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Original Article

Heavy oil fractions induce negative influences on mouse immune system

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ABSTRACT — It is well known that heavy oil such as pollutant caused serious influences on the marine ecosystem. We may suffer from various disorders in our body via intake of marine foods polluted with heavy oil. However the influences of heavy oil on our immune system have not yet been clarified. Here we show the effects of heavy oil extracts, water-soluble fraction (WSF), methanol-soluble fraction (MSF) and ethanol-soluble fraction (ESF), on immunoglobulin production of mouse splenocytes. All extracts increased IgA productivity of splenocytes. In oral administration, shrinkage of the immune organs such as spleen or thymus was observed in only WSF-administrated mice at least during 7 days. The amount of IgG production level in splenocytes cultured medium and sera were reduced by each extract administration. A flowcytometry method, to monitor splenocytes of WSF-administrated mice, has been set up using double staining with B and T cell-specific surface antibody. The results from cell population analysis indicated that B cells, including plasma cells producing antibody were reduced. The decrease in IgG level in sera was caused by reduction of plasma cells in spleen. Hence, it is suggested that reduction of Ig production was affected by the chemical compounds contained in WSF possibly such as polycyclic aromatic hydrocarbons (PAHs) through the estrogen receptor expressed in lymphocytes.

Key words: Immune response, Oral administration, Splenocyte, Estrogenic activity, Flowcytometry, WSF

INTRODUCTION

Heavy oil pollution causes serious adverse influence on the marine ecosystem. High incidence of heavy oil spills from shipwrecks of heavy oil tankers is one of the major sources of marine heavy oil pollution. In recent years, a Russian tanker, the Nakhodka, was wrecked in the Japan Sea. A large quantity of heavy oil that leaked out from this accident spread along the coastline of the northern part of Japan. Many researchers have investigated the influence of this catastrophic event on marine ecosystem (Kasai *et al.*, 2001; Yamamoto *et al.*, 2003; Hayakawa *et al.*, 2006). The influence was examined from various viewpoints of the marine ecosystem, typically dealing with target species such as mussel, fish and sea birds (Griffin and Calder, 1977; Lemiere *et al.*, 2004; Alonso-Alvarez *et al.*, 2007; Nakayama *et al.*, 2008). On the other hand, there are few reports on the influence of the heavy oil on human health. The effects of chemical substances contained in heavy oil on human health, especially on the immune system, have not yet been well clarified. Although direct exposure to heavy oil itself may be low, it cannot be ignored that there is a possibility that the high concentration of chemical substances contained in heavy oil can occur through bioaccumulation.

Polycyclic aromatic hydrocarbons (PAHs) such as benzopyrene are well known as chemicals contained in heavy oil (Díez *et al.*, 2005; Hayakawa *et al.*, 2006; Navas *et al.*, 2006; Saco-Alvarez *et al.*, 2008). These chemicals act as carcinogens or mutagens (Arcaro *et al.*, 2001). Therefore exposure to heavy oil can be a serious health hazard. Here, we focused on the influence of water-sol-

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uble substances, including the secondary metabolites in heavy oil on terrestrial mammals. In order to assess the *in vivo* effect, in our previous studies, water-soluble fraction (WSF) was orally administrated to mice. We found that the chemical compounds in WSF of heavy oil were induced by the abnormalities in reproductive organs (Nishimoto *et al.*, 2008, 2009). Humans are usually exposed to many kinds of chemical substances in their surroundings. Exposure to these chemicals via inhalation or skin contact could jeopardize the immune system and lead to immune system diseases such as sick house syndrome (Gao *et al.*, 2008). The IgG and IgA in our body play a very important role in cellular and mucosal immunity (Heiner, 1984; Ochs and Wedgwood, 1987; Fagarasan and Honjo, 2003; Cerutti, 2008). Hence, in this paper, we focused on the mammalian immune system, and investigated the potential influence of the chemical substances contained in heavy oil on mouse splenocytes.

Major population of cells in spleen is comprised of B and T lymphocytes. B lymphocytes could be grouped into mature B cells ($B220^+$) and antibody-producing plasma ($B220^{low}$) cells, and $CD3^+$T lymphocytes, are separated into helper T ($CD4^+$) cells and cytotoxic T ($CD8^+$) cells. Helper T cells and cytotoxic T cells play critical roles in acquired immunity (Singh *et al.*, 1999; Zhu and Paul, 2008). Many points still remain unclear in the effects of heavy oil WSF on mouse immune system. To clarify these points, we examined the Ig production levels in mouse splenocytes and peripheral blood serum by enzyme-linked immunosorbent assay (ELISA), and the cell population in spleen by flowcytometry analysis.

