## Immunosuppressive Activity of the Ethanol Extract of Sedum sarmentosum and Its Fractions on Specific Antibody and Cellular Responses to Ovalbumin in Mice

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The immunosuppressive activity of the ethanol extract of *Sedum sarmentosum* (EESS) and its fractions was studied with respect to specific antibody and cellular response to ovalbumin (OVA) in mice. ICR Mice were immunized subcutaneously with OVA on days 0 and 14. Beginning on the day of immunization, the mice were administered intraperitoneally (ip) with EESS and it fractions at a single dose of 0.25, 0.5, and 1.0 mg, and cyclosporin A at a single dose of 0.1 mg at intervals of 7 days. On day 28, splenocyte proliferation and specific antibody level in serum were measured. EESS significantly suppressed concanavalin A (Con A)-, lipopolysaccharide (LPS)-, and OVA-induced splenocyte proliferation in the immunized mice in a dose-dependent manner. The OVA-specific serum IgG, IgG1, and IgG2b levels in the immunized mice were also markedly reduced by EESS. Among four fractions of EESS, the BuOH fraction consisting mainly of flavonoid glycosides showed the highest suppressive activity. The results suggest that EESS could suppress the cellular and humoral immune response in mice, and deserve further research to be developed as immunosuppressant.

**Introduction.** – Sedum sarmentosum BUNGE (Crassulaceae) is well-known as a traditional Chinese medicine widely used in the treatment of acute and chronic hepatitis, jaundice with damp-heat pathogen, pyocutaneous disease, and difficulty in micturition [1]. Extensive pharmacological studies established that it possessed hepatoprotective [2-5], immunosuppressive [6-9], antiproliferative [10], estrogenic [11], angiotensin-converting enzyme inhibitory [12], and antioxidant [13] activities. Its main chemical constituents were flavonoids [12][14-17], sterols [16][18], triterpenes [2][16][19], alkaloids [10][20], cyanogenic glycosides [21-23], and megastigmanes [5][24][25].

S. sarmentosum is a reputable hepatoprotective drug and has been widely used to treat various types of hepatitis. It was anticipated that S. sarmentosum should have a bearing on the immune system, as the pathologic mechanisms of hepatitis in general is closely associated with both immune function disorder and allergies of a living being. The immune system is involved in the etiology as well as pathophysiologic mechanisms of this disease. One of the main causes of hepatocyte damage in a variety of hepatic diseases involves T cell-mediated cellular immunity [26]. Immunotherapy has also been used for suppressing the over-activated immune reaction that causes hepatic damage. The H<sub>2</sub>O extract of S. sarmentosum has been reported to possess immuno-suppressive properties [7], and sarmentosin, a H<sub>2</sub>O-soluble cyanogenic glycoside, was

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originally considered as the active constituent of the drug [22][23]. However, pharmaceutical granules that lack this compound still show remarkable activity [2][20]. Thus, there is a need for comprehensive, systematic, and multi-disciplinary evaluation of various claims to make effective use of this drug.

During the course of our studies on immunosuppressive properties of traditional Chinese medicines [27-30], we found that the EtOH extract of *S. sarmentosum* (EESS) exhibited distinctive immunosuppressive activity on the immune response of mice. While the hepatoprotective activities and active constituents of *S. sarmentosum* have been studied by many investigators [2-5], to our knowledge, its immunological actions have not been well-documented in spite of its increasing usage [2]. In this article, as a preceding step to identify active principles, EESS was successively fractionated with different organic solvents, and the immunosuppressive potentials of EESS and its fractions were investigated on the cellular and humoral immune responses of mice *in vitro* and *in vivo*.

**Results and Discussion.** – Immunosuppressive agents are the mainstay treatment for patients that have received organ grafts and are becoming increasingly important in the treatment of a variety of immunologically related diseases including rheumatic arthritis, systemic lupus erythematosus, and hepatitis [31]. There are, however, many problems with both the concept and reality of long-term immunosuppression as a therapeutic modality, both in terms of the nonspecific toxicity of the drugs that are currently available, and the increased risk of infections and tumors arising from global suppression of the immunosuppressants. The immunosuppressants with lower side effects are still a challenge to the medical system. Suppression of immune response by medicinal plant products as a possible therapeutic measure has become a subject of scientific investigation recently.

