Regulation of Human Immune Gene Expression as Influenced by a Commercial Blended Echinacea Product: Preliminary Studies

R.K. RANDOLPH,*[,1] K. GELLENBECK,* K. STONEBROOK,* E. BROVELLI,* Y. QIAN,* D. BANKAITIS-DAVIS,† AND J. CHERONIS†

Nutrilite Health Institute, Access Business Group, LLC, Buena Park, California 90622 and †Source Precision Medicine, Boulder, Colorado 80301

Consumption of Echinacea at the first sign of symptoms has been clinically shown to reduce both the severity and duration of cold and flu. Quantitative polymerase chain reaction optimized for precision and reproducibility was utilized to explore *in vitro* and *in vivo* changes in the expression of immunomodulatory genes in response to Echinacea. *In vitro* exposure of THP-1 cells to 250 μg/ml of Echinacea species extracts induced expression (up to 10-fold) of the interleukin-1α, interleukin-1β, tumor necrosis factor-α, intracellular adhesion molecule, interleukin-8, and interleukin-10 genes. This preliminary result is consistent with a general immune response and activation of the nonspecific immune response cytokines.

***In vivo* gene expression within peripheral leukocytes was evaluated in six healthy nonsmoking subjects (18–65 years of age). Blood samples were obtained at baseline and on Days 2, 3, 5, and 12 after consuming a commercial blended Echinacea product, three tablets three times daily (1518 mg/day) for two days plus one additional dose (506 mg) on day three. Serum chemistry and hematological values were not different from baseline, suggesting that liver or bone marrow responses were not involved in acute responses to Echinacea. The overall gene expression pattern at 48 hr to 12 days after taking Echinacea was consistent with an antiinflammatory response. The expression of interleukin-1β, tumor necrosis factor-α, intracellular adhesion molecule, and interleukin–8 was modestly decreased up through Day 5, returning to baseline by day 12. The expression of interferon-α steadily rose through Day 12, consistent with an antiviral response. These preliminary data present a gene expression response pattern that is consistent with Echinacea's reported ability to reduce both the duration and intensity of cold and flu symptoms.** Exp Biol Med 228:1051–1056, 2003

Key words: THP-1 cells; interleukin-1; interferon-alpha; cichoric acid; quantitative PCR; dietary supplement

[1] To whom requests for reprints should be addressed at Clinical Investigations, Nutrilite Health Institute, Access Business Group, LLC, 5600 Beach Boulevard, Buena Park, CA 90622. E-mail: keith.randolph@accessbusinessgroup.com

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Echinacea, also known as the American coneflower or purple coneflower, enjoys a long history of traditional use by native North Americans for its resistance-boosting properties and wound-healing benefits (1). In 1997 Echinacea was the number-one-selling herbal supplement in natural food stores in the United States. It was also number 13 on the list of most frequently prescribed single entity phytomedicines in Germany according to 1996 sales (2).

Three species of Echinacea are used medicinally: *E. angustifolia, E. pallida*, and *E. purpurea*. Though these three species of Echinacea have different chemistries, all have been shown to have immune-modulating activity. It appears that the activity is due to a combination of cichoric acid, alkylamides, and polysaccharides, which are present in all three species, but in differing amounts. A number of clinical trials have documented the efficacy of *E. purpurea* and *E. pallida* for lessening the severity and duration of infections, particularly those of the upper respiratory tract (3–9). In most of these trials, efficacy of Echinacea has been demonstrated at a dose equivalent to 900 mg of dried herb per day. Although the predominance of research supports the efficacy of Echinacea, not all clinical research has yielded positive results (10–12). One important consideration, as reported in a recent critical review by a group of physicians, is the recommendation that Echinacea should not be consumed on a continual basis. Rather, it should be consumed as needed for early symptoms of infection (13).

How does Echinacea modulate immune system function to alleviate the severity and duration of colds and flu? Animal experiments and *in vitro* studies have demonstrated that Echinacea and its isolated constituents possess several physiological properties including stimulation of phagocytosis (14–19), stimulation of cytokine production (20–26), enhancement of B lymphocyte antibody production (27, 28), and antiinflammatory activity (29–31). Whether these mechanisms are also operative in humans has not yet been determined.

