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Effects of Kampohozais (Sho-saiko-to, Ninjin-to, Hochu-ekki-to and Juzen-taiho-to) on Immune Responses in Mice

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Summary. **We have investigated the effects of Kampohozais (Traditional herbal drugs: Sho-saiko-to 小柴胡湯, Ninjin-to 人参湯, Hochu-ekki-to 補中益気湯, and Juzen-taiho-to 十全大補湯) on immune responses in mice. Ninjin-to augmented the natural killer (NK) cell activity by a single intraperitoneal administration at doses of either 50, 100 or 200 mg/kg. Sho-saiko-to also augmented this activity at a dose of 100 mg/kg, but suppressed it at a dose of 200 mg/kg. An oral administration of Sho-saiko-to and Ninjin-to once a day at a dose of 250 mg/kg augmented the NK cell activity 7 and 20 days later, respectively. The effect of the remaining two Kampohozais was negligible. Four Kampohozais each exhibited a stronger mitogenic activity on B cell-rich population than on T cell-rich population, suggesting that these Kampohozais act as B cell mitogens. Treatment of mice with Sho-saiko-to by oral administration once a day for 13 days at a dose of 250 mg/kg increased the number of splenic antibody-producing cells against sheep erythrocytes, accompanied with an increase in both serum hemagglutinin and hemolysin titers.**

INTRODUCTION

Kampohozais (Traditional herbal drugs) are suitable for long term oral administration without adverse reactions. Recently, some Kampohozais have received attention as immune activators, and clinical applications have been attempted for the treatment of viral hepatitis, respiratory diseases, opportunistic infections and certain cancers.[1-3] To research the possibility of clinical applications of Kampohozais for the prevention of the onset of AIDS (acquired immunodeficiency syndrome) in certain hemophilia patients in Japan, we have investigated the effects of Kampohozais: Sho-saiko-to 小柴胡湯, Ninjin-to 人参湯, Hochu-ekki-to 補中益気湯 and Juzen-taiho-to 十全大補湯, on immune responses in mice by the evaluation of NK cell activity, mitogenic activity on the lymphocytes and antibody production.

MATERIALS AND METHODS

Kampohozais: Germ-free lyophilized powders prepared from hot water extracts of Sho-saiko-to, Ninjin-to, Hochu-ekki-to and Juzen-taiho-to were kindly supplied from Tsumura Co., Ltd (Tokyo, Japan). These Kampohozais were weighed under aseptic conditions and dissolved in sterile phosphate-buffered saline (PBS) or sterile distilled water for intraperitoneal (i.p.) or oral administration, respectively.

Mice: Specific-pathogen free C3H/He and ICR males aged six weeks obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) were used.

NK cell-mediated cytotoxicity: C3H/He mice were treated with a single i.p. administration of 0.5 ml of Kampohozais at doses of 50, 100, 200 mg/kg or PBS. Two days later, the pooled spleen cells were prepared from five mice from each group. To evaluate the efficacy of oral administration, mice were treated once a day for 7 or 20 days at doses of 250 or 500 mg/kg. On the day following the last administration, the pooled spleen cells were prepared by the same manner as mentioned above. The cytotoxicity of NK cells in the pooled spleen cells was measured agaist the YAC-1 cells using a 4-h ^{51}Cr release assay. Spleen cell

preparation, target cell labeling, *in vitro* assay conditions and calculation of % cytotoxicity have been described previously.[4] All experimental points indicate the mean±S.D. of triplicate samples.