In an effort to search for new immunosuppressants, in this study, the immunosuppressive potentials of EESS and its fractions were investigated on the cellular and humoral immune responses of mice *in vitro* and *in vivo*, and tricin-7-O- $\beta$ -D-glucopyranoside (1), a flavonoid glycoside, was used for quantitative analysis of the BuOH fraction with the highest suppressive activity.



Tricin-7-O- $\beta$ -D-glucopyranoside (**1**) (Glc =  $\beta$ -D-glucopyranosyl)

1. *Effect of EESS on Splenocyte Proliferation* in vitro *and* in vivo. Among the T-lymphocytes, helper T-cells induce B-lymphocytes to secrete antibodies, and cytotoxic T-lymphocytes help phagocytes to destroy ingested microbes and to kill intracellular microbes. Humoral immunity, however, is mediated by antibodies, which are produced

by B-lymphocytes. The capacity to elicit an effective T-cell immunity can be shown by the stimulation of lymphocyte proliferation response. It is generally known that Con A stimulates T-cells, and LPS stimulates T-cell-dependent B-cell proliferation. As shown in Fig. 1, EESS significantly suppressed Con A- and LPS-stimulated splenocyte proliferation in vitro in a concentration-dependent manner. The effects of EESS on mitogen- and OVA-stimulated splenocyte proliferation in OVA-immunized mice are shown in Fig. 2. As a positive control, cyclosporin A (CsA) significantly inhibited Con A- and OVA-induced splenocyte proliferation in the OVA-immunized mice, but not affected LPS-stimulated splenocyte proliferation. Con A-induced splenocyte proliferation in the OVA-immunized mice was significantly suppressed by EESS at three doses (p < 0.05 or p < 0.01). EESS also significantly inhibited the cellular proliferations elicited by the B-cell mitogen LPS and OVA at a dose of 0.5 and 1.0 mg (p < 0.01 or p < 0.010.001). Moreover, LPS- and OVA-induced splenocyte proliferation in the mice treated with EESS at these two doses was significantly lower than that in the CsA-treated mice (p < 0.05, p < 0.01, or p < 0.001). The results indicated that EESS could significantly suppress the activation potential of T- and B-cells in mice in vitro and in vivo.



Fig. 1. Effect of the EtOH extract of S. sarmentosum (EESS) on mitogen-stimulated splenocyte proliferation in vitro. Splenocyte proliferation was measured by the MTT method and shown as a stimulation index (SI; see Exper. Part). The values are presented as means  $\pm$  S.D. (n=4). Significant differences with 0 µg/ml were designated as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001. CsA: cyclosporin A (positive drug).

2. Effect of EESS on the OVA-Specific Serum Antibody Response in OVA-Immunized Mice. The serum OVA-specific antibody levels in the OVA-immunized mice were measured two weeks after the last immunization using ELISA. As shown in Fig. 3, the amount of OVA-specific IgG and IgG1 in the serum was significantly decreased by EESS at a dose of 1.0 mg compared with OVA control (p < 0.001). Significant reduction in total serum IgG2b levels were observed in mice treated with EESS at a dose of 0.5 and 1.0 mg (p < 0.001). Moreover, the OVA-specific IgG, IgG1, and IgG2b levels in the serum from the mice treated with EESS at a dose of 1.0 mg were significantly lower than those measured for the mice exposed to CsA (p < 0.05 or p <



Fig. 2. Dose-dependent inhibitory effect of the EtOH extract of S. sarmentosum (EESS) on OVA- or mitogen-stimulated splenocyte proliferation in OVA-immunized mice. ICR Mice were immunized subcutaneously twice with OVA on day 0 and 14, and administered ip with EESS at a single dose of 0.25, 0.5, and 1.0 mg, and cyclosporin A (CsA, positive drug) at a single dose of 0.1 mg per animal at intervals of 7 days for a total of 5 doses. Splenocyte proliferation was measured by the MTT method and shown as a stimulation index (SI; see *Exper. Part*). The values are presented as means  $\pm$  S.D. (n = 5). Significant differences with OVA control groups were designated as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; those with CsA groups as a: p < 0.05, aa: p < 0.01, and aaa: p < 0.001.



