RHINOVIRUS INFECTION OF THE HUMAN AIRWAY EPITHELIUM: IN VITRO CHARACTERIZATION OF VIRAL REPLICATION, INFLAMMATORY RESPONSE, AND IMMUNE-MODULATING EFFECTS OF ECHINACEA

by

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ABSTRACT

Rhinoviruses (RVs) are the leading cause of upper-respiratory tract infections in humans. To date relatively little is known about the mechanism of RV infection and no cure or prevention exists. Mounting evidence shows that RV replicates very little in its host airway epithelial cells leading researchers to hypothesize that the illness associated with RV infection is the result of the host's immune response, but not necessarily RV replication. This study characterized RV infection in vitro in terms of viral replication, viral RNA, and pro-inflammatory cytokine/chemokine secretion over the course of a typical infection using two distinct airway epithelial cell lines (BEAS-2B and A549) and two different receptor-utilizing RV serotypes (RV14 and RV1A). Cells were infected with known amounts of RV, sampled over 1 week, and assayed for infectious virus, RV14 RNA, and interleukin (IL)-6 and/or IL-8 secretion. For BEAS-2B and A549 cells viral replication peaked between day 1 (D1) and D2 post-infection for both RV14 and RV1A, and no significant viral replication was observed after D3. Stimulation of IL-6 and IL-8 was typically not observed before D2 and remained elevated up to D7. Overall, BEAS-2B cells were more susceptible to RV infection than A549, and similar trends were observed for RV14 and RV1A, except RV14 failed to replicate in the A549 cells. Furthermore, UV inactivation of both RV serotypes completely inhibited viral replication and IL-6 secretion in the BEAS-2B model, suggesting the necessity of genetically intact virus to stimulate the IL-6 response. Finally, the effects of two chemically distinct Echinacea extracts on viral replication and IL-6 secretion were investigated in the BEAS-2B model. Neither of the Echinacea extracts had any effect on RV replication, nor did they stimulate IL-6 secretion in uninfected cells. However, Echinacea treatment of RV infected cells significantly affected IL-6 secretion, but a different trend was observed between RV serotypes, and for the two herb preparations. Overall, RV infection of airway epithelial cells results in relatively low levels of RV replication but a pronounced pro-inflammatory cytokine/chemokine response which is the likely cause of cold symptoms and a potential target for therapeutics.
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<tr>
<td>RV</td>
<td>rhinovirus</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disorder</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
</tr>
<tr>
<td>VLDLR</td>
<td>very low density lipoprotein receptor</td>
</tr>
<tr>
<td>HID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% human infectious dose</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infectious dose</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effects</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-B</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>D(0-7)</td>
<td>days after RV infection/inoculation</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>MEM</td>
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CO-AUTHORSHIP STATEMENT

All of the work required for this thesis was completed by me independently; however, my supervisors (Dr. James Hudson, Dr. Robert Harris, and Dr. Colin Brauner) provided me with mentorship and supervision, and will be listed as co-authors on the publications arising from this project.
Chapter 1: Literature Review and Thesis Objectives

INTRODUCTION

The most prevalent infection in humans is the acute upper-respiratory tract infection, also known as the common cold (Monto, 2002). Over 100 Rhinoviruses (RVs) persist in our world today and are the leading cause of such respiratory tract infections (Arruda et al., 1997; Johnston et al., 1995; van Gageldonk-Lafeber et al., 2005). In healthy individuals RV infections are typically short-lived and self-limiting; however, for susceptible groups such as infants, the elderly, and the immuno-compromized RV infection can be life-threatening. Furthermore, for people already affected by diseases such as asthma or chronic obstructive pulmonary disorder (COPD), RV infection is known to cause serious exacerbations of those conditions (Bardin, 1992; Fraenkel et al., 1995; Gern, 2002; Gern & Busse, 1999; Greenberg, 2002; Grunberg & Sterk, 1999; Mallia et al., 2006; Message & Johnston, 2004; Seemungal, 2001; Seemungal, 2000; Teran et al., 1997). In the case of asthma, recent studies suggest that early childhood RV infection may even play a causative role in the development of the disease (Gern, 2004; Singh et al., 2007).