MATERIALS AND METHODS

Sample preparation

Heavy oil was obtained from Dr. Shin-Ichi Kitamura, Center for Marine Environmental Studies (CMES) in Ehime University. To study the solvent-dependent manner because the soluble components are dependent on each solvent, the sample was extracted with distilled water (DW), methanol, and ethanol, respectively. Heavy oil was added to each vehicle (10%, v/v) and gently mixed for 20 hr following the methods described previously (Stephens *et al.*, 1997). Collected water phase sample was called "WSF". In the same way, methanol and ethanol extracts were called "Methanol-soluble fraction (MSF)" and "Ethanol-soluble fraction (ESF)", respectively. These samples were withdrawn and supplied to *in vitro* and *in vivo* assay systems. For oral administration, portions of MSF and ESF were evaporated with these solvents, and samples were then suspended with dimethyl sulfoxide (DMSO). These solutions were stored at −20°C storage and thawed at room temperature prior to administration.

Cell culture and condition

Mouse splenocytes and estradiol-sensitive cells were used to investigate immunoglobulin (Ig) productivity and estrogenic activity of heavy oil samples. For splenocytes preparation, mice were killed by cervical dislocation, and spleens were excised from the cavity. Spleens were strained with 40 nm-pore nylon mesh, and collected cells were hemolyzed with hemolysis buffer (155 mM NH_4Cl, 15 mM $NaHCO_3$, 1 mM EDTA-2Na, pH 7.3). After washing twice, lymphocytes from spleen were obtained. For *in vitro* assay in naïve mice, splenocytes were cultured in ERDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with WSF, MSF and ESF at various concentrations for 24 hr. For *in vitro* assay in WSF, MSF and ESF administrated mice, splenocytes were cultured in ERDF medium without supplement for 24 hr. After the cultivation, all supernatants were collected for the measurement of Ig production. Estradiol-sensitive T47D-KBluc cells were purchased from ATCC (Manassas, VA, USA). These cells were transformed cells stably expressing estradiol responsive elements (Wilson *et al.*, 2004). T47D-KBluc cells were normally maintained with 10% fetal bovine serum (FBS)/ERDF medium. All cells were cultured in ERDF medium at 37°C under 5% CO_2 in humidified air condition.

Animals and oral administration

Female albino mice were purchased from the Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Mice were housed in a room maintained at 24°C on a 12 hr light/dark cycle in specific pathogen-free facility, and provided tap water and diet *ad libitum*. All animal experiments were carried out in accordance with protocols approved by the Ehime University Animal Care and Use Committee and were performed in accordance with applicable guidelines and regulations. Three female mice (8 weeks old) per group were administered WSF, MSF, ESF or DMSO as a control. Each mouse was administrated with 20 µl volume of each sample per day at various concentrations for 7 or 28 days (only WSF) continuously. At 7, 14, 21 and 28 days, body weight and excised tissue weight were measured and the sera were separated from collected blood.

ELISA assay

All antibodies were purchased from Zymed Laboratories (Carlsbad, CA, USA). The amount of IgA, IgG, and IgM in the culture medium was measured by

ELISA (Miyazaki *et al.*, 1998; Sugahara *et al.*, 2008). For Igs detection, rabbit anti-mouse IgM, IgA and IgG were used to fix mouse IgM, IgA and IgG, respectively. These antibodies were diluted 1,000 times with 50 mM sodium carbonate-bicarbonate buffer (pH 9.6). Each well of ELISA plate was treated with 100 µl of the antibody solution for 2 hr at 37°C. Following blocking with 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) for 24 hr at 37°C, each well is treated with 50 µl of culture supernatant or mouse anti-sera diluted with 1% BSA/PBS for 1hr at 37°C. For mouse Igs detection, the secondary antibodies, HRP-conjugated goat anti-mouse IgA, HRP-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-mouse IgM were used, respectively. The well was treated with 100 µl of each second antibody diluted 1,000 times with 1% BSA/PBS for 1 hr at 37°C. For staining, 100 µl of 0.6 mg/ml of 2,2'-azino-bis(ethylbenzothazoline-6-sulfonic acid diammonium salt) dissolved in 0.03% H_2O_2-0.05M citrate buffer (pH 4.0) was added to wells. The absorbance at 415 nm was measured after the addition of 100 µl of 1.5% oxalic acid for termination of the coloring reaction. The wells were washed three times with 0.05% Tween20-PBS between each step.

