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# Cytokine Production in Leukocyte Cultures During Therapy With Echinacea Extract

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We measured the levels of the cytokines IL-1-α, IL-1-β, IL-2, IL-6, TNF-α, and IFN-γ in culture supernatants of stimulated whole blood cells derived from 23 tumor patients undergoing a 4-week oral treatment with a spagyric extract from *Echinacea angustifolia, Eupatorium perfoliatum,* and *Thuja occidentalis* (Echinacea complex). All patients had had curative surgery for a localized solid malignant tumor. Blood was taken before treatment and after 2 and 4 weeks of therapy. Twelve untreated tumor patients at the same clinical stage, also after curative surgery, served as a control group. In the blood cell cultures of all patients, a rather wide range of cytokine levels was found. After therapy with Echinacea complex, no significant alteration in the production of the cytokines could be seen in comparison to the controls, and also the leukocyte populations remained constant. We conclude that at this application and dosage, the therapy with Echinacea complex has no detectable effect on tumor patients' lymphocytes activity as measured by their cytokine production. 

**Key words:** whole blood cell cultures, cytokine production, Echinacea complex

## INTRODUCTION

Echinacea belongs to the most commonly used plants in medical treatment and extracts have been applied for immunostimulation for many years. However, the clinical application of this drug is based mainly on delivered practical experiences rather than on the results of controlled clinical studies. Since the cell-mediated immune response of an individual is controlled by a variety of soluble cytokines, in past years measurements of these substances in blood cell culture supernatants have been used not only for determining the cellular immunological potential of tumor patients (1–5) but also for the immunological monitoring of the effect of different therapies in oncology (6–8).

The current report focuses on the measurement of the cytokines IL-1-α, IL-1-β, IL-2, IL-6, TNF-α, and IFN-γ produced by cultured mitogen stimulated peripheral blood cells of patients treated with an extract from *Echinacea angustifolia, Eupatorium perfoliatum,* and *Thuja occidentalis*. The aim of this study was to evaluate whether the immunological parameters tested are changed under the treatment with these biological substances.

## MATERIALS AND METHODS

### Patients and Therapy

Twenty-three patients (10 males, 13 females), aged 28–81 years (mean 54 years), were entered into the study after curative surgery for different solid malignant tumors (breast n = 8, colorectal n = 8, renal n = 2, lung n = 1, prostate n = 2, corpus n = 1, melanoma n = 1). These patients received orally 3 ml per day of Echinacea complex (Spagomed, Heimisbach, Switzerland). Echinacea complex is a spagyric extract (HAB, 1978) of *Echinacea angustifolia* tinctura madre (TM) 40%, *Eupatorium perfoliatum* TM 40% and *Thuja occidentalis* TM 20%. Blood was taken before therapy and then after 2 and 4 weeks of treatment.

The control group consisted of 12 patients (3 males, 9 females), ages 44–70 years (mean 58 years) with curatively excised malignant tumors (breast n = 5, colorectal n = 3, lung n = 3, prostate n = 1). From these patients, without phytotherapy, blood was taken at the same intervals.

### Blood Samples

Ten ml of heparinized venous blood was taken from each donor and the samples, kept at room temperature, were used within 3 hours. A 0.5 ml aliquot was removed for total and differential leukocyte counts.

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### Whole Blood Cell Cultures

Cultures were performed as previously described with a system for which optimal conditions and kinetics of cytokine production were established (9). In brief, heparinized venous blood was diluted 1/10 with RPMI 1640 (Seromed, Berlin, Germany), which was supplemented with 50 U/ml penicillin (Seromed) and 50 µg/ml streptomycin (Seromed) and distributed in 0.5 ml aliquots in 12 mm polystyrol tubes.