Echinacea appears to be safe when taken at the equiva-

lent of 900 mg dried herb per day. No serious adverse effects have been reported in the clinical trials of Echinacea. Allergic reactions have been observed (32, 33) and Echinacea is not recommended for people with autoimmune disorders (34).

For the present work, a quantitative polymerase chain reaction (PCR, ABI Prism 7700 TaqMan) (35) system optimized for precision and reproducibility (Source Precision Medicine) was used to observe the effects of Echinacea concentrates and product formulations on select inflammation-related genes (Source's Precision Profile™ for Inflammation). Preliminary *in vitro* experiments utilized THP-1 cells—a human monocytic cell line derived from a monocytic leukemia. Echinacea concentrates were initially screened for their effects on the expression of 24 inflammation-related genes. A more focused panel of eight inflammation-related genes demonstrating good responsiveness in the initial screen was further evaluated as a function of Echinacea extract concentration. *In vitro* studies were followed up with a pilot scale human clinical trial involving six healthy subjects (3 male, 3 female) orally dosed with NUTRILITE Triple Guard Echinacea. A focused panel of inflammation-related genes was used to evaluate the *in vivo* effects of Triple Guard Echinacea on the whole blood of healthy subjects.

Materials and Methods

Gene Expression Methodology. All cellular and molecular biology was performed at Source Precision Medicine, Boulder, CO. For these studies, mRNA was isolated from THP-1 cells using the Ambion, Inc. (Austin, TX) RNAqueous Kit. mRNA from clinical whole blood samples was isolated using the PreAnalytix (Hombrechtikon, Switzerland) PAXgene™ Blood RNA Kit. First strand cDNA synthesis was performed using Applied Biosystems TAQMAN Reverse Transcription reagents. Primers and probes for the 25-gene loci were designed and validated by Source Precision Medicine.

Quantitative PCR was performed using the ABI Prism 7700 Sequence Detection System (Foster City, CA). Relative gene expression was measured by normalizing the experimental mRNA to an endogenous control (18S mRNA) and comparing to a calibrator (untreated THP-1 cells or untreated subjects). The relative gene expression measurement was captured in the following equation: $2^{-\Delta\Delta Ct}$ (where ct = cycle threshold or the cycle number at which PCR product is first detected, ΔCt = normalized sample value, and $\Delta\Delta Ct$ = normalized difference between a treated sample and the calibrator). Four replicate gene expression determinations were conducted for both *in vitro* and *in vivo* studies. Replicate values in all cases were within a 3% range of the mean and had a skew of less than 1.5% of the median.

Cell Culture Methodology. *In vitro* mRNA was isolated from cultured THP-1 cells (ATCC, Manassas, VA). The cell cultures were maintained at 37°C, 5% CO_2, and 1X RPMI 1640 w/o phenol red (Gibco-BRL), and Echinacea extracts were added directly to and dissolved in RPMI cell culture medium immediately prior to initiation of experiments. For experiments, triplicate wells of cells were incubated with RPMI solubilized extracts of *E. purpurea* herb, *E. purpurea* root, or *E. angustifolia* root at final concentrations up to 250 μg/ml for 6 hrs.

The term "Echinacea" is used in the marketplace to refer to a number of different species and plant parts. The use of specific species and plant parts varies among commercial products, and it was anticipated that this variation in composition would be measurable in an *in vitro* gene expression assay. The first *in vitro* study was undertaken to evaluate the gene expression response (24-gene Precision Profile for Inflammation) of THP-1 cells treated with a 250 μg/ml concentration of Echinacea. Concentration-dependent responses were also determined for *E. purpurea* herb extract at 10 μg/ml, 50 μg/ml, and 250 μg/ml.

The second *in vitro* study was conducted to confirm the consistency in gene expression response among three different lots of Echinacea herb extract standardized by cichoric acid content (1.5% to 1.8%). A focused panel of eight inflammation-related genes demonstrating good responsiveness in the initial screen was utilized for this study. Echinacea preparations were tested for endotoxin (LPS, lipopolysaccharide) contamination (a common bacterial contaminant that is known to elicit a gene expression response) using the LAL/endotoxin reaction assay (Associates of Cape Cod).