Reporter gene assay

For reporter gene assay, charcoal-treated FBS (cFBS) was used for cell culture. Briefly, FBS was prepared with 50 mg/ml activated charcoal (Sigma, St. Louis, MO, USA) for 0.5 hr rotation. The treated FBS were then filtered through a 0.22 µm membrane filter. Following the pre-culture with 5% cFBS/ERDF medium for 3 days, T47D-KBluc cells were cultured with WSF, MSF and ESF in ERDF medium containing 5% cFBS for 48 hr (Wilson *et al.*, 2004). Culture treated with 17β-estradiol (Sigma) at 0.1 nM was used as control. The luciferase assay was carried out using the Luciferase Assay System (Promega, Madison, WI, USA) and luminescence was measured by Luminescencer-JNR (ATTO, Tokyo, Japan) according to the instructions provided by the manufacturer.

Flowcytometry analysis

All antibodies for FACS analysis were purchased from BioLegend, Inc. (San Diego, CA, USA). For B and T lymphocytes frequencies measurement in spleen, splenocytes were stained with the anti-CD3ε and anti-B220. While for helper and cytotoxic T lymphocytes frequencies measurement, splenocytes were stained with the anti-CD4 and anti-CD8a. Briefly, splenocytes were labeled on ice for 30 min with appropriate antibodies, followed by two washes in FACS buffer (2% FBS/PBS, 0.01% sodium azide). The labeled cells were resuspended in FACS buffer and the data were acquired using FACScalibur with CellQuest (BD Biosciences), and then analyzed using WinMDI software (TSRI, La Jolla, CA, USA).

Statistical analysis

Results are expressed as means ± standard deviation (S.D.). Tukey's test was used to assess the statistical significance of the difference between control and treated groups. Each value of $p < 0.05^*$ or $p < 0.01^{**}$ is considered to be statistically significant.

RESULTS

In vitro effects of heavy oil extracts on mouse splenocytes

In order to examine the effect of heavy oil extracts on immune cells, for *in vitro* assay, splenocytes were prepared from naïve mice. The cells were inoculated at 7.5 x 10^5 cells/ml and cultured for 24 hr in ERDF medium supplemented with each heavy oil extract at various concentrations. The amounts of Ig produced by splenocytes in the culture medium were quantified by ELISA. As shown in Fig. 1, all extracts enhanced IgA production in dose-dependent manner. However, IgG production was not promoted. From this result, it was found that each extract might induce production of selectively different Ig subclasses in primary splenocytes.

Ig production of splenocytes in heavy oil extracts-administrated mice

To investigate the effects of heavy oil extracts on immune system *in vivo*, we orally administered WSF, MSF and ESF at 10^n (n = 0-2) diluted concentration to mice for 7 days. The body weights of each heavy oil extracts-administrated mice did not change or slightly reduced (data not shown). Splenocytes prepared from each extracts administrated-mice were inoculated at 7.5 x 10^5 cells/ml and cultured for 24 hr in ERDF medium. As shown in Fig. 2A, IgA production increased at lowest concentration of fractions in WSF- and ESF-administrated mice. However, IgA production of splenocytes was reduced in each extract-administrated mice at the highest concentration of fractions. On the other hand, IgG production was significantly suppressed at all concentration of fractions in WSF-administrated mice (Fig. 2B).

