IMMUMNOLOGICAL EFFECTS OF AN ECHINACEA PURPUREA EXTRACT IN PATIENTS WITH BREAST CANCER

U. Elsässer-Beile[1], M. Rostock[2], P. Bühler[1], U. Wetterauer[1], M. Schumacher[3] and H. Bartsch[2]

[1] Department of Urology, University of Freiburg, Germany [2] Tumor Biology Center at the University of Freiburg. [3] Institute of Medical Biometry, University of Freiburg

Summary

Echinacea extracts have been applied for immunostimulation for many years. The clinical application though is based mainly on delivered practical experiences rather than on the results of clinical studies. Since the cell-mediated immune response is controlled by a variety of soluble cytokines, the measurement of these substances may be a means for determining the cellular immunological potential. The current report focuses on the measurement of *ex vivo* cytokine production of patients treated orally with an aqueous extract from *Echinacea purpurea* in order to evaluate the alteration of immunological parameters during the treatment. 115 patients aged between 32 and 73 years, were entered into the study after a curative treatment of breast cancer. These patients were randomised in three groups: Group I: no therapy (n=35), Group II: therapy with 8 ml *Echinacea* extract daily for 3 weeks (n=43) and Group III: therapy with 24 ml *Echinacea* extract daily for 3 weeks (n=37). From all patients heparinized blood was taken at days 0, 14 and 21. For cell culture the blood was stimulated with PHA at 10 µg/ml or PWM at 5 µg/ml for 2 days. In the supernatants the levels of the cytokines IL1-alpha, IL2, IL6, IFN-gamma and TNF-alpha were determined by ELISA. Plasma levels of soluble cytokine receptors for IL2 and TNF were determined by ELISA. Measurement of the *ex vivo* leukocyte cytokine production in the blood cell cultures of the patients of Group II revealed a slight, yet not significant increase of IFN-gamma between day 1 and day 21 in comparison to the control group without treatment. In the blood cell cultures of the patients treated with 24 ml Echinacea extract daily a significant increase of IL1-alpha, IFN-gamma, TNF-alpha and TNF-p55 receptor could be observed between day 1 and day 21. From these results it can be concluded that orally applied Echinacea seems to have a dose- dependent immunostimulatory effect on monocytes and lymphocytes.

Key words: Echinacea extract, cytokine induction, cancer patients, breast cancer

Introduction

The purple coneflower (*Echinacea purpurea* and *E. angustifolia*) has advanced to one of the most popular phytomedicines and *Echinacea* preparations are marketed and applied worldwide to provide early treatment for colds and serve as immunostimulants. During the last years, several clinical trials were conducted with *Echinacea* preparations and it appears that certain galenicals shorten the duration and severity of colds and infections of the upper respiratory tract, when given as the first symptoms become evident (1,2). However, there is still no clarity as to what extend the substances really inherit the proclaimed therapeutic potential, since other clinical trials have produced negative results (3). The reason for this may be the variable phytochemical profiles of distinct *Echinacea* products, depending on the harvested plant material and extraction protocols, as well as the varying doses in clinical trials. This makes a rational comparison of clinical trials with various *Echinacea* products virtually impossible. Nonetheless, *Echinacea* preparations are also admininstered to tumour patients as adjuvant therapy, despite this non-proven effect (4).

Since the cell-mediated immune response of an individual is controlled by a variety of soluble cytokines, the measurement of these substances in *ex vivo* lymphocyte cultures has been used for the immunological monitoring of different therapies in oncology (5).

The current report focuses on the measurement of various cytokines produced by cultured mitogen-stimulated peripheral blood cells of patients before and during the treatment with an aqueous extract from *Echinacea purpurea* at different doses. The aim of this study was to evaluate the change of certain immunological parameters under this treatment.

Methods

Patients: 115 patients aged between 32 and 73 years, were entered into the study after a curative treatment of breast cancer. The patients were required to have no chemotherapy or radiation at least 4 weeks before and throughout study. These patients were randomly distributed in three groups: Group I: control group with no therapy (n=35), Group II: therapy with 8 ml *Echinacea* extract daily for 3 weeks (n=43) and Group III: therapy with 24 ml *Echinacea* extract daily for 3 weeks (n=37). One charge of an aqueous extract from *Echinacea purpurea* was used throughout the study (Madaus, Cologne, Germany).

Blood samples: From each patient 10 ml of heparinized venous blood was taken at days 0, 14 and 21. The samples, kept at room temperature, were analysed within 3 hours. A 1 ml aliquot was removed for total and differential leukocyte counts and for flow cytometry analysis.

Whole blood cell cultures: The blood was cultured as previously described with a system for which optimal conditions and kinetics of cytokine production had been established. 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, either phytohemagglutinin (PHA; Wellcome, Burgwedel, Germany) at a final concentration of 10 µg/ml or pokeweed mitogen (PWM; Sigma, Deisenhofen, Germany) at 5 µg/ml were added, followed by an incubation of the cell cultures at 37°C in a humidified atmosphere of 5% CO2. After 2 or 4 days of culture without change of medium, 320 µl of the supernatant were removed from each tube to be assayed for cytokine levels.

