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Effects of Echinaforce® treatment on *ex vivo*-stimulated blood cells

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ABSTRACT

The herb *Echinacea purpurea*, also called purple coneflower, is regarded as an immune modulator. This study examined changes in cytokine production in blood samples from 30 volunteers before and during 8-day oral administration with an ethanolic extract of fresh *Echinacea purpurea* (Echinaforce®). Daily blood samples were *ex vivo* stimulated by LPS/SEB or Zymosan and analysed for a series of cytokines and haematological and metabolic parameters. Treatment reduced the proinflammatory mediators TNF-α and IL-1β by up to 24% ($p < 0.05$) and increased anti-inflammatory IL-10 levels by 13% ($p < 0.05$) in comparison to baseline. This demonstrated a substantial overall anti-inflammatory effect of Echinaforce® for the whole group ($n = 28$). Chemokines MCP-1 and IL-8 were upregulated by 15% in samples from subjects treated with Echinaforce® ($p < 0.05$). An analysis of a subgroup of volunteers who showed low pre-treatment levels of the cytokines MCP-1, IL-8, IL-10 or IFN-γ ($n = 8$) showed significant stimulation of these factors upon Echinaforce® treatment (30–49% increases; $p < 0.05$), whereas the levels in subjects with higher pre-treatment levels remained unaffected. We chose the term "adapted immune-modulation" to describe this observation. Volunteers who reported high stress levels ($n = 7$) and more than 2 colds per year experienced a significant transient increase in IFN-γ upon Echinaforce® treatment (>50%). Subjects with low cortisol levels ($n = 11$) showed significant down-regulation of the acute-phase proteins IL1-β, IL-6, IL-12 and TNF-α by Echinaforce® (range, 13–25%), while subjects with higher cortisol levels showed no such down-regulation. This is the first *ex vivo* study to demonstrate adapted immune-modulation by an Echinacea preparation. While Echinaforce® did not affect leukocyte counts, we speculate that the underlying therapeutic mechanism is based on differential multi-level modulation of the responses of the different types of leukocytes. Echinaforce® thus regulates the production of chemokines and cytokines according to current immune status, such as responsiveness to exogenous stimuli, susceptibility to viral infection and exposure to stress.

Introduction

Virally transmitted respiratory tract infections are the most common diseases in Western countries. On average, adults suffer from 2 to 4 colds per year, whereas children can be affected up to 12 times annually (Fendrick, 2003).

Echinacea purpurea, also known as purple coneflower, is a medicinal plant. *Echinacea* extracts are currently used to prevent and treat influenza infections as well as the common cold (Blumenthal et al., 2007; Woelkart et al., 2008). A meta-analysis of *Echinacea* studies concluded that it is clinically beneficial (Schoop et al., 2006a; Shah et al., 2007; Linde et al., 2006), and various pharmacodynamic actions have been proposed (Gertsch et al., 2004). However, current knowledge about the mechanism of action of *Echinacea* is based mainly on *in vitro* research, and studies have reported different effects due to the use of different preparations from the same plant species (Gertsch et al. 2004; Rininger et al. 2000). *In vitro* studies have limited usefulness, as they do not reflect the bioavailability of phytochemical compounds, which are often investigated at non-physiological concentrations. In addition, a single dose of the test compound is commonly used in *in vitro* cultures, making this an inadequate model for investigating the effects of multiple dosing to simulate prophylactic intake of the compound. *Ex vivo* studies are better models for investigating drug actions, as they better reflect the effects of digestion, resorption and metabolism. Notably, the combined use of different plant parts may enhance synergistic activity: when investigating the effects of complex plant extracts, it is necessary to investigate the effect of the extract of the whole plant during and after administering the extract to the whole

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organism. To date, few studies have investigated the effects of repeated oral administration of *Echinacea purpurea* extracts on a series of chemokines and cytokines in humans using an *ex vivo* model.

