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Effect of oral administration of freshly pressed juice of *Echinacea purpurea* on the number of various subpopulations of B- and T-lymphocytes in healthy volunteers: Results of a double-blind, placebo-controlled cross-over study ☆

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Abstract

Background: In a recent double-blind placebo-controlled crossover-study the ''immune stimulatory'' effects (activation of macrophages leading to enhanced phagocytosis and production of several cytokines) of *Echinacea purpurea* preparations (EPP) which were observed in vitro experiments and following parenteral administration could not be confirmed following oral application of the drug in healthy volunteers. The aim of the present study was to investigate whether or not oral EPP has any effect on important lymphocyte-subpopulations.

Subjects and methods: Forty healthy male volunteers (age range 20–40 years) participated in the study. They received either a commercially available pressed juice of *E. purpurea* herbs or placebo juice using a double-blind placebo-controlled cross-over design with two treatment periods of 14 days. The total number of lymphocytes and 12 subgroups of lymphocytes were determined by using Flow-cytometry.

Results: After 1 week of treatment with verum the mean value of the total number of lymphocytes decreased slightly (-6%, $p = 0.033$) compared to the initial value. Treatment for 1 and 2 weeks with EPP had only minor effects on two of the 12 subtypes of lymphocytes. No significant changes were observed in the verum period for the following types of cells: T- and B-lymphocytes, CD4+- and CD8+-T-lymphocytes including the subgroups of ''naive'' and ''memory'' CD4+- and CD8+-T-lymphocytes as well as the natural killer cells. Using a modified version of the Wilcoxon–Mann–Whitney-*U*-test, which is claimed to be optimal for the evaluation of the results of studies with a cross-over design, a significant difference was found for the number of CD8+-T-lymphocytes and natural killer cells corresponding to either a decrease during treatment with verum or an increase in the number of these cells in the placebo period.

Conclusion: Oral administration of EPP for 1 and 2 weeks has only minor effects on two out of 12 lymphocyte subpopulations determined in the study. The small differences observed in the number of CD8+-T lymphocytes and natural killer cells are only of questionable physiological relevance.

Keywords: Echinacea; Lymphocyte subpopulation; Immunmodulation; Cluster of determination

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Introduction

For about 2–3 decades, drugs containing Echinacea have gained increasing popularity as 'immune stimulating' agents in European countries, particularly not only in Germany but also in the United States (Barrett, 2003; Bauer, 1999; Cook et al., 2002; Foster, 1991; Hobbs, 1994;). Recently, it has been reported that Echinacea preparations have become one of the best selling herbal drugs in the USA (Richman and Witkowski, 1999). Based on several earlier uncontrolled clinical trials it had been concluded that Echinacea preparations ameliorate the symptoms and shorten the duration of colds and upper respiratory infections (Bauer, 1999; Bauer and Wagner, 1990; Foster, 1991). These beneficial effects of Echinacea preparations have been substantiated by the results of more recent controlled double-blind clinical trials (rev. in Barrett, 2003; Goel et al., 2004).

The manifold studies in which an influence of Echinacea preparations on leukocyte count, stimulation of the phagocytic activity and/or increased release of cytokines were found were all performed using in vitro experiments and ex vivo cell preparations following parenteral application of the drugs (Barrett, 2003; Bauer, 1999; Burger et al., 1997; Bauer and Wagner, 1990; Foster, 1991) or certain constituents of the Echinacea preparations, such as purified polysaccharides (Luettig et al., 1989; Roesler et al., 1991; Stimpel et al., 1984). When using these types of application, the Echinacea preparation or purified constituents, always come in direct contact with the cells of the immune system under investigation. In contrast to these experimental study designs, commercially available Echinacea preparations are nearly exclusively offered for oral medication. Using the oral route of administration it has to be taken into account that certain constituents of Echinacea preparation might not be absorbed by the intestinal mucosa or might be destroyed by digestion, thereby eliminating the stimulatory effect on the phagocytic activity and other parameters of the non-specific immune system observed in vitro or following parenteral administration.

In order to clarify whether phagocytic activity and production of cytokines are stimulated by oral application of a commercially available *Echinacea purpurea* preparation (EPP) we recently performed a double-blind, placebo-controlled crossover study in healthy male volunteers. The EPP did not enhance phagocytic activity of either polymorphonuclear leukocytes (PMNL) or monocytes in venous blood when compared to placebo (Schwarz et al., 2002). The EPP did also not influence the production of TNFα and interleukin-1β (IL1β) by monocytes.