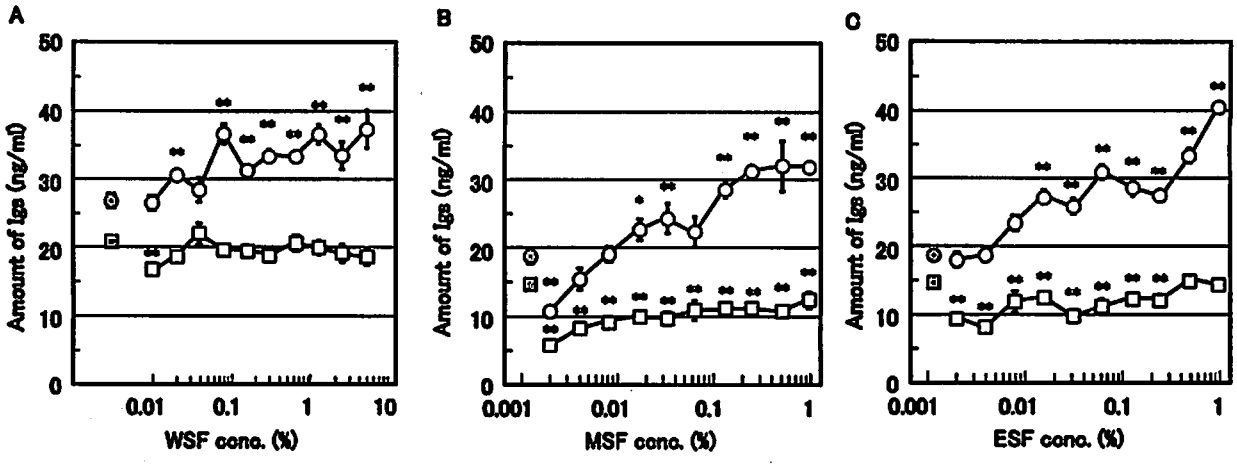

Fig. 1. *In vitro* effects of heavy oil extracts on immunoglobulin production and cytotoxicity of splenocytes in naïve mice. Mouse splenocytes were inoculated at 7.5 x 10^5 cells/ml in ERDF medium supplemented with or without three kinds of heavy oil extracts at various concentrations and cultured for 24 hr. Amounts of IgA and IgG production measured by ELISA were indicated in each figure. IgA and IgG were indicated as open circle (○) and open square (□), respectively. Shaded figures showed DW or DMSO as control for each. These results are expressed as the mean ± S.D. of two independent measurements. Significant difference with appropriate control or each sample (**; $p < 0.01$, *; $p < 0.05$).

The amount of Ig contained in the sera of heavy oil extracts-administrated mice

Ig subclass productivity differed among splenocytes prepared from different heavy oil extracts-administrated mice for 7 days. To examine whether Ig productivity in peripheral blood has the same tendency as in splenocytes, we measured the amount of Ig in sera collected from each extract-administrated mice by ELISA. As shown in Fig. 3A, the amount of IgA decreased but significant differences and dose-dependent manner were not shown in MSF- and ESF-administrated mice, respectively. In WSF-administrated mice, IgA production was roughly constant. On the other hand, it was indicated in Fig. 3B that the amount of IgG was reduced in WSF-administrated mice at the highest concentration. From the results in Figs. 2 and 3, it was revealed that the highest concentration of WSF in this experiment decreased the amount of IgG in the sera with decreasing of IgG productivity in splenocytes.

Chemical substances contained in heavy oil extracts have estrogenic activity

T47D-KBluc cells were stably expressed estrogen responsive elements bound to luciferase gene, and luciferase gene expression responded to the 17β-estradiol concentration (Wilson *et al.*, 2004). Previously, we found that chemical compounds contained in WSF have estrogenic activity (Nishimoto *et al.*, 2008, 2009). To investigate whether MSF and ESF have estrogenic activity, we carried out the luciferase reporter gene assay.

Following pre-culture, T47D-KBluc cells were inoculated at 2.0 x 10^5 cells/ml and cultured for 48 hr in ERDF medium supplemented with each heavy oil extract at various concentrations. For the fluorescence relativity, luminescence intensity of 0.1 nM β-estradiol was calculated as 1. As indicated in Table 1, the relative estrogenic activity in MSF- or ESF-treated cells was lower than that of the control or WSF-treated cells. In MSF-treated cells, estrogenic activity was enhanced at low concentration.

Histological effect of heavy oil extracts on orally administrated mice

From the results of reporter gene assay, we found that the estrogenic activity in WSF was stronger than that in MSF or ESF. Moreover, from previous experiments, the atrophy of spleen and thymus in only WSF-administrated mice for 7 days was observed. In order to examine the histological effects of WSF for a long duration, mice were orally administrated with WSF continuously for 28 days. The mice were divided into three mice for each administration group, and the experiment was repeated twice independently. The body weights of WSF-administrated mice were slightly reduced (data not shown).

