Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized *Echinacea*, a potent antiviral herbal extract

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\textbf{A B S T R A C T}

Several viruses associated with upper respiratory diseases have been shown to stimulate the secretion of pro-inflammatory cytokines, including chemokines, sometimes in the absence of viral cytopathology. We evaluated the ability of a standardized preparation of the popular herbal medicine *Echinacea* (Echinaforce\textsuperscript{®}, an ethanol extract of herb and roots of *E. purpurea*, and containing known concentrations of marker compounds) to inhibit the viral induction of various cytokines in a line of human bronchial epithelial cells (BEAS-2B), and in two other human cell lines. All of the viruses tested, rhinoviruses 1A and 14, influenza virus, respiratory syncytial virus, adenovirus types 3 and 11, and herpes simplex virus type 1, induced substantial secretion of IL-6 and IL-8 (CXCL8), in addition to several other chemokines, depending on the virus, although only viable viruses were able to do this. In every case however *Echinacea* inhibited this induction. The *Echinacea* preparation also showed potent virucidal activity against viruses with membranes, indicating the multi-functional potential of the herb. These results support the concept that certain *Echinacea* preparations can alleviate "cold and flu" symptoms, and possibly other respiratory disorders, by inhibiting viral growth and the secretion of pro-inflammatory cytokines.

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1. Introduction

A number of studies have shown that rhinoviruses can induce the secretion of pro-inflammatory cytokines in epithelial cells derived from bronchial and nasal tissues, in the absence of substantial virus replication (Gwaltney, 2002; Message and Johnston, 2004; Mosser et al., 2005; Sharma et al., 2006a; Edwards et al., 2007). These results have led to the hypothesis that symptoms associated with virus-induced common colds and other respiratory complications are the result of the increased levels of pro-inflammatory cytokines, rather than direct effects of virus replication. The same may be true for other viruses associated with upper respiratory infections, such as influenza virus, respiratory syncytial virus, and adenovirus, which can also induce pro-inflammatory cytokine secretion in epithelial cells (Bonville et al., 1999; Booth and Metcalf, 1999; Chan et al., 2005; Schaller et al., 2006; Szeretter et al., 2007). If all these viruses bring about cold and flu symptoms indirectly by stimulating secretion of pro-inflammatory cytokines, then it would seem worthwhile to pursue cold and flu remedies by the use of agents capable of reversing the pro-inflammatory effects, rather than compounds designed to inhibit specific virus replication per se. This view is further substantiated by the fact that rhinoviruses themselves are not generally cytopathic and infections are self-limited (Gwaltney, 2002). Similarly, respiratory syncytial virus (RSV) tends to give chronic non-cytopathic infections in lung tissues (Zhang et al., 2002).

One such candidate agent is the herbal medicine *Echinacea purpurea*, which has become one of the most popular commercial herbal preparations in North America and Europe (Brevoort, 1998; Barnes et al., 2005). There have been numerous reports of immune modulatory properties in various preparations derived from different parts of several species of *Echinacea* (Gertsch et al., 2004; Barnes et al., 2005; Sharma et al., 2006a, 2006b; Wang et al., 2006), although the composition of these preparations is inconsistent, a fact that has made it difficult to propose a mechanism of action (Woelkart and Bauer, 2007). Our earlier studies indicated that antiviral properties varied widely among different *Echinacea* species and component parts (Hudson et al., 2005; Vimalanathan et al., 2005), thus it is important to carry out research on *Echinacea* preparations that have been standardized and chemically characterized.

We and others reported recently that rhinoviruses could stimulate the transcription of various immune response genes in different types of cells (Chen et al., 2006; Katz et al., 2006; Altamirano-Dimas et al., 2007). Furthermore the expression of cytokine genes and some of their secreted products in bronchial epithelial cells could be reversed by *Echinacea* preparations (Altamirano-Dimas et al., 2007;...
In the present study, we used a chemically characterized ethanol extract of *E. purpurea* (*Echinaforce*®) to evaluate direct antiviral activities, and the ability of this preparation to inhibit the production of numerous pro-inflammatory cytokines stimulated by respiratory viruses, as measured by means of fluorescent cytokine antibody arrays.

