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Evaluation of the stimulatory effect of *Epimedium alpinum* L. methanolic extract on the immune response *in vivo*

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The effect of the methanolic extract of the underground parts of *Epimedium alpinum* L. (MEEA) on the immune response to Keyhole Limpet Hemocyanine (KLH) or alloantigens *in vivo* was studied in AO rats. Immunization of experimental animals with KLH or allogeneic lymphocytes together with MEEA was followed by an increase in cellularity of draining lymph nodes (LN) and enhanced proliferation of LN lymphocytes after their restimulation with specific antigens *in vitro*, compared to control rats immunized without MEEA. These effects correlated with an increase in relative values of B, MHC class II$^+$, CD25$^+$ and CD71$^+$ cells, whereas percentages of T cells and both subsets of T cells (CD4$^+$ and CD8$^+$) were not significantly altered. As a consequence of higher LN cellularity, total numbers of all cell subsets in the MEEA-treated group of rats were significantly increased, compared to the corresponding control. The addition of MEEA together with KLH *in vitro* to LN lymphocytes of rats immunized with KLH or KLH and MEEA *in vivo* was manifested by significant increase (0.1 μg/ml of MEEA) and decrease (50 μg/ml and 100 μg/ml of MEEA) of cell proliferation, respectively. However, when LN lymphocytes from rats, immunized *in vivo* with KLH and MEEA, were stimulated *in vitro* with MEEA together with an anti-αβ T cell receptor monoclonal antibody (R73), their proliferation was siginificantly inhibited. Taken together, obtained results suggest that MEEA possesses immunostimulatory activity *in vivo*, but some components from the extract exert immunosuppressive effects *in vitro* on previously *in vivo* activated T cells.

1. Introduction

Different Asian *Epimedium* species have been widely used in traditional medicine. During the last decades, detailed chemical and pharmacological investigations on these plants indicated prenylated flavonoids as the most important constituents (Wu et al. 2003). Another characteristic constituent of *Epimedium* species, quaternary aporphine alkaloid magnoflorine (Hegnauer 1964; Chen et al. 1996), has been identified as immunologically active compound (Mori et al. 1994).

In the flora of Europe there are only two wild *Epimedium* species, while in Serbia this genus is represented by only one species, *Epimedium alpinum* L. This work is a part of our study on the pharmacological activities of the methanolic extract of root and rhizome of *E. alpinum* (MEEA). Dried extract (8.13 : 1) was obtained by maceration of defetted plant material with methanol and evaporation of filtrate under reduced pressure; it contained 7.65% of total flavonoids, calculated as hyperoside and 9.17% of alkaloid magnoflorine (Kovačević et al. 2006; Došlov-Kokoruš et al. 2006).

Here we report studies on the effect of MEEA on the antigen-specific immune response *in vivo* in rats, using Keyhole Lympet Hemocyanine (KLH) or allogeneic lymphocytes as immunogens. In addition, the response of *in vivo* activated T cells to the extract *in vitro* was also evaluated.

2. Investigations and results

Our previous *in vitro* experiments (Kovačević et al. 2006) showed immunomodulatory effects of MEEA on T cell functions *in vitro*. To confirm these effects *in vivo*, we immunized rats with KLH in CFA with or without MEEA. As shown in Fig. 1A, 9 days following immunization a significant increase in LN cellularity was observed in rats treated with a single dose (500 μg/rat) of MEEA, compared to control. LN lymphocytes from these animals proliferated more strongly *in vitro* after restimulation with KLH, compared to control animals immunized with KLH/CFA alone (Fig. 1B).