Most interestingly, a marked atrophy of spleen and thymus in WSF-administrated mice at 28 days was observed (Figs. 4A and B). As indicated in Table 2, significant

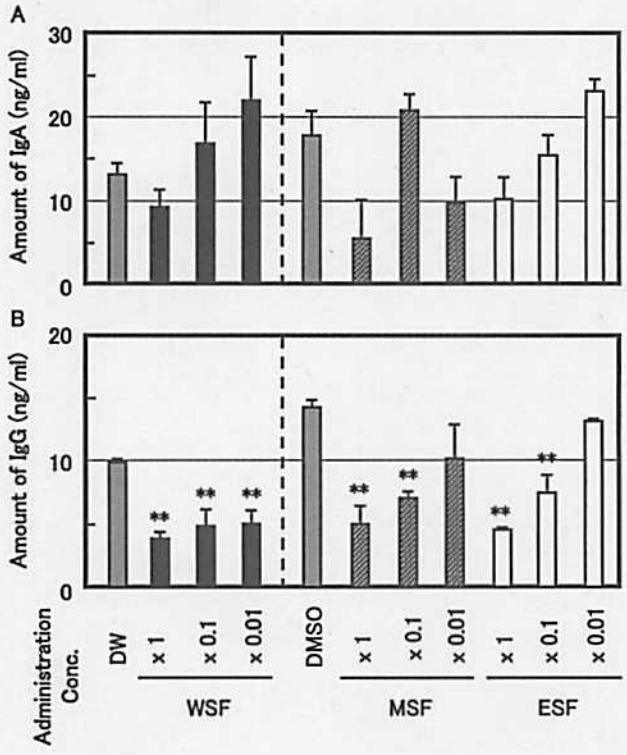

Fig. 2. Immunological effect on each heavy oil extract-administrated mice.
Mice were administrated with WSF, MSF and ESF for 7 days. For control, DW and DMSO were used. Splenocytes prepared from administrated mice were inoculated at 7.5 x 10^5 cells/ml in ERDF medium. Cells were cultured for 24 hr. IgA and IgG production measured by ELISA were indicated in (A) and (B), respectively. These results are expressed as the mean ± S.D. of two independent measurements. Significant difference with appropriate control or each sample (**; $p < 0.01$).

weight loss occurred in these immune organs of WSF-administrated mice compared to that of the control mice. Spleen and thymus were shrunk more than 20% in weight within 7 days from the start of WSF administration. The atrophy was quickly induced at administration period for 7 days. Once atrophy in these organs has developed, shrinking rate was constant or gradually increased thereafter.

Population analysis of splenocytes from WSF-administrated mice

The chemical compounds in WSF caused atrophy of spleen and thymus. In order to reveal the reason for atrophy of spleen, we analyzed the population of T and B lymphocytes in spleen. B220 is expressed on the surface of mature B lymphocytes. The activated B cells differentiated into Ig producing plasma cells. As shown in Fig. 5A, plasma cells in $B220^-$ region reduced approximately 4.5% in spleen of WSF-administrated mice. T lymphocytes are mainly distinguished into helper and cytotoxic type, CD4 and CD8, respectively. Moreover, $CD3^+$ T lymphocytes increased approximately 8.2% compared to that in the control mice (Fig. 5B). To analyze the reason for the increasing $CD3^+$ T lymphocytes population, splenocytes were stained for both CD4 and CD8. As shown in Fig. 5C, only $CD4^+$ T lymphocytes increased for approximately 8.2% whereas $CD8^+$ cells population remained constant compared to control.

DISCUSSION

When chemical compounds are taken into the body through foods such as contaminated fish, some part of them is metabolized and excreted, while some are accumulated in our body for a long time. Heavy oil is a typical contaminant in marine ecosystem and many fishes are contaminated with chemical substances contained in heavy oil. To assess the risk of effects of heavy oil intake