Human Clinical Trial Methodology. The protocol for the *in vivo* study was approved by the Western Institutional Review Board (Olympia, WA) and was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines. Six subjects (3 male and 3 female) between 18 and 65 years of age, nonsmoking, normally active, and judged to be in good health based upon an interview and physical examination, participated in the trial. On Day 1, a fasting baseline blood draw was taken and NUTRILITE Triple Guard Echinacea tablets were then consumed as three tablets three times daily for two days with an additional dose on the morning of the third day for a total of 21 tablets per subject. Subject body weight ranged from 55 to 79 kg (mean 67 kg), equating to a dosage level of 23 mg Echinacea/kg body weight on Days 1 to 2 and 8 mg/kg body weight on Day 3. mRNA was isolated from whole blood samples drawn at baseline (prior to Echinacea dosing) as well as 48 hr, 72 hr, 5 days, and 12 days (post Echinacea dosing). Relative gene expression was measured by comparing the 48-hr, 72-hr, 5-day, and 12-day responses relative to the baseline draw response. Blood samples were also collected at each time point for standard blood chemistries (Na+, K+, Cl-, P, Ca++, Mg+, glucose, bicarbonate, total protein, albumin, BUN, creatinine, AP, AST, ALT, LDH, UA, total bilirubin, and creatine kinase) and hematological parameters (hemoglobin/hematocrit, WBC, RBC, platelets, lymphocytes, neutrophils, monocytes, eosinophils, and basophils).

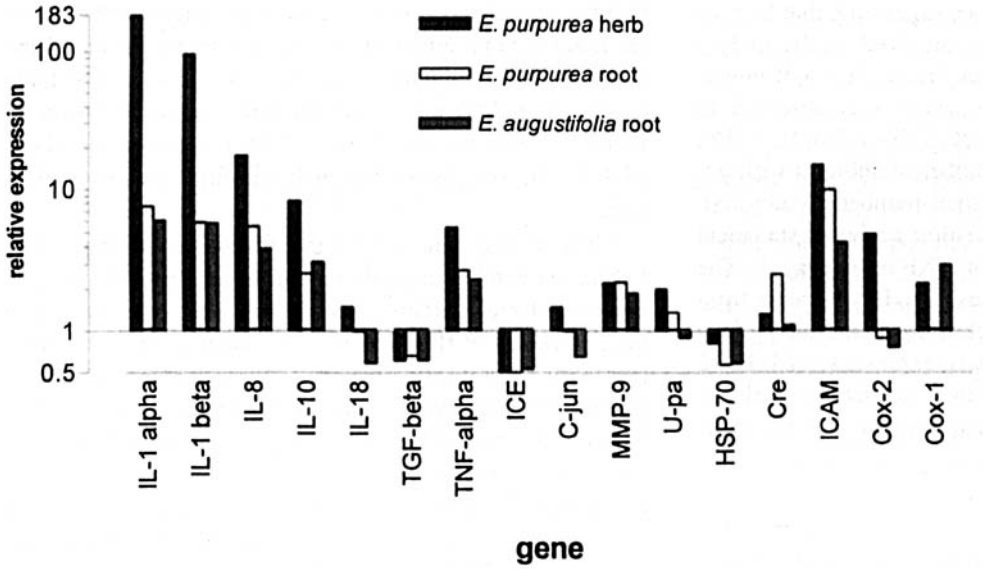

Figure 1. Gene expression response of THP-1 cells treated with select Echinacea extracts. THP-1 cells were incubated for 6 hr with 250 µg/ml extracts of *E. purpurea* herb, *E. purpurea* root, or *E. angustifolia* root. The average gene expression response of Echinacea-treated THP-1 cells is presented relative to the untreated control. Replicate variation in gene expression in all cases was less than 3%.

Statistical Analysis. Relative gene expression at each treatment time point was compared with control (*in vitro*) or baseline (clinical) results by one-way analysis of variance (ANOVA).

Results

***In Vitro* Gene Expression Results.** The gene expression response of THP-1 cells treated with three different *Echinacea* extracts is shown in Figure 1. All three Echinacea extracts elicited a similar gene expression response pattern. The *E. purpurea* herb extract exhibited the largest response overall, although it did not achieve statistical significance relative to the other Echinacea extracts ($P = 0.1$). In general, this gene expression response pattern (upregulation of IL-1α, TNF-α, ICAM, IL-8, and IL-10) is consistent with an activated antiviral state (36). Gene expression responses were concluded not to be due to endotoxin as Echinacea preparations tested negative for bacterial endotoxin by the Limulus assay.