Mitogenicity assay: The total spleen cell, B cell-rich and T cell-rich population obtained from 10 ICR mice were cultured in an RPMI-1640 medium supplemented with 10% heat inactivated (56℃ for 30 min) fetal bovine serum with or without various concentrations of Kampohozais in a 96-well plate (5×10^5 cells/well) at 37℃. After 48 h incubation, 0.5 μCi of [methyl-^{3}H] thymidine (specific activity: 2 Ci/mM, Amersham, Tokyo, Japan) was added to each well and the cells were further cultured for 20 h at 37℃. Thereafter, the acid-insoluble fraction was collected on a glass filter and the radio activity was measured.[5] A B cell-rich population was prepared by combined treatment with the anti-Thy-1.2 antibody and complement (Cedarlane, Ontario, Canada). A T cell-rich population was prepared by a method using anti-mouse immunoglobulin G (IgG) anti-body (MBL, Tokyo, Japan)-coated plastic flasks according to the method of Quintans et al.[6] Concanavalin A (Con A) and lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma, St. Louis, Mo., USA) were used as T cell- and B cell-specific mitogens, respectively. All experimental points indicate the mean±S.D. of triplicate samples.

Assay of hemolytic plaque forming cells (HPFCs) in spleen cells and titration of hemagglutinin and hemolysin in pooled sera: ICR mice were treated with an oral administration of Sho-saiko-to once a day for 13 days at various doses. On the 4th day of the treatment, mice were intravenously injected with 10^6 sheep red blood cells (SRBC) in 0.2 ml PBS. On the 14th day of the drug treatment, hemolytic plaque forming cells (HPFCs) in the pooled spleen cells (4×10^5 cells) from 5 mice of each group were measured by two different methods of Imai et al.[7]: one for the detection of immunoglobulin M (IgM)-producing cells was designated the direct method, in which a sufficient amount of complement was directly added to the agar media containing a mixture of spleen cells and SRBC; the other for the detection of IgG-producing cells in addition to IgM-producing cells was designated the indirect method, in which the anti-mouse IgG antibody was reacted before adding the complement. All experimental points indicate the mean±S.D. of quadriplicate samples. At the same time, hemagglutinin and hemolysin titers of pooled sera from 5 mice of each group were assayed against SRBC.[7]

RESULTS

The effect of Kampohozais on the NK cell activity

According to the i.p. administration, Sho-saiko-to and Ninjin-to in the four tested Kampohozais augmented NK cell activity. While Sho-saiko-to had such an effect at a dose of 100 mg/kg, it instead suppressed it at a dose of 200 mg/kg (Fig. 1A), whereas Ninjin-to increased this activity at all tested doses (Fig. 2A). On the other hand, according to the oral administration once a day for 7 days at a dose of 250 mg/kg, only Sho-saiko-to increased NK cell activity (Fig. 1 B). At the higher dose (500 mg/kg), the four Kampohozais exhibited negligible effects (data not shown). When mice were treated with Ninjin-to, Hochu-ekki-to or Juzen-taiho-to at a dose of 250 mg/kg for as long as 20 days, only Ninjin-to augmented the NK cell activity (Fig. 2B). No augmentation or suppression of the NK cell activity were observed in these mice treated with Hochu-ekki-to and Juzen-taiho-to by any manner of administration or at any dosages of drugs (data not shown).

Mitogenic activity of Kampohozais on the spleen cells

In preliminary experiments to evaluate the mitogenic activity on the total spleen cells from ICR mice cultured in the presence of 1.25 to 50 μg/ml of Kampohozais, the highest ^{3}H-thymidine incorporation was observed at a dose of either 5 or 10 μg/ml in all the Kampohozais by a 3.1 to 3.8-fold increase compared with that of a drug-free culture (2618±612 cpm). Mitogenic effects of 5 and 10 μg/ml were almost similar, but those of the remaining doses were negligible in all the Kampohozais. In the presence of LPS (20 μg/ml) and Con A (1 μg/ml), 5.6- and 21.3-fold increases, respectively, were obtained in thymidine incorporation. Thus, we investigated which B or T cell-rich population was more sensitive to the mitogenic effect of 10 μg Kampohozais/ml. As shown in Fig. 3, the relative rates of ^{3}H-thymidine incorporations in the presence of Con A were 2.7 and 41.7 for B and T cell-rich populations, respectively. In the case of LPS, these values were 8.2 and 2.0 for B and T cell-rich populations, respectively. These data indicate that isolated cell populations might allow us to test the specificity of the mitogenic effect with respect to B or T cell mitogen. As a result, tested Kampohozais each increased the ^{3}H-thymidine incorporation 4.1 to 5.3-fold only in a B cell-rich population, suggesting that all the Kampohozais used in this