Clinical investigations have reported the immunomodulatory effects of extracts of (mainly) freshly harvested *Echinacea purpurea*: prolonged 14-day treatment with single daily doses has an anti-inflammatory effect via regulation of TNF-α, interleukins, leucocytes and hsp70 as well as via superinduction of superoxide during some viral infections (i.e. common colds) (Randolph et al., 2003; Woelkart et al., 2006; Goel et al., 2004, 2005; Guitto et al., 2008; Agnew et al., 2005). Jurcic et al. (1989) observed increased phagocytosis after oral administration of an alcoholic extract of *Echinacea purpurea*, with peak induction of 120% after 5 days compared to placebo. Some preparations increased the number of leucocytes, neutrophils and monocytes, as well as the percentage of natural killer cells (Goel et al., 2005; Agnew et al., 2005); others induced no changes, and so the data remain inconclusive.

The trials cited above involved small cohorts and, in many cases, only single time point measurements i.e. before and after treatment with *Echinacea*. Some studies investigated isolated immune mediators in serum, while others employed *ex vivo* stimulation protocols to investigate the effects of *Echinacea* on immune cell response.

The aim of this study was to investigate the effects of repeated daily doses of a commercial *Echinacea* extract, Echinaforce®, on the production of several immune mediators in a heterogeneous group of subjects using an *ex vivo* stimulation model. Adapted effects were also observed in a subanalysis of subjects with a higher susceptibility to colds and exposure to stress and who were classified as either "strong" or "weak" immune producers based on their production of immune mediators. Sampling time points, nutritional and stress status and the presence of infections and adverse events were taken into account when assessing the effects of Echinaforce®.

Materials and methods

Study design and patients

After study approval (Eudract number 2005-004013-15) by the appropriate ethics committees (Bute Medical School, University of St. Andrews and Fife and Forth Valley Local Research Ethics Committee, NHS Five) and by the Medicines and Healthcare Products Regulatory Agency (MHRA), healthy subjects ($n = 30$) were enrolled in the study after providing written informed consent. The age range of the 12 women and 18 men was 18–57 years, and each reported ≥2 colds per year. The subjects were studied once during a period of increased stress (during examinations) and again 5 weeks after the stressful situation. The stress levels of the participants during these two periods were assessed by the perceived stress score-10 questionnaire (PSS-10) (Cohen et al., 1983), which takes into account live events and the ability to cope in the previous 4 weeks. We included subjects that were experiencing heightened stress in order to investigate the effects of Echinaforce® on a population expected to have compromised immune responses. Subjects were asked not to take any other medications or therapies, to restrain from vigorous physical activity and to avoid excessive drinking or smoking during the study periods. The time points for visits were fixed at the hour of the first blood donation; a delay in subsequent visits of more than 1.5 h was considered a violation of the protocol. A study diary was provided to each subject for recording cold symptoms during the treatment periods. Compliance was monitored throughout the study.

Table 1
Phytochemical profile of the hydro-ethanolic extract of *Echinacea purpurea* (Echinaforce®) used in this study.

Compound	Concentration (ug/ml)
Caffeic acid	0
Caftaric acid	264.4
Chlorogenic acid	40.2
Cichoric acid	313.8
Cynarin	0
Echinacoside	6.9
Dodeca tetraene	35.9

Treatment

Echinaforce® is a hydro-alcoholic extract made from the freshly harvested herbs and roots of *Echinacea purpurea* in a 95:5 ratio. Batch 018451 was tested for activity i.e. inhibition of LPS-induced production of TNF-α *in vitro* in peripheral blood mononuclear cells (PBMC) (data not shown). This preparation has been demonstrated to be endotoxin-free and to contain alkylamides in bioavailable form with pharmacological activities (Gertsch et al., 2004; Woelkart et al., 2006). The levels of several markers used to characterize Echinacea preparations are shown in Table 1. Caffeic acid, cynarin and polysaccharide were not detected in the preparation. After two days of baseline measurements, treatment commenced with oral administration of 4 1-ml doses per day of Echinaforce® for 5 days, increasing to 10 1-ml doses per day for 3 days. The study thus lasted 10 days for each subject for each study period (i.e. the stressful period and the non-stressful period). All subjects reported to the study site daily during the study period for blood sampling and to report potential adverse events.