Fig. 3. Dose-dependent inhibitory effect of the EtOH extract of S. sarmentosum (EESS) on OVA-specific IgG, IgG1, and IgG2b antibodies in OVA-immunized mice. ICR Mice were immunized subcutaneously twice with OVA on day 0 and 14, and administered ip with EESS at a single dose of 0.25, 0.5, and 1.0 mg, and cyclosporin A (CsA, positive drug) at a single dose of 0.1 mg per animal at intervals of 7 days for a total of 5 doses. OVA-Specific IgG, IgG1, and IgG2b antibody levels in the serum were measured by an indirect ELISA test (see *Exper. Part*). Results are presented as means ± S.D. (n=5). Significant differences with OVA control groups were designated as \*\*: p < 0.01, and \*\*\*: p < 0.001; those with CsA groups as a: p < 0.05 and ama: p < 0.001.

0.001). Collectively, findings indicate that EESS could significantly result in the reduction of serum antibody production in mice immunized with OVA. The levels of IgG1 and IgG2b were determined to define the T-helper-cell phenotype induced by the different immunization protocols, since production of IgG1 is favored by Th2 type of cytokines, and of IgG2b by Th1 type of cytokines [34]. Thus, it is likely that EESS at a suitable dose is effective on Th1- and Th2-cells, as associated sensitively with a decrease of IgG1 and IgG2b levels. Meanwhile, significant reduction in total serum IgG2b levels were observed in mice treated with EESS at a dose of 0.5 mg compared with the OVA control group (p < 0.001). There were, however, no significant differences in the total serum IgG and IgG1 levels between EESS-treated mice and control mice at the same dose. It suggests a more sensitive suppression of Th1 response by EESS than Th2 response. Both Th1 and Th2 responses are suppressed by EESS, suggesting that EESS may contain multiple immunosuppressive compounds toward different T-cell cytokine responses.

3. Immunosuppressive Activity of Four Fractions of EESS. Since EESS exhibited more distinctive immunosuppressive activity on a specific antibody and cellular response against OVA in mice, it was successively fractionated with different organic solvents to afford four fractions: petroleum ether (PE), AcOEt, BuOH, and aqueous (aq.) fractions, with the yield being 24.6, 7.9, 25.1, and 42.4% of EESS weight, respectively. These fractions were compared for suppressive potentials on the cellular and humoral immune responses to OVA in mice when given together with OVA. As shown in Fig. 4, the four fractions significantly decreased Con A- and OVA-stimulated splenocyte proliferation in the OVA-immunized mice (p < 0.05, p < 0.01, or p < 0.001). Splenocytes isolated from mice treated with the BuOH fraction at a dose of 1.0 mg, and stimulated by Con A or OVA show a lower proliferative response than that observed for the mice exposed to another fraction at the same dose (p < 0.01 or p < 0.001). The effects of four fractions on the serum OVA-specific antibody response in OVAimmunized mice were shown in Fig. 5. The amount of OVA-specific IgG and IgG1 in the serum was significantly decreased by AcOEt, BuOH, and aq. fractions in a dosedependent manner compared with the OVA control (p < 0.05, p < 0.01, or p < 0.001). Moreover, reducing effect of these three fractions against IgG antibody response were more significant than that of EESS at same dose (p < 0.05, p < 0.01, or p < 0.001). However, no significant differences (p > 0.05) were observed in the serum IgG and IgG1 level between the PE fraction group and the OVA group. All four fractions significantly suppressed the production of the serum IgG2b antibody in the OVAimmunized mice compared with control group (p < 0.05, p < 0.01, or p < 0.001). The IgG2b antibody level in serum from the mice treated with the BuOH fraction at a dose of 1.0 mg was significantly lower than that measured for the mice exposed to EESS at the same dose (p < 0.01). Collectively, findings indicate that the BuOH fraction is most effective in inhibition of the cellular and humoral immune responses of mice to OVA among the four fractions.

