at 2% level at the expense of sucrose. All procedures received approval by the University Laboratory Animal Care and Use Committee as confirming to standards for treatment of laboratory animals.

**Mitogenic response of splenic lymphocytes in rats.** After a 3-week feeding period, rats were anesthetized with diethyl ether and blood was collected from aorta. With aseptic techniques, the spleen was removed and disrupted by teasing and spleen cell suspension was passed through sterile nylon mesh. Cells were washed in RPMI 1640 medium. The single cells (3 × 10⁶ cells/ml) were resuspended in RPMI medium containing 10% fetal calf serum, 0.03% L-glutamine, 25 mM 4-
(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and antibiotic solution (100 U/liter penicillin G, 10.0 mg/liter streptomycin sulfate, 0.25 mg/liter fungizone). Blastogenic response of splenocytes to the mitogens, phytohemagglutinin (PHA, GIBCO, Grand Island, NY), concanavalin A (Con A, Sigma Chemicals, St. Louis, MO) or pokeweed mitogen (PWM, GIBCO, Grand Island, NY) was assessed by [methyl-\(^3\)H]thymidine (20 Ci/mmol, Amersham Japan, Tokyo) incorporation. Splenocytes (4.5 \times 10^5 cells) were incubated with mitogens in 200 \mu l of the culture medium at 37°C, with 5% CO\(_2\) for 72 h. Doses of mitogen per dish were 10 \mu g for Con A, 50 \mu l PHA (diluted to 3 fold of original solution with water) and 50 \mu l PWM (diluted 20 fold of original solution with water). Six hours before harvesting the cells, 0.5 mCi of [\(^3\)H]thymidine was added to each well. Cultures were harvested on a glass fiber filter and the incorporation of [\(^3\)H]thymidine was determined by a liquid scintillation counter (LSC-900, Aloka, Tokyo).

**Preparation of murine splenocytes and peripheral blood cells for flow cytometry.** Mice were anesthetized with diethyl ether and blood was obtained by heart puncture with heparinized syringe. The blood obtained from 4 mice per group was pooled. Spleen was removed from individual mouse and flow cytometric analysis was performed on individual samples using 4 mice per dietary group. To prepare a single cell suspension, spleen was minced and passed through a stainless steel mesh screen using the Eagle's minimum essential medium containing 10% fetal bovine serum. After hemolyzing remaining red blood cells in the suspension with one to two volumes of isotonic NH\(_4\)Cl solution, the splenocytes were collected by centrifugation at 500 \times g for 10 min. The cells were washed and resuspended at 1.0 \times 10^7 ml in phosphate-buffered saline (PBS). Peripheral blood lymphocyte and monocyte fraction were prepared by the density gradient centrifugation using Ficoll according to the manufacturer (Pharmacia) (7), and the cells were resuspended at 1.0 \times 10^6/ml PBS.

Antibodies (monoclonal or affinity purified polyclonal) used for labeling and the fluorescent dye employed are given in Table 1. The following T cell markers were monitored: 1) Thy1.2, a glycoprotein found on murine T cells and a major marker for defining the T cells; 2) L3T4, a marker specific for murine T helper cells

| Antibodies to lymphocyte and macrophage surface antigens |
|----------------------------------------------------------|
| PE-Mouse anti-mouse Thy1.2\(^1\)                         |
| FITC-Rat anti-mouse L3T4: CD4\(^4\)                      |
| Mouse anti-mouse Lyt2.2: CD8\(^3\)                      |
| FITC-Rat anti-mouse Ly5: B220\(^5\)                     |
| FITC-Rat anti-mouse macrophage: Mac 1\(^1\)             |
| Rat anti-mouse macrophage: F4/80\(^9\)                  |
| FITC-Goat anti-mouse IgM (\(\mu\) specific)\(^1\)       |
| PE-Goat anti-mouse IgM (\(\mu\) specific)\(^1\)         |