For mitogenic stimulation, phytohemagglutinin (PHA; Wellcome, Burgwedel, Germany) at a final concentration of 10 µg/ml or pokeweed mitogen (PWM; Sigma, Deisenhofen, Germany, 0.5–5 µg/ml) were added, followed by an incubation of the cell cultures at 37°C in a humidified atmosphere of 5% $CO_2$. After 2 or 4 days of culture without change of medium, 320 µl of supernatant was removed from each tube to be assayed for cytokine levels.

### Determination of Cytokines

Enzyme-linked immunoassays (ELISA) were applied for qualitative and quantitative determinations of the cytokines (Hoffmann-La Roche, Basel, Switzerland). These tests are based on the sandwich principle and are performed in one step. Since a strict correlation was found between assay performance of the naturally occurring cytokines and their recombinant counterparts, the latter were used as standards.

### Interferon-γ ELISA

IFN-γ levels in the supernatants were determined as previously described (10). In brief, the IFN-γ containing supernatants and the samples for the IFN-γ standard curve were distributed together with a horseradish peroxidase-labeled monoclonal antibody to IFN-γ (clone 69) in microtiter plates previously coated with the same clone 69 monoclonal antibody against IFN-γ. After incubation for 24 hours, unbound material was removed by a washing step, and the amount of bound peroxidase (pod) was determined by a short incubation with tetramethylbenzidine. On stopping the reaction with sulfuric acid, the color changed to yellow and its intensity was determined at 450 nm by a computerized multichannel photometer (Flow, Meckenheim, Germany). The amount of human IFN-γ was calculated from the standard curve, prepared with recombinant IFN-γ. This ELISA has an assay range of 50–1,000 pg/ml IFN-γ.

### Interleukin-1-α ELISA

For this test, microtiter plates were coated with a polyclonal goat antihuman IL-1-α antibody. For the detection of protein bound IL-1α, the pod-linked Fab-fragment of a polyclonal goat antihuman-IL-1-α antibody was used. The standard was recombinant IL-1-α (assay range: 10–100 pg IL-1-α/ml).

### Interleukin-1-β ELISA

In this test, the first immobilized antibody was a polyclonal goat antihuman-IL-1-β antibody. For the detection of bound IL-1-β, the pod-linked Fab-fragment of a polyclonal goat antihuman-IL-1-β antibody was used. The standard was prepared with recombinant IL-1-β. The test has an assay range of 100–1,000 pg/ml IL-1-β.

### Interleukin-2 ELISA

For this test, microtiter plates were coated with two monoclonal mouse antihuman IL-2 antibodies (clone 3D5 and clone 7B1) and a third pod-linked monoclonal mouse antihuman IL-2 antibody (clone 13A6) was used for detection of bound IL-2 (assay range: 50–1,000 pg/ml IL-2).

### Interleukin-6 ELISA

In this test, the first immobilized antibody was a monoclonal mouse antihuman IL-6 antibody (clone 16). For the detection of bound IL-6, a pod-linked sheep antihuman-IL-6 antibody was used. The standard was prepared with recombinant IL-6. The test has an assay range of 100–1,000 pg/ml IL-6.

### TNF-α ELISA

In this test, the first immobilized antibody was a monoclonal mouse antihuman-TNF-α-antibody (clone 6b) and the second a pod-coupled polyclonal rabbit antihuman-TNF-α-antibody. Recombinant TNF-α was used as standard. The assay range was 20–500 pg/ml TNF-α.

### Determination of T-cell Subsets by Flow Cytometry

Cells were stained with conjugated monoclonal antibodies of the Simultest™ reagents (Becton Dickinson, Heidelberg, Germany) and measured by flow cytometry using the SimulSET™ software (Becton Dickinson). In brief, 100 µl of heparinized blood was mixed and incubated at 20°C with the appropriate amount of each Simultest™ reagent in separate tubes. Contaminating erythrocytes were then removed by addition of FACS$^R$ Lysing Solution (Becton Dickinson) to the tubes. Samples were subsequently fixed with 1% formaldehyde (Riedel de Häen, Seelze, Germany).