To date, relatively little is known about the mechanism of RV infection and no cure or prevention exists. Mounting evidence shows that RV replicates very little in its host airway epithelial tissues (Lopez-Souza et al., 2004), leading researchers to hypothesize that the illness associated with RV infection is the result of the host’s immune response to some viral trigger, but not necessarily to the level of viral replication itself. Bearing this in mind, new therapies which modulate the immune system and mitigate RV associated symptoms are becoming increasingly interesting to scientists. One such candidate therapy is the natural herb extract Echinacea. Echinacea use has recently gained widespread popularity in Western culture, and commercial products are widely available to consumers. Although the quality of many commercial formulations is questionable, there is growing evidence that Echinacea has diverse effects on the immune system (Brousseau & Miller, 2005; Brush et al., 2006; Currier & Miller, 2000; Goel, 2005; Sharma et al., 2006). In terms of mitigating RV-related illness, Echinacea may down-regulate the inflammatory response provoked by viral infection; however, its mechanism of action is largely unknown (Sharma et al., 2006).
In my experiments, I aimed to characterize RV infection in immortalized human airway epithelial cells and to evaluate the effects of Echinacea extracts on RV infected and uninfected cells. This first chapter provides background material relevant to this thesis and concludes with general objectives and hypotheses. In Chapter 2 (In Vitro Characterization of Rhinovirus Infection in Airway Epithelial Cells) I characterized RV infection in two distinctive airway epithelial cell models using two different receptor-utilizing RV serotypes. I measured levels of viral replication, viral RNA, and cell secretion of pro-inflammatory cytokines and chemokines over the course of a typical infection and compared those parameters over time, between cell models, and between RV serotypes. In Chapter 3 (The Effects Ultraviolet Inactivated Rhinovirus on Airway Epithelial Cells) I investigated the potential viral trigger for cell inflammatory mediator secretion by using ultraviolet (UV) inactivated RV to assess its ability to infect cells and/or elicit an inflammatory response. In Chapter 4 (The Effects of Echinacea Extracts on Rhinovirus Infected and Uninfected Airway Epithelial Cells) I examined the effects of two chemically distinct Echinacea extracts on infected and uninfected bronchial epithelial cells in terms of viral replication, viral RNA, and cell pro-inflammatory cytokine secretion. Finally, Chapter 5 presents a general discussion of my findings, overall significance and conclusions, and future directions for investigation.

The Host: The Airway Epithelium

The human airway epithelium is a pseudostratified cell layer which provides a physical barrier between the internal and external environments of the air passages while playing a vital role in processes such as the maintenance of lung fluid balance, mediation of smooth muscles, clearance and metabolism of irritants and pathogens, and activation of the inflammatory response. The human airway epithelium consists of at least 8 morphologically different cell types which can be functionally subdivided into: columnar ciliated epithelial cells, mucous cells, and basal cells (Jeffery & Reid, 1975; Spina, 1998). The ciliated cells are in contact with the external environment and are the predominant airway epithelial cell type (Halama et al., 1990). The cilia of these cells beat in a rhythmic fashion to move any trapped debris out of the respiratory tract to be coughed out or swallowed. The mucous cells (e.g. goblet and Clara cells) secrete acidic-mucin granules
into the airway lumen which mix with water forming an outer mucus lining (Stinson & Loosli, 1978). Finally, the basal cells are attached to the underlying basement membrane and anchor the other cells of the epithelium. They are also the primary stem cells to the other cell types (Knight & Holgate, 2003). The cells themselves function as a barrier to the outside environment, and furthermore paracellular diffusion is restricted by the formation of tight junctions between the apices of adjacent cells (Qu, 2005). The cells of the airway epithelium also secrete many important molecules such as: lipid mediators, growth factors, broncho-constricting peptides, arachidonic acid metabolites, cytokines, and chemokines, which play diverse roles in ensuring proper respiratory functioning (Knight & Holgate, 2003). The human respiratory tract is susceptible to infection by many bacteria, fungi, and viruses. The most common viruses to infect the airway epithelial cells are the RVs often resulting in acute upper-respiratory tract infections known as the common cold.

