The *in vivo* effect of *Echinacea purpurea* succus on various functions of human blood leukocytes

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Abstract

17 healthy human volunteers were treated for 7 days with 6 ml of Echinacea purpurea succus daily (IMMUNAL drops, LEK Slovenia) or placebo. Blood from cubital vein was collected twice- before and after treatment. The following parameters were evaluated: (1) release of growth factors by mononuclear leukocytes (human leukocyte-induced angiogenesis, H LIA test); (2) in vitro mononuclear leukocytes response to mitogen PHA, (3H thymidine); (3) chemiluminescent activity of granulocytes, (4) CD4 and CD8 lymphocyte markers.

Echinacea administration resulted in stimulation of proliferative and angiogenic activity of blood mononuclear leukocytes, stimulation of blood granulocytes activity and increase of CD4/CD8 ratio.

Key words: *Echinacea purpurea, in vivo treatment, human volunteers, blood leukocytes.*

(Centr Eur J Immunol 2003; 28 (3): 126–130)

Introduction

Echinacea purpurea and *Echinacea angustifolia* (Asteraceae family) belong to the most important herbal remedies with immunostimulatory properties. Commonly called the purple coneflower, Echinacea is also a favorite garden plant.

These medicinal plants originate from the North American continent, where they have been used by the Indians for centuries in folk medicine for curing burns, snake bites, severe colds, and all kinds of pains and infections. Later, Echinacea was adopted by white settlers. Between 1887-1895 Echinacea was introduced to Americans by pharmacists John King and John Uri Lloyd and at beginning of the 20-th century, it had become the best-selling tincture in the United States [1, 2]. By 1895 Echinacea products had become available in Germany. In the late 1930s, Gerhard Madaus started commercial cultivation of Echinacea purpurea in Germany. The majority of pharmacological and clinical studies performed since 1939 have involved fresh juice of the flowering Echinacea purpurea [3].

The name Echinacea is derived from the Greek word „echinos" (hedgehog) and refers to the prickly, conical receptacle of the plants. Many compounds of Echinacea extracts (polysaccharides, alkamides, polyphenols, glycoproteins) posses immunomodulatory, anti-oxidative and anti-inflammatory activity [4, 5]. Echinacea is one of the most powerful and effective remedies against many kinds of bacterial and viral infections. A lot of papers describe, on experimental models, stimulation by Echinacea extracts of various parameters of cellular and humoral immunity [6-13].

Different species of Echinacea, the part of the plant processed and the processing procedure are variables influencing its effectiveness and mode of action. However, many experts consider the fresh-pressed juice of Echinacea purpurea to be the best preparation, having the greatest level of clinical support.

Despite this statement and widespread use, the clinical value of Echinacea products is still questioned. *Placebo*-controlled randomized studies of the effect of high doses given for long time gave negative results [14-17]. On the other hand, clinical studies of short-term therapeutic administration in respiratory tract infections, or of topical application in skin diseases, were promising [18-25]. In our previous studies performed in mice we have found that *Echinacea purpurea* preparations may vary in their

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immunomodulatory effect, and some of them suppressed lymphocyte reactivity to mitogen PHA in doses recommended by producers, being stimulatory in lower doses [26, 27]. It was in agreement with the results of Coeugniet et al [23] performed in humans. So, we believe that it is very important to determine proper dose of each new Echinacea product before introducing it to human therapy.

The aim of our present study was to evaluate the effect of succus *Echinacea purpurea* originated from Slovenia (IMMUNAL drops), given for 7 days to healthy human volunteers in dose recommended by producer, on various parameters of cellular immunity mediated by blood leukocytes.

Material and methods

Study was performed in 17 healthy male volunteers, 22-35 years old. Blood from cubital vein was obtained twice, before and after 7-days treatment.

The following materials were studied:

1. IMMUNAL drops, (LEK), 6 ml daily (Lot. 000172501C, caffeic acid deriv. 5.47 mg%, 9 persons).
2. *Placebo*, 1 tablet daily (sample 58/01, 8 persons).

