Arch. Pharm. Res. **14**(3), 217-224 (1991)

Immunomodulating Activities of Water Extract from *Xanthium strumarium* (II): Immunostimulating effects of the water layer after treated with chloroform

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(Received June 8, 1991)

Abstract □ One of water and/or methanol extracts from 14 herbal drugs which were screened using murine splenocytes showed immunosuppressive activities previously. After water extract from *Xanthium strumarium* was treated with chloroform, 100 μg/m*l* of water layer (XS-WC1) has very strong immunostimulating activities tested by ^{3}H-thymidine incorporation (control vs 100 μg/m*l*, 4962 cpm vs 69515 cpm). MLR also appears to be stimulated strongly (control vs 100 μg/m*l*, 26345 cpm vs 78688 cpm). When 100 μg/m*l* of XS-WC1 and 0.8 μg/m*l* of concanavalin A (ConA) were added, more ^{3}H-thymidine were incorporated significantly, compared with 0.8 μg/m*l* of ConA only. In contrast with ConA, results from 5 μg/m*l* of lipopolysaccharide (LPS) and 100 μg/m*l* of XS-WC1 were not different, compared with 5 μg/m*l* of LPS only. These results indicated that responses of XS-WC1 to B cell and T cell may be different. XS-WC1 was injected intraperitoneally (10 mg/kg, 50 mg/kg, 100 mg/kg) for 4 days or 10 days and tested secretion of IgM or IgG by direct and indirect hemolytic plaque-forming cell assays, respectively. Numbers of hemolytic plaques for both IgM and IgG were increased significantly. Especially, secretion of IgGs was increased more than 10 times. After administration of XS-WC1 for 7 days (50 mg/kg, 100 mg/kg) splenomegaly due to graft vs host reaction was observed. Human lymphocytes separated from whole blood by Ficoll-Hypaque method were also proliferated after treatment of 10 μg/m*l* and 50 μg/m*l* of XS-WC1. As seen in murine lymphocytes, human lymphocyte proliferation was increased synergistically after treatment with both of XS-WC1 and phytohemagglutinin (PHA). It appears that XS-WC1 may have potential immunostimulating activities and that it remains to be purified further for isolation of active components.

Keywords □ Murine lymphocytes, human lymphocytes, ^{3}H-thymidine incorporation, MLR, graft vs host reaction, hemolytic plaque-forming cell assay, immunostimulating activities, chloroform treated aqueous extracts of *Xanthium strumarium*

It was reported that *Panax ginseng* and *Angelica gigantis* have immunostimulating activities in humoral or cell-mediated immune responses[2,3,21,23]. Even though *Xanthium strumarium* has been used for treatment of acute or chronic rhinitis, rheumatic arthritis or eczema clinically in oriental medicine[4], its immunomodulating effects were not studied systematically. Chemical and physical properties of several components of it and its anticancer activities were published[5]. Previously, we reported that the water extract from *Xanthium strumarium* appeared to be remarkable immunosuppressive by *in vitro* assays[1]. Splenomegaly from graft vs host reaction (GVH) observed in the previous paper gives us puzzling questions because it was thought not to be immunosuppressive. After treatment of the water extract from *Xanthium strumarium* with chloroform, aqueous layer (XS-WC1) showed immunostimulating activities surprisingly. Mitogenic effects, interaction with ConA and LPS, and mixed lymphocyte

reaction (MLR) were tested in order to examine *in vitro* immune reaction. Direct and indirect hemolytic plaque assays and GVH were performed to confirm *in vivo* effect on immune system. Effects of XS-WC1 on human lymphocytes separated from whole blood by Ficoll-Hypaque method were evaluated in this paper.

EXPERIMENTAL METHODS

Animal

BALB/c and C57BL/6 mice kept in specific pathogen free condition were provided by Toxicology Research Center, Korea Research Institute of Chemical Technology, Taejeon and were 8-12 weeks old when they were used for all experiments. Animal rooms were controlled by 12 hour light and dark cycle at 22℃ and 55-60% humidity. Animals were fed solid food (Samyang Company, Seoul, Korea) and water *ad. libitum*.