### Statistical Analysis

The results in the treated patients and untreated control group were statistically evaluated using analysis of variance with repeated measurements with the two "within factors" time and mitogenic stimulation. With this method the values of each patient were tested individually. Due to the skewed distribution, all values were logarithmically transformed in preparation for variance analysis.

## RESULTS

### Cytokine Levels in the Whole Blood Cell Cultures Before and After Therapy With Echinacea Complex

Upon stimulation with the mitogens PHA and PWM in the cell cultures of blood taken from the tumor patients a rather wide range of cytokine levels was found. Since the values followed a skewed distribution data are presented as box plots and not as means with standard deviations. The Q1-Q3 ranges and median values of the 23 Echinacea complex treated patients are given in Table 1. Almost identical values were obtained from the blood cell cultures of the untreated control patients.

Comparison of the cytokine levels in the cell cultures from blood taken before and after 2 weeks and 4 weeks Echinacea complex therapy showed no significant difference for IL-1-α, IL-1-β, IL-2, IL-6, TNF-α, and IFN-γ. Box plots for IL-1-α and IFN-γ are shown in Figures 1 and 2.

When the cytokine values were compared in the pre- and posttherapeutic cell cultures of the same patient, no significant difference was found. Also, in the blood cell cultures of the untreated control patients of whom blood was taken at the same intervals, no change in the production of the tested cytokines could be observed.

### Differential White Blood Cell Counts and T-cell Subpopulations Before and After Echinacea Complex Therapy

During or after therapy with Echinacea complex, no significant changes were seen for the absolute counts of leukocytes, lymphocytes, monocytes, and granulocytes or for the lymphocyte subpopulations, i.e., the counts of CD4- and CD8-positive cells, for HLA-Dr positive cells and NK cells, as compared to the pretherapeutical values (Table 2).

## DISCUSSION

Echinacea preparations have been administered for immunostimulation for >80 years. Two species are listed in the European Pharmacopoea: Echinacea angustifolia and Echinacea purpurea. They differ in morphology and chemical composition. Most chemical analyses were done with Echinacea angustifolia, whereas biological activity was tested with Echinacea purpurea. However, in all reports concerning the biological effects, in vitro or animal systems were used, but they were not shown or tested in controlled clinical studies [11].

In the present study, a spagyric extract from *Echinacea angustifolia, Eupatorium perfoliatum,* and *Thuja occidentalis* was used for an oral treatment of tumor patients after curative excision of a primary malignant tumor. The effect of this therapy was monitored by measuring in vitro the leukocyte cytokine production after mitogenic stimulation. This parameter was shown to reflect rather well the actual cellular immunological activity of a patient (5,12). Our system of enzymoimmunological cytokine determinations in whole blood cell cultures has already been used in other therapy studies with immunomodulatory substances such as recombinant IL-2 or IFN-α where major alterations of the cellular immune system could be shown (8). However, in the present study we found no changes in the cytokine production of cultured mitogen-stimulated blood cells from patients who had received a therapy with Echinacea extract. Also, no changes in leukocyte and lymphocyte subpopulations cell counts were seen. Therefore we conclude that the *Echinacea* extract had no measurable influence on the cellular immunological activity at least at the used application and dosage.

To our knowledge this is the first study investigating the effect of therapeutically administered *Echinacea* extract on the cytokine production of human lymphocytes. Prior authors studied mostly the direct effect of Echinacea preparations or purified polysaccharides on different human leukocyte populations in vitro where both an enhancement of granulocyte and monocyte phagocytosis (13–16) and a secretion of IL-1, IL-6, and TNF-α by monocytes could be demonstrated (17). An effect on the unspecific immunity and an enhancement of resistance against systemic infections with *Candida albicans*

