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Immunomodulatory effect of shikonin derivatives isolated from *Lithospermum canescens* on cellular and humoral immunity in Balb/c mice

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The immunomodulatory activity of acetylshikonin (ACS) and isobutyrylshikonin (IBS) was studied in female and male inbred Balb/c mice, and in F1 hybrids (Balb/c × C3H). ACS and IBS were isolated from *Lithospermum canescens* Lehm. (Boraginaceae) roots. Splenocytes from mice fed 40 μg of ACS had higher proliferative potential in cultures with PHA than corresponding controls and also higher migratory *in vitro* activity than splenocytes obtained from control animals. ACS at a 40 μg daily dose stimulated G-v-H reaction but inhibited it at a 200 μg dose. IBS at a 40 μg dose significantly increased humoral response.

1. Introduction

Shikonin derivatives, among them acetylshikonin (ACS) and isobutyrylshikonin (IBS), are an important group of secondary metabolites in *Lithospermum canescens* Lehm. (Boraginaceae). This species is a common prairie plant of Canadian origin.

Biological studies of shikonin undertaken by many authors, mainly in the Orient, have shown a broad spectrum of significant activities of this naphthoquinone. A detailed review on shikonin, alkannin and other naphthoquinones, their biosynthesis, chemistry, synthesis, cell cultures and medicinal properties, has been presented by Papageorgiou et al. (1999). From the medicinal point of view, the following biological activities are important features playing an essential role in therapy: antibacterial and antifungal, antiamebic, antitumor, wound-healing and anti-inflammatory effects, immunostimulating properties and also inhibition of topoisomerase I and II, protection from UV-radiation, stimulation of peroxidase, and inhibition of microsomal monooxygenase (Wagner et al. 1988; Papageorgiou at al. 1999). The inhibition of angiogenesis *in vivo* and *in vitro* induced by shikonin has been reported by Hisa et al. (1998). Yoon et al. (1999) described the induction of apoptosis in the HL 60 human premyelocytic leukemia cell line by shikonin isolated from the root of *Lithospermum erythrorhizon.* In view of the wide and varried biological spectrum of shikonin and its derivatives, extracts containing the pigments of *Lithospermum erythrorhizon* roots (Ko-Shikin) and *Arnebia euchroma* (Nan-Shikon) are marketed in Japan. An ointment called "Shiun-ko" containing shikonin and its derivatives is used to treat burns and hemorrhoids in Japan (Sasaki et al. 2002). As regards European studies, "Heliderm", a wound-healing product, gave very good results in clinical trials in terms of granulation and epithelization (Papageorgiou at al. 1999). The first phytochemical analysis of *L. canescens* was undertaken by Wiedenfeld et al. (1998, 2003) and Pietrosiuk et al. (2003). In the latter work, among other unidentified compounds two shikonin derivatives, acetylshikonin and isobutyrylshikonin, were found and determined in transformed roots of *L. canescens* growing *in vitro*. The aim of our work was to examine the influence of the compounds acetylshikonin and isobutyrylshikonin isolated from *Lithospermum canescens* roots on some parameters of cellular and humoral immunity in Balb/c mice.

2. Investigations, results and discussion

This study of the immunomodulatory activity of acetylshikonin (ACS) and isobutyrylshikonin (IBS) was performed on female and male inbred Balb/c mice, and on F1 hybrids (Balb/c × C3H) weighing ca 20 g, 7–9 weeks old.

Mice received 40 μg or 200 μg ACS or IBS daily for 7 days orally in 40 μl 10% alcohol (feeding with use of Eppendorf pipette). These doses correspond to 20 or 100 mg given to a 70 kg person (applying the factor 7 for differences between mouse and human in the ratio of surface area to body mass). Mice in the control group received 10% ethyl alcohol. The doses used were determined on the basis of our own preliminary investigations and literature data (Kim et al. 2001).

ACS and IBS were isolated from roots of *L. canescens.* To obtain the dye fractions the crumbled roots (197.0 g) were extracted with *n*-hexane in a Soxhlet apparatus for 72 h at 70 °C. The solvent was evaporated from the extract solu-

tion under reduced pressure. The separation of the individual compounds was carried out using a flash chromatography method (stationary phase: Kieselgel 60, eluent: n-hexane-CH_2Cl_2 (90 : 10 to 5 : 95), detection $\lambda = 212$ nm, nitrogen pressure (1–1.5 bar). For checking purity of the isolated compounds, HPLC and UV spectral analyses were performed in a DIONEX HPLC system equipped with an automated sample injector (ASI-100) and UVD 340S detector under the following conditions: gradient elution – acetonitrile (40–0 ml)/0.04 M orthophosphoric acid (60–100 ml); flow rate 1.5 $cm^3\ min^{-1}$; column: EC 250/4.6 Nucleosil® 120–7 mm C_{18} (Macherey – Nagel), and monitoring eluent at 215, 278, 514 and 320 nm. Structures of the isolated compounds were determined using the ^{13}C and ^{1}H NMR method (Pietrosiuk et al. publication in preparation).