Interferon-gamma ELISA: IFN-gamma containing supernatants and the samples for the IFN-gamma standard curve were distributed together with a horse radish peroxidase-labelled monoclonal antibody to IFN-gamma (clone 69) in microtiter plates previously coated with the same monoclonal antibody against IFN-gamma. After 24 hours incubation, unbound material was removed by a washing step and the amount of bound peroxidase (pod) was determined by a short incubation with tetramethylbenzidine. After stopping the reaction with sulfuric acid, the colour intensity was determined at 450 nm. The amount of human IFN-gamma was calculated from the standard curve, prepared with recombinant IFN-gamma. This ELISA has an assay range of 50 - 1,000 pg/ml IFN-gamma.

Interleukin-1-alpha ELISA: For this test microtiter plates were coated with a polyclonal goat anti-human IL1-alpha antibody. For the detection of protein-bound IL1-alpha, the pod-linked Fab-fragment of a polyclonal goat anti-human-IL1-alpha antibody was used. The standard was recombinant IL1-alpha (assay range: 10 - 100 pg IL1-alpha/ml).

Interleukin-2 ELISA: For this test microtiter plates were coated with two monoclonal mouse anti-human IL2 antibodies (clone 3D5 and clone 7B1) and a third pod-linked monoclonal mouse anti-human IL2 antibody (clone 13A6) was used for detecting bound IL2 (assay range: 50 - 1,000 pg/ml IL2).

Interleukin-6 ELISA: In this test, the first immobilised antibody was a monoclonal mouse anti-human IL6 antibody (clone 16). For the detection of bound IL6, a pod-linked sheep anti-human-IL6 antibody was used. The standard was prepared with recombinant IL6. The test has an assay range of 100 -1,000 pg/ml IL6.

TNF-alpha ELISA: In this test, the first immobilized antibody was a monoclonal mouse anti-human-TNF-alpha-antibody (clone 6b) and the second a pod-coupled polyclonal rabbit anti-human-TNF-alpha-antibody. Recombinant TNF-alpha served as standard. The assay range was 20 - 500 pg/ml TNF-alpha.

TNF-p55 receptor ELISA: For this test, microtiter plates were coated with a monoclonal mouse anti-hu-TNF-p55 receptor antibody (clone htr-20). In a single step reaction the plasma was incubated with pod-bound TNF in the microtiter wells. For the detection of bound TNF-p55-receptor pod-bound recombinant human TNF-alpha was used. (Assay range: 100 - 1,000 pg TNF-p55-receptor/ml).

IL2 receptor ELISA: This test worked on the same principle as the TNF receptor test. The first immobilised antibody was a monoclonal mouse anti-hu-IL2 receptor antibody (clone 7G7) and the second a pod-coupled human monoclonal anti-T-cell antibody. Recombinant IL2 was used as standard. The test has an assay range of 50 - 2,000 pg/ml IL2 receptor.

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 Lysing Solution (Becton Dickinson) to the tubes. Samples were subsequently fixed with 1% formaldehyde.

Statistical analysis: The results in the treated patients and untreated control group were statistically evaluated using SAS-Version 6.09. Due to the skewed distribution, all values were logarithmically transformed in preparation for variance analysis. The changes of the immune parameters during the time course were analysed with the sign test. For quantification of the substance's influence a covariance analysis was carried out.

Results

Comparison of ex vivo cytokine production in the blood cell cultures and plasma TNF-p75 receptor levels during the 3 week therapy with Echinacea extract

Upon stimulation with the mitogens PHA and PWM in the cell cultures of blood taken from the tumour patients a rather wide range of cytokine levels was found. The values followed a skewed distribution and therefore were logarithmically transformed for statistical analysis. The development of the cytokine and receptor values during the *Echinacea* extract therapy (days 0, 14, 21) of the two therapy groups II and III was statistically compared to that of the untreated Group I of control patients.