Preparation of herbal extracts

As shown in Fig. 1, *Xanthium strumarium* was extracted with boiling water and treated with chloroform (E.P. grade, Oriental Chemical Co., Seoul, Korea). The water layer was separated and concentrated by a rotary evaporator (Buchi, Switzland) at reduced pressure. It was lyophillized by freezing dryer (FTS system, USA) overnight and used as experimental drugs being designated XS-WC1 which will be used in rest of this paper. XS-WC1 was dissolved in complete medium including RPMI 1640 (GIBCO, NY, USA), 10% fetal calf serum (Hyclon, Utah, USA), 20 mM HEPES, 2.5×10^{-5} M mercaptoethanol (Sigma St. Louis, MO, USA), 50 units/m*l* penicillin and non-essential amino acid (GIBCO, NY, USA) and sterilized through 0.2 μm filter (Acrodisc, MI, USA). The concentration of XS-WC1 was always adjusted with complete medium.

Mitogenic effects

In order to examine proliferation of normal murine or human lymphocytes by the drugs, ^{3}H-thymidine incorporation experiment was done using single cell suspension of murine spleen described in the previous paper[6] or human whole blood[7]. Human O type blood was purchased from Red Cross, Taejeon and separated by Ficoll-Hypaque (Sigma, MO, USA) to collect lymphocytes. After

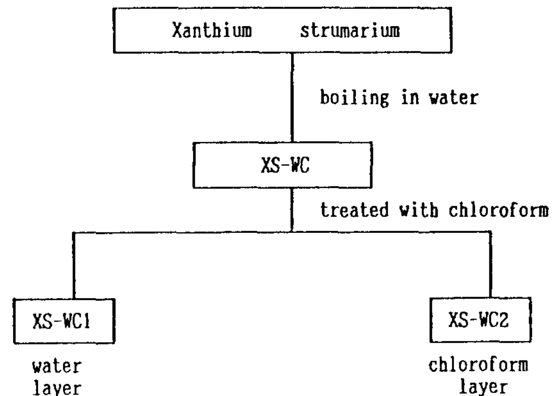

Fig. 1. Scheme of extraction procedure. See in experimental methods.

washing with washing medium including 2% FCS three times, cell pelletes were resuspended in complete medium. Viability was tested by trypan blue exclusion method. Tested lymphocytes were always viable at least over 90%. The single cells (2×10^5 cells/well) and adjusted concentration of XS-WC1 were cultured in 96 well plate (Nunclon, Rockside, Denmark) for 44 hours at 37℃ in 5% CO_2. After 4 hour pulse with ^{3}H-thymidine (New England Nuclear, MA, USA), cells were collected with a cell harvester (Cambridge, MA, USA) and counted by a scintillation counter (Packard, IL, USA)[8]. All experiments were done in triplicate. Prednisolone (10 μg/m*l*, Sigma, MO, USA), LPS (20 μg/m*l*, Difco, MI, USA) and ConA (5 μg/m*l*, GIBCO, NY, USA) were used as controls of murine lymphocyte experiment and PHA (GIBCO, NY, USA) and pokeweed mitogen (PWM, GIBCO, NY, USA) were used as those of human lymphocyte experiment.