The following tests were performed in the study:

1. Cellular immunity investigation *ex vivo* – Mitogen-induced (PHA) splenocyte proliferation assay (*in vitro* culture).
2. *Ex vivo* spleen cell chemokinesis assay by Sandberg (*in vitro* culture).
3. Cellular immunity investigation – *ex vivo-in vivo*: local Graft-versus-Host reaction of spleen cells (LIA test).
4. Anti-SRBC antibody production.

After 7-days' feeding, some mice were immunized with SRBC, others were sacrificed (Morbital), spleens were excised and splenocytes were isolated under sterile conditions by straining through a steel sieve and centrifugation on Histopaque 1077 to remove erythrocytes.

Proliferation activity in unstimulated and in PHA-stimulated cultures was measured by the ^{3}H thymidine incorporation test. Splenocytes from mice fed 40 μg of ACS had a higher proliferative potential in cultures with PHA than corresponding controls. However, this effect disappeared for splenocyte cultures from 200 μg treated groups of mice. In fact, in the presence of 2 μg of PHA, splenocytes from this group of mice presented a significantly lower response than those from the control group. Table 1 presents results obtained from experiments performed on splenocytes in 72-hour cell cultures *in vitro*.

Splenocytes of mice fed ACS had higher migratory *in vitro* activity than splenocytes obtained from control animals, but only in the group fed 40 μg of solution. The 200 μg dose gave significant inhibition of chemokinetic response of splenocytes. Table 2 presents results of experiments performed on splenocyte 24-hour *in vitro* cultures. ACS in 40 μg daily doses stimulated G-v-H reaction it and in 200 μg dose inhibited (Table 3). The effect on antibody production is presented in the Fig. IBS at a 40 μg dose significantly increased this parameter of humoral immunity.

Table 1: Effect of 7 days *in vivo* ACS administration on mouse spleen cell proliferative reaction to mitogen PHA in 72-hour cell culture *in vitro* (x cpm ± SE)

ACS daily dose (μg)	PHA concentration (μg/ml)			
	0	0.5	1.0	2.0
0	63 ± 6.5 n = 11	68 ± 5.9 n = 11 ***	332 ± 50 n = 11 *	3189 ± 222 n = 11 **
40	59 ± 6 n = 5	157 ± 16 n = 6	513 ± 73 n = 5	4125 ± 240 n = 6 **
200	63 ± 2.0 n = 5	78 ± 4.5 n = 6	238 ± 27 n = 6	2461 ± 156 n = 6 ↓

* 0.05 < p < 0.1; ** p < 0.05; *** p < 0.01

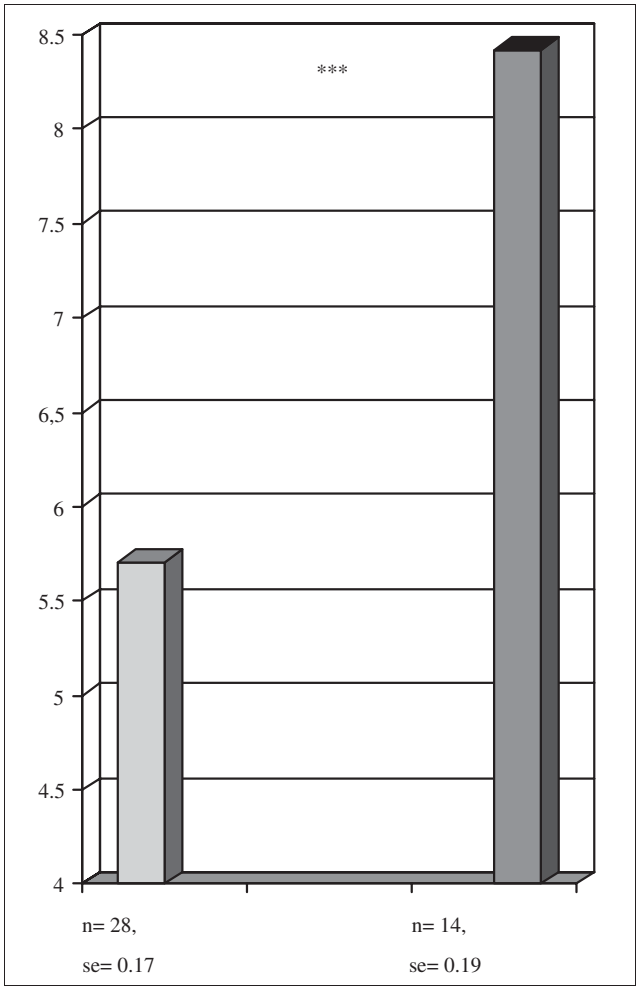

Fig: Effect of isopropylshikonin on antibody production in mice. ▒ Control ■ Isobutyrylshikonin, *** p < 0.001, se – standard error of the mean, n – number of tested sera