Ex vivo stimulation

Within 15 min of collection, blood samples were *ex vivo*-stimulated with either Zymosan® – (333 μg/ml) or LPS (lipopolysaccharide, variant O55:B5 from *E. coli* at 100 ng/ml)/super-antigen SEB (staphylococcal enterotoxin B at 25 ng/ml) in pre-coated tubes for 24 h at 37 °C. After incubation, the serum was separated from the sediment using a valve septum (Instant Leukocyte Culture System, ILCS®, EDI GmbH, Reutlingen, Germany) and stored at −20 °C until analysis. ILCS® was developed specifically to minimize variability in conventional leukocyte cell cultures.

After stimulation with Zymosan®, blood samples were analysed for elastase and after stimulation by LPS/super-antigen SEB, blood samples were analysed for interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, macrophage chemotactic protein-1 (MCP-1), tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ). These analyses were performed by EDI GmbH, an EN ISO 13485-certified facility (11.2000).

Efficacy evaluation

The effects of Echinaforce® were expressed as cytokine production during treatment/mean cytokine production on days 1 and 2 prior to Echinaforce® administration (baseline). The resulting stimulation indices were analysed for statistical significance based on $a = 0.1$ (*) or $a = 0.05$ (**). The effects are reported in the figures for either individual days or for the whole treatment period, each relative to baseline levels. Only results obtained from subjects who strictly adhered to the protocol were used for analysis ($n = 28$). Wilcoxon tests for paired differences were used to detect overall effects (entire study population), and Wilcoxon two-sample rank sum tests were used to detect differences between subgroups, e.g. adapted effects.