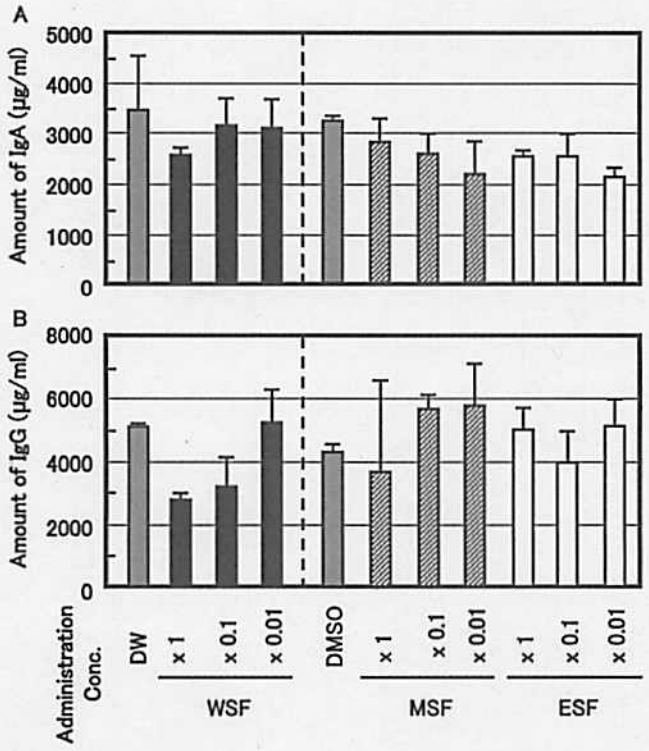

Fig. 3. Immunological effect on peripheral blood in each heavy oil extract-administrated mice.
Mice were administrated with WSF, MSF and ESF for 7 days. DW and DMSO were used as controls. The amounts of IgA and IgG production in peripheral sera measured by ELISA were indicated in (A) and (B), respectively. These results are expressed as the mean ± S.D. of two independent measurements.

Table 1. Relative estrogenic activity in heavy oil extracts

Concentrations (%)	0.0001	0.005	0.05	0.2	2.5
WSF	1.21	1.20	1.83	1.60	1.36
MSF	0.69	0.59	0.33	0.31	0.03
ESF	0.25	0.31	0.36	0.41	0.23

Relative estrogenic activities indicate that luminescence intensity of 0.1 nM β-estradiol at was calculated as 1. Luciferase assay data was obtained from four times independently repeated experiments.

via food web, we examine the effect of chemical compounds contained in heavy oil on mice. In previous study, we found that chemical substances in heavy oil induced cystoma-like formation and atrophy of prostate gland (Nishimoto *et al.*, 2009). It was suggested that the chemicals in heavy oil affects reproductive organs in mammals. It has been known that some chemicals could trigger allergic diseases (Hess, 2002; Hasegawa *et al.*, 2009) and induced immnotoxicity in the immune organs (Gao *et al.*, 2008). Hence, in this study, to investigate whether the chemical substances in heavy oil could affect other organs aside from reproductive tissues, we focused on the immune tissues.

To examine whether heavy oil extracts has effects on mouse immune cells, we used splenocytes from naïve mice. IgA production of splenocytes treated with WSF,

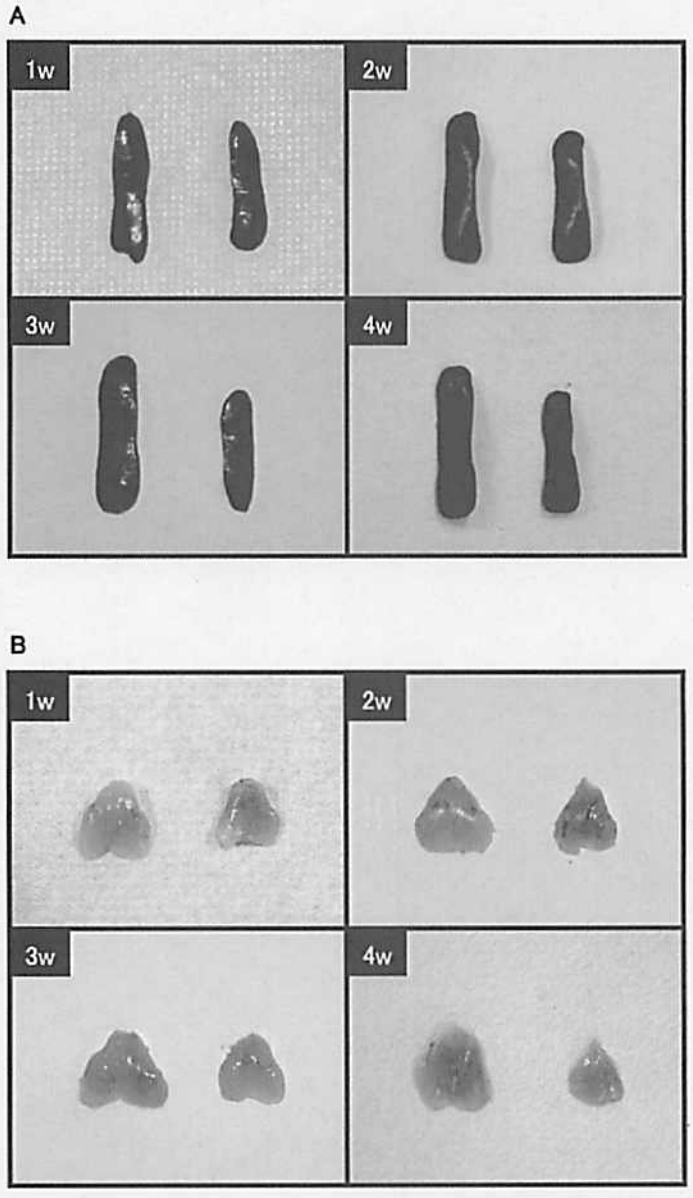

Fig. 4. Pathological abnormality of immune tissues in WSF-administrated mice.
Mice were orally administrated with WSF for 7, 14, 21 and 28 days. Spleen (A) and thymus (B) was excised from peritoneal and thoracic cavity, respectively, at each collection date. Three mice were divided into each administration group. This experiment was carried out twice independently.

