PHYTOTHERAPY RESEARCH
Phytother. Res. (2009)
Published online in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.3051

The Efficacy of Echinacea in a 3-D Tissue Model of Human Airway Epithelium

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

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

We evaluated the antirhinovirus efficacy of a standardized preparation of *Echinacea purpurea* (Echinaforce®) in a 3-dimensional organotypic model of normal human airway epithelium (EpiAirway™ tissue). Individual replicate tissue samples, maintained as inserts in culture for 3 days or 3 weeks, were infected with rhinovirus type 1A (RV1A), *Echinacea* alone, a combination of the two, or medium only. None of the treatments affected the histological appearance or integrity of the tissues, all of which maintained a high level of cell viability and preservation of cilia. RV infection resulted in increased mucopolysaccharide inclusions in the goblet cells, but this feature was reversed by *Echinacea* treatment. This result was confirmed by measurements of mucin secretion, which was stimulated by RV but reversed by *Echinacea*, suggesting that mucus production during colds could be ameliorated by *Echinacea*. We did not find evidence of virus replication, although the RV-infected tissues secreted substantial amounts of the pro-inflammatory cytokines IL-6 and IL-8 (CXCL8), and this response was reversed by *Echinacea* treatment. These results confirmed previous findings derived from studies of bronchial and lung epithelial cell lines, namely, that RV infection results in a substantial inflammatory response in the absence of virus replication. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Echinacea; Asteraceae; 3-D airway tissue; organotypic; anti-inflammatory; cytokines; rhinovirus; influenza virus.

INTRODUCTION

Rhinoviruses (RVs) are the major causative agents of common colds and other upper respiratory syndromes (Gwaltney, 2002; Message and Johnston, 2004; Schaller *et al*., 2006). Recent studies have shown that RV infection in cultured epithelial cells, and in nasal epithelial tissues *in vivo*, result in substantial induction of pro-inflammatory cytokines and chemokines, in the absence of significant virus replication (Mosser *et al*., 2005; Schaller *et al*., 2006; Edwards *et al*., 2007; Sharma *et al*., 2008). Thus the typical symptoms of a common cold, such as sneezing, coughing, runny nose, stuffed nasal passages, and fever (Gwaltney, 2002), are not the direct result of viral pathology, but rather the indirect effect of the secreted chemokines, which attract inflammatory leukocytes to the site of infection. Consequently, successful treatment of colds might be obtained by appropriate use of an anti-inflammatory agent.

A promising agent is the herbal medicine derived from *Echinacea purpurea* (Asteraceae), which has become one of the most popular commercial herbal preparations in North America and Europe (Barnes *et al*., 2005). There have been many 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; Matthias *et al*., 2007; Sharma *et al*., 2008), although the composition of these preparations is inconsistent – a fact that has made it difficult to propose a general mechanism of action (Woelkart and Bauer, 2007; Vohra *et al*., 2009). Thus it is important to carry out research on *Echinacea* preparations that have been standardized and chemically characterized; this is what we have attempted to do in our recent studies on Echinaforce®, a standardized ethanol extract of *E. purpurea* (Sharma *et al*., 2008; 2009).

In addition, it is important that the cell culture models used to evaluate anti-infectious agents reflect conditions *in vivo* as far as possible (Nickerson *et al*., 2007). We evaluated this condition by means of a commercial source of normal human airway epithelial tissue, which can be propagated *in vitro* under defined conditions such that tissue architecture and differentiation patterns are preserved (Klausner *et al*., 2007). Such a system more closely resembles *in vivo* tissue, and might be more appropriate than cell lines for the analysis of *Echinacea* and RV infection. Our objective was to assess the effects of RV infection, and the standardized *Echinacea* preparation, on various parameters of tissue integrity and cytokine induction.