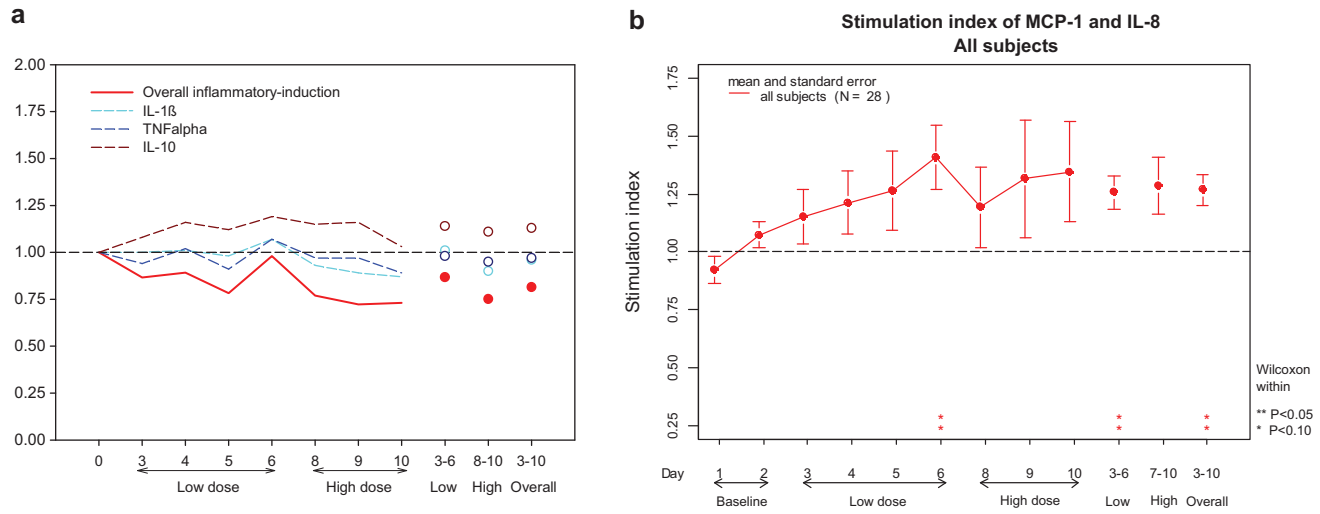

Fig. 1. (a and b) *Ex vivo*-stimulated whole blood cultures from Echinaforce®-treated subjects ($n = 28$) show increased production of an anti-inflammatory protein, IL-10 (a), and the chemotactic mediators MCP-1 and IL-8 (b), with concomitant down-regulation of pro-inflammatory TNF-α and IL-1β. Effects of Echinaforce® – in comparison to baseline – are expressed for every day, as well as for the two treatment phases ("low" and "high") and the whole treatment period ("overall"). Error bars indicate the standard error of the means (SEM).

Cytokines and chemokines were analysed not only as isolated parameters but also in groups regarding their respective actions (e.g. TNF-α, IL-1β and IL-10 levels for overall inflammatory induction or MCP-1 or IL-8 for chemotactic processes (Bry and Hallman, 1991; Dinarello, 1997)). Effects on the whole population (overall effects) are given, but also on immunologically distinct groups of subjects, selected by immune performance, by stress levels and by endogenous cortisol production (adapted effects in subgroups). Due to complexity reasons we decided to leave out the discussion of different dosing effects by the drug.

Results

A total of 30 subjects were included in the study. The mean age was 24.1 ± 11.7 years, the mean mass was 67.7 ± 13.8 kg, the mean height was 171.4 ± 9.5 cm and the mean body mass index was 23 ± 3.7. Results from 28 subjects were used in the per protocol analysis: compliance could not be calculated in one subject, and one subject dropped out of the study due to difficulty in providing blood samples.

Evaluation of individual stress levels showed a mean PSS-10 score of 19.1 ± 7.6 in the first period (during examinations) and a mean PSS-10 score of 12.0 ± 5.0 in the second (low stress) period. None of the subjects reported cold symptoms over the entire treatment period. There were also no significant or clinically relevant changes in the differential cell blood counts during the treatment period.

Overall effects (whole group)

The synergistically acting, pro-inflammatory cytokines IL-1β and TNF-α were reduced by up to 24% ($p < 0.05$) with concomitant augmentation of the anti-inflammatory factor IL-10 in comparison to the respective baseline values (13%, $p < 0.05$). Qualitative integration of these events revealed that Echinaforce® had an anti-inflammatory effect, although the contributions of single cytokines were small (overall inflammatory induction; Fig. 1).

A similar effect was noted for the production of IL-8 and MCP-1. Both were weakly up-regulated in parallel by ~15%, but when combined these factors showed significant induction in terms of chemotactic parameters. Fig. 1 illustrates the pharmacodynamic effects on inflammatory and chemotactic processes during Echinaforce® treatment. Analysis of all subjects revealed trends for elastase and IL-6, but the trends did not appear to be biologically relevant (data not shown).

Adapted effects (subgroups)

Evaluation of the whole group (overall effects) demonstrated relatively small changes in individual chemokine and cytokine levels that, taken together (related cytokines), had a biologically important effect on the immune system. To identify the adapted effects related to Echinaforce® treatment, subjects were classified into two subgroups, "strong" and "weak" producers, according to cytokine production at baseline.

Data from 8 strong producers i.e. subjects with high constitutive cytokine production, were compared to data from weak producers in order to evaluate the immune system response to Echinaforce® treatment in these subgroups. Echinaforce® extract induced initially low IFN-γ, IL-8, IL-10 and MCP-1 production by 18%, 35%, 28% and 49%, respectively [expressed as average induction for the whole treatment period ($p < 0.05$)]. In strong producers, there were no changes in these immune mediators during Echinaforce® treatment (Fig. 2). In contrast, initially high levels of TNF-α and IL-1β decreased with Echinaforce® treatment, starting on the very first day (Fig. 3).