Since EPPs might exert their effects by influencing other parts of the immune system it was decided to determine the number of various subpopulations of lymphocytes in the blood samples which were taken during the periods of treatment with verum and placebo in the trial mentioned above (Schwarz et al., 2002).

Material and methods

Subjects

Healthy male volunteers, age range 20–40 years were eligible for the study. Exclusion criteria and data on antropomorphic characteristics and on laboratory values of the subjects were published recently (Schwarz et al., 2002). The study was approved by the Ethics Committee of the Robert-Bosch-Hospital. Written informed consent was obtained from all participants.

Study design, treatment, and randomization

The study was performed using a double-blind placebo-controlled cross-over design with two treatment periods of 14 days each and a wash-out period of 4 weeks. During each of the treatment periods the participants ingested either a freshly pressed juice of *E. purpurea* herbs or placebo juice 12 ml daily. The study medication was the commercially available ESBERITOX® MONO of SCHAPER&BRUEMMER, Salzgitter, Germany. Esberitox® mono is a herbal medicinal product which underwent intensive quality controls including quantitative analyses. It contains 22% (v/v) of ethanol. The extent of the quality controls is laid down in the regulatory dossier for marketing authorization for Esberitox® mono liquid. This medicine was approved by the Bundesinstitut für Arzneimittel und Medizinprodukte in Germany in May 2000 for regular medicinal use. As a matter of confidentiality, detailed quality control data may not be published in order to safeguard the costly R&D data against copyists.

The placebo juice produced by the manufacturer also consisted of an ethanol/water solution containing 22% (v/v) of ethanol with artificial colour and taste adapted to that of the verum but without any Echinacea extracts. The participants were interviewed at days 0, 7 and 14 of each study period regarding regular intake of the study medication and any sign of adverse effects or symptoms of infection. Details concerning randomization, the procedure of follow-up and deviations from the study protocol were published recently (Schwarz et al., 2002). In five of the participants an acute nasopharyngeal infection occurred in one of the treatment periods (three in the verum period and two in the placebo period). These five subjects were not included in the per protocol analysis (Schwarz et al., 2002).

Blood sampling

Venous blood was collected between 8:00 and 9:00 on day 0, 7 ,14 and 42, 49 and 56. Additional blood samples for standard laboratory values including safety measurements were obtained on the first and the last day of each treatment period (day 1/42 and day 14/56; data in Schwarz et al. (2002)).

Determination of lymphocyte subpopulations

Lymphocyte subpopulations were determined using a flow-cytometer (Epics XL, Beckman Coulter, Krefeld, Germany) within 1 h following blood sampling. Lymphocyte subpopulations were identified by using the following cluster determinants (CD): CD-3, CD-4, CD-8, CD-14, CD-19, CD-56, CD-45RA, CD-45RO, HLA-DR (Immunotech Coulter Company, France). Studies on the variability from day-to-day revealed that the percentage of these subpopulations changed less than 2.7% in the mean within 1 week.

All other haematological parameters and clinical chemical analysis were performed by standard methods in the department of laboratory medicine of the Robert-Bosch-Hospital.

Statistical analysis

All values are presented as mean ± standard deviation (SD) unless otherwise specified. To test the efficacy of the treatment with verum the Wilcoxon–Mann–Whitney-*U*-test using the modification of Lehmacher (Lehmacher, 1991) was applied. This modification is claimed to be optimal for the evaluation of the results of studies with cross-over design to test the efficacy of the treatment: Intra-individual differences between the data of treatment period II (adjusted to the baseline data, day 42) and treatment period I (adjusted to the baseline data, day 0) were calculated. The differences of groups 1 and 2 were compared with the Wilcoxon–Mann–Whitney-*U*-test. In addition, data of measurements after 1 and 2 weeks of the treatment period were compared to the baseline for verum and placebo, respectively, using the *T*-test for matched pairs (Wilcoxon for matched pairs). Statistical analysis was performed for the intention-to-treat (ITT) population which includes all participants who ingested the medication at least once ($n = 40$), and the per protocol (PP) group which includes the subjects of the ITT-population without those five subjects who did not repeat the treatment period after having had the acute infection. Statistical analysis was performed using the software STATISTICA Version 5; (Edition 97 (3.C.3)).

Results

Forty healthy male volunteers (mean values ±SD: age 28 ± 5.8 years; BMI 22.9 ± 2.1) participated in the study.