In the following experiments it was checked how MEEA modulates *in vitro* proliferation of LN lymphocytes from immunized animals. The addition of MEEA, together with KLH, *in vitro* to cultures of LN lymphocytes from rats immunized with KLH alone, resulted in siginificant increase (0.1 μg/ml of MEEA) and decrease (50 μg/ml and 100 μg/ml MEEA) of cell proliferation, respectively (Fig. 2A). Similar influence of MEEA *in vitro* was observed

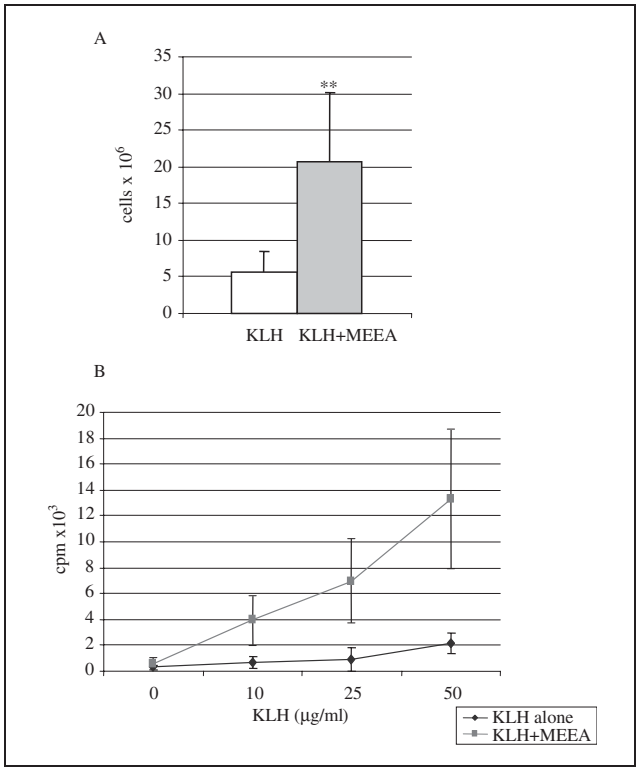

Fig. 1: Effect of MEEA on cellularity of draining LN after immunization with KLH in CFA (A) and proliferation of LN lymphocytes *in vitro* after restimulation with KLH (B)
Results are given as mean ± SD for five animals/group.
** = $p < 0.01$ compared to KLH alone (A).
Statistical significance of differences for each KLH concentration between experimental and control group (B) is $p < 0.005$

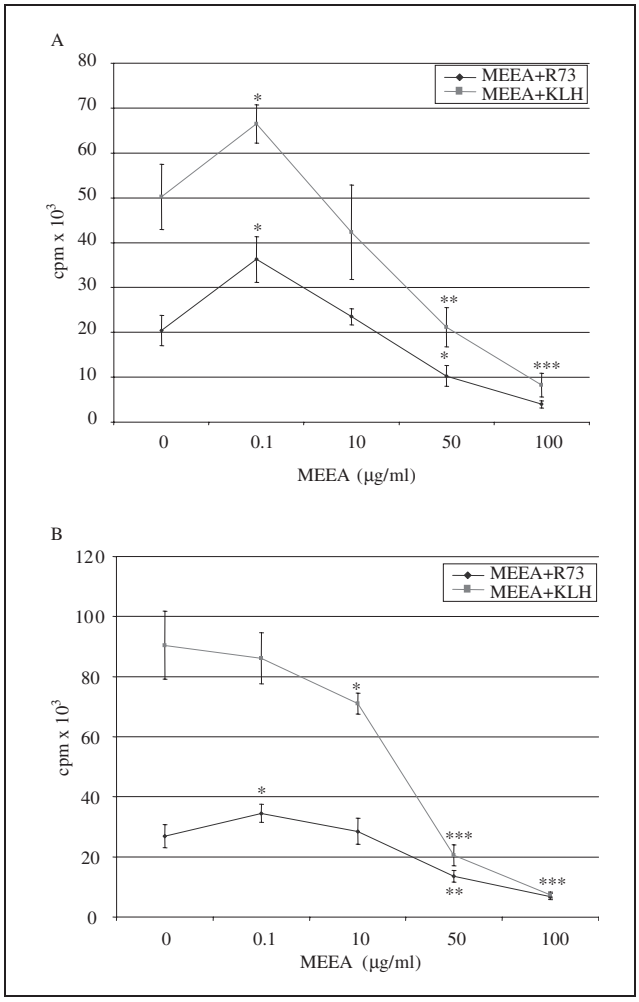

Fig. 2: Proliferation of LN lymphocytes *in vitro* in the presence of MEEA together with KLH or R73 mAb, following *in vivo* treatments of rats with KLH (A) or KLH + MEEA (B).
Concentrations of R73 mAb was 1 µg/ml and KLH 50 µg/culture. Cell proliferation was determined as described in Experimental. Values are given as mean ± SD of triplicates (one representative experiment).
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$, compared to corresponding controls (without MEEA)

