IMMUNOSTIMULATORY EFFECT OF IMMUNOSTIM PLUS – A STANDARDIZED FIXED COMBINATION OF SCHIZANDRA CHINENSIS WITH ELEUTHEROCOCCUS SENTICOSUS EXTRACTS ON LYMPHOCYTE-DEPENDENT CELLULAR IMMUNITY IN MICE

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Abstract

The in vivo effects of fructus Schizandraceae and radix Eleutherococci combined extracts on various parameters of lymphocyte activity in Balb/c mice were studied. Feeding mice 300, 600 or 1200 µg of the extracts per day for 7 d, produced the activation of chemokinetic activity of spleen lymphocytes, enhancement of proliferative response of splenocytes and blood lymphocytes to mitogens, stimulation of the graft-versus-host activity of splenic lymphocytes, and stimulation of SRBC antibody production.

Key words: mice, Schizandra, Eleutherococcus, lymphocytes, cellular immunity, humoral immunity.

Eleutherococcus senticosus (siberian ginseng) is a commonly used herbal preparation with adaptogenic, anti-stress, and immunomodulatory properties (3, 5, 8, 10, 18, 19). Schizandra chinensis (Magnoliaceae), Chinese herb, has a long history of its medical use as adaptogen and anti-oxidant, and because of its hepatoprotective properties. It was effective against viral and chemical induced hepatitis (12). However, data concerning the solitary immunotropic effects of Shizandra, or immunotropic effects from the combination of Siberian ginseng with Shizandra are lacking. The aim of this study was to evaluate, for the first time, the effect of these two medicinal plant extract combinations, for the important parameters of in vivo lymphocyte function in mice.

Material and Methods

Animals and experimental design. The study was performed on 8-10 week old inbred Balb/c mice, weighing about 20 g, of both sexes, delivered from a breeding colony from the Polish Academy of Sciences, and Warsaw and Mazurian (Olsztyn) universities. The studied material was Immunostim Plus (Herbapol Lublin, Poland), 300 mg capsules, composed of dried extract of Eleutherococcus senticosus radix (155 mg), dried extract of Schizandrae chinensis fructus (100 mg), and adjunctive substances (50 mg). The mice received Immunostim for 7 d in daily dose of 600 µg. The dose corresponded to 300 mg given to a 70 kg person (applying the counter 7 for differences between the mouse and human in relation of body surface to body mass), and was suggested by the producer as a daily therapeutic dose. This dose was used in all our experiments. The effect of higher (1 200 µg) or lower (300 µg) dose of Immunostim was also evaluated in some experiments. Mice received orally (feeding with the use of Eppendorf pipette) Immunostim dissolved in 45 µL of water or in 10% ethanol, and the same dose of 10% ethanol or water as corresponding controls.

On day 8, some mice were immunised with 10% SRBC (0.2 mL intraperitoneally), and bled 7 d after the immunisation. The remaining group of mice was anaesthetized with chloral hydrate, bled from the retroorbital plexus, and euthanized. Their spleens and blood samples were used for cellular immunity tests, and the Splenocytes were isolated from mice under sterile conditions. The splenocytes were isolated from mice under sterile conditions. The splenocytes were isolated from mice under sterile conditions.
conditions by straining through stainless sieve and cotton gauze, and then centrifuged on Lymphoprep in order to remove erythrocytes.

Spleen cell chemokinesis (spontaneous migration) assay in vitro was performed according to the Sandberg method (9) with our own modifications (2). Briefly, isolated splenocytes were resuspended in a Parker culture medium with 5% inactivated foetal calf serum (FCS), at the final concentration of 30x10^6 cells/mL. Afterwards, siliconized capillary tubes were filled with cell suspension, sealed with plasticine, centrifuged (5 min, 450 g), and then fixed on the glass plates. After 24 h incubation (37°C, 5% CO_2), the distances of migration were measured in millimetres at a magnification of 6.5 x, and presented as migration units (1 M.U. = 0.18 mm).

Examination of antibody level. The antibody level was evaluated by using the haemagglutination assay, in inactivated (56°C, 30 min) sera. After performing serial serum dilutions, 0.5% SRBC was added, and the mixture was incubated for 60 min at room temperature, then centrifuged (10 min, 150 g) and finally shaken. The Haemagglutination titer was evaluated under a light microscope; and the last dilution where at least 3 cell conglomerates were present in at least 3 consecutive fields at objective magnification 20x, were considered positive.