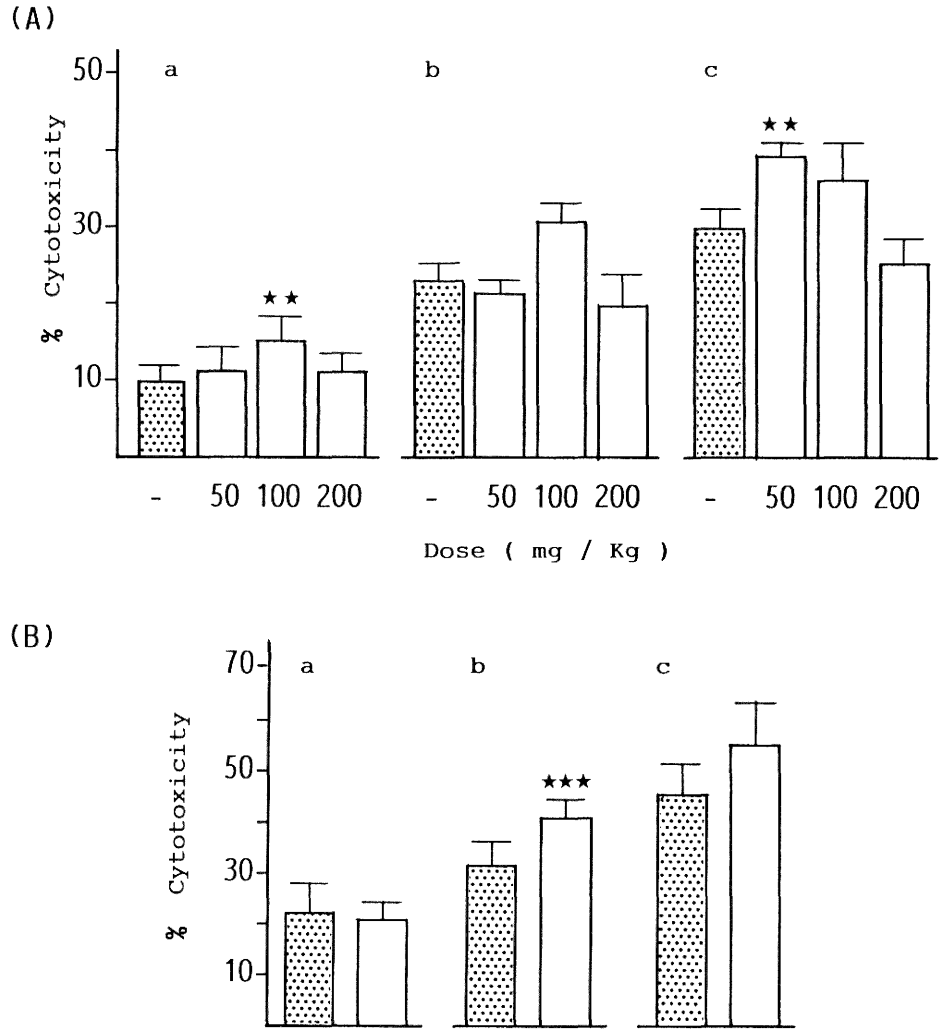

Fig. 1. Effect of Sho-saiko-to on the NK cell activity. Mice were treated with a single i.p. administration at doses of 50, 100 or 200 mg/kg (**A**), or an oral administration at dose of 250 mg/kg once a day for seven days (**B**). On the second day (**A**) or the next day (**B**) after the last administration, NK cell activity in the pooled spleen cells was assayed at E: T ratios of 20:1 (a), 501 (**b**) and 100:1 (**c**). All experimental points indicate the mean (thick bar)±S.D. (thin bar) of triplicate samples. Double and triple stars indicate statistically different results from the control (dotted bars) with $p<0.05$ and 0.01, respectively.

study might act as B cell mitogens.

