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Echinacea as an Antiinflammatory Agent: The Influence of Physiologically Relevant Parameters

M. Sharma[1], R. Schoop[2] and J. B. Hudson[1]*

[1]Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, Canada
[2]A. Vogel Bioforce AG, Roggwil, Switzerland

Numerous *Echinacea* preparations are available on the market for the prevention and treatment of cold and 'flu symptoms and inflammatory conditions associated with infections. Most of these preparations are consumed orally in the form of aqueous or ethanol extracts and tinctures. Since the recommended consumption normally involves a brief local exposure to the diluted preparation at an unspecified time in relation to the actual infection, then it is important that experimental models for the evaluation of *Echinacea* reflect these limitations. A line of human bronchial epithelial cells, in which rhinoviruses stimulate the production of pro-inflammatory cytokines, was used to evaluate several relevant parameters. The chemically characterized *Echinacea* preparation (*Echinaforce*®) was capable of inhibiting completely the rhinovirus induced secretion of IL-6 (interleukin-6) and IL-8 (chemokine CXCL-8) in these cells, regardless of whether the *Echinacea* was added before or after virus infection, and in response to a range of virus doses. This inhibitory effect was also manifest under conditions resembling normal consumption with respect to the duration of exposure to *Echinacea* and the *Echinacea* dilution. It is concluded that under real life conditions of *Echinacea* consumption, the virus-induced stimulation of pro-inflammatory cytokines can be effectively reversed or alleviated.

Keywords: Echinacea; antiinflammatory; cytokines; chemokines; IL-6 (interleukin 6); IL-8 (CXCL8).

INTRODUCTION

Different species and parts of *Echinacea* (Asteraceae) plants have been used traditionally in North America for the treatment of various symptoms of 'colds' and 'flu', as well as other applications (Barnes *et al*., 2005). A number of well known marker compounds have been characterized, including polysaccharides, specific caffeic acid derivatives and alkylamides (Bauer, 1998; Binns *et al*., 2002), and these have all demonstrated biological activities in various tests *in vitro* and *in vivo* (Bauer, 1998; Vimalanathan *et al*., 2005; Sharma *et al*., 2006; Woelkart and Bauer, 2007; Altamirano-Dimas *et al*., 2007). The variety of these bio-activities suggests that the interactions between *Echinacea* and cells are complex, and may involve different levels of action and different compounds.

Many clinical trials have been conducted in individuals suffering from natural or experimentally induced rhinovirus infections, but with variable results (Barnes *et al*., 2005; Schoop *et al*., 2006; Woelkart and Bauer, 2007; Shah *et al*., 2007). This variability may be due to differences in the therapeutic products and experimental protocols. Thus the question of clinical efficacy will remain unresolved until some of the variables have been considered systematically. There has also been considerable discussion about the timing of *Echinacea* consumption, in relation to cold or 'flu symptoms, what kind of formulation should be consumed, and in what dosage. It should be possible to resolve most of these issues by judicious use of appropriate model systems.

Rhinoviruses have been implicated as major players in common colds and various types of allergic rhinitis and bronchial syndromes (Message and Johnston, 2004; Schaller *et al*., 2006). However, numerous studies have shown that rhinovirus infection in cultured epithelial cells, and in nasal epithelial tissues *in vivo*, results in relatively low levels of virus replication and cytopathology, apparently due to the small number of cells supporting virus replication (Gwaltney, 2002; Mosser *et al*., 2005), yet in spite of this there is substantial induction of secretion of certain pro-inflammatory cytokines and chemokines, particularly IL-6 and IL-8 (Message and Johnston, 2004, Sharma *et al*., 2006; Schaller *et al*., 2006; Edwards *et al*., 2007). Thus the typical symptoms of a common cold, such as sneezing, coughing, runny nose, stuffed nasal passages, etc. (Gwaltney, 2002), are not the direct result of viral pathology, but rather the indirect stimulation of pro-inflammatory cytokines and chemokines, which are secreted and attract the various inflammatory leukocytes to the site of infection. Consequently, the successful treatment of colds and 'flu might be obtained by appropriate use of an antiinflammatory material. This can be evaluated in an experimental cell culture system, provided it bears resemblance to the *in vivo* situation.