MATERIALS AND METHODS

***Echinacea* source.** The test material was Echinaforce® (A. Vogel Bioforce AG, Roggwil, 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 several caffeic acids and alkylamides (Sharma *et al*., 2008). A 1:100 dilution of Echinaforce, as used in the experiments, contained the following concentrations of compounds, in μg/mL: caf-

* Correspondence to: JB Hudson, Department of Pathology and Laboratory Medicine, C-360, 2733 Heather Pavilion, Vancouver *BC*, Canada. V5Z 3J5
E-mail: jbhudson@interchange.ubc.ca

Received 13 July 2009
Revised 11 September 2009
Accepted 14 September 2009

taric acid, 2.64; chlorogenic acid, 0.40; cichoric acid, 3.14; echinacoside, 0.07; tetraenes (as PID 8/9), 0.36; and no detectable caffeic acid, cynarin, or polysaccharide. In addition, the preparation was free of detectable endotoxin (as determined by means of a commercial assay kit from Lonza Walkersville Inc., MD, USA; lower limit of detection 0.1 unit/mL),

EpiAirway™ tissue. A total of three, separate, normal human airway epithelial tissues (code AIR-100), from three different donors, were obtained from MatTek Corp (Ashland, MA, USA). Each tissue had been divided into 24 individual inserts, in culture trays, for propagation in a minimum volume of Dulbecco Minimal Essential Medium (DMEM). We transferred the inserts to 6-well culture trays for experimental purposes. Handling and maintenance, in a 5% CO_2 atmosphere at 37°C, were carried out according to the instructions supplied. The first two tissues were used within 3 days of receipt, and the inserts of the third tissue were maintained for 3 weeks to allow further growth and differentiation.

Cells and viruses. BEAS-2B human bronchial epithelial cells, originally obtained from ATCC (American Type Culture Collection, Rockville, MD, USA), were used as described previously (Sharma *et al*., 2008). The H-1 sub clone of HeLa cells (ATCC), were grown in DMEM + 5% fetal bovine serum (FBS). No antibiotics or antimycotic agents were used. Rhinovirus type 1A (RV1A, from ATCC) was propagated and assayed, by plaque assay, in H-1 cells. The stock virus had a titer 1×10^8 pfu/mL.

Infection and treatment of tissues. Medium was removed from the tissue inserts. Triplicate cultures were then exposed for 60 min to RV1A (either from the top only or from both top and bottom) to give a multiplicity of infection of approximately 1 pfu/cell (several control inserts were removed, trypsinized, and counted for viable cell numbers). Controls received medium in place of virus. The inocula were then carefully removed and replaced by either *Echinacea* (1:100 dilution in DMEM) or medium alone. After 24 or 48 h, supernatants and tissues were removed and separated, and processed according to the tests to be performed. Viable cell measurements were made by trypan blue staining on trypsinized cells, and by MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] staining according to the assay protocol supplied with the tissues (Klausner *et al*., 2007).

Virus assays were made on tissue and supernatant samples by plaque assay in H-1 cells, as described previously (Sharma *et al*., 2008). Cytokine measurements on supernatants were performed by means of ELISA kits from e-Bioscience, San Diego CA, USA (for IL-6 and TNFα), and R&D, Minneapolis MN, USA, for IL-8. Mucin 5AC measurements were made with reagents from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). In the absence of a suitable mucin standard, we expressed results as relative OD_{540} readings (Absorbance at 540 nm).

Histology. Individual tissues were placed into 10% neutral buffered formalin prior to processing through to paraffin wax, stained with hematoxylin and eosin (H & E), or periodic acid Schiff (PAS, for mucopolysaccharides). Microscopic examination was made by means of the Ariol image system at the Center for Translational and Applied Genomics (CTAG) histology laboratory, Vancouver, and further tissue analyses (for measurements of tissue thickness) were made with the aid of the Applied Imaging software.

RESULTS

Three separate groups of EpiAirway tissues (originally from different donors) were used for the three experiments. In the first two experiments, cultures were used within 3 days of their establishment, when the tissues comprised 3–4 layers of epithelial cells, with a clearly visible layer of cilia. In the third experiment, the tissues were maintained for 3 weeks before treatments, resulting in further growth such that 6–8 cell layers were present, together with the layer of cilia.

Histology and tissue integrity

In all cases, the histological appearance of the tissues was unaffected by virus infection or by *Echinacea* treatment, or by the combination of the two (Figs 1A–1C, H & E). The cilia appeared normal, goblet cells were evident, and structurally the overall appearance of the tissues was the same. The only exception was the case of RV-infected tissues, which generally showed more inclusions within the goblet cells, particularly in PAS stained tissues (Figs 2A–2C, PAS staining), indicating excessive mucus production and secretion. This effect on the goblet cells however was reversed by *Echinacea* treatment, and was further confirmed by the measurements of MUC 5AC secretion (Table 3). Figure 3 shows the typical appearance of the 3-week-old tissues – in this case, RV infected. Apart from the additional growth of these tissues, there were no apparent changes due to RV infection or *Echinacea* treatment. Thus tissue integrity was conserved, and cilia and goblet cells were always present. In addition, the RV-infected tissues again showed evidence of abundant inclusions in the goblet cells (Fig. 3).