The effect of Sho-saiko-to on antibody production against SRBC

The effect of Kampohozais on antibody production against SRBC was investigated using Sho-saiko-to as a representative of the four Kampohozais tested in this study (Fig. 4). By the indirect method for the assay of IgG-producing cells in addition to IgM-producing cells, a significant increase in the number of HPFCs was observed at a dose of 250 mg/kg, but not at doses of 100 and 750 mg/kg. By the direct method for the assay of IgM-producing cells, a low but recognizable increase was obtained at a dose of 250 mg/kg, probably reflecting the relatively longer period such as 10 days after immunization. Concomitant with an increase in the number of HPFCs, the serum hemagglutinin and hemolysin titers increased 8 to 4-fold respectively, at a dose of 250 mg/kg compared with those of untreated mice.

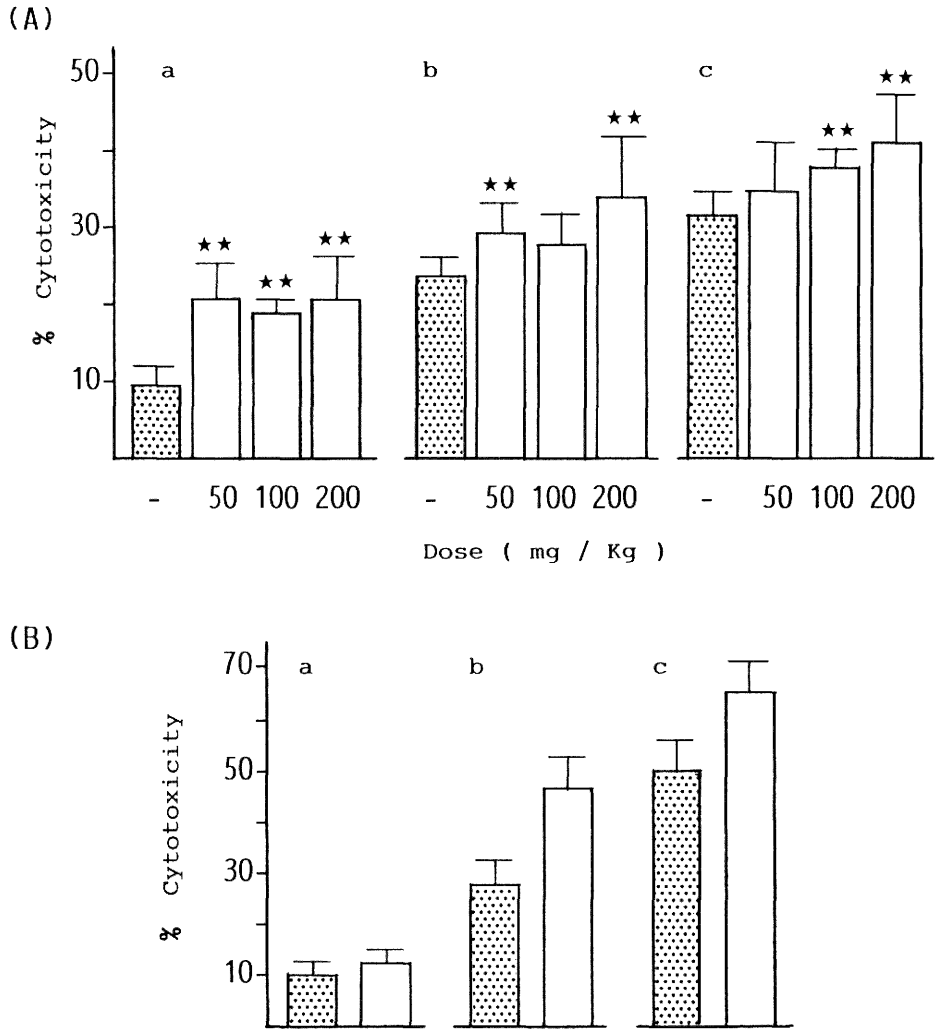

Fig. 2. Effect of Ninjin-to on the NK cell activity. Mice were treated with a single i.p. administration at doses of 50, 100 or 200 mg/kg (**A**), or an oral administration at a dose of 250 mg/kg once a day for 20 days (**B**). On the second day (**A**) or the next day (**B**) after the last administration, NK cell activity in the pooled spleen cells was assayed at E:T ratios of 20:1 (**a**), 50:1 (**b**) and 100:1 (**c**). All experimental points indicate the mean (thick bar)± S.D. (thin bar) of triplicate samples. Double stars indicate statistically different results from the control (dotted bars) with $p<0.05$.

DISCUSSION

We demonstrated that two Kampohozasis, Sho-saiko-to and Ninjin-to, augmented the NK cell activity. However, the remaining two, Hochu-ekki-to and Juzen-taiho-to, did not, in accordance with previous reports.[3,8] In the case of a single i.p. administration, Ninjin-to augmented the NK cell activity at all doages of 50 to 200 mg/kg. Sho-saiko-to also augmented this activity only at a dose of 100 mg/kg, but suppressed it at a dose of 200 mg/kg. On the other hand, a successive oral administration of Sho-saiko-to was able to augment the NK cell activity in a shorter period compared with Ninjin-to. These data suggest that the manner of augmentation of the NK cell activity of the two Kampohozais is different with respect to doses and duration of the treatment. In order to evaluate an *in vitro* mitogenic activity of Kampohozais, the splenic lymphocytes were cultured in the presence of 1.25 to 50 μg/ml of Kampohozais. The four Kampohozais each showed mitogenic activity only within a narrow dosage range (5 to 10 μg/ml; data not shown). Hence, 10 μg/ml was used, and the four Kampohozais at this dose showed mitogenic activity selectively on the B cell-rich population.