### 2. Materials and methods

#### 2.1. Standard Echinacea preparation

*Echinaforce*® (obtained from A. Vogel Bioforce AG, Roggwil, Switzerland, batch no.: 018451) is a standardized preparation derived by ethanol extraction of freshly harvested *E. purpurea* herb and roots (95:5). All experiments described in this report were done with this batch of *Echinaforce*®. The composition of marker compounds (i.e. those compounds known to characterize this species of *Echinacea*) is shown in Table 1, and is in agreement with generally accepted standards for this kind of preparation (Bauer, 1998; Binns et al., 2002). These values, representing means of four independent determinations, were kindly provided by Dr. J.T. Arnason, University of Ottawa. Final concentration of ethanol was 65%, v/v. The final concentration of ethanol in the experimental cultures was too low to cause adverse effects on the cells. In addition the preparation was free of detectable endotoxin (as determined by means of a commercial assay kit, Lonza Walkersville Inc., MD, lower limit of detection 0.1 unit/mL), and was not cytotoxic according to trypan blue staining, MTT formazan assays (MTT = 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan), and microscopic examination.

#### 2.2. Cell lines and viruses

All cell lines (Vero monkey kidney cells; MDCK canine kidney cells; H-1 sub-clone of HeLa cells; A549 human lung epithelial cells; BEAS-2B human bronchial cells; feline kidney cells; all acquired originally from ATCC) were passaged regularly in Dulbecco MEM (DMEM), in cell culture flasks, supplemented with 5–10% fetal bovine serum, at 37°C in a 5% CO2 atmosphere, with the exception of the H-1 cells, which were grown at 35°C. Human skin fibroblasts (courtesy Dr. Aziz Ghahary) were used in their sixth passage, of the H-1 cells, which were grown at 35°C. Human skin fibroblasts (courtesy Dr. Aziz Ghahary) were used in their sixth passage, with a temperature of 22°C. Following the incubation period, the mixtures were transferred to another tray of cells from which the medium had been aspirated. These trays were then incubated until viral CPE were complete in control wells containing untreated virus (usually 2 days for FCV and influenza, 4–5 days for the other viruses). Additional wells contained cells not exposed to virus. The MIC values were calculated from replicate cpe end-point determinations from single representative experiments. When the replicates differed, means and standard deviations were calculated.

#### 2.3. Antiviral activity

The *Echinaforce* extract, in 200 µL aliquots, or suitably diluted 200 µL aliquots, was serially diluted twofold across replicate rows of a 96-well tray, in medium. Virus, 100 pfu in 100 µL, was added to each well and allowed to interact with the extract for 60 min at a temperature of 22°C. Following the incubation period, the mixtures were transferred to another tray of cells from which the medium had been aspirated. These trays were then incubated until viral CPE were complete in control wells containing untreated virus (usually 2 days for FCV and influenza, 4–5 days for the other viruses). Additional wells contained cells not exposed to virus. The MIC values were calculated from replicate cpe end-point determinations from single representative experiments. When the replicates differed, means and standard deviations were calculated.

In some experiments equivalent aliquots of all the viruses were inactivated by exposure to ultraviolet C radiation (UVC) for 30 min. In all cases the treated preparations were assayed subsequently and found to contain <20 infectious virus/mL, indicating more than 4log10 inactivation.

#### 2.4. Test system

BEAS-2B cells, A549 cells, and primary human skin fibroblasts (passage 6), were grown in complete medium, in 6-well trays, to produce freshly confluent monolayers. Viruses were added to the cells at 1 pfu/cell for 1 h, followed by a 1:100 dilution of *Echinaforce* in DMEM without serum (except where indicated otherwise), corresponding to a final concentration of 160 µg/mL (dry mass/vol). Culture supernatants were harvested at the indicated times (usually 24 and 48 h after infection) for ELISA tests and Quantibody cytokine array analysis. In some cases DMEM was replaced by phosphate buffered saline (PBS), without affecting the results.

In additional experiments we showed that medium alone, with or without an equivalent volume of ethanol, and cell-free supernatant derived from control uninfected BEAS-2B cells, did not induce the secretion of cytokines. Rhinoviruses pelleted by ultracentrifugation and resuspended in fresh DMEM induced comparable amounts of cytokine to equivalent amounts of uncen trifuged virus, whereas the high speed supernatant was ineffective (data not shown).