The gene expression response of THP-1 cells treated with increasing *E. purpurea* concentrations is shown in Figure 2. Concentration-dependent upregulation of IL-1α, IL-1β, IL-8, IL-10, TNF-α, and ICAM was observed.

A comparison of THP-1 cells treated with three different lots of Echinacea standardized to cichoric acid content demonstrated a nearly identical gene expression response in the same focused eight-gene panel (data not shown). The similar qualitative and quantitative response to different lots of Echinacea herb suggests that it is feasible to standardize a complex preparation to yield a consistent biological response.

Human Clinical Trial Results. Serum chemistry and hematological values for each subject did not significantly differ ($P > 0.05$) from baseline at any time during the

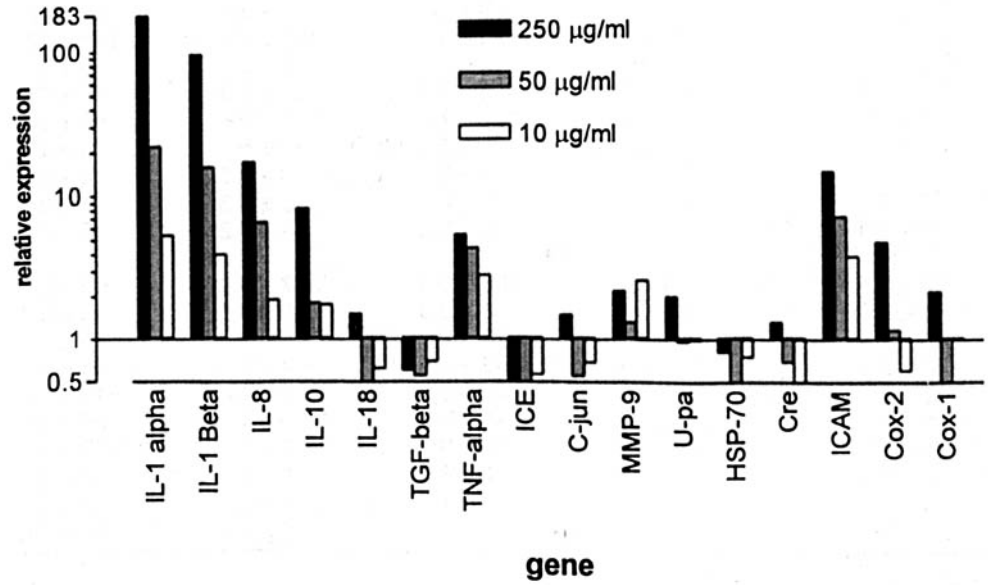

Figure 2. Gene expression response of THP-1 cells treated with increasing concentrations of *E. purpurea* herb. THP-1 cells were incubated for 6 hr with 10, 50, and 250 µg/ml concentrations of *E. purpurea* herb. The average gene expression response of *E. purpurea* herb treated THP-1 cells is presented relative to the untreated control.

12-day observation period (Table I), suggesting that liver or bone marrow responses were not involved in the body's response to Echinacea in this time frame. A small downregulation of acute inflammation genes was observed in most, but not all subjects (IL-1β, 4/6; TNF-α, 5/6; IL-8, 3/6; Cox-2, 4/6; and ICAM-1, 4/6; number of subjects with decreased relative gene expression/total number of subjects). The magnitude of this downregulation achieved statistical significance ($P = 0.04$) only for TNF-α on Day 5. The overall gene expression pattern was a modest decrease from baseline to Day 5 and a trend back toward baseline by Day 12 (Fig. 3). Conversely, the relative expression of IFN-α2 increased steadily through day 12 in all six subjects, achieving statistical significance relative to baseline on Day 12 ($P = 0.02$).