To carry out such an analysis, the rhinovirus infected BEAS-2B epithelial cell system was used, together with a standardized and chemically characterized preparation of *Echinacea purpurea*, to evaluate the role of various experimental parameters relevant to a natural infection involving symptoms of the 'common cold'.

* Correspondence to: Dr J. B. Hudson, Department of Pathology and Laboratory Medicine, University of British Columbia, C-360 Heather Pavilion, 2733 Heather Street, Vancouver V5Z 1M5, Canada.
E-mail: jbhudson@interchange.ubc.ca

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MATERIALS AND METHODS

***Echinacea* source.** The test material was *Echinaforce*® (A. Vogel Bioforce AG, Switzerland), a 65% ethanol extract of freshly harvested aerial parts of *Echinacea purpurea* supplemented with 5% *E. purpurea* roots. This preparation was essentially free of polysaccharides, and contained the following caffeic acids and alkylamides (caffeic acid 0, caftaric acid 264.4 μg/mL, chlorogenic acid 40.2 μg/mL, cichoric acid 313.8 μg/mL, cynarin 0, echinacoside 6.9 μg/mL, PID 8/9 36.3 μg/mL). The composition was determined (courtesy J. T. Arnason, University of Ottawa) by HPLC as described in Binns *et al*. (2002).

Cells and viruses. BEAS-2B human epithelial cells, originally obtained from ATCC, were grown in Dulbecco MEM (DMEM) in 10% fetal bovine serum. For the experiments, the cells were sub-cultured in 6-well trays, and when confluent the medium was changed to DMEM, HBSS (Hanks balanced salt solution), or PBS (phosphate buffered saline), without serum. Under these conditions the cultures remained viable for at least 5 days. The H-1 sub clone of HeLa cells (ATCC) and the human lung epithelial cell line A-549 (ATCC), were grown in DMEM + 5% fetal bovine serum. Human skin fibroblasts (courtesy Dr Aziz Ghahary) in their sixth passage were also cultivated in DMEM with 10% serum. No antibiotics or antimycotic agents were used.

Rhinovirus types 1A and 14 (RV 1A and RV 14, from ATCC) were propagated and assayed, by plaque assay, in H-1 cells. The stock viruses had titers of between 2×10^7 and 1×10^8 pfu/mL.

Test system. Details of the test system were described previously (Sharma *et al*., 2006). BEAS-2B cells, and other cells, were grown in complete medium, in 6-well trays, to produce confluent monolayers. The medium was then replaced with PBS (phosphate buffered saline) or other serum-free media for the experiments. Virus was added to the cells at a multiplicity of infection 1.0 infectious virus per cell (1 pfu/cell), unless noted otherwise, for 1 h at 35 °C, followed by a 1:100 dilution of *Echinaforce* in PBS. Culture supernatants were harvested at the indicated times for measurement of cytokines, by ELISA tests.

ELISA assays were carried out according to the instructions supplied by the companies (either Immunotools, Germany, or e-Bioscience, USA).

RESULTS

Kinetics of pro-inflammatory cytokine secretion in different media

In order to establish a relevant, consistent and reliable culture system in which to evaluate antiinflammatory activity, the study compared the kinetics of secretion of several cytokines in uninfected and RV- infected BEAS-2B cells in different media (MEM, the normal culture medium; HBSS, Hank's balanced salt solution; PBS, phosphate buffered saline) over a period of several days. During this time the level of IL-6 and IL-8 secretion changed relatively little in uninfected cells, particularly in PBS or HBSS. Virus infection, however, resulted in substantial cytokine induction, first observed at 4 h after infection, but in greater amounts at 24–96 h, for both IL-6 and IL-8 (Figs 1 and 2). After this time the levels of cytokines declined.