Influence on the graft-versus-host reaction. Local graft-versus-host reaction (lymphocyte-induced angiogenesis test, LIA) was performed according to Sidky and Auerbach (11). Briefly, mice were fed Immunostim Plus, as described above, next the spleens were dissected, and spleen cell suspensions were grafted intradermally (5 x 10^5 cells in 0.05 mL of Parker medium per graft) into F1 mice (Balb/c x DBA2). Before performing the injections, the mice were anaesthetised with 3.6% chloralhydrate (0.1 mL per 10 g of b. w.). Both flanks of each mouse were finely shaved with a razor, and 2-3 injections were localised on each flank. Cell suspensions were supplemented with 0.05 mL/mL of 0.01% trypan blue, in order to facilitate recognition of injection sites later on.

The grafted spleen cells recognised DBA2 antigens, and produced many immunological mediators including proangiogenic growth factors (immunological angiogenesis). A number of newly formed blood vessels were the indirect measure of this aspect of lymphocyte reactivity.

After 72 h, the mice were treated with a lethal dose of Morbital (Biowet, Pulawy, Poland). All newly formed blood vessels were identified and counted under microscopic dissection on the inner skin surface, at 6x magnification, using criteria suggested by the authors of the method. Statistical analysis was done by Student’s t-test.

Isolation of cells from blood. Three groups of mice were bled (control and mice fed 600 µg or 1 200 µg of Immunostim Plus), and blood from 5 mice was pooled in each group. Leukocytes were isolated from blood by centrifugation at 200 g for 30 min at 4°C on the Gradiisol L gradient (Aqua-Medica, Poland), washed 3 times in PBS, and then resuspended in RPMI 1640 medium (Sigma), supplemented with 10% FCS (Gibco-BRL) at a stock concentration of 2 x 10^6 cells/mL of medium. The viability of the cells was checked by supravital staining with 0.1% w/v of trypan blue.

Proliferative response of splenic and blood lymphocytes. Proliferative responses of lymphocytes stimulated by mitogen concanavalin A (ConA), phytohaemagglutinin (PHA), or lipopolysaccharide (LPS) were determined by MTT assay (7, 13, 20). MTT [3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide] (Sigma) was dissolved in PBS at concentration of 5 mg/mL and filtered. On 96-well culture plates (Costar, USA) 100 µL of blood lymphocytes in RPMI 1640 containing 10% FCS, 2 mmol L-glutamine, 0.02 mmol 2-mercaptoethanol, 1% Hepes buffer, and penicillin/streptomycin (100 U/100 µg/mL), were mixed with 100 µL of RPMI 1640 containing mitogens ConA (5 µg/mL), PHA (10 µg/mL) or LPS (20 µg/mL). Three cultures from each pool of leukocytes were established. After 72 h incubation at 37°C in 5% carbon dioxide atmosphere (Asab Incubator, Sweden), 50 µL of MTT solution was added into each well, and the plates were incubated for 4 h at 37°C. After incubation, the plates were centrifuged (140 g, 15°C, 5 min), supernatants were removed, 100 µL of DMSO (Sigma) was added into each well, and then incubated for 15 min at room temperature. After incubation, the solubilized reduced MTT was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech).

The results from 3 cultures were pooled. The mean values and standard errors from pooled experiments were used for comparison between the groups by Student’s t-test.

Results

The results from the effect of Immunostim Plus feeding on chemokinetic activity of mouse spleen lymphocytes in vitro, and on the graft-versus-host activity in vivo, after grafting these cells intradermally to F1 hybrids, are presented on the Table 1. As can be seen from the Table, highly significant stimulation of splenocyte migration in vitro cultures, and significant in vivo stimulation in local graft-versus-host response (LIA tests), for both tested Immunostim doses were found. Highly significant stimulation of splenic and blood lymphocyte proliferative response to mitogens and stimulation of SRBC antibody production, were also observed (Table 2 and Fig.1). The presented results indicate that Immunostim Plus may be used to improve antibody production and lymphocyte-mediated cellular immunity. All the tested doses displayed stimulatory properties.
Table 1
Stimulatory *in vivo* effect of IMMUNOSTIM-PLUS on the *in vitro* chemokinetic and *in vivo* graft- versus- host activities of Balb/c mice splenic lymphocytes