using R73 mAb, as a polyclonal stimulator of T cells, instead of KLH. As expected, under such conditions, the proliferation of T cells was higher than cellular proliferation in the presence of KLH (Fig. 2A). When LN lymphocytes from rats immunized *in vivo* with KLH and MEEA were used, a similar effect of MEEA on KLH-induced T-cell proliferation *in vitro* was found, as seen in the previous control group of rats. However, when these cells were restimulated with R73 *in vitro*, MEEA only inhibited proliferation of lymphocytes. The inhibitory effect was observed using concentrations of 10 µg/ml–100 µg/ml of MEEA (Fig. 2B).

Further it was studied the effect of MEEA on the alloantigen response *in vivo*. Rats were immunized with allogeneic lymphocytes alone or with the addition of three different doses of MEEA (1.25 mg/rat; 375 µg/rat or 50 µg/rat). After 6 days, proliferation of LN lymphocytes (responders) in mixed leukocyte reaction was tested, using allogeneic lymphocytes as stimulators.

Results presented in Fig. 3 show that lymphocytes from rats treated with MEEA in doses of 1.25 mg/rat and 375 µg/rat proliferated more strongly *in vitro* than control lymphocytes; the effect was stronger at a lower stimulator/responder cell ratio. The lowest dose (50 µg/rat) did not significantly influence the alloantigen immune response.

Finally, phenotypic characteristics of LN cells in rats immunized with KLH and MEEA were studied and compared with the corresponding control (rats immunized with KLH, only). As it is presented in the Table, relative values (%) of B cells (sIg^+ cells), MHC class II^+ cells, IL-$2R^+$ cells and $CD71^+$ cells were higher in the experimental group of rats, whereas the percentages of T cells

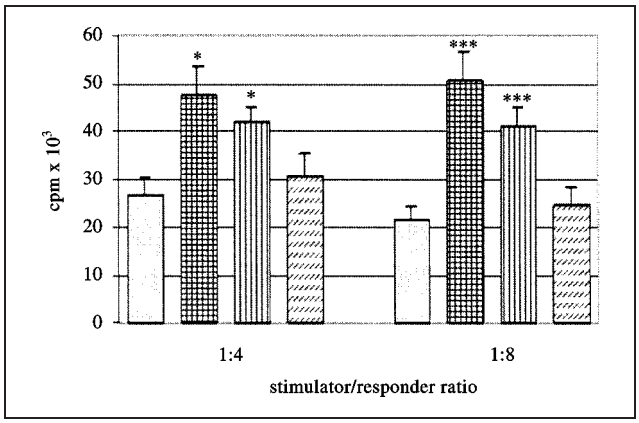

Fig. 3: Effect of MEEA on the immune response to allogeneic cells *in vivo*. Rats were immunized with allogeneic lymphocytes alone or with three different doses of MEEA, as described in Experimental. LN lymphocytes from immunized animals were restimulated *in vitro* with allogeneic lymphocytes at two different stimulator/responder ratios. Values are given as mean cpm ± SD of triplicates of one representative experiment.
* = $p < 0.05$; *** = $p < 0.005$, compared to the control (without MEEA). ☐ control; ▦ MEEA 1.25 mg; ▥ MEEA 0.375 mg; ▨ MEEA 0.05 mg

Table: Effect of MEEA on phenotypic characteristics of LN lymphocytes in rats immunized with KLH