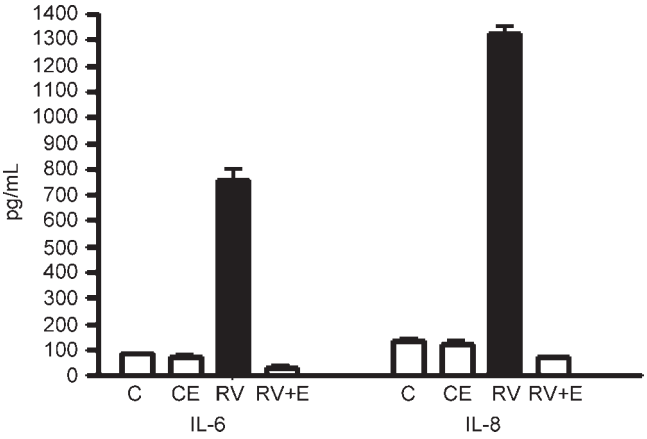

Figure 1. Effect of *Echinacea* on RV-induced cytokine secretion. BEAS-2B cells were grown to confluent monolayers, and half the cultures were infected with RV 1A (1 pfu/cell) for 1 h. The media on both infected and uninfected cultures were then replaced with phosphate buffered saline with or without *Echinacea* at 1:100 dilution. After 48 h, culture supernatants were removed for assay of IL-6 and IL-8. C, control (uninfected) cultures without *Echinacea*; CE, control cultures plus *Echinacea* treatment; RV, infected cultures without *Echinacea*; RV + E, infected cultures plus *Echinacea* treatment.

Figure 1 shows the comparison between the relative amounts of IL-6 and IL-8 at 48 h after infection. Although the maximum amount of induced cytokine was usually somewhat higher in the presence of complete medium, the differential between control and virus infected cells was consistently larger in PBS and HBSS (data not shown). Therefore it was decided to use PBS for most of the subsequent experiments. In addition as simple a medium as possible was used to avoid possible interactions between medium and *Echinacea* components.

Figure 1 also shows the dramatic inhibitory effect of *Echinacea* on induced IL-6 and -8, which were often reduced to control levels. Similar results were obtained for any of the time points chosen, 24–96 h (not shown). However, there was no significant effect of *Echinacea* on the levels of these cytokines in control uninfected cells (Fig. 1).

These results were unaffected by the passage number of the BEAS-2B cells; passage 8 and passage 54 cells showed similar responses to RV infection and to *Echinacea* inhibition (not shown). In addition rhinovirus type 1A (RV 1A), which uses a different cellular receptor from RV 14 (LDL instead of ICAM-1), showed results similar to RV 14.

Furthermore similar results were obtained when the BEAS-2B cells were replaced by A549 human lung epithelial cells, or by human skin fibroblast cells (data not shown).

Time of addition of the *Echinacea*

Since the exact time of a natural rhinovirus infection can never be certain, or the interval between infection and

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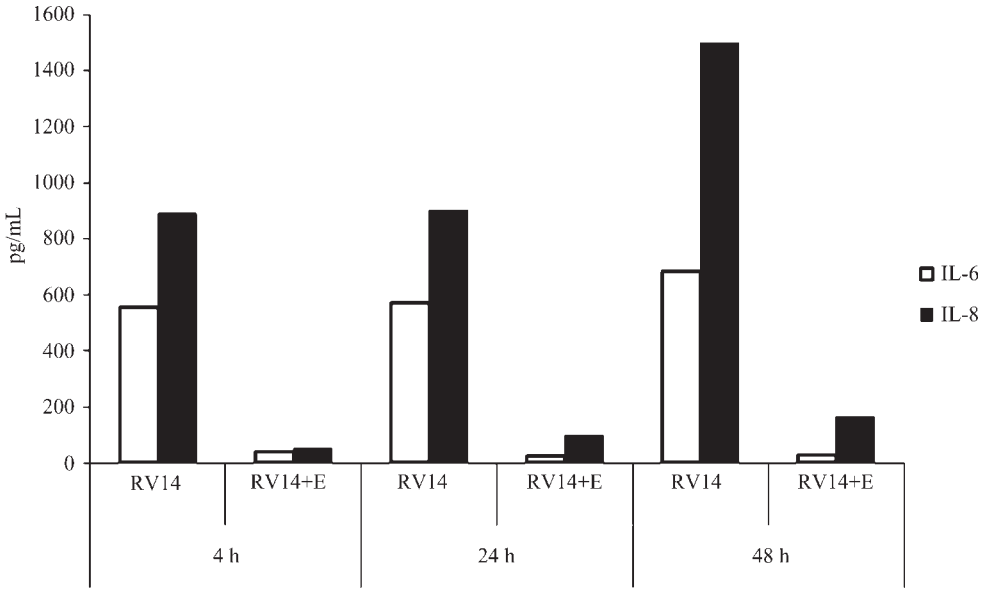