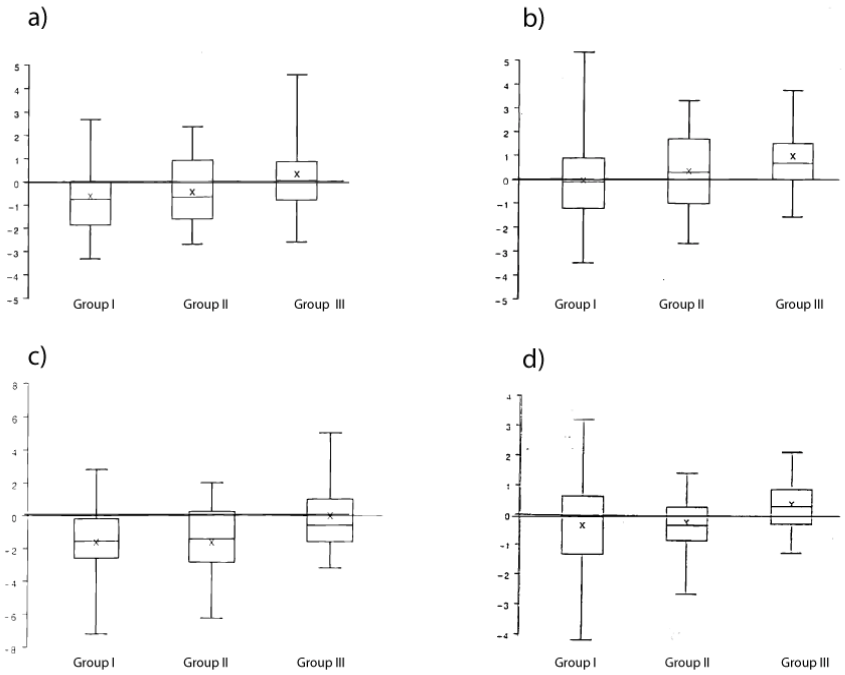

Fig. 1: Changes of cytokine production by lymphocytes and TNF-p55 receptor plasma levels of patients undergoing therapy with *Echinacea* extract (Group II: 8 ml and Group III: 24 ml) and untreated patients (Group I) between day 1 pretherapeutically and day 21 after therapy. Box-plots of the difference of the logarithmically transformed values of day 0 and day 21 are shown. a) IFN-gamma (mitogen: PWM 5 µg/ml), b) IL1-alpha (mitogen PHA) c) TNF-alpha (mitogen: PWM 5 µg/ml) d) TNF-p55 receptor plasma levels.

In the blood cell cultures of the patients of Group II with low dose *Echinacea* extract a slight, yet not significant increase of IFN-gamma was found compared to the controls. With regard to the other cytokines, no differences could be observed. In the blood cell cultures of the patients of Group III (24 ml *Echinacea* daily) a significant increase of IL1-alpha (after PHA-stimulation), IFN-gamma (after PWM-stimulation) and TNF-alpha (after PWM-stimulation) could be shown between day 1 and day 21 in comparison to the control group. Data are shown in Fig 1 a) – c). Additionally, in this Group II also the plasma levels of soluble TNF-p55 receptor increased significantly during the experimental time between day 0 and day 21 (Fig 1 d).

Differential white blood cell counts and T-cell sub-populations before and after Echinacea complex therapy

During the therapy with *Echinacea* extract no significant changes were observed in either of the two doses groups. Changes could neither be shown for the absolute counts of leukocytes, lymphocytes, monocytes and granulocytes nor for the lymphocyte sub populations i.e. the counts of CD4- and CD8-positive cells, for HLA-DR positive cells and NK cells, as compared to the pretherapeutical values.

Discussion

Echinacea represents one of the most common traditional American herbal medicines that are commonly believed to act as an immunostimulant. It has been suggested that preparations are able to stimulate both innate immune responses and T-cell response.

In vitro investigations with distinct *Echinacea* extracts have reported stimulatory effects on macrophages (6). Further authors report activation of natural killer cells, as well as non-specific induction of pro-inflammatory cytokines (7, 8). In yet another study the cytokine gene expression pattern of lymphocytes and monocytes was measured by quantitative PCR and was shown to be consistent with an anti-inflammatory response (9). Recently, the role of *Echinacea* alkylamides as potent immunomodulators and their action via the cannabinoid receptor CB2 has been identified (10).

In a previous study we have investigated the *ex vivo* cytokine induction in leukocyte cultures of patients with different solid tumours during a four week treatment with a low dose *Echinacea* extract.

In this study no significant cytokine induction was found and it was suggested that the dosage of the preparation might be too low to see immunological effects (11). Therefore, the present study was conducted with both a low dose and a high dose of an *Echinacea* extract for oral treatment of patients with breast cancer after curative therapy. Similarly to the first study, the effect of this therapy was monitored by measuring the leukocyte cytokine production pattern after mitogenic stimulation. These parameters were shown to reflect the actual cellular immunological activity quite well (12). We found significant changes of cytokine production after *Echinacea* therapy with a high dose of 25 ml extract per day, but not with a low dose of 8 ml per day. Besides the macrophage-derived cytokines IL1-alpha and TNF-alpha we also found a significant increase of IFN-gamma, which is known to be relevant for T-cell response.

In order to address the question whether the reported cytokine modulations might be physiologically relevant for the use of *Echinacea* extract, further studies are required. Facing the wide use of these preparations by cancer patients for complementary medicine, also recommendations for dosage and duration of therapy are necessary. However for all studies close attention should be paid to the use of defined and standardized preparations.

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