The following immunological parameters were studied:

- Proliferative activity of blood mononuclear cells (MNC) stimulated by mitogen PHA [28, 29].
  Briefly: MNC were isolated from heparinized blood on Lymphoprep gradient, washed thrice with PBS, resuspended in culture medium (RPMI 1640 (Difco) enriched with L-glutamine, FCS and antibiotics) at a concentration of $2x10^6$/ml and cultured in microplates with PHA at concentration 0, 1, 2 or 5 mcg/ml. Following 48 h incubation at 37°C in a humidified 5% CO_2 atmosphere, 0.2 uCi of tritiated thymidine was added for the next 18 hours. Afterwards, the cultures were harvested using multiple sample harvester (Scatron, Norway) and the incorporated radiolabel was counted using liquid scintillation counter (Rack-Beta, LKB, Sweden). Mean of quadriplicate count was calculated and expressed as counts per minute (cpm).
- Angiogenic activity of blood MNC (human leukocyte-induced angiogenesis, HLIA assay) according to [30, 31]. Briefly: MNC isolated as above were resuspended in Parker medium and injected intradermally into 6-7 weeks old female inbred Balb/c mice ($5x10^5$ cells in 0.05 ml per inoculum). Before performing injections the mice were anaesthetized with 3,6% chloralhydrate (0,1 ml per 10 g of body weigh). Both flanks of each mouse were finely shaved with razor blade, on each flank we localized 2-3 injections. Cell suspensions were supplemented with 0.05 ml/ml 0,01% trypan blue in order to facilitate recognition of injection sites later on. After 72 hours mice were killed (Morbital) their skin was separated from underlying tissues, injection sites were localized on the inner side of skin, and newly-formed blood vessels were counted in dissecting microscope at 6x magnification. Identification was done according to the criteria proposed by Sidky and Auerbach (tortuosity and divarications).
- Chemiluminescent (CL) activity of blood granulocytes, according to [32].
  Briefly: 0.05 ml of heparinized blood was diluted 1: 4 with PBS supplemented with 0.1% of glucose and 0.1% of BSA. 0.05 ml of such diluted blood was added to 0.2 ml of luminol solution (10^{-5} M) in PBS and placed in the scintillation counter (Rackbeta 1218, LKB Wallac) in the „out of coincidence" mode for spontaneous CL measurement. Then, cells were activated by addition of 0.02 ml of fMLP (Sigma) in final concentration 10^{-7} M. Chemiluminescence of stimulated cells was then measured for 15 min. The results were calculated as maximal CL value (in cpm) for 1000 granulocytes.
- Analysis of blood mononuclear cells subpopulations was done by monoclonal antibody staining of $CD4^+$ (T helper/inducer lymphocytes), $CD8^+$ (T suppressor/cytotoxic lymphocytes), and $CD19^+$ (B lymphocytes), using DAKO APAP KIT System 40, USA, in Lymphoprep- isolated blood mononuclear cell suspensions, according to producer instructions.

Statistical analysis of results was performed by Student t test.

Results

Table 1 presents the effect of IMMUNAL drops on proliferative activity of blood MNC in mitogen- stimulated cell cultures. In this test system, we observed statistically significant increase of stimulatory index, calculated by dividing values of cultures with mitogen by values of mitogen-free cultures. *Placebo* had no effect (Table 2).

Table 3 presents the results of experiments performed for estimating angiogenic activity of blood MNC, chemiluminescent activity of granulocytes and CD4/CD8 lymphocyte ratio. Significant (granulocytes activity) and highly significant stimulation (blood MNC) was observed in comparison to the values obtained before treatment. In *placebo* group, no stimulation was observed. MNC of persons treated with IMMUNAL drops presented significantly higher CD4/CD8 lymphocyte ratio after treatment, in comparison to the values of *placebo* as well as to the values obtained before treatment. Also T/B lymphocytes ratio was significantly higher after treatment (10.7 se: 1.2) than before treatment (6.1 se: 0.56), with no difference in *placebo* group.

Discussion

In our present study we focused on the effect of IMMUNAL drops on 4 important parameters of cellular

Table 1. The effect of **IMMUNAL** (succus, 6ml daily) 7-days administration to 9 human volunteers on the reactivity of their blood lymphocyt to mitogen PHA in *in vitro* culture (3H thymidine, scintillation counter); n=number of cultures; I.S. = stimulation index calculated in comparison to corresponding mitogen-free cultures

	PHA concentration					
	1 μg/ml		**2 μg/ml**		**5 μg/ml**	
	Before IMMUNAL	**After IMMUNAL**	**Before IMMUNAL**	**After IMMUNAL**	**Before IMMUNAL**	**After IMMUNAL**
n	53	54	54	54	53	54
mean I.S. ±SE	39±1.6	53.1±2.7	42.5±2.1	62.8±51	43.4±2.6	61.4±5.2
Statistical significance of difference		p<0.001 ↑		p<0.001 ↑		p<0.001 ↑