Markers	KLH	KLH + MEEA	KLH	KLH + MEEA
	%		$\times 10^6$	
αβTCR	68.4 ± 3.7	63.3 ± 4.6	12.6 ± 1.2	19.8 ± 2.0***
CD4	45.5 ± 2.6	42.4 ± 3.4	8.1 ± 0.8	13.0 ± 1.1***
CD8	32.2 ± 3.0	33.4 ± 3.1	5.8 ± 0.9	10.2 ± 1.4***
sIg	34.1 ± 1.9	43.8 ± 2.9**	6.3 ± 1.0	13.3 ± 1.2***
CD25	1.3 ± 0.3	4.3 ± 1.3***	0.2 ± 0.2	1.3 ± 0.4***
CD71	5.9 ± 0.8	15.2 ± 2.1***	1.1 ± 0.2	4.7 ± 0.5***
MHC class II	44.2 ± 4.1	56.4 ± 3.8***	8.1 ± 1.4	17.3 ± 2.1***

LN lymphocytes were isolated at day 9 following immunization of rats with KLH alone or KLH + MEEA, as described in Experimental. Phenotypic analysis was determined by flow cytometry. Values (relative and absolute numbers of cell subsets) are given as mean ± SD for 5 animals in each group.
** = $p < 0.01$; *** = $p < 0.005$, compared to corresponding controls

($\alpha\beta TCR^+$ cells), as well as both T cell subsets ($CD4^+$ and $CD8^+$) did not significantly differ from the control. However, absolute numbers of all cell subsets were higher in the experimental group, due to increased LN cellularity. The increase of B cell number (relative to control) was higher than the increase in the number of T cells.

3. Discussion

After *in vitro* confirmation of immunostimulatory activity of methanolic extract of underground parts of *E. alpinum,* evidence for this activity was obtained *in vivo* experiments, in a model of antigen-specific proliferation of lymphocytes. A single dose of MEEA (500 μg), applied together with KLH *in vivo*, increased the number of T and B cells as well as enhanced antigen-specific proliferation of LN lymphocytes. In addition, application of some doses of MEEA (1.25 mg/rat and 375 μg/rat) significantly augmented alloreactivity *in vivo*. Similar experiments, but on different models, have been performed earlier with the extracts of *Epimedium hunanense*, *Epimedium sagittatum* and *Epimedium koreanum* (Liang et al. 1997; Chen et al. 1995; Kim et al. 2001; Liao et al. 1995).

The unresolved question arising from our's and from these results is whether other cells but lymphocytes also respond to immunomodulatory activity of *Epimedium* extracts. Present results regarding increased numbers of MHC class II^+ cells (B cells and professional antigen-presenting cells) and previous findings that components from *Epimedium* species stimulated phagocytosis by the mononuclear phagocyte system (Iinuma et al. 1990) favor the hypothesis that antigen-presenting cells could also be a target to the action of MEEA.

It is known that different components with immunoenhancing activity have been isolated from *Epimedium* extracts. Among them, flavonoids, such as epimedin C and icariin, baohuoside-1 have been best characterized (Liang et al. 1997; Xu et al. 1987; Li et al. 1994; He et al. 1995; Li et al. 1991). MEEA contained a high quantity of total flavonoids (7.65%, calculated as hyperoside) and, probably, those constituents influenced immunoenhancing activity of the investigated extract.

Analysis of the obtained results indicates that a dominant immunosuppressive mechanism of MEEA *in vitro* was a consequence of apoptosis induction. This phenomenon is in accordance with previous publications showing that certain plant alkaloids possess cytotoxic and cytostatic activities (Chen et al. 1997). The best characterized alkaloid with such activities is magnoflorine and it was isolated and confirmed as a predominant alkaloid in *E. alpinum*; MEEA contained high quantity of magnoflorine (9.17%) (Došlov-Kokoruš et al. 2006).

Magnoflorine has been shown to suppress local graft-versus-host reaction in mice by interfering with the induction phase of the cellular immune response (Mori et al. 1994). This alkaloid could be responsible for the suppression of T cell proliferation *in vitro* in the presence of higher concentrations of MEEA, especially when MEEA was added, together with a polyclonal T-cell activator (R73 mAb), to previously KLH/MEEA-stimulated LN lymphocytes *in vivo*.