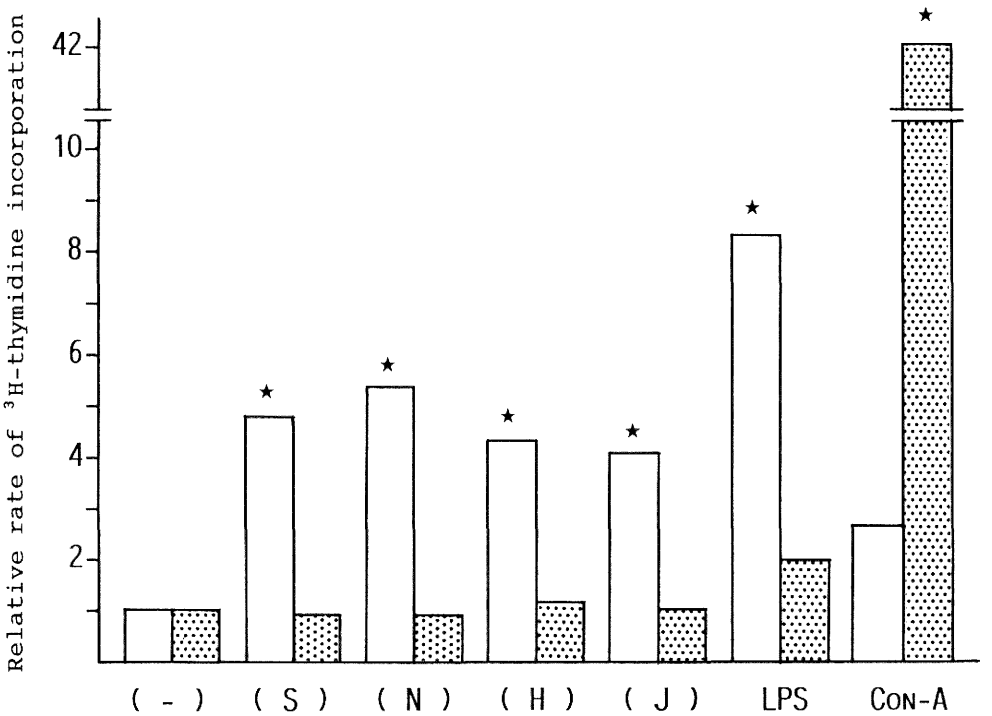

Fig. 3. Mitogenic activity of Kampohozais on the splenic B cell and T cell-rich populations. Mitogenic activities of Sho-saiko-to (S), Ninjin-to-(N), Hochu-ekki-to (H) and Juzen-taiho-to (J) (each 10 μg/well) on the B cell (open bars)- and T cell (dotted bars)- rich populations from ICR mice were assayed. LPS (20 μg/well) and Con A (1 μg/well) were used as the controls for B cell and T cell-specific mitogens, respectively. The relative rate of thymidine incorporation in the ordinate is calculated as 1.0 for the mean of triplicate drug-free cultures (537 ± 67 and 943 ± 98 cpm for B and T cell-rich populations, respectively). Single star indicates statistically different results from the drug-free culture (−) with $p<0.01$.

Sho-saiko-to also increased the number of antibody-producing cells in the spleen of ICR mice after successive oral administrations only at a dose of 250 mg/kg. In accordance with our results, Iwana et al. reported that the long term oral administration of Hochu-ekki-to, Juzen-taiho-to and Toki-shakuyaku-san stimulated antibody production in ICR mice within an optimal dose range.[1)]

Furthermore, Kumazama et al. demonstrated that the polysaccharide isolated from Angelicae radix (Toki) activated the murine B cells polyclonally and differentiated to antibody-producing cells even in the absence of either the helper T cell or macrophages *in vitro*.[9)] In the four Kampohozais used in this study, Toki was included in Hochu-ekki-to and Juzen-taiho-to, but not in Sho-saiko-to and Ninjin-to. Hence, it is suggested that some components other than Toki can show the mitogenic activity and stimulate antibody production in the latter two Kampohozais. On the other hand, it has been shown that the augmentation of NK cell activity rather suppressed the antibody production of B cells.[10,11)] In contrast, this study demonstrated that Sho-saiko-to stimulated both NK cell activity and antibody production. Because Sho-saiko-to contains at least seven components, such immune action of Sho-saiko-to might be explained as follows: certain components augmented NK cell activity, whereas the other components negated the suppressive effect of NK cells on the antibody production. Alternatively, the stimulation of antibody production by the mechanism revealed in Kumazawa's report[9)] might be independent of the regulatory effect of NK cells. However, further studies on each component of Sho-saiko-to are necessary.

In the early stage of microbial (bacteria, viruses and parasites) infections, nonspecific host defense factors such as interferons, macrophages and NK cells represent the main line of defense.[12)] On the other hand, it is well known that antibodies show a neutralizing activity against microbial infections in an antigen-specific manner. Considering that most AIDS patients die of opportunistic infections, the augmentation of nonspecific host defense factors and serum antibody titers are important to prevent op-