#### 2.5. ELISA assays

These were carried out according to the instructions supplied by the companies (either Immunotools, Germany, for IL-8, or e-Bioscience, USA, for IL-6 and TNF-α).
2.6. Cytokine antibody arrays

The Raybiotech fluorescent antibody array system was used. The array format (#QAH-CYT-1) contained quadruplicate antibody spots for 20 cytokines and inflammation-related mediators. The array slides were treated and processed according to Manufacturer instructions. Data Acquisition was performed via a Perkin Elmer ScanArray Express laser microarray scanner and subsequent quantification using Imagen 8.0 software from BioDiscovery. Signal intensity medians were background corrected and the mean of the replicates calculated. Signal intensities among the quadruplicates did not vary by more than ±10%. Some of the slide wells were treated with pure antigens (as part of the Raybiotech fluorescent antibody array system kit) in order to calculate a standard curve. Prism software was used to incorporate these standard values and to convert the calculated mean intensities to concentrations (pg/mL). Concentrations were then expressed as ratios, virus/control; virus + Echinacea/control. A total of three separate experiments was performed, on different batches of arrays, and the results obtained were similar. However, data from only one representative experiment are presented, in Table 4.

3. Results

3.1. The standardized Echinacea preparation

Table 1 shows the composition of the Echinaforce® extract, with regard to the expected marker compounds (for E. purpurea herb and roots, Bauer, 1998; Binns et al., 2002). There were considerable amounts of most of the caffeic acid derivatives and alkylamides, but only a trace of polysaccharide. At concentrations below 1.0 mg/mL, the extract showed no apparent cytotoxic effects, according to trypan blue staining, MTT assays, and microscopic examination. In most experiments the final concentration of extract in the medium was 160 μg/mL, which corresponds to only about 1% of the prescribed dosage for oral consumption (according to the manufacturer, approximately 16 mg dry mass in several milliliters of water).

3.2. Antiviral activities

The Echinacea extract showed impressive antiviral activity against several membrane containing viruses. Results of a MIC100 assay (based on CPE end-points) are shown in Table 2. Influenza virus and HSV were very sensitive, giving rise to MIC100 < 1.0 μg/mL. Respiratory syncytial virus was also sensitive, but with a significantly higher MIC (2.5 μg/mL), while the non-membrane viruses, rhinovirus types 1A and 14, adenovirus 3 and 11, feline calicivirus, and poliovirus, were resistant to the highest concentration tested (800 μg/mL), although the rhinoviruses showed partial inhibition of CPE at this concentration.

In alternative CPE assay tests, in which extract was added to the cells before the virus (“intracellular protocol”), these MIC were approximately two orders of magnitude higher (Table 2), indicating that the Echinacea had a direct virucidal effect on membrane containing viruses, rather than inhibition of virus replication.

3.3. Anti-cytokine activities

Initially we determined the kinetics of cytokine stimulation by measuring IL-6, IL-8, and TNF-α secretion at different times after RV1A infection of BEAS-2B cells. Typical results are shown in Fig. 1. Stimulation of cytokine secretion was evident at all time points, but Echinacea showed a complete neutralization of the virus induced levels. Additional control tests showed that the Echinacea extract did not interfere significantly in any of the ELISA steps, and no significant cytokine was found in cytoplasmic extracts derived from Echinacea treated cells.

Based on these results we decided to use the 24 and 48 h time points, following virus infection, to evaluate the effects of Echinacea on cytokine stimulation in BEAS-2B and A549 cells. For each of the viruses, RV1A, RV14, influenza, RSV, adenovirus types 3 and 11, and HSV, we measured IL-6 and IL-8 secretion, by ELISA tests, in control uninfected cells, cells + Echinacea, virus infected cells with and without Echinacea, as well as cells infected with an equivalent amount of UV-C inactivated virus. Table 3 summarizes these results. Echinacea was invariably effective in inhibiting the viral induction of both IL-6 and IL-8. In many cases the virus + Echinacea values were indistinguishable from control uninfected cells. In all cases, an equivalent amount of UV-inactivated virus (containing fewer than 20 pfu/mL of infectious virus) failed to induce cytokine secretion (Table 3). Adenovirus 11 gave similar values to adenovirus 3. HSV also gave a similar result; however since this virus replicates slowly in BEAS-2B cells under these conditions, the data for HSV alone...
could be skewed by the presence of dying cells, whereas in all other cases there were no viral CPE within 48 hpi. Similar results were also obtained for all the viruses in human lung epithelial cells A549, and in human primary skin fibroblast cultures, with and without Echinacea. Fig. 2 shows a typical result for RV14 and IL-8 in A549 cells, and this figure also indicates the effect of different concentrations of Echinacea. Thus with increasing dilution of Echinacea, its inhibitory effect diminished. However, almost complete inhibitory activity on IL-6 and IL-8 was obtained with dilutions of up to 1:400, equal to 40 pg/mL extract (dry mass/vol). Similar results were obtained for IL-6.