Since the results of all parameters were nearly identical when expressed as mean ± SD compared to the median values and 25–75% range all values are presented as mean ± SD. The results of the ITT-analysis and of the PP-analysis hardly differed. The data, therefore, presented in the following paragraphs are preferentially those of the ITT-analysis. Only a few data from the PP-analysis will be presented (Table 3) to demonstrate either the similarity of the results or some minor differences compared to the results of the ITT-analysis.

Data on total numbers of lymphocytes, B- and T-lymphocytes and of CD4-+ and CD8-+ (CD8+) lymphocytes are listed in Table 1. The values of the absolute number and the percentage of total lymphocytes and of B- and T-lymphocytes obtained in the

Table 1. Total lymphocytes, B- and T-lymphocytes and CD4- and CD8-positive T-lymphocytes on day 0,7 and 14 (mean values ±SD)

	Verum			Placebo		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 21
Leukocytes (Giga/l)	5.7 ± 1.0	5.5 ± 0.0	5.5 ± 1.1	5.6 ± 1.1	5.5 ± 1.0	5.3 ± 0.9
Lymphocytes (%)	37.7 ± 7.0	36.5 ± 6.7	38.0 ± 7.5	36.0 ± 7.3	36.9 ± 6.5	35.6 ± 6.9
Lymphocytes (cells/µl)	2125 ± 509	1994 ± 510*	2013 ± 538	1991 ± 473	2003 ± 488	1887 ± 488
B-lymphocytes (cells/µl)	236 ± 130	227 ± 132	222 ± 99	223 ± 122	224 ± 95	210 ± 126
T-lymphocytes (cells/µl)	1560 ± 471	1492 ± 440	1496 ± 448	1492 ± 397	1496 ± 412	1396 ± 431
CD4 pos. T-lymphocytes (cells/µl)	911 ± 321	891 ± 293	886 ± 285	883 ± 268	886 ± 270	820 ± 296
CD8 pos. T-lymphocytes (cells/µl)	594 ± 238	539 ± 212	545 ± 242	534 ± 208	541 ± 222	503 ± 207
Ratio % CD4pos./%CD8pos.	1.83 ± 0.74	1.82 ± 0.70	1.84 ± 0.76	1.86 ± 0.73	1.84 ± 0.71	1.86 ± 0.77

Significant difference versus day 0: $^{*}p < 0.05$.

treatment periods with verum and placebo did not differ significantly. A minor decrease (−6%) in the total number of lymphocytes was observed in the verum period on day 7 ($p = 0.033$) and day 14 ($p = 0.062$) compared to day 0 (Table 1). In the mean values of the subgroups of CD4+ and CD8+ cells and in the ratio CD4+ : CD8+ cells no significant changes occurred in the verum and placebo periods. There was also no significant change in the mean value of the number of natural killer cells in both treatment periods (Table 2).

Using the statistical analysis of Lehmacher (SAL), however, a significant difference after 1 week of treatment was found in the number of CD8+ cells ($p < 0.05$) and of natural killer cells ($p = 0.027$) corresponding in both cases to either a decrease with verum treatment or an increase with placebo treatment (Table 3).

In the ITT analysis the number of both "naive" CD4+ cells and "memory" CD4+ cells were decreased after 1 and 2 weeks of treatment in both the verum and the placebo period (range of decrease: 5.4–9.5% and 5.6–11%, respectively; Table 2). Comparable differences were observed in the PP analysis (data not shown). For the number of "naive" CD8+ cells a significant decrease (−8.8%, $p = 0.03$) was observed only after 1 week of treatment in the verum period (Table 2). By comparing the values of the CD8-+ cells after 1 and 2 weeks of verum treatment with those of the placebo period no significant differences were found (Table 2). There were also no significant differences for the "naive" and "memory" CD4-+ and CD8-+ cells between the values of the verum and placebo period when the SAL method was used for statistical analysis (data not shown).