Adapted effects were also apparent when participants were classified based on their self-reported susceptibility to cold infections and their PSS-10 stress levels, accounting for the negative impact of the latter on the performance of the immune system.

When subjects were treated with Echinaforce® during a stressful period (the first part of the study; during examinations), there was a significant increase in IFN-γ of over 25%, with peak induction of 50% ($p < 0.05$) after one day of high-dose treatment and subsequent decrease to baseline on the last day of treatment. This effect was not observed in volunteers whose perceived stress (PSS-10) dropped by more than 5 points in the second part of the study (treatment during a non-stressful time) (Fig. 4). The same induction on IFN-γ was noted in subjects who reported having more than 2 colds per year i.e. who had greater susceptibility to infections leading to the common cold (data not shown).

Safety was assessed by adverse events reporting and by other laboratory measurements. Specifically, a differential blood cell count was measured on 4 days under treatment and was compared

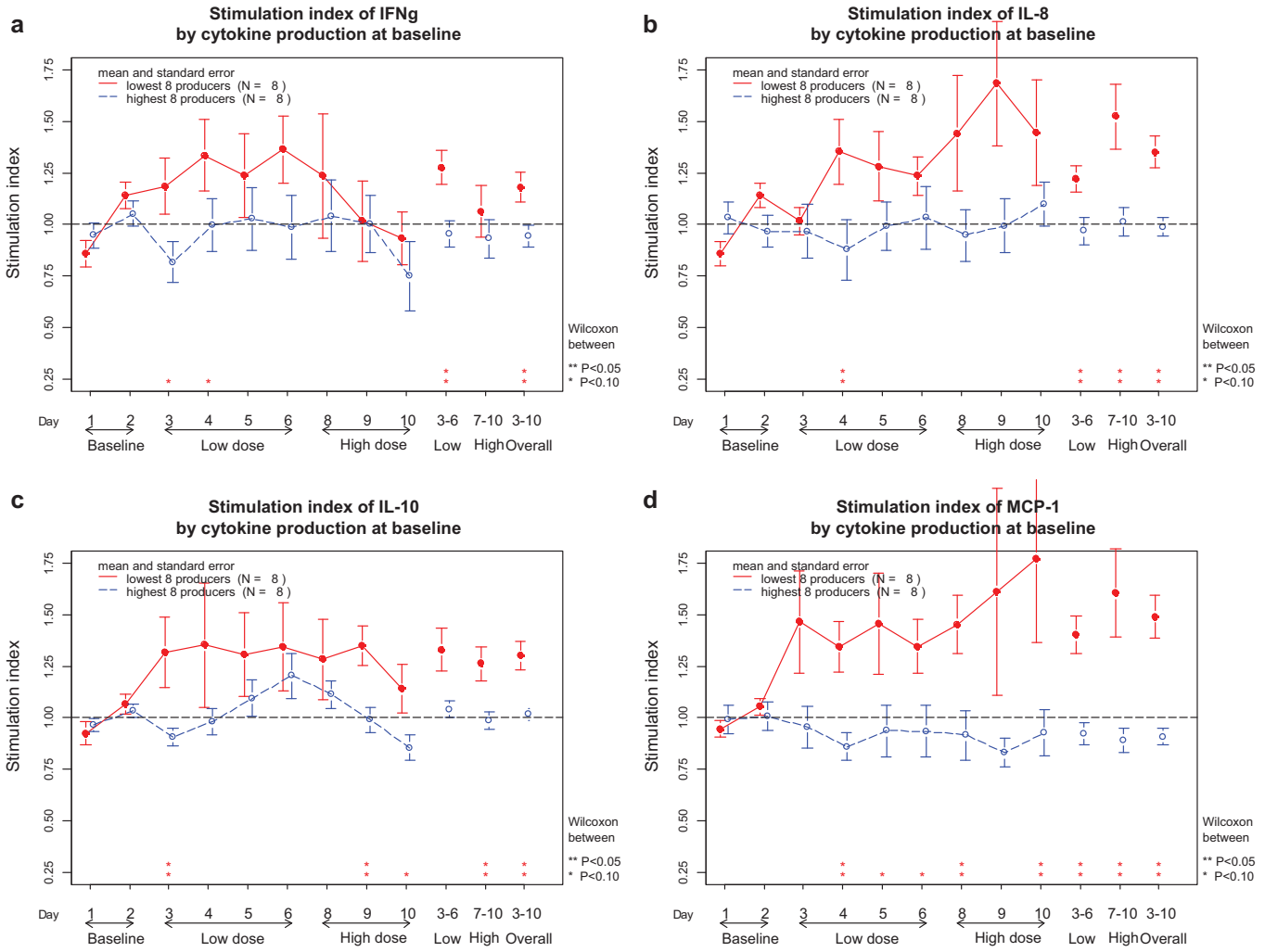

Fig. 2. (a–c) Cytokine levels prior to treatment with Echinaforce® were used to classify subjects as "strong" from "weak" producers as a measure of immune system performance. IFN-γ, IL-8, IL-10 and MCP-1 were clearly up-regulated by Echinaforce® treatment in weak producers (solid red line). Despite considerable variation in data (SEM), the increases in all four factors were statistically significant. The response in subjects who showed high cytokine production at baseline remained unaffected (dashed blue line). Echinaforce® therefore showed immunomodulatory action. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

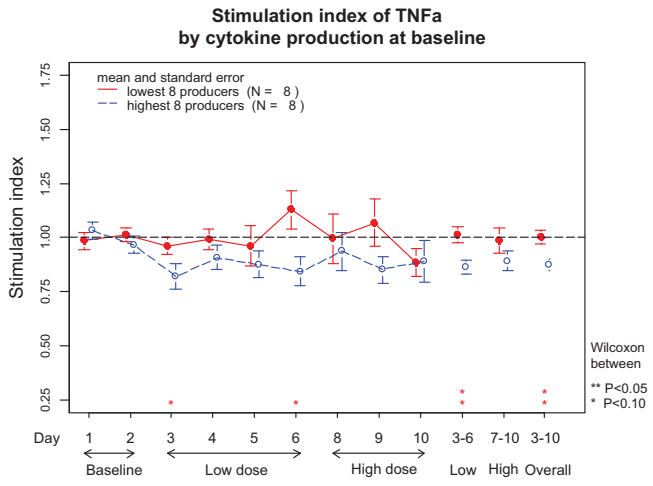

Fig. 3. TNF-α decreased during Echinaforce® treatment in strong producers, showing anti-inflammatory effects (blue dashed line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

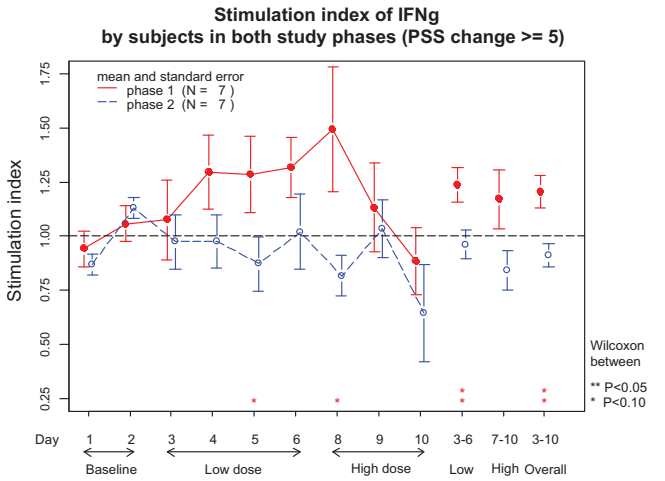

Fig. 4. IFN-γ was induced rapidly by Echinaforce® treatment and returned to baseline production at the end of the high-dose treatment. This was observed only in subjects with increased stress or higher susceptibility to cold infections.