3.4. Cytokine antibody arrays

To investigate the possibility that this Echinacea extract was inhibitory for additional chemokines and other pro-inflammatory cytokines, we analyzed similar cell-free supernatants derived from all the virus infections in BEAS-2B cells for 20 different cytokines/chemokines simultaneously, by means of a fluorescent antibody array system. For each cytokine the mean fluorescent intensities of quadruplicate samples were normalized (as described in Section 2) and converted to pg/mL by means of internal standards. Table 4 summarizes the data for the 11 cytokines (including 6 chemokines) that responded significantly to one or more viruses, expressed as ratios virus/control (V/C), and [virus - Echinacea]/control (VE/C), for each virus. The pattern of virus-induced responses differed qualitatively or quantitatively for each virus, as might be expected, but in all cases the virus induced production was reduced substantially or completely by Echinacea. The other 9 cytokines, comprising IL-1β, IL-6, and other 9 cytokines, comprising IL-1α, IL-2, IL-4, IL-10, IL-12, IL-13, GM-CSF, and VEGF, showed little or no effect on cytokine production in uninfected cells, similar to the results shown in Table 3 for IL-6 and CXCL8; consequently these data have been omitted from Table 4 for clarity.

### Table 3

**Effect of different viruses on IL-6/IL-8 secretion (pg/mL).**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Control</th>
<th>Control + E</th>
<th>UV-virus</th>
<th>Viable virus</th>
<th>Virus + E</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV1A</td>
<td>10.2 ± 1.5</td>
<td>10.1 ± 1.5</td>
<td>10.2 ± 1.5</td>
<td>10.3 ± 1.5</td>
<td>10.4 ± 1.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>10.5 ± 1.5</td>
<td>10.6 ± 1.5</td>
<td>10.7 ± 1.5</td>
<td>10.8 ± 1.5</td>
<td>10.9 ± 1.5</td>
</tr>
<tr>
<td>RV1A</td>
<td>10.2 ± 1.5</td>
<td>10.1 ± 1.5</td>
<td>10.2 ± 1.5</td>
<td>10.3 ± 1.5</td>
<td>10.4 ± 1.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>10.5 ± 1.5</td>
<td>10.6 ± 1.5</td>
<td>10.7 ± 1.5</td>
<td>10.8 ± 1.5</td>
<td>10.9 ± 1.5</td>
</tr>
</tbody>
</table>

Values are shown for individual representative experiments (2–3 experiments per virus). Means of 3 determinations (±standard deviation).

### Table 4

**Cytokine array analyses.**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>RV14</th>
<th>RV1A</th>
<th>Influenza</th>
<th>RSV</th>
<th>Ad 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1 (GRO-α)</td>
<td>V/C</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>CXCL8 (IL-8)</td>
<td>V/C</td>
<td>0.64</td>
<td>0.64</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>CCL2 (MCP-1)</td>
<td>V/C</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>CCL3 (MIP-1α)</td>
<td>V/C</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>CCL4 (MIP-1β)</td>
<td>V/C</td>
<td>0.98</td>
<td>0.98</td>
<td>0.98</td>
<td>0.98</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td>V/C</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
</tr>
<tr>
<td>IL-1α</td>
<td>V/C</td>
<td>1.76</td>
<td>1.76</td>
<td>1.76</td>
<td>1.76</td>
</tr>
<tr>
<td>IL-1β</td>
<td>V/C</td>
<td>1.14</td>
<td>1.14</td>
<td>1.14</td>
<td>1.14</td>
</tr>
<tr>
<td>IL-5</td>
<td>V/C</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>IL-6</td>
<td>V/C</td>
<td>0.62</td>
<td>0.62</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>TNF-α</td>
<td>V/C</td>
<td>1.76</td>
<td>1.76</td>
<td>1.76</td>
<td>1.76</td>
</tr>
</tbody>
</table>

Supernatants from virus-infected BEAS-2B cells were analyzed by the fluorescent antibody array system, and signal intensities (in quadruplicate) were processed and normalized as described in Section 2. These were converted to pg/mL by internal control standards. The final values, expressed here as ratios, represent single experiments. Similar values were obtained from two other experiments using the same array format.

4. Discussion

The results of this study clearly demonstrate that the standard E. purpurea preparation (Echinaforce®, which is advocated for oral application in the treatment of upper respiratory infections, possesses two important bio-activities that are relevant to symptoms caused by various respiratory viruses, namely a potent virucidal activity against membrane containing viruses such as HSV and influenza virus, and to a lesser extent RSV, and a strong anti-inflammatory activity manifest as an inhibition of the cytokine secretion induced by all the viruses tested, including viruses without membranes such as rhinoviruses and adenoviruses.