\(^1\)Caltag Laboratory Inc., South San Francisco, CA.  \(^2\)Serotec, Oxford.  \(^3\)Cedarlane Laboratory Ltd., Ontario.
(Th); and 3) Lyt2.2, a marker characteristic of murines suppressor cells (Ts) and cytotoxic T cells (Te). Ly5 was used as murine B cell marker, a glycoprotein specifically found on all B cells from the earliest pre-B cell stage to plasma cell stage. Macrophage markers were as follows: 1) Mac 1, a marker for a receptor of complement 3 present in macrophages and granulocytes, 3) F4/80, a marker for mature antigen of differentiated murine macrophages. For flow cytometry, a cell suspension (50 µl) was mixed with PBS (40 µl) and a primary antibody (10 µl), incubated at room temperature for 20 min and washed twice with PBS. When secondary antibody is required, the washed cells were resuspended in PBS (90 µl) and incubated with an appropriate secondary antibody (10 µl) at room temperature for 20 min, and washed with PBS. These labeled cells were suspended finally in PBS (200 µl). Flow cytometric analysis was performed by using Epics Profile II Flow Cytometer (Coulter Electronics Inc., FL). Orthogonal fluorescence of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) was measured using a 525 nm interference bandpass filter and 575 nm long bandpass filter, respectively. All fluorochromes were exited with the 488 nm and 15 mW output of an argon laser. Lymphocytes and macrophages were selected for analysis gating with forward angle light scatter vs. orthogonal light scatter. The percentage of positive cells for each phenotype marker using single-color was determined using a cytogram gate defined as a box consisting of low forward angle light scatter vs. orthogonal fluorescence. The percentage for positive cells for each marker in two-color flow cytometric analysis was determined by multiple gating. These cells were simultaneously examined for the presence of phycoerythrin and fluorescein isothiocyanate.

Statistical analyses. Data from both experiments were analyzed using Student’s t-test (8).

RESULTS

Mitogenic response

There were no differences in body weight gain between the dietary treatment groups (data not shown), although the rats in the C. xanthorrhiza group ate less than those in the control did (15.3±0.4 vs. 18.3±0.4 g; p<0.05). There were no diet effects on relative spleen weights (data not shown).

In a preliminary experiment, we have determined the maximum proliferative responses of lymphocytes from normal rats to Con A (40, 10, and 2 mg), PHA (1:2, 1:3 and 1:5 dilution) and PWM (1:5, 1:10, and 1:20 dilution). Maximal stimulation for Con A, PHA, and PWM was observed at concentrations of 10 mg/ml, at the dilution of 1:3 and 1:20/ml, respectively. At these maximal mitogen concentrations, lymphocyte proliferative responses from cultures stimulated with PHA, Con A and PWM were all significantly higher in rats fed C. xanthorrhiza than in those fed the control diet (Fig. 1).
Fig. 1. Effects of *C. xanthorrhiza* on phytohemagglutinin (PHA)-, concanavalin A (Con A)-, and pokeweed mitogen (PWM)-stimulated lymphocyte proliferation in rats. Each bar shows M±SE for 5 rats per group. *Significantly different from the control group at p<0.05.

**Flow cytometric analysis**

There were no differences in body weight gains between the dietary treatment groups (data not shown). Since 4 mice were housed in a cage, food intake was not determined, but both groups of mice consumed the foods at a comparable amount in each cage.

As shown in Fig. 2, the proportion of Thy1.2 positive cells differed in the presence of FITC labeled Ly5 (left in Fig. 2) and FITC labeled L3T4 (center in
Fig. 3. Effects of *C. xanthorrhiza* on phenotypic distribution of splenic T cells in mice. *L3T4* positive cells in the presence of FITC (L3T4) and PE (Thy 1.2). **Lyt 2.2** positive cells in the presence of PE (Goat anti-mouse IgM). Each bar shows M±SE for 4 mice per group. *Significantly different from the control group at p<0.05.

Fig. 4. Effects of *C. xanthorrhiza* on ratio of T/B cells, Th/T, and Th/Ts in mice. *Thy 1.2/Ly5*: ratio of PE (Thy 1.2) positive to FITC (Ly5) positive cells. **L3T4/Thy1.2**: percentages of FITC (L3T4) positive cells in PE (Thy 1.2) positive cells. ***L3T4/Lyt2.2**: ratio of FITC (L3T4) positive cells to PE (Goat anti-mouse IgM) positive cells. Each bar shows M±SE for 4 mice per group. *Significantly different from the control group at p<0.05.

Fig. 2). Although this discrepancy is not clear, *C. xanthorrhiza* showed an increased proportion of the splenic nucleated cells bearing the T cell marker Thy 1.2 throughout the dietary period: at 5 weeks (left), and 3 and 4 weeks (center), respectively. When changes in B cell proportion were expressed with respect to the percentage of splenic nucleated cells (right in Fig. 2), a significant elevation in cells
Fig. 5. Effects of *C. xanthorrhiza* on phenotypic distribution of splenic and blood macrophages in mice. **Mac 1 positive cells in the presence of FITC (Mac 1). F4/80 positive cells in the presence of FITC (Goat anti-mouse IgM). Each bar for splenic macrophage distribution shows M±SE for 4 mice per group, and the bar for blood macrophage distribution shows a value for pooled sera. + Single value due to loss of sample.

bearing Ly5 was noted for mice fed the medicinal plant for 3 weeks.