Table 2. Natural killer cells and subgroups of CD4pos. and CD8pos. T-cells according to the presence of cluster determinants CD45RA, CD45RO, HLA-DR (mean number of cells per µl ± SD)

	Verum			Placebo		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 21
Natural killer cells	281 ± 144	252 ± 123	272 ± 140	258 ± 139	264 ± 115	243 ± 103
CD4pos/CD45RApos (naïve T-helper cells)	598 ± 263	541 ± 237*	554 ± 233*	558 ± 258	528 ± 260	510 ± 250*
CD4pos/CD45ROpos (memory T-helper cells)	428 ± 106	385 ± 138**	395 ± 107	410 ± 93	387 ± 92*	365 ± 101
CD8pos/CD45RApos (naïve T-suppressor cells)	556 ± 217	507 ± 198*	539 ± 230	518 ± 202	513 ± 213	482 ± 196
CD8pos/CD45ROpos (memory T-suppressor cells)	215 ± 102	191 ± 104	199 ± 101	210 ± 95	197 ± 97	187 ± 83
HLA-DRpos. T-lymphocytes (cells/µl)	40 ± 14	39 ± 15	36 ± 13	37 ± 15	36 ± 15	34 ± 15
HLA-DRpos. T-lymphocytes (cells/µl)	41 ± 25	44 ± 32	39 ± 29	41 ± 25	42 ± 29	36 ± 31

Significant difference versus day 0: $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.005$.

Table 3. Differences of the number of CD8pos. T-lymphocytes and natural killer cells after 1 week of treatment. Statistical analysis using Wilcoxon–Mann–Whitney *U*-test in the modification of Lehmacher (Lehmacher, 1991)

Variable	Mean difference		*p*-Value
	For	Mean ± SD	
CD8 pos. T-lymphocytes (number of cells/µl)	Whole group	−41 ± 202	ITT: $p = 0.048$
	Subgroup 1[a]	−115 ± 217	
	Subgroup 2	+21 ± 171	
	Whole group	−36 ± 198	PP: $p = 0.198$
	Subgroup 1	−99 ± 234	
	Subgroup 2	+6 ± 162	
Natural killer cells (number of cells/µl)	Whole group	−22 ± 125	ITT: $p = 0.027$
	Subgroup 1	−63 ± 120	
	Subgroup 2	+11 ± 122	
	Whole group	−16 ± 124	PP: $p = 0.096$
	Subgroup 1	−47 ± 124	
	Subgroup 2	+6 ± 122	

[a]Subjects taking the Echinacea preparation in the first treatment period = subgroup 1 ($n = 20$), subjects taking placebo in the first treatment period = subgroup 2 ($n = 20$).

No changes of the number of HLA-positive lymphocytes were observed during the treatment periods with verum and placebo (Table 2). The SAL did not give any evidence for an effect of the verum or placebo treatment on the number of these cells.

Discussion

Echinacea preparations are widely used in general practitioners' prescriptions and in self-medication in Germany and some European countries (Barrett, 2003; Bauer and Wagner, 1990; Cook et al., 2002; Foster, 1991; Hobbs, 1994; Melchart et al., 1994). According to several surveys they are also the leading phytomedicines in North America (Barrett, 2003; Cook et al., 2002; Richman and Witkowski, 1999). Echinacea preparations are preferentially offered and used as ''immunostimulating'' agents for the treatment and prevention of various infectious disorders (Barrett, 2003; Bauer and Wagner, 1990; Cook et al., 2002; Foster, 1991; Hobbs, 1994). They have been suggested to be especially beneficial for the treatment of colds and infections of the upper and lower respiratory system (Bauer, 1999; Bauer and Wagner, 1990; Grimm and Müller, 1999; Hobbs, 1994). In a recent critical review nine controlled double-blind treatment trials with random assignment of participants to receive either an Echinacea preparation or placebo were identified (Barrett, 2003). In eight of the nine trials, the Echinacea preparation was suggested to be better than the placebo in decreasing the severity of symptoms and/or reducing the duration of colds and upper-respiratory infections. This beneficial effect was again confirmed in a double-blind, placebo-controlled trial using a standardized Echinacea preparation (Goel et al., 2004).

Until recently Echinacea extracts and constituents of the commercially available Echinacea preparations were assumed to induce their beneficial effects primarily by stimulating certain components of the nonspecific immune system. The most important pharmacological effects under discussion were the stimulation of the phagocytic activity of PMNL and other phagocytes (Barrett, 2003; Bauer, 1999; Bauer and Wagner, 1990; Parnham, 1999) and the activation of phagocytes to produce proinflammatory cytokines (TNFα, IL-1 and -6) and other mediators (Bauer, 1999; Burger et al., 1997; Luettig et al., 1989; Parnham, 1999; Stimpel et al., 1984). While the latter effects of Echinacea preparations were found in numerous in vitro experiments or in ex vivo cell preparations following parenteral application of the Echinacea preparation no enhancement of the phagocytic activity of PMNL or monocytes from peripheral blood were observed following oral treatment of the standardized EPP used here for 1 or 2 weeks to human volunteers in this double-blind placebo-controlled study (Schwarz et al., 2002). There was also no stimulation of the release of TNFα and IL-1 from isolated blood monocytes following the Echinacea treatment (Schwarz et al., 2002).