Figure 2. Time course of RV-induced cytokines ± *Echinacea*. BEAS-2B cells were grown to confluent monolayers, and half the cultures were infected with RV 14 (1 pfu/cell) for 1 h. The media on both infected and uninfected cultures were then replaced with phosphate buffered saline with or without *Echinacea* at 1:100 dilution. Periodically, culture supernatants were removed for assay of IL-6 and IL-8. For simplicity only the values for RV-infected cultures are shown. RV14, infected cultures without *Echinacea*; RV14 + E, infected cultures plus *Echinacea* at 1:100 dilution.

appearance of symptoms, then it is necessary to know if the *Echinacea* can work at any time during this period.

In the standard treatment (Fig. 1), *Echinacea* was added immediately after virus infection, and incubated for an additional 24–48 h. However, when the addition of *Echinacea* was delayed for 4, 24 or 48 h after virus infection, and the exposure to *Echinacea* continued for a further 24 h, the virus-induced cytokine induction was still inhibited (Fig. 2) for both cytokines. In addition *Echinacea* can also be added 24 h before infection, and still inhibit subsequent RV-induced stimulation of cytokines (see below).

Echinacea dose effect

A common question about *Echinacea* consumption is: how much is appropriate for its success in counteracting cold symptoms?

To determine if the inhibitory capacity of *Echinacea* was dose dependent, as might be expected, the anticytokine effect of different doses was examined, using the same experimental conditions described above. The previous tests (Fig. 1) utilized a 1:100 dilution of *Echinacea*, equivalent to a final concentration of 160 µg/mL. However inhibition was still observed with dilutions up to 1:400, and occasionally at 1:800, but there was clearly a dose response effect, as shown in Fig. 3. This suggests that the preparation could be diluted considerably and should still be active. None of the *Echinacea* doses used in these studies appeared to have adverse effects on the cells.

Effect of virus concentration (multiplicity of infection, MOI)

Another variable that could affect the outcome of a cold is the amount of virus acquired in the infection.

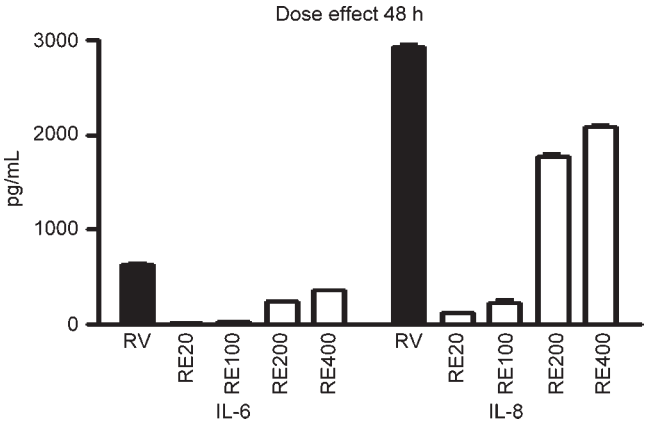

Figure 3. *Echinacea* dose effect. BEAS-2B monolayers were produced as usual, and half the cultures were infected with RV 14, 1 pfu/cell, followed by exposure of infected and uninfected cultures to various dilutions of *Echinacea* for 48 h, in PBS. Culture supernatants were then removed for the assay of IL-6 and IL-8. Data are shown only for the infected cultures; all the control values were very low (<100 pg/mL). RV, virus only with no *Echinacea*; RE 20-RE 400, infected cultures plus indicated dilution of *Echinacea*.