The effect of *C. xanthorrhiza* on the T cell-subset population is shown in Fig. 3. Analysis of the L3T4 marker revealed a statistically significant increase in the proportion of the splenic Th cells in mice fed the medicinal plant for 4 weeks. The proportion of T cells bearing Lyt2.2 indicated no significant change in cytotoxic or suppressor T cells between the *C. xanthorrhiza* and control groups throughout the dietary period.

Figure 4 shows a ratio of T/B (left) and Th/Ts (right) cells, and a percentage of Th in T (center) cells. *C. xanthorrhiza* significantly increased the ratio of T/B cells at 5 weeks, and decreased the percentage of Th in T cells at 3 weeks. No significant effect was observed for Th/Ts cell ratio.

As shown in Fig. 5, *C. xanthorrhiza* did not significantly alter the proportion of
the splenic macrophages bearing each marker Mac 1 and F4/80. The proportion of Mac 1 positive peripheral blood cells tended to be higher in mice fed C. xanthorrhiza as compared with control group throughout the feeding period.

DISCUSSION

In the present study, the proliferative response of splenocytes to PHA, Con A, and PWM was markedly higher in rats fed C. xanthorrhiza after 3-week dietary study. Since both PHA and Con A are mitogens for T cells, and PWM is for T and B cells, this medicinal plant seems to affect B as well as T cell functions. The murine spleen is a readily accessible tissue for study, containing predominantly lymphocytes representative of all the pertinent mature lymphoid subsets and containing a relatively low number of macrophages and other leukocytes (<5%). We did not determine the effect of macrophage depletion on lymphocyte responses, but others have shown that the removal of monocytes or monocyte-derived macrophages had variable effects on responses of purified lymphocytes to different types of mitogen (9). In fact, Bash proposed that T cell mitogens may be affected by changes in the synthesis or function of cytokines produced by T cells and macrophages (10). Interleukin-1 of macrophages has been shown to enhance activation and proliferation of T cells (11).

Subsequently, the effect of C. xanthorrhiza on lymphocyte population was examined by using flow cytometry. For this purpose mice were selected instead of rats since various markers for surface antigens of lymphocytes and macrophages are available for mice. With respect to T cells, the proportion of T cells (based on Thy1.2 expression) increased in mice fed the medicinal plant throughout the feeding period (from 3 to 5 weeks). Proportion of B cells (based on Ly5 expression) also increased in mice fed the plant for 3 weeks, but not in mice fed for longer period. Thus, the effect of medicinal plant on the blastogenesis of B cells may be transient. The different responses of T and B cell population and the ratio of T to B cells to the feeding period suggest that the T cell function as compared to the B cell function seems to be more sensitive to the medicinal plant.

It is known that certain subclasses of T cells vary with regard to sensitivity to irradiation, glucocorticoids and immunosuppressive drugs (12). Significant changes in the proportion of lymphocyte subset can lead to imbalance that affects both the response and regulation of the immune system. In this study, on the contrary to the consistent elevation of T cell population throughout the feeding period, C. xanthorrhiza transiently modified the proportions of T cell subsets: an elevation of Th cells only at 4 weeks with no changes for Ts cells. In addition, C. xanthorrhiza transiently decreased the percentage of Th in T cells at 3 weeks and exhibited no significant change in the ratio of Th/Ts cells. Thus, this medicinal plant appears not to modify the proportion of Th to Ts, but it remains to be possible that the plant may increase T cell subsets other than Th and Ts cells.

Since the effect of the diet on macrophages populations may differ according to
their location, macrophage populations of spleen as well as peripheral blood were examined. In this study, *C. xanthorrhiza* exerted no significant effects on the populations of macrophages bearing markers Mac 1 and F4/80 in these locations. Although the population bearing Mac 1, which react with other than macrophages (13), in the peripheral blood tended to be higher in mice fed *C. xanthorrhiza*, such an effect was not observed for macrophages bearing F4/80, which are assumed to represent mature macrophages (14). *C. xanthorrhiza* rather caused a decreased tendency of the macrophage population expressing the latter marker with an increase of feeding period in the spleen as well as peripheral blood.

Summarizing the results from mitogenic response of lymphocytes in rats and flow cytometric analysis in mice, *C. xanthorrhiza* affected T and B cell-derived immune functions. The effect of this medicinal plant in infected animals remains to be determined.

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