Models of the Airway Epithelium

In order to facilitate laboratory research, many in vitro models of the human airway epithelium have been developed by scientists. The most commonly used models are cultured cell lines derived from native airway epithelial cells. These cell lines have typically been transformed by viruses, or derived from cancerous growths, rendering them immortalized and amenable to repeated culture. Examples of immortalized airway epithelial cell lines include: SV40 adenovirus transformed bronchial epithelial (BEAS-2B) cells (Ke et al., 1988), and human adenocarcinoma derived type II alveolar-like (A549) cells (Lieber et al., 1976). The above cell lines retain much of their native cell functions including the ability to form monolayers and the capability to secrete various biological molecules (Ke et al., 1988; Lieber et al., 1976; Reddel et al., 1988; Shapiro et al., 1978; Smith, 1977). When grown submerged in culture medium immortalized cells undergo limited differentiation and thus resemble basal epithelial cells most closely (Albright et al., 1990). They also lack distinct apical and basolateral membranes; however, junctional complexes can be observed (Albright et al., 1990). Overall, immortalized cell lines are an approachable and widely accepted model for many scientific experiments.

It is possible to induce further differentiation in the aforementioned cell lines by manipulating their growth environment. For example, culturing BEAS-2B or A549 cells on
permeable supports or specialized membranes with an air-liquid interface produces monolayers with apical and basolateral sides and increases the expression of tight junction proteins (Blank et al., 2006).

Finally, with access to live airway epithelial tissues scientists can establish primary cultures by processing and plating cells using specialized procedures. Primary cultures are typically created from nasal, tracheal or bronchial tissues derived from surgical procedures such as polypectomies (Donninger et al., 2003; Lopez-Souza et al., 2004; Suzuki et al., 2002). Obviously, such cultures are the truest in vitro models of the airway epithelium retaining native epithelium characteristics most closely. However, live tissues are often difficult to obtain (especially in sufficient amounts), culture techniques are more sensitive and complicated, and cells only allow for 1-2 passages before undergoing terminal differentiation. Furthermore, primary tissues derived from surgeries may be affected by the presence of pre-operative drugs such as anesthetics.

All of the above models are susceptible to RV infection at least to some degree. Lopez-Souza et al. (2004) found that the least differentiated cells (immortalized cell cultures) were the most vulnerable to RV infection and the most differentiated cells (primary cultures) were much more resistant. Undifferentiated cultured cells produced viral titers 30 to 130 times that of their differentiated primary culture counterparts, while immortalized cells grown on permeable supports produced intermediate viral concentrations. A similar trend was observed for RV induced inflammatory mediator secretion and suggests that undifferentiated airway cell models may represent a somewhat exaggerated response to RV infection.

Rhinovirus (RV)

RVs belong to the Picornaviridae family and are icosahedral with a protein capsid surrounding genetic material encoded in single stranded RNA (Bella & Rossman, 1999). Their genetic information carries a positive polarity and is translated into a polyprotein upon entry into the cytoplasm. This polyprotein is then automatically cleaved by the cell and further processed by viral proteases, eventually forming the viral capsid proteins and non-structural proteins involved in the replication process (Reithmayer et al., 2002). To date, over 100 RV serotypes have been identified based on the presence of unique and
specific antigens (Bella & Rossman, 1999). The RV capsid is about 30nm in diameter and consists of 60 copies of four distinct viral proteins called: VP1, VP2, VP3, and VP4. The first three viral proteins form the outer protein shell while VP4 exists interiorly in contact with the single stranded RNA. The RV structure is characterized by protuberances on a fivefold vertex with depressions called “canyons” between the vertices (Bella & Rossmann, 1999; Kim & Kim, 1989; Oliveira et al., 1993; Rossmann, 1989; Zhao et al., 1996).