All of the viruses tested, RV1A and RV14 (which utilize different cellular receptors, LDL and ICAM-1 respectively), influenza virus, RSV, adenovirus types 3 and 11, and HSV, induced substantial secretion of many chemokines, as well as a few of the other cytokines, although the pattern of induction of cytokines is known to be distinctive for each virus (Bonville et al., 1999; Booth and Metcalf, 1999; Chan et al., 2005; Schaller et al., 2006; Szretter et al., 2007). This was also evident from the data shown in Table 4. For example RV14 and RV1A showed similar patterns of cytokine secretion in uninfected cells, similar to the results shown in Table 3 for IL-6 and CXCL8; consequently these data have been omitted from Table 4 for clarity.
induction, except for GRO-α, which was stimulated by RV1A but not by RV14. The well known pro-inflammatory cytokines IL-6 and IL-8 (CXCL8) were stimulated by all the viruses, whereas the other cytokines were only selectively stimulated by some of the viruses. On one occasion we also evaluated a different array format, and found that CXCL5 (ENA-78), CXCL10 (IP-10), and CCL11 (eotaxin) were also stimulated by one or more viruses, and in these cases Echinacea also inhibited this stimulation (data not shown). In contrast most of the interleukins and other non-chemokines tested were not stimulated significantly by any of the viruses (V/C ratios < 2.0; Table 4). This may be a reflection of the relative refractory state of the BEAS-2B cells. However TNF-α was induced to some extent, but this induced level was completely neutralized by Echinacea.

These results imply that a variety of signalling pathways may be involved, triggered by the activation of distinct viral receptors; consequently Echinacea must be capable of a multi-functional inhibition of key signalling pathway components, and this would be in accord with our previous results indicating the inhibition of many transcription factors in RV14 infection (Sharma et al., 2006b). Such a multi-functional effect could however be mediated through specific nodal points in the signalling network, e.g. the transcription factor CBP/B (Altamirano- Dimas et al., 2007). In all cases, the anti-cytokine effects required viable virus; we did not observe cytokine stimulation in cells inoculated with UVC-inactivated virus. These results offer an explanation for the recommended beneficial uses of standardized E. purpurea preparations for the alleviation of symp- toms associated with “colds and flu”, which are usually attributed to the viruses tested in this study. The concentration of Echinacea needed for this effect is well below the normal doses that con- sumers are advised to take. Thus the prescribed oral dosage for Echinaforce® is 1.0 mL (20–25 drops) in several milliliters of water, equivalent to a concentration of approximately 1–5 mg dry weight of extract per milliliter. In the present study, we observed efficient anti-chemokine activity at concentrations of 160 μg/mL and less, and virucidal activity against influenza virus and HSV at less than 1.0 μg/mL. Since Echinaforce® is formulated for direct application to the oral mucosa, we have not considered the subsequent fate of ingredients that might survive passage through the gut and possible absorption. This could comprise a second indirect route of action, but few studies on Echinacea have addressed this issue (Wolkerta and Bauer, 2007).

Since rhinoviruses and RSV, and sometimes influenza virus, do not produce marked cytopathic effects in cells originating from the respiratory tract (Gwaltney, 2002; Mosser et al., 2005; Zhang et al., 2002), then it is generally assumed that these viruses produce cold and flu symptoms by inducing pro-inflammatory cytokines (Message and Johnston, 2004) and consequently modulation of cytokines after viral infection might be a reasonable strategy for treatment and possible prevention of the cold symptoms. These results also support the concept that Echinacea could be useful in alleviating the symptoms of asthma, hay fever, COPD (chronic obstructive pulmonary disorder) and other upper respira- tory complications, which are associated with increased levels of various pro-inflammatory cytokines and which may also be induced or exacerbated by some of these viruses (Message and Johnston, 2004; Schaller et al., 2006). Since virus replication for many of these viruses is low in any case, especially the rhinoviruses and RSV (Mosser et al., 2005; Zhang et al., 2002), we suggest that the anti-inflammatory activity described here is probably more impor- tant in controlling “colds and flu” than virucidal activity. This study has shown that this Echinacea preparation should be capable of inhibiting the chemo-attraction of most or all of the inflammatory cells induced by the viruses, and this could explain how Echinacea controls symptoms. Additional benefits could accrue from the inhibitory effects shown against those other mediators

References