MSF and ESF was enhanced in a dose-dependent manner (Fig. 1). However IgG production remained constant.

Moreover, the amount of IgA produced by splenocytes from WSF-administrated mice at low concentration of fractions was increased. On the other hand, the amount of IgG produced by splenocytes from each extract-administrated mice significantly decreased irrespective of the fraction concentration. Through the differences in the Igs productivity in splenocytes between naïve and administrated mice, we suggest that splenocytes from naïve mice

Table 2. Relative atrophic rate of various tissues in WSF-administrated mice

Administration period	Spleen	Thymus	Liver	Kidney
1 week	75.5 ± 4.2 **	54.4 ± 2.4 **	86.8 ± 1.6 *	93.3 ± 6.5
2 week	80.7 ± 5.9 **	79.8 ± 16.1	77.3 ± 4.9 **	96.4 ± 6.6
3 week	65.0 ± 15.9	68.2 ± 4.5	70.1 ± 4.5 **	88.3 ± 2.7 *
4 week	69.1 ± 9.5	53.7 ± 13.1 *	73.7 ± 8.7 *	90.0 ± 7.1

Relative atrophic rate was calculated based on the tissue weight of control mouse for criteria. The value are expressed as mean ± S.D. for 3 to 6 mice per administration period. One (*) and two (**) asterisk marks indicate statistical significance at $P < 0.05$ and $P < 0.01$ vs control, respectively.

directly and rapidly responded without observed effects by metabolite. These different responses may be induced by not only chemical metabolites but also chemical compounds contained in each extract in administrated mice, because solvent-dependent differences were shown on the productivity and concentration of Igs.

The amount of IgG in the sera from WSF-administrated mice decreased approximately 60% compared to the control level. Both the *in vitro* effect and the amount of Igs in sera i.e., IgG production has the same tendency. The IgG in our body plays a very important role in cellular immunity (Heiner, 1984; Ochs and Wedgwood, 1987). Since these results showed that IgG level reduction occurred in the sera from WSF-administrated mice, it followed that chemical components in WSF may have IgG-oriented effects.

An intensive estrogenic activity was exhibited in WSF thought there was also estrogenic activity in MSF and ESF. With regard to the relationship between estrogenic activity and IgG production, we found that the chemical compounds showing intense estrogenic activity suppressed IgG production. Therefore chemical compounds exhibiting estrogenic activity are more soluble in water than in methanol or ethanol. There are many kinds of chemical substances contained in heavy oil, and the well known major components are PAHs. Some PAHs are water soluble, and have estrogenic activity and carcinogenicity (Singhal *et al.*, 2008). Estrogen receptors (ERs) are expressed in lymphocytes (Pernis, 2007; Tai *et al.*, 2008), chemical compounds such as PAHs that have estrogenic activity may affect immune responses of lymphocytes by signal transduction via ERs.

We observed atrophy of spleen and thymus in only WSF-administrated mice. T and B cell maturation progress in white pulp of spleen, and many antibodies are produced from plasma cells. Immature T cells develop in thymus, and naïve T cells migrate to spleen or other lymph nodes. To confirm the reason for atrophy of spleen, we analyzed lymphocytes population in spleen. We found that $B220^+$ B cells number decreased and $CD3^+$ T cells number were increased, and the numbers of $CD4^+$ T cells increased whereas populations of $CD8^+$ T cells were almost constant. It was suggested from FACS analysis that decrease in B cell population was correlated with the reduction of IgG production in sera or spleen from WSF-administrated mice. Since the number of plasma cells producing Ig was decreased, we presumed that the amount of IgG was low.

There are several reasons for atrophying spleen. In conclusion, we suggest that one of the reasons of spleen atrophy is the reduction in the population of plasma cell producing IgG. Chemical substances such as PAHs having estrogenic activity in WSF may affect Ig secreted from lymphocytes via ERs.