Interaction with mitogens[9]

Mixture of ConA or LPS (*E. coli* 055 : 135) with XS-WC1 were tested using murine lymphocytes and mixture of PHA or PWM with XS-WC1 were tested using human lymphocytes. These experiments were done at moderate mitogenic level of each mitogen, for example, at 0.8 μg/m*l* of ConA, 5 μg/m*l* of LPS, 2% of PHA and 1% of PWM. As described above in the mitogenic effect, single cells and mixture of each mitogen and XS-WC1 were cultured together in 96 well plate at 37℃ in 5% CO_2. After 4 hour pulse of ^{3}H-thymidine, cells were harvested and counted by a scintillation counter. All experiments

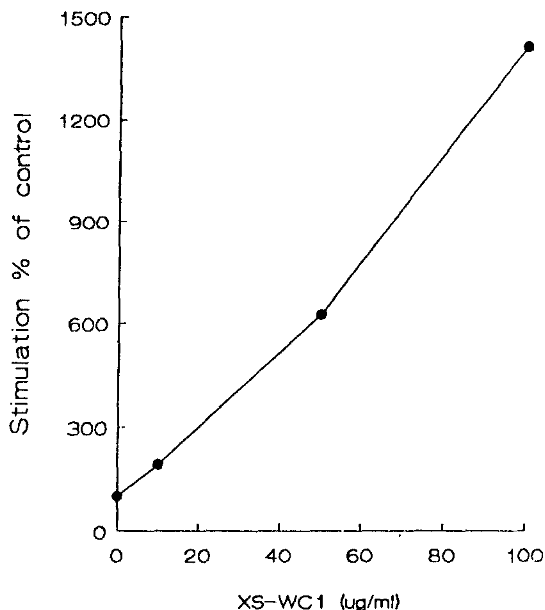

Fig. 2. Stimulating effect of XS-WC1 on the mouse lymphocyte proliferation.
The data were represented as the percentage of stimulation based on response in the control. XS-WC1 was added when culture was started. Radioactivity of prednisolone treated group was 169 cpm and those of LPS and ConA treated group were 122395 cpm and 12959 cpm, respectively.

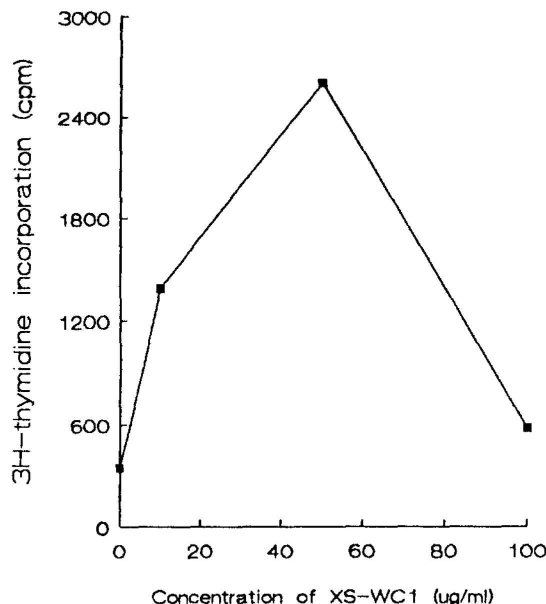

Fig. 3. Stimulating effect of XS-WC1 on human lymphocyte proliferation.
The data were represented as mean cpm of [^{3}H]TdR uptake in triplicate. XS-WC1 was directly added when culture was started. The data were represented as the percentage of reaction based on response in the control. Radioactivity of PHA and PWM treated groups were 18977 cpm and 15422 cpm, respectively.

were done in triplicate.

Mixed lymphocyte reaction (MLR)

It was tested whether XS-WC1 can influence one-way MLR[10] which consists of responder cells (C57 BL/6, H-2^b) and mitomycin C treated stimulant cells (BALB/c, H-2^d). Responder cells and stimulant cells were prepared as the same as the above in mitogenic effect and then only stimulant cells were treated with mitomycin C (0.5 μg/m*l*, Boeringer Mannheim Biochemical, NJ, USA) which is an inhibitor of DNA synthesis. After 30 min incubation with mitomycin C at 37°C, the cells were washed three times and resuspended in complete medium and used as stimulant cells. A mixture of two strain cells (5×10^5 of each strain cells) was incubated with adjusted concentration of XS-WC1 at 37°C in 5% CO_2 for 68 hours. After 4 hour pulse with ^{3}H-thymidine, the cells were harvested and counted with a scintillation counter. All experiments were done in triplicate. Prednisolone was used as a control drug (10 μg/m*l*).