4. Experimental

4.1. Animals

Experiments were performed on Albino Oxford (AO) rats, male, 10 weeks old and Dark Agouti (DA), female, 12 weeks old, bred at the vivarium of the Institute of Medical Research, Military Medical Academy, Belgrade, under conventional laboratory conditions.

Investigated extract was dissolved either in physiological saline for *in vivo* administration or in culture medium for *in vitro* experiments.

4.2. Immunization protocols

Rats were immunized by injection of KLH (Sigma, Munich, Germany) (2 mg/ml) emulsified in the complete Freund's adjuvant (CFA) with or without MEEA (10 mg/ml) in a total volume of 100 μl, into footpads. The dose of MEEA per rat was 500 μg. To measure the alloantigen-induced immune response, splenocytes (1×10^6/rat) from DA rats were mixed with physiological saline (control) or MEEA (25 mg/ml, 7.5 mg/ml or 1 mg/ml) in a total volume of 100 μl and injected into footpads of AO rats. After 6 days (immunization with allogeneic cells) or 9 days (KLH experiment) draining popliteal lymph nodes (LN) were removed and cells were prepared.

4.3. Preparation of LN lymphocytes

LN lymphocytes from immunized animals were aseptically removed, pressed through stainless meshes placed in Petri dishes with addition of phosphate buffered saline (PBS) + 5% fetal calf serum (FCS), filtered through nylon gauze to remove large debris and clumps and then washed twice by centrifugation ($580 \times g$) with RPMI medium (Sigma, Munich, Germany) + 5% FCS. Cells were counted and their viability was determined by trypan blue dye exclusion. Cell viability was usually higher than 95%.

4.4. Proliferation assay

LN lymphocytes were resuspended in complete RPMI medium and adjusted to a concentration of 2×10^6 cells/ml. Cells were cultivated for 96 h in 96-well plates (ICN, Costa Mesa, CA, USA) (200 μl/well), in an incubator with 5% CO_2, at 37 °C with different concentrations of KLH, soluble R73 (an αβ T cell receptor) (TCR) monoclonal antibody (mAb) (1 μg/ml) (Serotec), or different concentrations of MEEA with KLH (50 μg/culture) or R73 mAb. LN lymphocytes (2×10^5 cells/well) from animals immunized with allogeneic lymphocytes were restimulated *in vitro* with two different concentrations of allogeneic (DA) splenocytes as stimulators (0.5×10^5/well or 0.25×10^5/well). DA splenocytes were treated with mitomycin C (25 μg/ml) (Sigma) for 45 min, and then washed twice with RPMI medium before addition to cultures. For each experiment adequate controls were used. Cellular proliferation was measured after an 18 h pulse with 1 μCi [^{3}H]

thymidine (5 Ci/mM; Amersham, Bucks, U.K.). Cells were harvested on glass fiber filters and radioactivity was measured by standard scintillation techniques, using a Beckman scintillation counter. The results were expressed as mean count per minute (cpm) of triplicate samples.

4.5. Cell fluorescence

LN lymphocytes were resuspended in phosphate buffered saline (PBS) supplemented with 2% FCS and 0.1% sodium azide (1×10^6/tube) and incubated for 45 min at 4 °C with following mouse mAbs to rat cell surface antigens: R73 (αβTCR), W3/25 (anti-CD4), OX-8 (anti-CD8), OX-39 (anti-CD25), OX-26 (anti-CD71) and OX-6 (anti-MHC clas II). Goat anti-rat Ig conjugated with FITC was used for detection of surface Ig (sIg). All antibodies were from Serotec, Oxford, U.K. After washing, in PBS/FCS/sodium azide, lymphocytes previously incubated with mAbs were incubated for 30 min at 4 °C with polyclonal goat anti-mouse Ig conjugated with FITC (Amersham, Bucks, U.K) (dilution 1 : 50) with addition of 3% normal rat serum. After washing twice in PBS, cells were resuspended in PBS and analyzed on the EPICS-XL MCL flow cytometer. The percentages of positive cells in lymphocyte-gated populations (90% of total cells) were determined by analyzing at least 10.000 events. Cells stained with secondary antibodies only were used as controls.

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