<table>
<thead>
<tr>
<th>IMMUNOSTIM dose (µg/mouse)</th>
<th>Number of tests</th>
<th>Mean migration distance ± SE</th>
<th>Number of tests</th>
<th>Mean number of newly-formed vessels ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24</td>
<td>5.67 ± 0.34</td>
<td>26</td>
<td>13.8 ± 0.33</td>
</tr>
<tr>
<td>300</td>
<td>24</td>
<td>11.29 ± 0.55***</td>
<td>17</td>
<td>16.8 ± 0.27**</td>
</tr>
<tr>
<td>600</td>
<td>24</td>
<td>9.58 ± 0.42***</td>
<td>17</td>
<td>15.1 ± 0.47*</td>
</tr>
</tbody>
</table>

*P<0.05  **P<0.01  *** P<0.001

Table 2
Stimulatory *in vivo* effect of IMMUNOSTIM-PLUS on the proliferation activity of mice splenic and blood lymphocytes in the *in vitro* 72 h cultures with mitogens (mean OD 620 nm ± SE)

<table>
<thead>
<tr>
<th>Daily dose (µg/mouse)</th>
<th>Spleen</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
<td>LPS</td>
</tr>
<tr>
<td>0</td>
<td>0.306 ± 0.002</td>
<td>0.315 ± 0.003</td>
</tr>
<tr>
<td>600</td>
<td>0.362 ± 0.01***</td>
<td>0.387 ± 0.006***</td>
</tr>
<tr>
<td>1200</td>
<td>0.468 ± 0.01***</td>
<td>0.418 ± 0.006***</td>
</tr>
</tbody>
</table>

***P<0.001

Fig. 1. Stimulatory effect of IMMUNOSTIM-PLUS on antibody production in mice.
Discussion

In our previous studies, a highly significant increase in granulocyte activity, without influence on leukocyte number in the blood in mice and pigs fed Immunostim Plus was observed (14, 15). These experiments revealed, for the first time, that the combination of *Schizandra chinensis* and *Eleutherococcus senticosus* extracts proved to be a strong immunostimulator of non-specific cellular defence, dependent on the first line cells, granulocytes. We also have found that Immunostim Plus, administered after tumour cell grafting, significantly diminished neovascular reaction induced in mice by syngeneic tumour cells (15).

Previously, we have also studied the effect of *Eleutherococcus senticosus* alone on lymphocyte-dependent cellular and humoral immune responses in mice (8). We have shown that this plant has immunomodulatory properties. The immunomodulatory effect of *Eleutherococcus* on lymphocyte-dependent immunity was also described by others (3, 10, 18).

In the present study, we obtained further evidence for strong immunostimulatory in vivo action of herbal remedy composed of *Schizandra chinensis* and *Eleutherococcus senticosus* extracts. This remedy augmented the most important classic parameters of lymphocyte-dependent immunity such as antibody production, and proliferative response to mitogens, chemokinetic activity, and the development of growth factors in the course of graft-versus-host reaction. We have studied this last effect using in vivo cutaneous test, which is a good and reliable method for the evaluation of angiogenic potential of various biological materials, such as immune or tumour cells, sera, and isolated growth factors. This test is also a good method to study the effect of various substances on proangiogenic activity (15-17).

In our earlier study, we reported the influence of *Eleutherococcus senticosus* on cellular and humoral response in mice. In some tests, the kinds of effect were dependent on initial activity of lymphatic cells (8). Also Schmolz *et al.* (10) suggested *Eleutherococcus senticosus* having immunomodulatory potency. They observed an inhibition at higher and stimulation at lower *Eleutherococcus* concentrations of G-CSF, IL-6, and IL-13 production in *in vitro* whole human blood cultures, enhancement of RANTES synthesis over a wide range of concentrations, and inhibition of the release of IL-4, IL-5, and IL-12. In the *in vivo* placebo-controlled human study, Bohn *et al.* (3) observed general enhancement of the activation state of T lymphocytes. Cao (4) reported stimulation of lymphokine-activated killer (LAK) cells by *radix Eleuterococcus* extract.

However, we have not found papers concerning the effect of *Schizandra* alone, or in a combination with *Eleutherococcus* on lymphocyte-dependent cellular immunity. There is one paper describing the use of a combination of these extracts in clinical studies. Amaryan *et al.* (1) performed a double blind, placebo-controlled, and randomised clinical trial of herbal ImmunoGuard in patients with familial Mediterranean fever. It was a combination of four herbal extracts, among them of *Schizandra* and *Eleutherococcus*. The authors obtained significant improvement of all features (duration, frequency, severity of attacks) in the verum group as compared with the placebo.

References

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