Measurements of the tissue thickness were made using Applied Imaging software. For this purpose, triplicate randomly selected areas of each of the stained sections were measured. However, there were no consistent differences between the treatments (Table 1). We concluded that overall the tissues were unaffected by virus infection or by *Echinacea* treatment.

These results were supported by measurements of cell viability by both trypan blue staining and MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] assays. An example of the MTT assay is shown in Table 2. All of the tissues, including the 3-week-old tissues, showed high cell viability, which was not affected by virus or *Echinacea* treatment.

Virus replication

We could not find evidence of significant virus replication in the infected tissues. Thus, at different times after

DOI: 10.1002/ptr

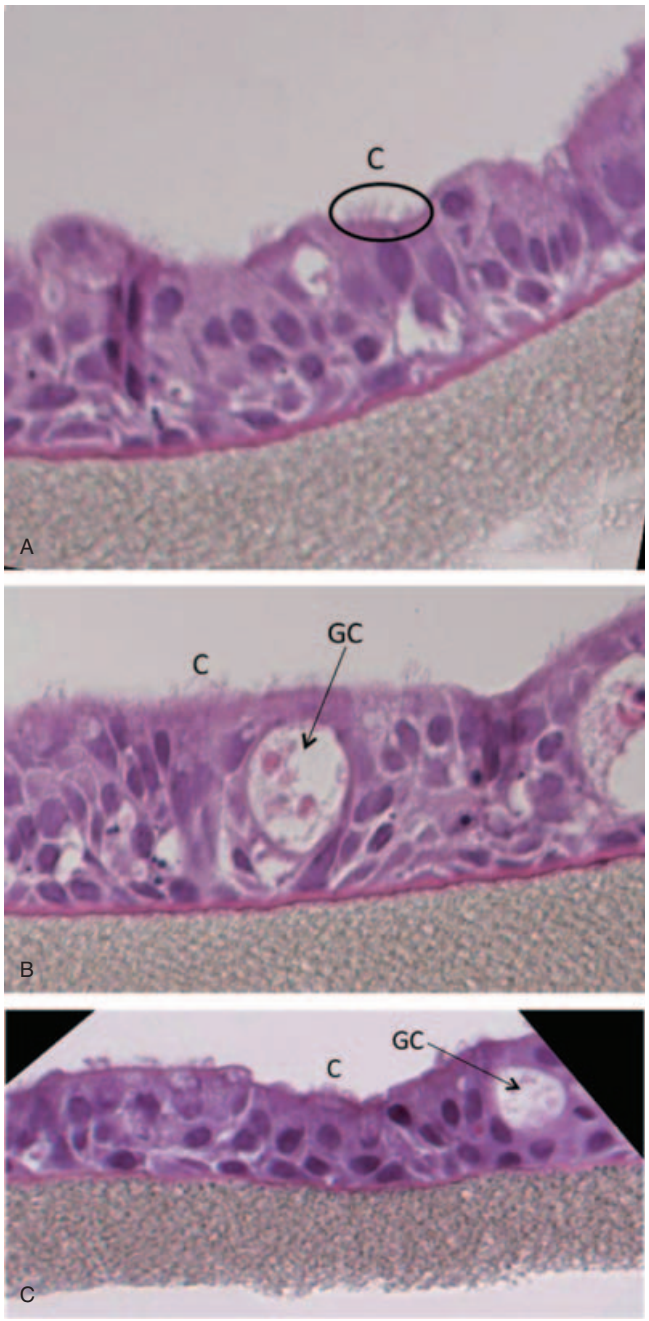

Figure 1. Sections of Epi-Airway tissues (3-day cultures) fixed on membrane supports and stained with H & E, magnification × 20; (A) and (B), sections through RV infected tissues; note presence of intact cilia layer C and prominent goblet cells GC; (C) RV + *Echinacea* treated. The cilia layer C is still conspicuous.