As the immunosuppressive activity was more evident in the BuOH fraction, this fraction was further analyzed. The BuOH fraction was proved to consist mainly of flavonoid glycosides. Plant-derived flavonoids are inhibitors of various intracellular processes, notably phosphorylation pathways, and potential inhibitors of cellular autoimmunity [35][36]. Suppression of immune response by flavonoids as a possible



Fig. 4. Effect of the EtOH extract of S. sarmentosum (EESS), and its fractions on Con A- and OVAstimulated splenocyte proliferation in OVA-immunized mice. ICR Mice were immunized subcutaneously twice with OVA on day 0 and 14, and administered ip with EESS at a single dose of 1 mg, or its fractions at a single dose of 0.25, 0.5, and 1.0 mg per animal at intervals of 7 days for a total of 5 doses. Splenocyte proliferation was measured by the MTT method and shown as a stimulation index (SI; see Exper. Part). The values are presented as means  $\pm$  S.D. (n=5). Significant differences with OVA control groups were designated as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001. PE: petroleum ether fraction; AcOEt: ethyl acetate fraction; BuOH: butanol fraction; aq.: aqueous fraction.

therapeutic measure has become a subject of scientific investigation recently [37][38]. Seven flavonoid glycosides, tricin-7-O- $\beta$ -D-glucopyranoside (1), luteolin-7-O- $\beta$ -D-glucopyranoside, myricetin-3-O- $\beta$ -D-glucopyranoside, quercetin-3-O- $\beta$ -D-glucopyranoside, quercetin-3-O- $\beta$ -D-glucopyranoside, quercetin-3-O- $\alpha$ -(6<sup>'''</sup>-caffeoylgluco-syl- $\beta$ -1,2-rhamnosyl)-7-O- $\alpha$ -L-rhamnopyranoside, and tricetin-3'-O- $\beta$ -D-glucopyranoside were isolated from the BuOH fraction using ordinary and reversed-phase silicagel, as well as *Sephadex LH-20* column chromatography. It was reported that



Fig. 5. Effect of the EtOH extract of S. sarmentosum (EESS), and its fractions on OVA-specific IgG, IgG1, and IgG2b antibodies in OVA-immunized mice. ICR Mice were immunized subcutaneously twice with OVA on day 0 and 14, and administered ip with EESS at a single dose of 1 mg, or its fractions at a single dose of 0.25, 0.5, and 1.0 mg per animal at intervals of 7 days for a total of 5 doses. OVA-Specific IgG, IgG1, and IgG2b antibody levels in the serum were measured by an indirect ELISA test (see *Exper. Part*). Results are presented as means  $\pm$  S.D. (n=5). Significant differences with OVA control groups were designated as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; those with EESS groups as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; those with EESS groups as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; those with EESS groups as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; those with EESS groups as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; those with EESS groups as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; those with EESS groups as \*: p < 0.05, \*\*: p < 0.01, and \*\*\*: p < 0.001; and \*\*\*: p < 0.01, and \*\*\*: p < 0.01. PE: petroleum ether fraction; AcOEt: ethyl acetate fraction; BuOH: butanol fraction; aq.: aqueous fraction.

compound **1** possessed inhibitory activities on proliferation of spleen T- and Blymphocytes of normal mice *in vitro* [39]. Therefore, **1** was selected as the indicating substance for quantitative analysis of the BuOH fraction. The content of **1** in the BuOH fraction was found to be  $3.44 \pm 0.05\%$ . Hence, the presence of flavonoid compounds principally could contribute favorably to the medicinal use of *S. sarmentosum*. Further researches of *in vitro* and *in vivo* immunosuppressive activity of the above isolated flavonoid glycosides are being carried out.

**Conclusions.** – EESS could significantly suppress Con A- and LPS-stimulated splenocyte proliferation *in vitro*, and reduce splenocyte proliferation and the production of antibodies in OVA-immunized mice. Among the fractions, the BuOH fraction consisting mainly of flavonoid glycosides is most effective in inhibition of the cellular and humoral response in mice. This result might be responsible, at least in part, for the use of *S. sarmentosum* for treating various types of hepatitis in traditional Chinese medicine.