Hemolytic plaque-forming cell (PFC) assay

Mishell and Dutton's method which was a modified Jerne's agar plaque technique was performed[11-16]. Sheep red blood cells (SRBC) were used as antigen of immunization and *in vitro* assay. BALB/c mice were immunized with 1×10^9 SRBC intravenously on day 0 and treated with 10 mg/kg, 50 mg/kg and 100 mg/kg of XS-WC1 intraperitoneally for 4 days from immunization day. Control group of the mice was treated with saline only. On day 4, spleens were removed and prepared as single cell suspension. Splenocytes and SRBC were mixed with 0.4% agar (FMC, MF, USA) and incubated in presence of guinea pig complement (GIBCO, NY, USA) for 2 hours in CO_2 incubator (Forma Scientific, Ohio, USA). Indirect hemolytic PFC assay was also done after 10 day intraperitoneal administration of XS-WC1. As secreting IgG needs anti-IgG

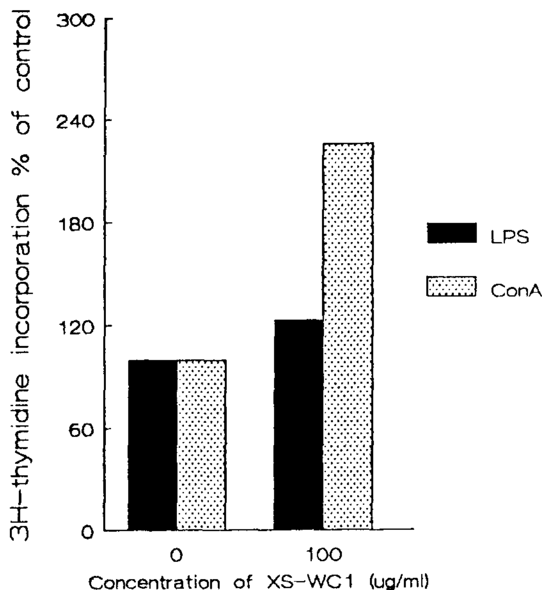

Fig. 4. Interaction of XS-WC1 with ConA or LPS. The data were represented as the percentage of reaction based on response in control. XS-WC1 was directly added to the culture at the initiation of assay.

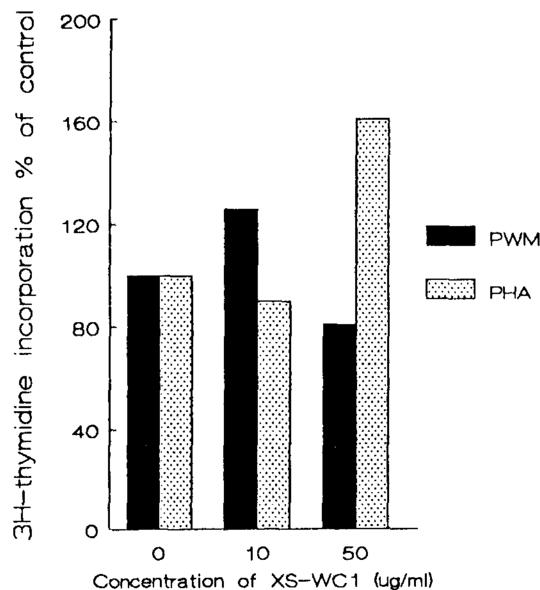

Fig. 5. Interaction of XS-WC1 with PHA or PWM. The data were represented as the percentage of reaction based on response in control. XS-WC1 was directly added to the culture at the initiation of assay.

in order to bind to guinea pig complement, a mixture of cells and SRBC was incubated for 1 hour with rabbit anti-mouse IgG prepared by several immunization of mouse IgG in our laboratory and then incubated with guinea pig complement. Hemolytic plaques were counted. Each group of the animal consisted of three mice and each experiment was tested in triplicate.