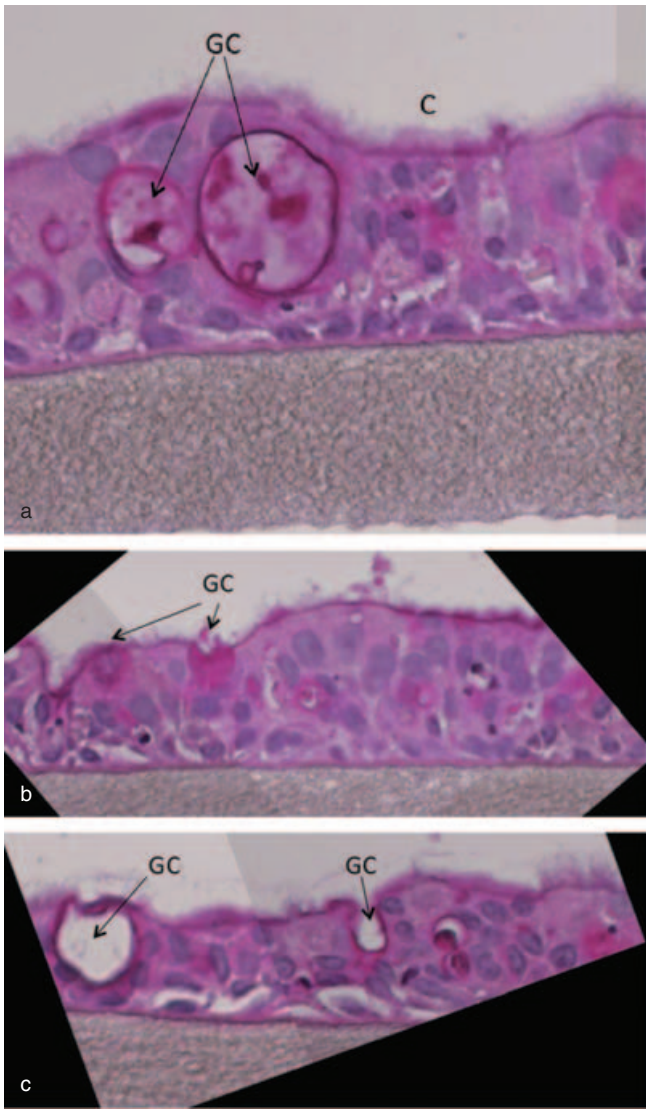

Figure 2. PAS fixed and stained tissues (3-day cultures), × 40; (A) RV infected, showing prominent filled goblet cells GC and intact cilia layer C; (B) same, showing a goblet cell secreting mucous to surface GC; (C) control untreated section showing relatively empty goblet cell GC.

Table 1. Tissue thickness

Tissue sample	Mean thickness ± SD (mm)
Control, untreated	48.86 ± 1.31
+*Echinacea*	48.26 ± 0.92
+RV (A)	50.45 ± 1.60
+RV (B)	47.12 ± 6.69
+RV (A) + *Echinacea*	47.53 ± 1.34
+RV (B) + *Echinacea*	39.0 ± 4.24

Each replicate tissue was infected by RV either from below (A), or from above and below (B), with or without *Echinacea*. Fixed and stained tissues (H & E) were analyzed with the Ariol Imaging system, and each of replicate individual tissues was measured at three different points along the section.

infection (24–72 h), the level of RV in supernatants or whole tissue samples were no greater than background levels due to residual virus inoculum (approximately 100 pfu/mL). In some samples the virus inoculum (1.0 mL) was applied only to the top of the tissues; in other cases, it was applied to both top and bottom, but this did not affect the result.

Cytokine induction

In contrast, supernatants from the RV-infected tissues showed significant stimulation of pro-inflammatory cytokines IL-6 and IL-8, and this stimulation was reversed by *Echinacea* treatment (Fig. 4). *Echinacea* treatment by itself did not stimulate cytokine secretion. These results were similar to those obtained by infection of cultured bronchial and lung epithelial cell lines

DOI: 10.1002/ptr

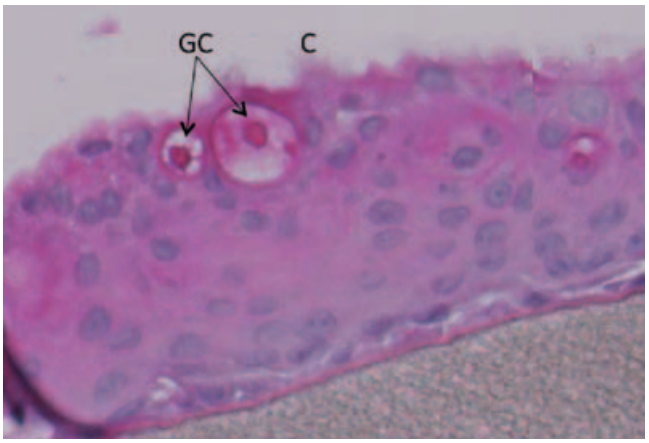

Figure 3. RV infected 3-week old tissue, PAS stained, showing intact tissue with conspicuous cilia C and goblet cells GC with mucopolysaccharide inclusions. × 40.