**TABLE 1. Cytokine Levels in Whole Blood Cell Cultures of 23 Patients Before and After 2- or 4-Week Therapy With Echinacea Complex[a]**

| Cytokine | Mitogen | Before therapy median (Q1–Q3) | After 2 weeks median (Q1–Q3) | After 4 weeks median (Q1–Q3) |
|---|---|---|---|---|
| IFN-γ [ng/ml] | PHA | 21 (10–45) | 28 (14–49) | 25 (13–42) |
| | PWM | 17 (8–42) | 21 (14–48) | 22 (11–43) |
| TNF-α [pg/ml] | PHA | 3,800 (2,200–5,100) | 3,500 (2,800–5,200) | 3,200 (2,300–5,400) |
| | PWM | 5,400 (2,800–7,500) | 4,600 (2,800–8,400) | 4,500 (2,800–9,500) |
| IL-1-α [pg/ml] | PHA | 87 (70–130) | 80 (67–150) | 95 (63–140) |
| | PWM | 290 (230–380) | 350 (250–400) | 320 (260–440) |
| IL-1-β [pg/ml] | PHA | 1,300 (840–2,300) | 1,900 (770–3,200) | 1,500 (680–2,000) |
| | PWM | 7,100 (4,300–11,000) | 7,100 (5,500–9,400) | 6,400 (5,600–9,700) |
| IL-2 [pg/ml] | PHA | 6,100 (3,900–9,200) | 7,100 (4,200–12,000) | 8,800 (4,800–12,000) |
| | PWM | 3,600 (1,500–6,500) | 4,700 (2,000–8,800) | 2,800 (1,600–6,000) |
| IL-6 [ng/ml] | PHA | 37 (27–47) | 42 (30–55) | 40 (31–50) |
| | PWM | 51 (41–69) | 67 (42–78) | 54 (48–68) |

[a]Mitogens: 10 μg/ml PHA, 5 μg/ml PWM.

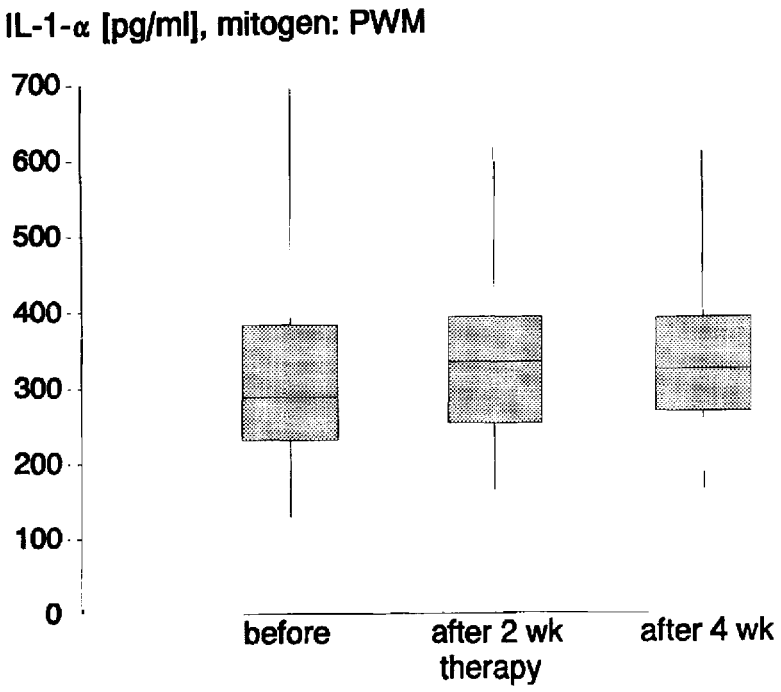


**Fig. 1.** Box plots of the IL-1-α values in mitogen-stimulated cell cultures from blood taken before and under therapy with Echinacea complex (mitogen: PWM 5 μg/ml, incubation time: 4 days, n = 23 patients).