Graft vs host reaction (GVH)

GVH was done by slightly modified Jan Klein method[17]. Splenocytes of C57BL/6 mice were prepared to single cell suspension as the same as the above in mitogenic effect. Ten million cells were injected intravenously into BDF1 (C57BL/6×DBA/2) as recipient. For 7 days, 50 mg/kg and 100 mg/kg of XS-WC1 were administered intraperitoneally. Seven days later, spleens were removed and weighed. Syngeneic control mice were injected with same amount of BDF1 splenocytes intravenously. Spleen index was calculated as following;

$$\text{Spleen Index(S.I.)} = \frac{\text{allogeneic spleen wt./body wt.}}{\text{syngeneic spleen wt./body wt.}}$$

Cyclophosphamide (100 mg/kg, Sigma, MO, USA) was used as a control durg. It was injected every other day intraperitoneally.

Statistical analysis

All the results of experiments were evaluated using the student' t-test.

RESULTS

Mitogenic effect

As seen in Fig. 2 and 3 XS-WC1 can stimulate proliferation of murine and human lymphocytes. Murine lymphocytes were proliferated most at 100 μg/m*l* but human lymphocytes were proliferated most at 50 μg/m*l* and less at 100 μg/m*l*. We observed less proliferation of murine lymphocytes at 500 μg/m*l* (the data were not shown). Even though proliferation of murine and human lymphocytes was less than well-known mitogen such as LPS, ConA, PHA, or PWM which are very toxic to clinical trials, substantial increase of proliferation which show over 10 times more at 100 μg/m*l* than control in murine lymphocytes makes us study further. As

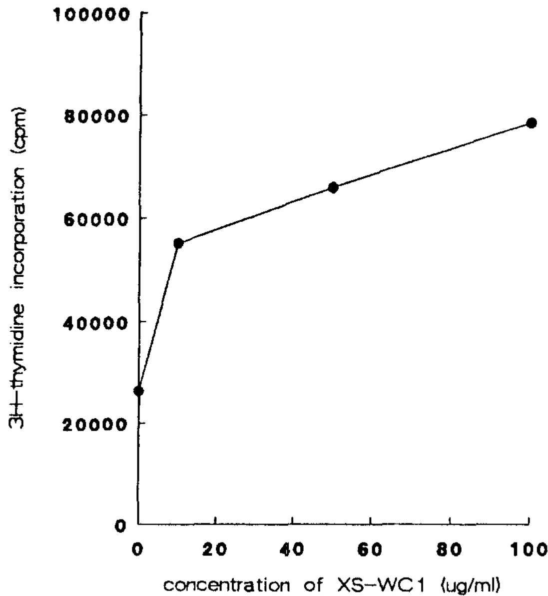

Fig. 6. Stimulation of mixed lymphocyte reaction of XS-WC1.

The data were represented as mean cpm of [^{3}H]TdR uptake. XS-WC1 was added when culture was started. C57BL/6 and BALB/c mouse cells were used as responder and stimulant, respectively. Stimulant cells were treated with mitomycin C (50 μg/m*l*). Radioactivity in prednisolone treated group was 209 cpm.