On the other hand, $CD4^+$ T cells performed important roles such as in immune diseases (Mellergård *et al.*, 2004; Zhu and Paul, 2008). We speculate that increase of $CD4^+$ T cells is attributed to the feedback stimulation of irregularly secreted cytokines by intracellular disturbance through ERs (Pernis, 2007; Tai *et al.*, 2008). Though we have to analyze the Th subsets in detail, it could be suggested that chemical compounds in WSF may modulate the balance between Th1 and Th2 in $CD4^+$ T lymphocytes (Nohara *et al.*, 2002; Kushima *et al.*, 2009).

Hence, considering these results it is possible that we may suffer from opportunistic infection or immune diseases such as allergy or rheumatoid arthritis by severe damage in immune system when chemical substances having estrogenic activity are orally taken into our body (Hess, 2002; Hasegawa *et al.*, 2009). However, the precise mechanism of the effect of WSF on immune system is not completely clarified. The detailed analysis of splenocytes and thymocytes population is now under investigation.

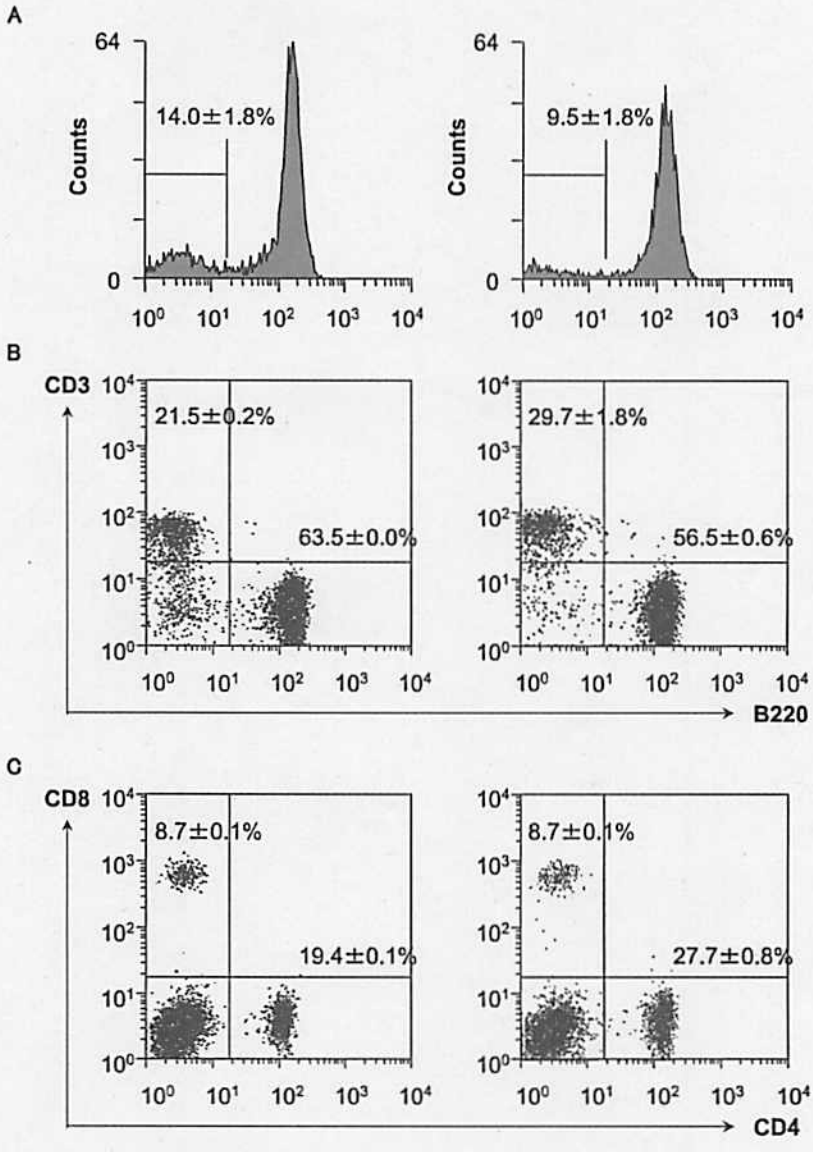

Fig. 5. Splenocytes population analysis in WSF-administrated mice.
Splenocytes were prepared from 14 days WSF-administrated mice. (A) High or low B220 expressed cells are indicated as mature B or plasma cells, respectively. (B) B and T cells population. (C) Cytotoxic and helper T cells belonging to T cells. These results are expressed as the mean ± S.D. of six individual mice in two independent experiments.

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