Discussion

These studies demonstrate that reproducible relative gene expression in response to Echinacea extracts can be obtained from both *in vitro* and *in vivo* experiments utilizing a quantitative PCR system optimized for precision. The *in vitro* data suggest that short-term (6-hr) exposure of THP-1 cells to Echinacea extracts increases the expression of inflammation-related genes. The data also suggest that different Echinacea product formulations modulate immune gene expression in qualitatively similar ways, but with notable quantitative differences. Additionally, a combination of Echinacea species and herb and root extracts stimulates THP-1 cells to a greater degree than a single species or plant part.

The *in vivo* data suggest that transient consumption of Echinacea exhibits a modest trend in reducing the expression of selected inflammation-related genes and increases the expression of IFN-α in healthy subjects. This is consistent with an antiinflammatory, antiviral response. These preliminary data are, to our knowledge, the first to shed light on the molecular mechanisms of action for Echinacea species extracts *in vivo*.

Although the *in vitro* and *in vivo* data presented are not sufficient to support conclusions for both short (<6 hour) and longer (≥24 hr) time frame responses to Echinacea in the human body, it is interesting to speculate that Echinacea may evoke an early pro-inflammatory response followed by a later antiinflammatory response. Such a bimodal time response is consistent with the documented effects of Echinacea at decreasing both the duration and intensity of symp-

Table I. Blood Chemistry and Hematological Values[a]

Measurement	Day 1 (Baseline)	Day 2	Day 3	Day 5	Day 12
Chemistry					
Na (mEq/L)	140 ± 2	138 ± 1	140 ± 2	140 ± 1	141 ± 1
K (mEq/L)	5 ± 0.8	5 ± 0.4	5 ± 0.7	5 ± 0.7	5 ± 0.4
Cl (mEq/L)	105 ± 3	104 ± 3	105 ± 2	105 ± 2	104 ± 1
Glu (mmol/L)	46 ± 8	50 ± 5	46 ± 4	46 ± 6	49 ± 4
CO2 (mMol/L)	21 ± 2	22 ± 0.6	22 ± 3	22 ± 2	22 ± 2
Ca ± ± (mg/dl)	10 ± 0.4	10 ± 0.4	10 ± 0.3	9 ± 0.3	9 ± 0.3
T Pro (g/dl)	8 ± 0.5	7 ± 0.5	7 ± 0.3	7 ± 0.5	7 ± 0.5
BUN (mg/dl)	15 ± 6	17 ± 7	18 ± 6	16 ± 5	17 ± 5
Creatinine (mg/dl)	1 ± 0.2	1 ± 0.2	1 ± 0.1	1 ± 0.1	1 ± 0.2
Alk Phos (U/L)	74 ± 29	72 ± 30	71 ± 27	69 ± 30	69 ± 31
AST (U/L)	21 ± 5	18 ± 5	21 ± 10	20 ± 11	20 ± 7
ALT (U/L)	22 ± 18	20 ± 16	23 ± 24	29 ± 38	23 ± 22
LDH (U/L)	189 ± 27	163 ± 21	198 ± 39	182 ± 33	177 ± 22
UA (mg/dl)	5 ± 1	5 ± 1	6 ± 2	5 ± 2	5 ± 1
T Bilirubin (mg/dl)	0.8 ± 0.2	0.9 ± 0.6	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.4
CK (U/L)	135 ± 55	114 ± 44	129 ± 75	139 ± 99	159 ± 103
GGT (U/L)	20 ± 5	19 ± 5	20 ± 5	19 ± 5	18 ± 4
Phos (mg/dl)	3 ± 0.6	3 ± 0.6	4 ± 0.6	3 ± 0.6	3 ± 0.7
Mg ± (mg/dl)	2 ± 0.2	2 ± 0.1	2 ± 0.2	2 ± 0.3	2 ± 0.1
Albumin (g/dl)	5 ± 0.3	5 ± 0.3	5 ± 0.2	4 ± 0.2	5 ± 0.3
Hematology					
Leukocytes (10^3/μL)	5.6 ± 1.6	5.0 ± 0.7	4.6 ± 0.8	4.7 ± 0.9	5.3 ± 0.7
Erythrocytes (10^6/μL)	5 ± 0.6	5 ± 0.6	5 ± 0.6	5 ± 0.6	5 ± 0.7
Hemoglobin (g/dL)	15 ± 2	15 ± 2	15 ± 2	15 ± 2	15 ± 2
Hematocrit (v/v %)	44 ± 5	44 ± 4	43 ± 5	44 ± 5	45 ± 6
Platelets (10^3/μL)	256 ± 54	273 ± 75	263 ± 60	267 ± 74	249 ± 57
Lymphocytes (%)	33 ± 10	37 ± 4	36 ± 3	38 ± 4	35 ± 6
Neutrophils (%)	58 ± 12	49 ± 6	52 ± 7	49 ± 3	52 ± 7
Monocytes (%)	5 ± 2	10 ± 4	8 ± 6	8 ± 3	8 ± 4
Eosinophils (%)	4 ± 2	5 ± 2	4 ± 1	4 ± 2	5 ± 3
Basophils (%)	0.3 ± 0.3	0.3 ± 0.2	0.3 ± 0.3	0.7 ± 0.4	0.7 ± 0.6