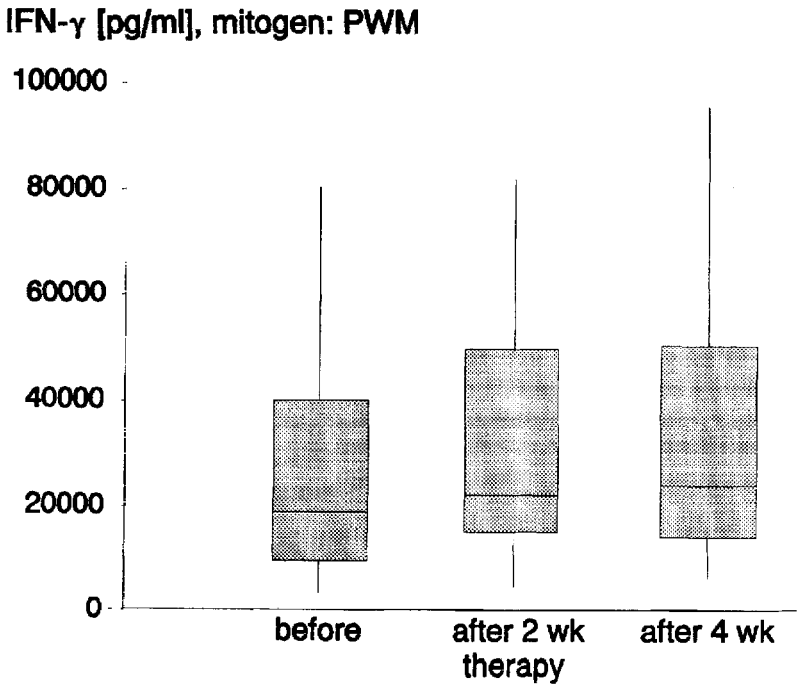


**Fig. 2.** Box plots of the IFN-γ values in mitogen-stimulated cell cultures from blood taken before and under therapy with Echinacea complex (mitogen: PWM 5 μg/ml, incubation time: 4 days, n = 23 patients).

**TABLE 2. Differential White Blood Cell Counts and T-cell Subpopulations in Peripheral Blood of 23 Patients Before and During Echinacea Complex Therapy**

| | Before therapy | | After 2 weeks | | After 4 weeks | |
|---|---|---|---|---|---|---|
| | median | Q1-Q3 | median | Q1-Q3 | median | Q1-Q3 |
| Leucocytes | 3,900 | 3,400–4,350 | 3,900 | 3,350–4,800 | 4,100 | 3,600–4,700 |
| Lymphocytes | 980 | 780–1,170 | 1,010 | 700–1,210 | 980 | 900–1,270 |
| Monocytes | 270 | 230–310 | 270 | 230–350 | 290 | 250–330 |
| Granulocytes | 2,650 | 2,540–2,810 | 2,570 | 2,420–2,890 | 2,750 | 2,580–2,910 |
| Lymphocyte subsets (% of all lymphocytes | | | | | | |
| $CD3^+$ T cells | 67 | 60–78 | 65 | 60–74 | 66 | 61–77 |
| $CD19^+$ B cells | 7 | 5–10 | 8 | 6–10 | 8 | 5–11 |
| $CD3^-/CD16^+/CD56^+$ NK cells | 23 | 18–28 | 21 | 15–28 | 21 | 15–27 |
| $CD3^+/CD4^+$ cells | 42 | 41–52 | 45 | 38–48 | 45 | 38–51 |
| $CD3^+/CD8^+$ cells | 37 | 32–43 | 36 | 31–44 | 38 | 30–43 |
| $HLADR^+$ T cells | 13 | 8–26 | 14 | 8–28 | 10 | 8–24 |

could also be shown in vivo after application of isolated polysaccharides from *Echinacea purpurea* in immunodeficient mice (18).

The reason for the missing effect of Echinacea complex therapy on leukocyte populations and cytokine production in our study may be twofold, namely, a too low concentration of active substances in the extract or a wrong route of application. Probably other preparations will give different results. Especially, nonspagyric Echinacea extracts and "high-dose" therapies should be investigated. At any rate, our method of measuring cytokine production in blood cell culture supernatants may assist in optimizing treatment regimens with biological immunomodulatory substances.

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