Table 2. Tissue viability by MTT assay

Tissue sample	OD_{540} (Absorbance) mean ± SD
Control, untreated	1.67 ± 0.21
+*Echinacea*	1.75 ± 0.13
+RV1A	1.84 ± 0.12
+RV1A + *Echinacea*	1.73 ± 0.09

Replicate tissues were treated as indicated, and at 48 h after infection were removed and processed for MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] staining, as described in Materials and Methods. Color development was measured on a plate reader at a wavelength of 540 nm.

(Sharma *et al.*, 2008; 2009). TNFα was not detected, and was not induced (above background level) in these supernatants.

Mucin production

Virus infection resulted in substantial mucin secretion (MUC-5AC, Fig. 5), but this was reversed by *Echinacea* treatment, in confirmation of the histological analysis. These results were similar to those obtained by infection of monolayers of BEAS-2B cell cultures (Table 3).

DISCUSSION

The EpiAirway™ tissue system represents a 3-dimensional, organotypic, model of intact normal human airway epithelial cells, and has been promoted as a valid animal-alternative model for the evaluation of pharmacological agents for toxicity and other parameters (Klausner *et al.*, 2007). For this study, the system has the advantage of retention of normal airway histology and function combined with ease of maintenance and manipulation. We were therefore able to evaluate the roles of RV infection, and *Echinacea* treatment, in simulated *in vivo* conditions. This is important because recent studies with *in vitro* cell cultures have questioned the importance of RV replication in the causation of cold symptoms (Message and Johnston, 2004; Sharma *et al.*, 2006; 2008), whereas several groups have reported

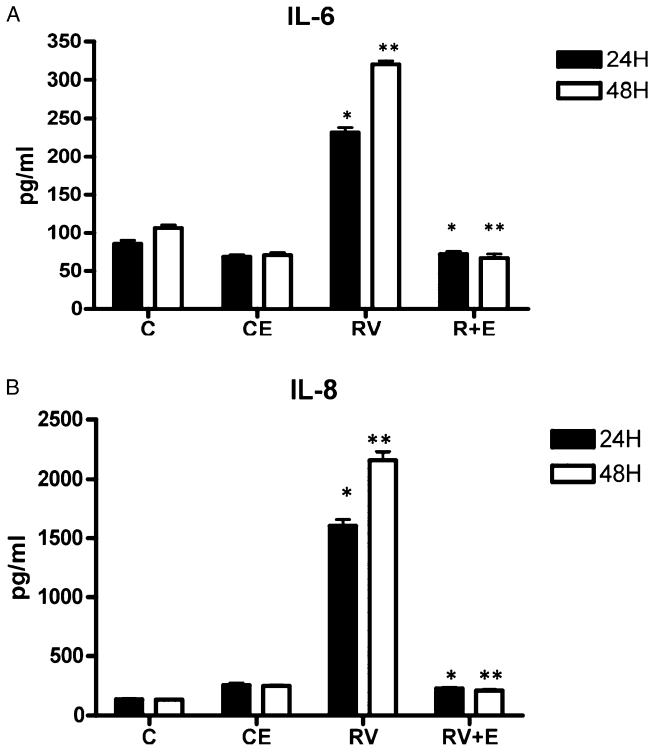

Figure 4. Cytokine secretion in tissue supernatants. Replicate supernatants of treated and control tissues, at 24 and 48 h pi. were assayed for IL-6 (A) and IL-8 (B) by ELISA. Some of the error bars are barely visible. TNFα was not detected. C = control uninfected and untreated; CE = control+ *Echinacea*; RV = infected but no *Echinacea*; R + E = infected and treated with *Echinacea*. Asterisks are shown for the significant differences ($p < 0.001$) between RV and [RV + E] for 24 h (single asterisks) and for 48 h (double asterisks).