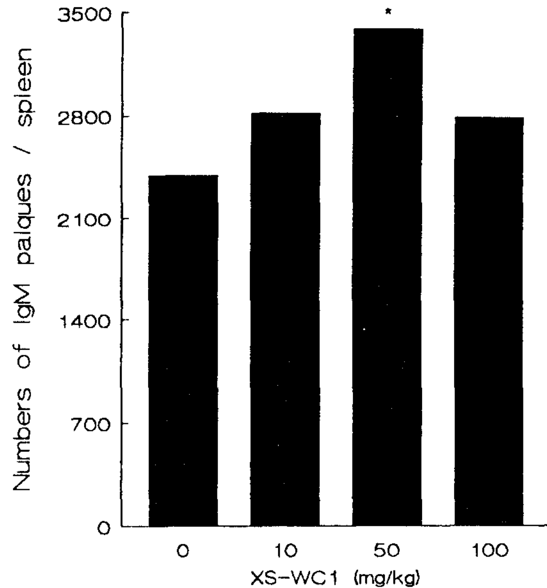

Fig. 7. Effect of XS-WC1 on the secretion of IgM tested by direct hemolytic plaque forming cell assay.

Results were represented as mean of three animals. Each animal was tested in triplicate. XS-WC1 was administered intraperitoneally for 4 days.

*$p<0.05$ as compared with control.

described in the previous paper[1], whole aqueous extract suppressed proliferation of murine lymphocytes almost same level as prednisolone did. But after treatment with chloroform, suppression fraction was moved into chloroform layer which showed remarkable suppression of lymphocyte proliferation (the data will be shown in next paper).

Interaction with well known mitogen

In order to examine how lymphocyte proliferation by XS-WC1 interact with LPS, ConA, PHA and/or PWM, mixture of XS-WC1 and either LPS or ConA for murine lymphocytes and mixture of XS-WC1 and either PHA or PWM for human lymphocyte were tested. Data were shown in Figs. 4 and 5. When 100 μg/m*l* of XS-WC1 and 0.8 μg/m*l* of ConA were tested, synergistic increase of murine lymphocyte proliferation was observed. As seen in murine lymphocytes, PHA which is T cell mitogen of human lymphocytes was influenced by 50 μg/m*l* of XS-WC1. Proliferation by LPS or PWM which is B cell mitogen was not changed by XS-WC1 which was used in these experiments.

Mixed lymphocyte reaction

To examine whether XS-WC1 can also influence allogeneic stimulation by two strains which are different in H-2 region, one-way MLR was performed. As seen in Fig. 6, even 10 μg/m*l* stimulated MLR twice. Maximum response was observed at 100 μg/m*l*. Higher concentration could stimulate less than 100 μg/m*l* (the data not shown).

Hemolytic plaque forming cell assay

All the above results support stimulation of immune response *in vitro*. Further proofs of stimulation of immune response *in vivo* were studied by hemolytic plaque forming cell assay for humoral response and GVH for cellular response. As IgM was secreted first and IgG was secreted later, time schedule of administration of XS-WC1 was adjusted as 4 day administration for IgM response and 10

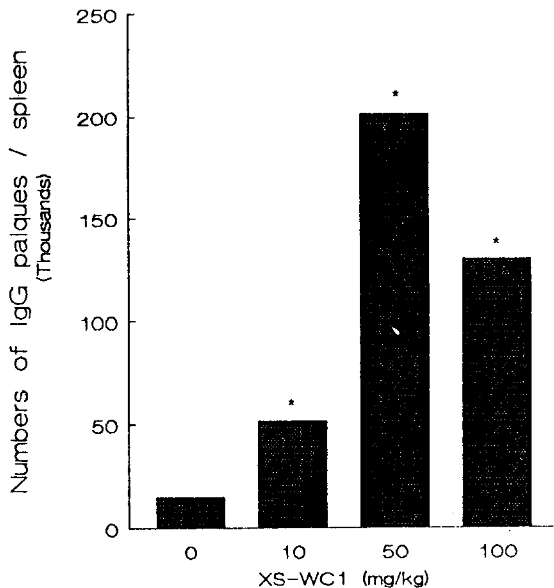

Fig. 8. Effect of XS-WC1 on the secretion of IgG tested by indirect hemolytic plaque forming cell assay. Results were represented as mean of three animal. Each animal was tested in triplicate. XS-WC1 was administered intraperitoneally for 10 days. Anti-IgG serum was obtained from rabbits after periodic immunization by subcutaneous injection of mouse whole serum which was collected in our laboratory.
*p<0.05 as compared with control.

day administration for IgG response. As seen in Fig. 7, IgM secretion was increased most at 50 mg/kg. Surprisingly, IgG response was stimulated more than 10 times when 50 mg/kg of XS-WC1 was administered intraperitoneally, as seen in Fig. 8.