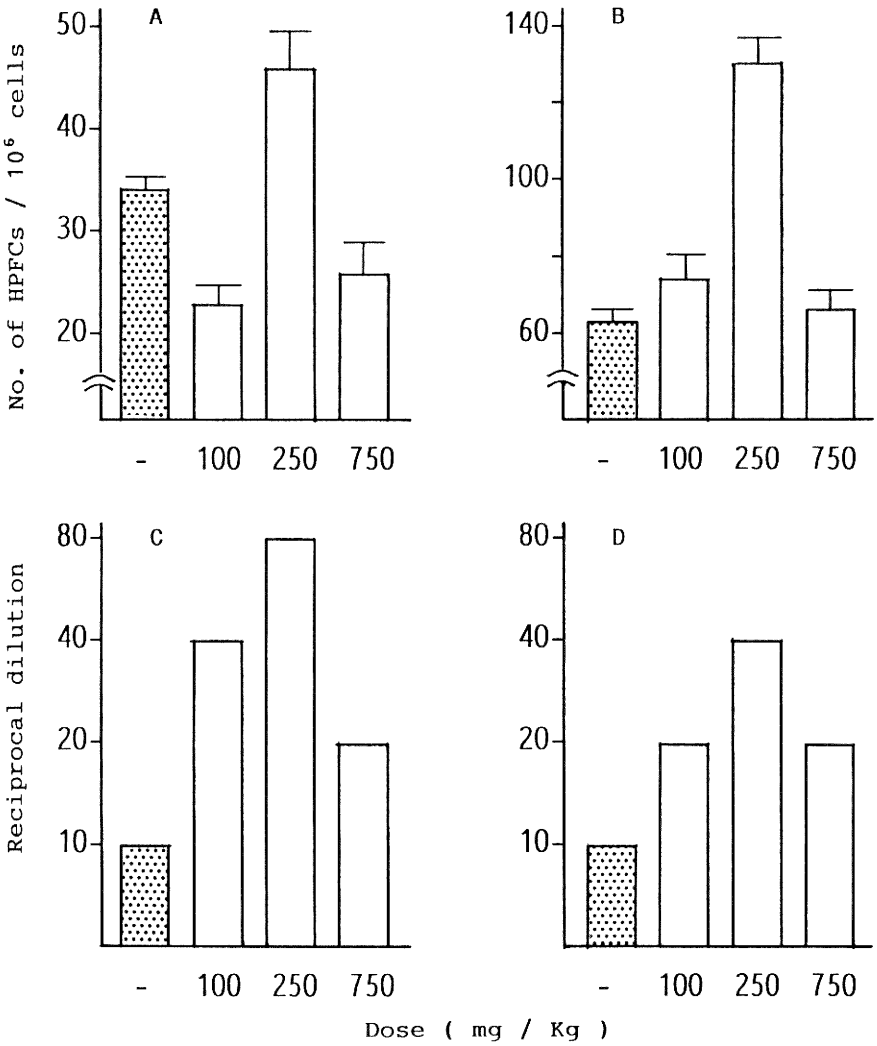

Fig. 4. Effect of Sho-saiko-to on antibody production. Hemolytic plaque forming cells (HPFCs) were assayed by direct (**A**) and indirect (**B**) methods. All experimental points indicate the mean (thick bar)±S.D. (thin bar) of quadruplicate samples. At the same time, hemagglutinin (**C**) and hemolysin (**D**) titers in the pooled sera were examined. Serum antibody titers in the ordinate are expressed as the highest reciprocal serum dilution showing complete hemagglutination or hemolysis.

portunistic infections in AIDS patients.

It is interesting to examine the effect of Kampohozais on the behavior of lymphocyte subsets. Although the data of flow cytometric analysis were not shown, Sho-saiko-to seemed to exhibit its activity primarily on the suppression of Lyt2-positive T cells, and the other Kampohozais seemed to exhibit their activities on the stimulation of both Lyt2 and L3T4 T cell subsets. To obtain more information and reach further insights into the mechanism of the immune actions of Kampohozais, we are considering assaying the induction of various lymphokines by the Kampohozais themselves and their components in addition to flow cytometric analysis of lymphocyte subsets.

Our results indicate that the Kampohozais used in this study have the potential for immune action in mice within an optimal dose range, but the manner for exhibiting the effects might be different for each Kampohozai. In light of these findings, it is suggested that studies on the clinical applications of these Kampohozais might prove significant for the prevention of the onset of AIDS in hemophilia patients.

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