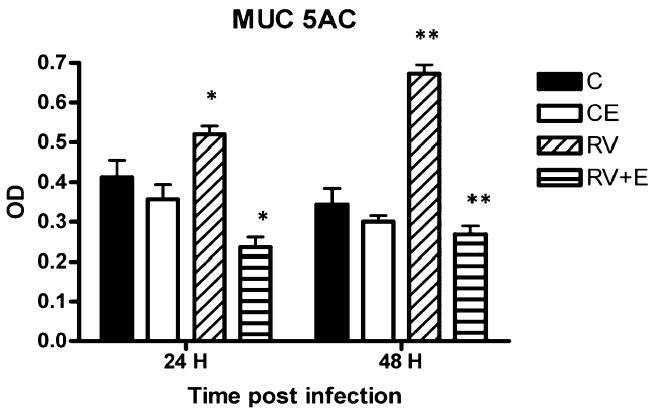

Figure 5. MUC-5AC Secretion. Tissues were treated with RV and/or *Echinacea*, and supernatants were harvested at 24 and 48 h pi. for assay of MUC-5AC. Results are expressed as OD (absorbance) values at 540 nm. Asterisks are shown for the significant differences between RV and [RV + E] for 24 h (single asterisks, *p* value = 0.001) and for 48 h (double asterisks, *p* value <0.001).

the induction of pro-inflammatory cytokines by the virus (Message and Johnston, 2004; Schaller *et al.*, 2006; Sharma *et al.*, 2006; Edwards *et al.*, 2007; Sharma *et al.*, 2009). Thus cold symptoms appear to be the result of the cytokine induction, and consequently inhibition of this induction represents an important target for prospective cold therapy.

The potential drawback of cell culture studies is the possibility that they might not faithfully represent what happens during a natural infection such as a cold

DOI: 10.1002/ptr

Table 3. MUC 5AC Secretion in BEAS-2B Cells

Culture supernatant	24 hours p.i.	48 hours p.i.
Control, untreated	0.21 ± 0.05	0.33 ± 0.05
Control + *Echinacea*	0.32 ± 0.07	0.25 ± 0.09
+RV	0.64 ± 0.05	0.72 ± 0.08
+RV + *Echinacea*	0.24 ± 0.07	0.21 ± 0.05

Replicate BEAS-2B cell cultures were treated as indicated and supernatants were harvested at 24 and 48 h pi. for measurement of relative mucin (MUC 5AC) secretion. Values (all from one experiment) are expressed as mean ± SD of OD_{540} readings.

(Nickerson *et al*., 2007). This point of view could be supported by the apparent variation in response of different types of epithelial cell to RV replication. Thus there are a small number of 'permissive' cell lines, mostly derived by subcloning HeLa cells (e.g., H-1 cells used in our laboratory), that fully support RV replication, and therefore can be used to propagate and assay RVs. In contrast most other epithelial cell lines only support relatively low levels of virus replication, or none at all, and without significant cytopathic effects, e.g., BEAS-2B and A549 cells, and primary cells derived from nasal and bronchial tissues (Gwaltney *et al*., 2002; Mosser *et al*., 2005). Furthermore, the state of differentiation of the cultured cells can influence virus replication (Lopez-Sousa *et al*., 2004). Therefore we considered it important to study the effects of RV infection, and *Echinacea* treatment, on the integrity and function of 'normal' tissues.

We found that tissues maintained in culture for 3 days, or 3 weeks, were essentially resistant to RV infection and to treatment with *Echinacea*, or the combination of the two. Normal tissue integrity was preserved as determined by histology and microscopic analysis, and the frequency of the cilia was unchanged. The only alteration was seen in the goblet cells of RV-infected tissues, which appeared to be more frequently filled with PAS-positive inclusions, presumably mucins. This response, however, was reversed by *Echinacea* treatment. These latter results were confirmed by measurements of MUC 5AC secretion, the dominant respiratory mucin. The mucin response probably explains the excessive mucus secretions often present in colds, and which may consequently be reversed by Echinaforce® treatment.

We did not detect RV replication in any of these tissues, in accordance with many of the *in vitro* studies (cited earlier). However, the pro-inflammatory cytokines IL-6 and IL-8 (chemokine CXCL8), recognized as good markers of an inflammatory response (Edwards *et al*., 2007; Sharma *et al*., 2006; 2009), were stimulated by RV infection, and this response was reversed by *Echinacea.* This result is in accordance with our previous findings in epithelial cell culture models (Sharma *et al*., 2008).