**Rhinovirus Receptors**

The RV viral binding sites have been determined by antibody binding studies (Bella & Rossman, 1999). Binding sites were located by analyzing mutant viruses that were unaffected by antibodies. Four specific antigenic areas were located and mapped onto the known structure of RV14. These antigenic sites correspond to the outer edges of the canyons and are the exposed parts of the virus (Bella & Rossmann, 1999; Rossmann, 1989). The viral binding site for receptors is largely found within the canyons although it has been shown that this binding does extend over the rims of the canyons (Bella & Rossmann, 1999; Smith et al., 1996).

The RVs are often classified by the type of cell receptor that they bind as identified by monoclonal antibody studies where antibodies recognized a 95kd glycoprotein on both human cells and mouse transfectants (Greve et al., 1989). Most RVs (more than 90) belong to the “major” group, sharing a common receptor called the intercellular adhesion molecule-1 (ICAM-1) (Bella & Rossmann, 1999; Greve et al., 1989; Staunton et al., 1989). The “minor” group consists of 10 serotypes which utilize receptors in the low density lipoprotein receptor (LDLR) family (Bella & Rossmann, 1999; Hofer et al., 1994). Finally, human RV87 does not bind to either of the above receptors and has recently been reclassified as an enterovirus based on genome sequences, although receptor-binding studies are lacking (Oberste et al., 2004; Uncapher et al., 1991).

It has been proposed that once bound to their receptors some viruses enter the cytoplasm by cellular endosomes (Bayer et al., 1998; Zeichhardt et al., 1985). Such a mechanism has been demonstrated for RV2 (a minor RV) and also for the foot-and-mouth disease virus (Baxt, 1987). This mechanism is characterized by the low pH internal to
endosomes which causes the release of capsid-bound RNA into the cellular environment (Baxt 1987; Bayer et al., 1998; Prchla et al., 1994; Zeichhardt et al., 1985).

Other studies have demonstrated that the ICAM-1 receptor (major RVs) is capable of uncoating both RVs and polioviruses at normal physiological pH in soluble form, suggesting the passage of the virus without and endocytic step (Perez & Carrasco, 1993).

**Intercellular Adhesion Molecule-1 (ICAM-1) Receptor**

The ICAM-1 receptors are transmembrane glycoprotein cell adhesion molecules (Bella & Rossmann, 1999). Exteriorly they consist of a row of immunoglobulin domains. It is these domains that are the basic building blocks of antibodies (Bella & Rossmann, 1999). The ICAM-1 ligand is a pair of integrin receptors that is largely found on leukocyte cells. In the leukocyte the ICAM-1 receptors’ main functions are to promote cell adhesion to the extracellular matrix and also to lymphocytes (Bella & Rossmann, 1999). This role is important in the inflammatory response when the expression of ICAM-1 receptors is elevated in endothelial cells, thus causing these cells to adhere to leukocytes passing in the blood and drawing leukocytes to locations of injury or infection (Bella & Rossmann, 1999). Although there is evidence that RV-stimulated cytokine secretion increases the permeability of lung endothelial cells (Sedgwick et al., 2002), it is unknown whether RV can or does directly infect the airway endothelium.