## **Experimental Part**

*General.* Ovalbumin (OVA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS), RPMI-1640 medium, and rabbit antimouse IgG peroxidase conjugate were purchased from *Sigma Chemical Co.* (USA), goat anti-mouse IgG1 and IgG2b peroxidase conjugate were obtained from *Southern Biotech. Assoc.* (Birmingham, AL, USA), fetal calf serum (FCS) was provided by *Hangzhou Sijiqing Corp.*, aluminum hydroxide gel (Alum) was purchased from *Zhejiang Wanma Pharm Co. Ltd*, cyclosporin A (CsA, positive drug) was provided by *Hangzhou Huadong Medicine Co. Ltd*, Zhejiang, China.

*Experimental Animals.* Female ICR mice (Grade II, 5 weeks old) weighing 18-22 g were purchased from *Zhejiang Experimental Animal Center* (Certificate No. 22-2001001, Hangzhou, China) and acclimatized for 1 week before use. Rodent laboratory chow and tap water were provided *ad libitum*, and maintained under controlled conditions at a temp. of  $24\pm1^\circ$ , humidity of  $50\pm10\%$ , and a 12-h light/12-h dark cycle. All the procedures were in strict accordance with the P. R. China legislation on the use and care of laboratory animals, and with the guidelines established by the *Institute for Experimental Animals* of Zhejiang University and were approved by the university committee for animal experiments.

*Plant Material.* The whole plant of *Sedum sarmentosum* BUNGE was collected in Hangzhou, Zhejiang province, China, in July 2004. A voucher specimen (No. 20040612) has been deposited with the *Laboratory of Nature Drug*, College of Animal Sciences, Zhejiang University, China, and identified by Prof. *Xiang-Ji Xue* at the College of Pharmaceutical Sciences, Zhejiang University.

Preparation and Analysis of Extract. The freshly collected plants of S. sarmentosum were dried at  $40^{\circ}$  in the dark and ground into powder. The material (3 kg) was extracted with 70% EtOH three times under reflux for 2 h. After filtration and centrifugation  $(1700 \times g, 30 \text{ min})$ , the combined soln. was concentrated under reduced pressure in a rotavapor at  $45^{\circ}$  and finally dried in high vacuum to afford *ca*. 157.15 g of viscous extract (EESS; yield 5.24% (*w/w*)). EESS was suspended in H<sub>2</sub>O and then fractionated successively with petroleum ether (PE), AcOEt, and BuOH until the org. solvent layer became colorless, leaving a residual aq. fraction. Each fraction was evaporated in high vacuum to yield the residues of PE, AcOEt, BuOH, and aq. fractions, resp. The BuOH fraction was dissolved with MeOH, filtered through a 0.22-µm *Millipore* filter, and then used for quant. analysis by HPLC. The content of compound **1** in the BuOH fraction was determined using a *Symmetry*<sup>®</sup> *C18* column (250 mm × 4.6 mm i.d., particle size 5 µm), MeOH/H<sub>2</sub>O 35:65 as mobile phase and *Waters 2996 PDA* detector on the *Water 600E* HPLC instrument.

A stock soln. with a concentration of 10 mg/ml was prepared by dissolving EESS in 0.89% saline. The soln. was sterilized by passing it through a 0.22-µm *Millipore* filter, and then analyzed for endotoxin level

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by a gel-clot *Limulus* amebocyte lysate assay (*Zhejiang A and C Biological*, Zhejiang, China). The endotoxin level in the EESS soln. was less than 0.5 EU (endotoxin units)/ml.