On the basis of these results, we can conclude that Echinaforce® is very effective in neutralizing the inflammatory response induced by RV, as well as some of the cold symptoms, such as excessive mucus production, and at the same time Echinaforce® has no detrimental effect on tissue integrity and number of cilia. In addition, this system validates the continued use of our previously described cell culture models for elucidation of the mechanisms of action of *Echinacea* in RV-infected epithelial cells.

REFERENCES

Barnes J, Anderson LA, Gibbons S, Phillipson JD. 2005. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell. *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench: a review of their chemistry, pharmacology and clinical properties. *J Pharm Pharmacol* **57**: 929–954.

Edwards MR, Hewson CA, Laza-Stanca V, Lau HTH, Mukaida N, Hershenson MB, Johnston SL. 2007. Protein kinase R, IkB kinase b, and NFkB are required for human RV induced pro-inflammatory cytokine production in bronchial epithelial cells. *Molecular Immunology* **44**: 1587–1597.

Gertsch J, Schoop R, Kuenzle U, Suter AL. 2004. Echinacea alkylamides modulate TNF-a gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS Lett* **577**: 563–569.

Gwaltney JM. 2002. Clinical significance and pathogenesis of viral respiratory infections. *Am J Med* **112**: 13S–18S.

Klausner M, Ayehunie S, Breyfogle BA, Wertz PW, Bacca L, Kubilus J. 2007. Organotypic human oral tissue models for toxicological studies. *Toxicol In Vitro* **21**: 938–949.

Lopez-Sousa N, Dolganov G, Dubin R, Sachs LA, Sassina L, Sporer H, Yagi S, Schnurr D, Boushey HA, Widdicombe JH. 2004. Resistance of differentiated human airway epithelium to infection by rhinovirus *Am J Physiol Lung Cell Mol Physiol* **286**: L373–L381.

Matthias A, Banbury L, Stevenson LM, Bone KM, Leach DN, Lehmann RP. 2007. Alkylamides from *Echinacea* Modulate Induced Immune Responses in macrophages *Immunological Investigations* **36**: 117–130.

Message SD, Johnston SL. 2004. Host Defense Function of the Airway Epithelium in Health and Disease Clinical Background. *J Leukoc Biol.* **75**: 1–13.

Mosser AG, Vrtis R, Burchell L, Lee WM, Dick CR, Weisshaar E, Bock D, Swenson CR, Cornwell RD, Meyer KC, Jarjour NN, Busse WW, Gern JE. 2005. Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Amer J Resp Critical Care Medicine* **171**: 645–651.

Nickerson CA, Richter EG, Ott CM. 2007. Studying Host-Pathogen Interactions in 3-D: Organotypic Models for Infectious Disease and Drug Development. *J Neuroimmune Pharmacol* **2**: 26–31.

Schaller M, Hogaboam CM, Lukacs N, Kunkel SL. 2006. Repiratory viral infections drive chemokine expression and exacerbate the asthmatic response. *J Allergy Clin Immunol* **118**: 295–302.

Sharma M, Arnason JT, Burt A, Hudson JB. 2006. *Echinacea* extracts modulate the pattern of chemokine and cytokine secretion in rhinovirus-infected and uninfected epithelial cells. *Phytotherapy Res* **20**: 147–152.

Sharma M, Schoop R, Hudson JB. 2008. *Echinacea* as an antiinflammatory agent: the influence of physiologically relevant parameters. *Phytother Res* DOI: 10.1002/ptr.2714.

Sharma M, Anderson SA, Schoop R, Hudson JB. 2009. Induction of pro-inflammatory cytokines by respiratory viruses and reversal by standardized *Echinacea*, a potent antiviral herbal extract. *Antiviral Res* **83**: 165–170.

Vohra S, Adams D, Hudson JB, Moore JA, Vimalanathan S, Sharma M, Burt A, Lamont E, Lacaze N, Arnason JT, Lee TDG. 2009. Selection of Natural Health Products for Clinical Trials: a Preclinical Template. *Can J Physiol Pharmacol* **87**: 371–378.

Woelkart K, Bauer R. 2007. The role of alkamides as an active principle of *Echinacea. Planta Med* **73**: 615–623.