One would reasonably expect greater amounts of virus to produce more intense symptoms.

To test this increasing concentrations of virus (RV14) were used, from 0.01 to 1.0 pfu/cell, which resulted in successively greater amounts of cytokine induction, for both IL-6 and IL-8 (Fig. 4), although even at a multiplicity of infection of 1.0 infectious virus per 100 cells there was still a substantial induction in cytokine secretion after 48 h. Nevertheless, in all cases *Echinacea* was able to reverse these responses and bring the cytokine levels down to the control levels previously indicated in Fig. 2. These data are shown for A-549 cells: but similar results were obtained for BEAS-2B cells.

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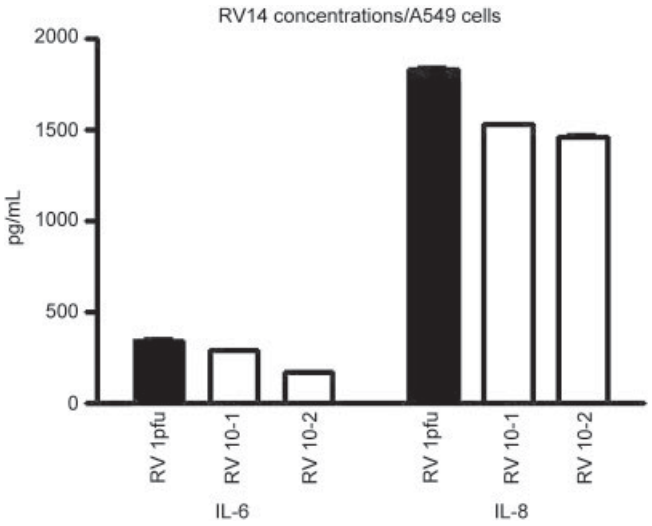

Figure 4. Effect of virus concentration. A549 cells were grown to confluent monolayers, and half the cultures were infected with RV 14, at various multiplicities of infection, 10^{-2}, 10^{-1} or 1.0 pfu/cell. After 48 h, supernatants were removed from all cultures for assay of IL-6 and IL-8. Data are shown only for infected cultures. Control values were all low (<200 pg/mL).

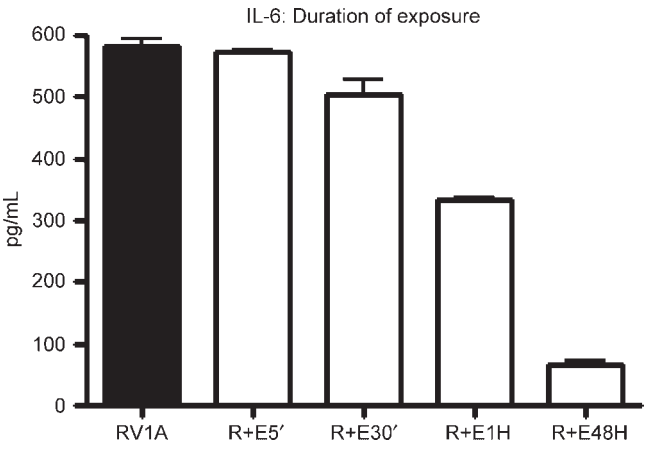

Figure 5. Duration of *Echinacea* exposure. BEAS-2B monolayers were produced as usual, and half the cultures were infected with RV 1A, 1 pfu/cell, followed by exposure of infected and uninfected cultures to *Echinacea* at 1:100 dilution for various times, followed by washing to remove excess *Echinacea*. All cultures were then incubated for the balance of the 48 h period, at which point supernatants were removed and assayed for IL-6 and IL-8. Data are shown only for the infected cultures; all the control values were very low (<200 pg/mL). RV 1A, virus only, no *Echinacea*; E5, E30, E1H, E48H, indicate times of exposure of infected cultures to *Echinacea* at 1:100 dilution.