[a] Table values represent the average ± SE.

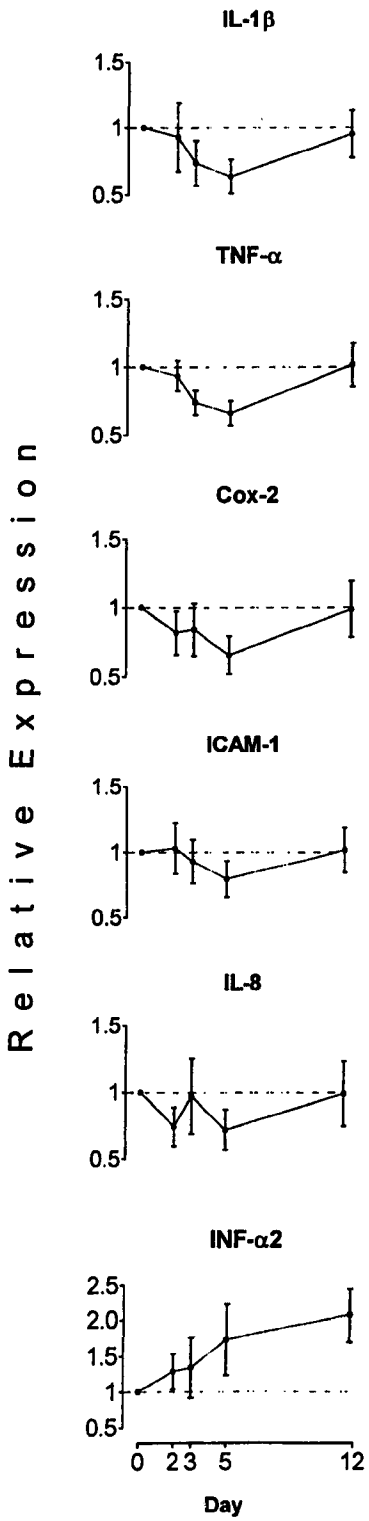

Figure 3. Time course of *in vivo* gene expression responses during (and following) a 3-day oral dosing schedule of a commercial blended *Echinacea* product. Messenger RNA was isolated from whole blood samples at 2, 3, 5, and 12 days and quantitative PCR was performed as described in Methods. The gene expression responses are presented relative to the initial pretreatment baseline draw. Data represent the average (± SE) relative gene expression for all subjects at each time point.

toms associated with cold and flu (37–43). Further research will be required to evaluate this hypothesis.

There are several important features to this work that should be noted. First, the overall gene expression response among subjects exhibited some trends that are worth further investigation. While these trends are consistent with the clinical efficacy of Echinacea, the data do not warrant extrapolation to the population at large, or to individuals combating infectious disease. This will require additional research in a larger group of individuals, including those responding to infection. Second, short-term gene expression responses comparable with those in the *in vitro* studies (e.g., less than 12 hr) were not evaluated in the clinical trial. It is unknown whether the acute effects observed *in vitro* would also be observed acutely *in vivo*.

Despite these limitations, the current data are intriguing and consistent with a growing body of literature supporting the effectiveness of Echinacea for shortening the duration and intensity of cold and flu symptoms. Further research with larger subject groups, shorter time intervals, broader gene panels, and healthy subjects versus those with a viral infection will provide more insight into the mechanism of action for Echinacea.

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