No reports are available in the literature about the effect of shikonin or its derivatives acetylshikonin and isobutyrylshikonin on lymphocyte proliferative, chemokinetic and graft-versus host activities, or on humoral immunity studied *in vivo*. Wagner et al. (1988) have described *in vitro*

Table 2: Effect of 7 days *in vivo* ACS administration on mouse spleen cell chemokinetic activity in 24-hour *in vitro* cell culture

ACS daily dose (μg)	Number of cultures	Stimulation/inhibition index ± SE	Statistical significance of difference from control
0 (control group)	56	1 ± 0.01	–
40	17	1.16 ± 0.06	p < 0.05 ↑
200	23	0.86 ± 0.01	p < 0.05 ↓

Table 3: Effect of 7 days ACS administration to Balb/c mice on the reactivity of their splenic lymphocytes in local G-v-H reaction in F_1 hybrids (LIA test)

ACS daily dose (μg)	Number of tests	Mean number of newly-formed blood vessels ± SE	Statistical significance of difference from control
0 (control group)	35	14.5 ± 0,53	–
40	9	20.4 ± 1.36	p < 0.01
200	20	12.7 ± 0.44	p < 0.05

stimulation of human granulocytes and lymphocytes by pico- and femtogram quantities of different compounds amongst which were alkannin and its enantiomer shikonin. Studies reporting anti-tumor and antiangiogenic effects and induction of apoptosis refer only to shikonin isolated from the Chinese herb *Lithospermum erythrorhizon* (Hisa et al. 1998; Kim et al. 2001; Yoon et al. 1999), and not from Canadian *Lithospermum canescens.* Thus, using a mouse experimental model, we show for the first time the modulatory effect of acetylshikonin and isobutyrylshikonin on cellular and humoral immunity. Results of our study indicated that the immunomodulatory effect of acetylshikonin depended on doses used. ACS stimulated lymphocyte-dependent cellular immunity in mice; it was stimulatory in a 40 μg daily dose, but inhibitory in a 200 μg dose. This might be connected with the stimulation of some populations of suppressor cells in the spleens of animals fed higher doses of ACS. This effect was visible in all parameters used to study cell-mediated immunity. IPS stimulated cellular and humoral response at a 40 μg daily dose. Cellular pharmacology studies of shikonin derivatives have suggested that shikonin is an effective inhibitor of protein-protein interaction and that this general inhibitory effect could account for various biological activities of this compound (Chen et al. 2002). On the other hand, an accelerating effect of shikonin on the proliferation of granulation tissues has been reported by Ozaki et al. (1998). We suggest that the source of conflicting results lies in the different response of immune system cells to various acetylshikonin doses; it stimulates various parameters of cellular immunity at lower doses and suppresses them at higher doses.

3. Experimental

3.1. Mitogen-induced (PHA) splenocyte proliferation assay

The mitogen-induced (PHK) splenocyte proliferation assay was done according to Kamiński et al. (1998). Briefly, spleen cell cultures were incubated in microplates (culture medium RPMI-1640 with L-glutamine, 10% FBS and antibiotics) without or with different concentrations of mitogen PHA: 0.5; 1.0; 2 and 5.0 μg/ml. After 48 h of incubation 10 μl of tritiated thymidine (^{3}HTdR 0.2 mCi) was added. After a further 24 h thymidine incorporated in cells was counted using a β-counter (RackBeta 1218, LKB Wallac).
The arithmetical mean of quadruplicate counts was calculated and expressed as counts per minute (cpm)

3.2. Spleen cells chemokinesis assay in vitro

The test was performed according to the Sandberg method (Sandberg 1976; Białas-Chromiec et al. 1999) with our modifications. Briefly, isolated splenocytes were resuspended in Parker culture medium with 5% inactivated FCS at a final concentration of 30×10^6 cells/ml. Afterwards, siliconized capillary tubes were filled with cell suspension, sealed with Plasticine, centrifuged (5 min, 450 × g) and fixed on glass plates. Cell levels were marked. After 24 h incubation (37 °C, 5% CO_2) the distances of migration were measured in millimeters (mm) at a magnification of 6.5 ×, and presented in migration units (1 M.U. = 0.18 mm).

3.3. Influence of root extract on Graft-versus-Host reaction

Local GvH reaction was performed according to Sidky and Auerbach (1975). Briefly, mice were fed with ACS as described above for 7 days, next spleens were dissected and after isolation spleen cells were grafted intradermally (2–4 × of 500 thousands of cells/mouse) into (Balb/c × C3H) F1 mice. The grafted cells recognized C3H antigens and produced many immunological mediators including proangiogenic agents (immunological angiogenesis). In this case the number of newly formed blood vessels was the measure of cell reactivity.

3.4. Antibody production

Mice were fed IPS for 7 days. On the 8th day mice received 0.2 ml 10% SRBC (Sheep Red Blood Cells) as an intraperitoneal injection. Seven days later they were bled from the retroorbital plexus. Antibody level was evaluated by a haemagglutination assay in inactivated sera (56 °C, 30 min).

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