to the values obtained before treatment (baseline). When baseline values were compared to those obtained during low- or high-dose Echinaforce® treatment, there were no changes in haemoglobin levels, haematocrit, erythrocytes, MCV, MCH, MCHC, leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils and thrombocytes. The metabolic markers GGT, bilirubin, creatinine, total cholesterol, HDL cholesterol and the total cholesterol:HDL cholesterol ratio remained stable during Echinaforce® treatment, with no clinically relevant changes. During Echinaforce® treatment, the mean C-reactive protein (CRP) level decreased from 15.50 ± 15.13 g/l at baseline to 7.10 ± 3.28 g/l. However, this did not reach statistical significance. No adverse events were observed aside from reddening of the skin at the puncture site.

Discussion

Cytokines and chemokines play critical roles in the immune response and contribute to symptoms during respiratory tract illness. Local production of immune mediators like IL-1β, IL-6, IL-8 and TNF-α increases during the common cold and during influenza infections in normal and asthmatic subjects (de Kluijver et al., 2003). Modulation of these mediators and of leukocyte activity might represent an effective strategy for the prevention and treatment of respiratory tract diseases and their consequences (Johnston, 1997).

The current study investigated a series of immune mediators that act specifically at different levels of the immune defence. Elastase, IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1, TNF-α and IFN-γ levels were measured under highly controlled conditions after *ex vivo* stimulation of whole blood from Echinaforce®-treated subjects. Overall, 8 days of treatment with Echinaforce® resulted in small but consistent effects on individual immune mediators that, in combination, resulted in biologically relevant effects on the immune system. Interestingly, IL-1β and TNF-α were both down-regulated, while IL-10 levels increased. The inverse regulation of pro- and anti-inflammatory cytokines clearly indicated an overall inhibition of inflammatory processes by Echinaforce®.

These results are in agreement with previous *in vitro* and *in vivo* studies that showed down-regulation of TNF-α plus parallel induction of IL-10 after exposure to Echinaforce® and other extracts (Gertsch et al., 2004; Randolph et al., 2003; Woelkart et al., 2006; Chicca et al., 2009; Kim et al., 2002). Since IL-10 is under the control of TNF-α, effects on IL-10 may be directly due to the effects of Echinaforce® or be caused indirectly by down-regulation of IL-1β and TNF-α. However, the rapid changes in IL-10 production in response to Echinaforce® suggest a direct effect.

Intriguingly, the effects of Echinaforce® on IL-1β and TNF-α were more pronounced when subjects were analysed according to their endogenous cortisol levels. The IL-1β and TNF-α levels decreased by up to 32% compared to baseline ($p < 0.05$) in subjects with lower levels of cortisol ($\leq$350 nmol/l, $n = 11$). The same was observed for IL-6 and IL-12 levels, which were not significantly altered in the whole population (data not shown). In contrast, subjects with cortisol levels >350 nmol/l ($n = 17$) were not affected by Echinaforce®.

The acute-phase proteins IL-1β, IL-6 and TNF-α are upregulated early in rhinovirus infection, and their production likely underlies the clinical manifestation of cold infections (Johnston, 1997; Subauste et al., 1995; Terajima et al., 1997). These proteins make epithelial cells susceptible to viral infection (Subauste et al., 1995) and are associated with rhinovirus-induced asthma exacerbation and airway hyperreactivity. IL-6, TNF-α and IL-1β are regulated directly by cortisol levels during viral infection (Dobbs et al. 1996). Echinaforce® therefore appears both to directly and indirectly reduce inflammation associated with viral infections, which might in turn ameliorate the inflammation-induced development of cold symptoms during acute phases of infection (Johnston, 1997; Gentile et al., 2003; Hakonarson et al., 1995). Thus, cortisol-dependent inhibition of acute-phase proteins reflects the specific effects of Echinaforce® in subjects with low endogenous production of anti-inflammatory control.