Graft vs host reaction

GVH is known to be the model of allogeneic transplantation. From Table I, we know XS-WC1 could also stimulate GVH response. When 50 mg/kg and 100 mg/kg of XS-WC1 were administered for 7 days, both groups showed splenomegaly compared with the control group ($p<0.05$). It was observed that XS-WC1 did not change the size of spleen in syngeneic group (the data not shown).

DISCUSSION

In oriental medicine, *Xanthium strumarium* was used for treatment of rheumatism which is known as one of autoimmune diseases but immunological studies on it were not done yet[4]. Therefore, original studies on it were focused on immunosuppressive activities as we described in the previous paper[1]. Interestingly, we discovered this herbal drug has various activities, not only immunosuppressive but also immunostimulating activities. It is easy to understand these phenomena, because plants contain plenty of active components which are either specific or common components. Only a few of them are pharmacologically active and interesting to develop as new drugs. The clue to dissect these opposite activities was treatment of chloroform. After extraction with chloroform as shown in Fig. 1, immunosuppressive components were moved into chloroform layer (XS-WC2) and immunostimulating components into aqueous layer (XS-WC1). From Figs. 2-5 and 6, we realized that XS-WC1 may proliferate murine and human lymphocytes *in vitro*. Especially, when XS-WC1 was added to T cell mitogens such as ConA or PHA, synergistic increase of proliferation was observed. But when it was added to B cell mitogen such as LPS or PWM, no influence of proliferation was observed. It indicated that stimulation of lymphocytes by XS-WC1 may be different in B and T cells. It remains to be characterized on mechanism of immunostimulating activities of XS-WC1 using functional studies and/or flow cytometry after separating different cell population. In order to confirm the *in vivo* effect, XS-WC1 was administered intraperitoneally and examined for humoral and cellular responses. As seen in Figs. 7 and 8 and Table I, both of *in vivo* immune responses were also stimulated. IgG secretion in the XS-WC1 treated group were increased remarkably. There are so many diseases which are caught when host immune responses are poor[18,19,22]. A few immune stimulating agents such as LPS are known, but they were not successful in clinical trial because most of them are toxic[20]. It is interesting enough to purify further to isolate active components.

CONCLUSION

Murine as well as human lymphocytes were proliferated by XS-WC1 *in vitro*. XS-WC1 increased lymphocyte proliferation by ConA or PHA synergistically but not lymphocyte proliferation by LPS or

Table I. Graft vs. host reaction of XS-WC1

Group	Donor	Recipient	Spleen wt./body wt.	Spleen index
Saline	C57BL/6	BDF1	3.97± 0.785	1.16
Saline	BDF1	BDF1	3.43± 0.0943	1.00
CP[a]	C57BL/6	BDF1	1.47± 0.170	0.429
XS-WC1[b]				
50 mg/kg	C57BL/6	BDF1	6.19± 0.957	1.81*
100 mg/kg	C57BL/6	BDF1	5.81± 0.163	1.69*

*p<0.05 as compared with control.
[a]Cyclophosphamide was intraperitoneally injected three times for 7 days at 100 mg/kg.
[b]XS-WC1 was intraperitoneally administered for 7 days.

PWM. After intraperitoneal administration of XS-WC1, both of IgG and IgM secretion were stimulated remarkably. Splenomegaly due to GVH was increased significantly after intraperitoneal treatment of XS-WC1. It appears that XS-WC1 may have strong immunostimulating activities.

ACKNOWLEDGMENT

We are thankful to Toxicology Research Center in KRICT for supplying the animals.

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