Compared to the numerous publications concerning the effect of Echinacea preparations on the number and function of phagocytes only little information is available regarding lymphocytes and lymphocyte subpopulations. In two clinical studies, an increase in mean total lymphocyte count in the peripheral blood was observed after i.m. or s.c. injection for 7 days with squeezed sap of *E. purpurea* (Coeugniet and Elek, 1987; Gaisbauer et al., 1986). Neither of these trials were placebo-controlled studies and both included a mixed population of patients. In both studies a transient tendency towards a decrease in the ratio of T-helper (CD4+): T-suppressor (CD8+) cells immediately after treatment was observed that normalized after a further 7 days (Coeugniet and Elek, 1987; Gaisbauer et al., 1986). In in vitro studies extracts of *E. purpurea* enhanced natural killer cell activity of peripheral blood mononuclear cells from healthy subjects and patients with the acquired immunodeficiency syndrome versus K562 cells (See et al., 1997). Conflicting results were obtained in two experimental studies following oral administration of Echinacea extracts. When male rats were fed a commercially available Echinacea product (50 or 225 mg/kg/day) for 6 weeks no changes in natural killer cell activity, T-cell-mediated delayed-type hypersensitivity or specific-antibody formation were observed. In female rats antibody formation was suppressed when the higher dose of the Echinacea preparation was given for 2 weeks (South and Exon, 2001). When a commercially available extract of *E. purpurea* root was administered via the chow to mice immunized with killed leukaemia cells in an erythroleukemic mouse model for 3 months the number of natural killer cells was significantly elevated compared to the values obtained in control animals receiving chow without the Echinacea product. In the early phase of the tumor development (9 days) the number of T- and B-lymphocytes in the spleen was markedly enhanced in the group fed the Echinacea product. The mechanisms of the stimulatory effect in the latter study remains to be clarified. Among the factors that might be involved are changes in the intestinal absorption of immune stimulating compounds present in the Echinacea preparation caused by the leukemia (Currier and Miller, 2002).

In the present study there was no increase in the overall number of lymphocytes or of any of the lymphocyte subpopulations tested. Regarding the mean values the only significant changes were small decreases after 1 week of verum treatment for the total lymphocyte count (−6%) and the subgroup of CD8+ lymphocytes expressing CD45-RA (−8,8%). When the intra-individual differences between the data of

treatment period II and treatment of period I (both adjusted to the baseline data; 14) were calculated after 1 week of treatment significant differences were found for the number of CD8 + -T-lymphocytes and natural killer cells which again corresponded either to a decrease caused by verum treatment or an increase caused by placebo. These slight changes in total lymphocyte count and in a few lymphocyte subpopulations are probably only of questionable biologic significance. This interpretation of the aforementioned minor differences is supported by the transient nature of these effects (no significant differences following 2 weeks of verum treatment). Further, the only small but significant change after 2 weeks of treatment, a decrease in the mean number of CD4 + -T-lymphocytes expressing CD45-RA, was observed after both treatment periods (verum period −7.4%, placebo period −8.6%; Table 2). Considering the number of parameters tested, the latter small differences seem more likely to be a result produced by chance than to be caused by a true verum or placebo effect.

In conclusion the results of the present study give no evidence of an Echinacea-induced increase in any of the lymphocyte subpopulations investigated. It is assumed, that stimulation of certain parts of the lymphocyte system by Echinacea preparations observed in in vitro experiments (See et al., 1997) or following parenteral application (Coeugniet and Elek, 1987; Gaisbauer et al., 1986) of the drug are caused by direct contact of the constituents with the cells of the immune system under investigation, a feature which markedly differs from the situation when Echinacea preparations are given orally. Effects on certain parameters of the specific immune system observed after prolonged oral application of Echinacea preparations in recent studies in rats (South and Exon, 2001) and mice (Currier and Miller, 2002), are non-uniform. Further studies are needed to clarify the mechnanism(s) which are responsible for the beneficial effect of Echinacea preparations observed in clinical studies (rev. in Barrett, 2003).

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