Substantial up-regulation of chemokines during Echinaforce® treatment was observed in the whole group. Increased chemokine production may have beneficial effects on respiratory infections by increasing the ability of peripheral lymphocytes to infiltrate the infected tissue (Subauste et al., 1995; Noah and Becker, 1993; Larsen et al., 1989). Neutrophils and macrophages are involved in localized immune reactions to respiratory viruses in the nasal lumen (Levandowski et al., 1988; Teran et al., 1997).

The results of the present study contradict some studies that reported that Echinaforce® potently blocks production of IL-8 by airway epithelial cells upon viral induction (Sharma et al., 2008); however, the results of the current study are in agreement with previous findings that Echinaforce® super-induces the production of IL-8 in systemic peripheral blood monocytes (unpublished data). We surmise that Echinaforce® increases migration of systemic leucocytes to the site of infection but blocks epithelial cell production of IL-8 that is associated with cold symptoms.

Since viruses are intracellular, an efficacious cellular immune reaction is essential for pathogen clearance. IFN-γ is a potent activator of macrophages, NK cell function and antigen-specific B-cell proliferation. In addition, IFN-γ shifts the immune reaction towards a Th1 response, which is crucial in overcoming viral infections (Romagnani, 1992). In the present study, marked up-regulation of IFN-γ by Echinaforce® was noted in weak cytokine producers, in subjects after a period of increased stress, and in subjects with an increased susceptibility to cold infections. The result that *Echinacea* induces IFN-γ is not new, but it has previously been observed only *in vitro* and in mice fed *Echinacea* over a 3-week period (Mishima et al., 2004; Hayashi et al., 2001). Glaser et al. (1986) found that increased stress is associated with decreased production of IFN-γ and a decline in NK cells and T lymphocyte activity in students during final examinations. Furthermore, stress induces a shift from Th1 towards Th2 immune responses, which would negatively affect the clearance of viral infections (Kiecolt-Glaser et al., 1995; Mittwoch-Jaffe et al., 1995). The beneficial effects of *Echinacea* on the production of IFN-γ, which strengthens anti-viral defences, could explain its traditional use, especially by people with increased susceptibility to infection and for recurrent infections.

The differential blood cell count during treatment with Echinaforce® did not differ from baseline measurements. This was also observed in a previous study in which Echinaforce® was administered to 80 subjects over a two-month period (Schoop et al., 2006b). We therefore assume that the effects of Echinaforce® on cytokine level resulted from changes in activity rather than changes in the number of blood cells (i.e. leukocytes, granulocytes or lymphocytes).

The results of the current study have implications for the clinical use of Echinaforce®. We hypothesize that Echinaforce® exerts adapted immune-modulatory effects by inducing chemotaxis and by inducing anti-viral and anti-inflammatory effects, especially (or even primarily) in subjects who are immunologically vulnerable. In addition, Echinaforce® acts specifically on pro-inflammatory proteins such as TNF-α by inhibiting excessive production. During this study, no cold symptoms were reported by the subjects, illustrating on a macroscopic level the cellular effects of Echinaforce® on the immune system.

In conclusion, we hypothesize that the efficacy of Echinaforce® in prevention and treatment of cold and influenzal infections is the result of its ability to reduce inflammatory processes, to increase leukocyte chemotaxis to the site of infection and to activate antivi-

ral defences. The beneficial effects of Echinaforce® were observed primarily in subjects with weak immune responses and in those with increased stress levels or greater susceptibility to infections leading to the common cold. The mechanism of action underlying these effects of Echinaforce® appears to be adapted immunomodulation.

Disclosure

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