In vitro Splenocyte Proliferation Assay. Spleen collected under aseptic conditions, in Hank's balanced salt soln. (Sigma), was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension, and the erythrocytes were lysed with NH<sub>4</sub>Cl (0.8% (w/v)). After centrifugation ( $1500 \times g$  at 4° for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium (RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2sulfanylethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% FCS). Cell numbers were counted with a hemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as described in [28]. Briefly, splenocytes were seeded into a 96-well flat-bottom microtiter plate (Nunc) at  $1 \times 10^7$  cells/ml in 100 µl of complete medium, thereafter Con A (final concentration 5 µg/ml), or LPS (final concentration 10 µg/ml), or RPMI 1640 medium with CsA and EESS (final concentration  $0.1-100.0 \mu g/ml$ ) was added giving a final volume of 200  $\mu$ l (tetraplicate wells). The plate was incubated at 37° in a humidified atmosphere with 5% CO<sub>2</sub>. After 44 h, 50 μl of MTT soln. (2 mg/ml) was added to each well and incubated for further 4 h. The plates were centrifuged ( $1400 \times$ g, 5 min), and the untransformed MTT was removed carefully by pipetting. 200 µl of a DMSO soln. (192 µl DMSO with 8 µl of 1N HCl) was added to each well, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630-nm reference after 15 min. The stimulation index (SI) was calculated based on the following formula: SI=the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

Administration and Immunization. The mice were immunized by the procedures as described in [28]. Six-week-old female ICR mice were divided into several groups, each consisting of five mice. Animals were immunized subcutaneously in one hind limb with 200  $\mu$ g of OVA and 1 mg of *Alum* in 0.2 ml of saline soln. on day 0. A boosting injection was given 2 weeks later. Beginning on the day of immunization, the immunized mice were administered ip with EESS and its fraction at a single dose of 0.25, 0.5, and 1.0 mg (the dose of fractions was standardized to yields found in EESS), and cyclosporin A (CsA, positive drug) at a single dose of 0.1 mg in 0.2 ml of saline soln. at intervals of 7 days. Saline-treated animals were included as blank control. The above-mentioned doses of EESS and its fractions did not cause any mortality and side effects, and was considered safe in this experiment. Two weeks after the last immunization, the splenocytes and sera were collected for proliferation assay and measurement of OVA-specific antibody.

In vivo *Splenocyte Proliferation Assay.* Splenocytes collected from the immunized mice prepared as described before were seeded into a 96-well flat-bottom microtiter plate (*Nunc*) at  $1 \times 10^7$  cells/ml in 100 µl of complete medium, thereafter Con A (final concentration 5 µg/ml), LPS (final concentration 10 µg/ml), OVA (final concentration 20 µg/ml), or RPMI 1640 medium were added to give a final volume of 200 µl (tetraplicate wells). The plates were incubated at 37° in a humidified atmosphere with 5% CO<sub>2</sub>. Splenocyte proliferation was assayed as described before.

Measurement of OVA-Specific Antibody. OVA-Specific IgG, IgG1, and IgG2b antibodies in serum were detected by an indirect ELISA as described in [28]. In brief, the 96-well microplates were coated with 100 µl/well of OVA soln. (50 µg/ml in 50 mM carbonate-bicarbonate buffer, pH 9.6) for 24 h at 4°. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20, and then blocked with 5% FCS in PBS at 37° for 1 h. After three washings, 100 µl of 1:200 diluted serum samples (or 0.5% FCS in PBS as control) were added to triplicate wells. The plates were then incubated for 2 h at  $37^{\circ}$ , followed by repeated washing  $(3 \times)$ . Next, horseradish peroxidase-conjugated antibody against IgG, IgG1, or IgG2b was added to the wells, and incubated for 2 h at 37°. After washing, the peroxidase activity was determined as follows: 100 µl of substrate soln., made from 10 mg of 'ortho-phenylenediamine' (=benzene-1,2-diamine) and 37.5 µl of 30% H<sub>2</sub>O<sub>2</sub> in 25 ml of 0.1M citrate-phosphate buffer (pH 5.0), was added to each well. The plate was incubated for 10 min at 37°, and the enzyme reaction was terminated by adding 50  $\mu$ l/well of an aq. 2N H<sub>2</sub>SO<sub>4</sub> soln. The optical density (*OD*) was measured with an ELISA reader at 490 nm, with a 595-nm reference. Data were expressed as the mean OD value of the samples minus the mean OD value of the blank control. Where sets of serum samples have been subjected to within and between group comparisons, ELISA assays were performed on the same day for all of the samples.

*Statistical Analysis.* The data were expressed as mean  $\pm$  S.D., and examined for their statistical significance of difference with *Student*'s *t*-test, *p* < 0.05 being considered significant.

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