Duration of exposure to *Echinacea*

In order to mimic the natural consumption of *Echinacea*, experiments were carried out in which *Echinacea* (at the usual 1:100 dilution) was added to the RV-infected cells for various times, and then the *Echinacea* washed off the cells. Cytokine secretion was then measured 24 h later. A short exposure of only 5 min did not significantly affect the RV-induction of cytokines; however, with increasing exposure to *Echinacea*, the more effective was the cytokine inhibition (Fig. 5). This suggests that under normal conditions of oral *Echinacea* consumption, inhibition of RV-induced cytokine secretion could be substantial, even with a high virus input.

Although the results presented in Fig. 5 indicate that a brief 5 min exposure to 1:100 *Echinacea* was not very effective, nevertheless the results in Table 1 show that a 5 min exposure to 1:10 *Echinacea* (a more realistic dose in practice) gave a significant reduction in IL-6.

Table 1. Exposure to Echinacea: different concentrations and times

Treatment	IL-6 (pg/mL) (mean ± SEM)
RV infection for 48 h, no Echinacea	830 ± 3.4
RV infection followed by Echinacea 1:100 for 48 h	64.7 ± 16.3
Pre-exposure to Echinacea 1:200 for 24 h, followed by RV infection for 24 h	46.2 ± 2.2
Pre-exposure to Echinacea 1:100 for 24 h, followed by RV infection for 24 h	21.3 ± 1.3
RV infection followed by Echinacea 1:10 for 5 min, incubated for 24 h	588 ± 12

DISCUSSION

The present results show that *Echinaforce*, at a concentration resembling its natural consumption, is capable of completely reversing the rhinovirus induced secretion of the pro-inflammatory cytokines IL-6 and IL-8. These cytokines are prominent in numerous types of infection and inflammatory conditions (Message and Johnston, 2004; Schaller *et al*., 2006). In previous studies (Sharma *et al*., 2006) it was shown that other pro-inflammatory cytokines, which are induced to a lesser degree in the epithelial cells, e.g. SCF, were also inhibited by *Echinacea* preparations.

The degree of virus induced cytokine secretion was affected, as might be anticipated, by the amount of virus added to the cells, and this situation will reflect the natural variation in virus 'doses' that individuals receive during infection. It is unlikely, however, that a person would be exposed to more than 1.0 pfu/cell, the maximum dose used here, during normal exposure, especially since the yields of rhinoviruses *in vivo* are relatively low compared with other viruses (Gwaltney, 2002; Mosser *et al*., 2005). Therefore it is believed that the efficacy of *Echinaforce* described in this study is more than adequate to counteract the inflammatory response in normal rhinovirus infections.

Considerable dilution of the *Echinaforce* was still compatible with its efficacy; consequently the prescribed dosage of commercial preparations (at least for *E. purpurea*) is supported by these experiments.

A number of experiments were performed designed to mimic the natural uses of *Echinacea*, in terms of timing and duration of application. *Echinaforce* was still very effective when applied before, as well as up to 48 h after, the virus infection, and the actual duration of exposure was not a limiting factor. Thus prophylactic and therapeutic usage is both supported. Therefore the recommendations for taking *Echinacea* preparations of this kind at the first sign of a cold are justified.

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The mechanisms of action of *Echinacea* are not understood, since previous studies (Barnes *et al*., 2005) have implicated possible roles for various constituents, such as polysaccharides, caffeic acid derivatives and alkylamides. *Echinaforce* is essentially free of polysaccharides, so they can be ruled out as major players in the cytokine effects, although it should be pointed out that *Echinacea* preparations enriched in polysaccharides do have profound effects on gene expression (Altamirano-Dimas *et al*., 2007). Some of the other constituents possess antiviral, antibacterial and other activities (Vimalanathan *et al*., 2005; Sharma *et al*., 2008), and consequently different compounds, including additional known constituents, may be involved in the overall mechanism of action.

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