Table 2. The effect of ***PLACEBO*** 7-days administration to 8 human volunteers on the reactivity of their blood lymphocytes to mitogen PHA in *in vitro* culture (3H thymidine, scintillation counter); n=number of cultures; I.S. = stimulation index calculated in comparison to corresponding mitogen-free cultures

	PHA concentration					
	1 mcg/ml		**2 mcg/ml**		**5 mcg/ml**	
	Before *PLACEBO*	**After *PLACEBO***	**Before *PLACEBO***	**After *PLACEBO***	**Before *PLACEBO***	**After *PLACEBO***
n	48	47	48	48	48	48
I.S. ±SE	45.5±2.7	41.6±2.6	46.1±3.4	46.7±3.8	43.4±3.6	45.6±4.1
Statistical significance of difference		n. s.		n. s.		n. s.

Table 3. The effect of **IMMUNAL** drops or *placebo* administration to human volunteers for 7 days on various parameters of their cellular immunity. The results are presented as stimulation indices (value after treatment/value before treatment) ±s.e.; n = number of tests

Chemiluminescent activity of blood granulocytes			**Angiogenic activity of blood lymphocytes**		**CD4/CD8 blood lymphocytes ratio**	
	n	**S.I. ±s.e.**	**n**	**S.I. ±s.e.**	**n**	**S.I. ±s.e.**
IMMUNAL	18	1.39±0.13	267	1.17±0.02	26	1.37±0.12
Placebo	16	0.77±0.11	238	1.04±0.01	22	0.83±0.09
Statistical significance of difference		p<0.05		p<0.001		p<0.01

immunity in human, mediated by blood granulocytes and mononuclear leukocytes (lymphocytes and monocytes). We obtained good stimulation of all parameters studied using daily dose recommended by producer (6 ml) given for 7 days.

The response of lymphocytes to the plant mitogen, PHA, represents an *in vitro* correlate of an *in vivo* immunological response and involves various subpopulations of mononuclear leukocytes. We suppose, that IMMUNAL drops may be used as a drug of choice in persons presenting lowered T-cell mediated immunity.

The human leukocyte-induced angiogenesis (HLIA) test is used in the laboratory diagnostics for evaluation of total reactivity of the subject's cellular immune system. Activated lymphocytes and monocytes release large spectrum of cytokines and growth factors, among them factors possessing angiogenic activity. These factors are important in various neovascular processes, including wound repair, fractures healing, healing of ischaemic heart and brain disease, e.t.c.

IMMUNAL drops increased angiogenic potential of mononuclear leukocytes of treated persons, what suggests, that IMMUNAL may be used as a complementary drug in these pathological conditions.

Very important are the results obtained in granulocytes chemiluminescence test. Polymorphonuclear leukocytes (PMNs) provide the first line of defense against microbial pathogens. The main bactericidal mechanism of these cells

is oxygen-dependent. The most important event in the killing process is the generation of the series of reactive oxygen species during the oxidative burst. This process leads to the emission of light proportional to free radical quantity – measured as chemiluminescence (CL). CL is widely accepted as a modality for the assessment of overall PMN metabolism.

The present study shows that treatment with IMMUNAL drops significantly increased this parameter of nonspecific cellular immunity.

The Comission E of the German Institute for Drugs and Medical Devices approves several Echinacea preparations for use in colds and other upper respiratory tract infections. We suppose, that *Echinacea purpurea* succus may have wider application. Stimulation by this drug of granulocytes activity may be important for fighting many bacterial and viral infections in various body organs, not only of respiratory tract ailments. Stimulation of angiogenic growth factors release by mononuclear leukocytes opens new promising fields of application of Echinacea in ischemic diseases, disturbed healing processes, chronic infections of bones, chronic ulcerations e.t.c. Our earlier studies revealed, that some antibiotics (for example clindamycin) combined with some herbal immunomodulators (aloe extract, peat preparation) exerted synergistic stimulatory effect in HLIA test [33]. As clindamycin is often used in patients with bones infections, it would be interesting to check whether such type of synergy and better clinical results might be obtained by adding *Echinacea purpurea* succus to the therapy regimen of these patients. Echinacea preparations, given for 7-10 days are safe drugs. Toxicity studies in rats gave negative results [34]. Contraindications are controversial. There are no medical reports of Echinacea administration worsening autoimmune diseases, and western medical herbalists use Echinacea in various autoimmune conditions. The contraindications in tuberculosis and AIDS are also speculative. There are even reports (although not controlled) of successful use of Echinacea in tuberculosis [35].

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