The major RVs have exploited the ICAM-1 receptors and utilized them as viral binding sites binding ICAM-1 receptors at the deepest canyon site on their surface (Olson et al., 1993). The binding of receptor and virus is the first step of infection which is followed by viral genetic uncoating and entry into the cell by crossing the plasma membrane. It has been demonstrated that major group RV infection causes a rapid up-regulation in membrane bound ICAM-1 expression in airway epithelial cells. (Grunberg, 2000; Papi & Johnston, 1999; Papi, 2002; Winther et al., 2002). For example, Papi, (2002) found that upon infection with RV, ICAM-1 expression in cell lines and primary cultures increased 4.2 to 6 times respectively, when compared to controls. This RV induced increase in ICAM-1 expression seems to occur mostly in basal cells and to a lesser extent in ciliated cells (Grunberg, 2000). ICAM-1 expression peaks 8-24 hours post-infection and gradually declines back to control levels usually by day 5 (Papi & Johnston, 1999; Winther et al.,
The up-regulation in ICAM-1 expression is correlated to the airway inflammatory response which can be triggered by irritants, pathogens, and as a complication from diseases such as asthma and COPD where this response is unnaturally exaggerated. Researchers have found a direct link between viral infection and the increase of inflammatory mediators (e.g. cytokines), leading to the up-regulation of ICAM-1 and other airway inflammatory response genes (Grunberg, 2000; Higashimoto et al., 1999; Papi, 2002; Winther et al., 2002).

Low Density Lipoprotein Receptors (LDLRs)

The LDLR receptors are comprised of: LDLR proper, very low density lipoprotein receptor (VLDLR), LDLR-related protein, megalin, and other membrane proteins (Hofer et al., 1994; Reithmayer et al., 2002). The ligands for this family of receptors are binding domains consisting of cysteine residue repeats, although particular ligands are quite remarkably divergent (Reithmayer et al., 2002). As such, the mechanism of receptor recognition is still largely unknown. The LDLR type RV2 and RV1A have been successfully replicated in mouse cell lines which also carry LDLR-type receptors (Lomax & Yin, 1989; Reithmayer et al., 2002). The LDLR receptors are strongly correlated with the endocytic mechanism (Brabec et al., 2006). It is thought that the low pH of the endosomes triggers the opening of virus induced pores in the plasma membrane thus allowing the viral RNA to enter the cell (Bayer et al., 1998; Prchla et al., 1995). The LDLR receptors have been definitively shown to bind some minor RV serotypes (Hofer et al., 1994). Interestingly, cells lacking or with suppressed LDLR receptor expression do allow limited viral entry of some serotypes suggesting the possibility of additional points of entry (Hofer et al., 1994).

The Common Cold: Infection & Illness

RVs are the cause of over 50% of upper respiratory tract infections in humans (Arruda et al., 1997; Johnston et al., 1995). Other viruses that can cause the common cold include the respiratory syncytial virus, adenoviruses, and coronaviruses (Arruda et al., 1997). Most adults experience an average of 2-4 colds a year, while children may experience 6-8 colds per year (Monto & Sullivan, 1993). Infections are more common in temperate climates.
during the colder months of the year (Couch, 1996; Monto, 2002). Individuals usually become infected by contact with contaminated surfaces or inhalation of large particle aerosols like those arising from coughing (D'Alessio et al., 1976; Dick et al., 1987; Gwaltney et al., 1978; Gwaltney & Hendley, 1982). While infection is usually initiated in the nasopharyngeal area, most of the airway tissues are susceptible to infection at least to some degree (Winther et al., 1986). Unlike enteroviruses, RVs have not been found to replicate in the gastrointestinal tract and are rapidly inactivated in the stomach (cited in Couch, 1996).

The 50% human infectious dose (HID_{50}) for RVs is generally regarded as low; ranging from 0.032 to 0.4 of the 50% tissue culture infectious dose (TCID_{50}) in human fibroblasts. However, this infectious dose is variable, for example; RV14 HID_{50} was found to be 5.7 times the fibroblast TCID_{50} (cited in Couch, 1996). Individuals with existing RV antibodies are resistant to infection and the viral dose required to cause illness is higher than for individuals lacking antibodies (Alper et al., 1996). The incubation period of RV leading to first viral shedding in vivo is 10-12 hours while the viral replication cycle duration is 6-8 hours (Harris & Gwaltney, 1996). Onset of illness typically begins with a “scratchy” throat, followed by symptoms including: nasal discharge, nasal obstruction, sneezing, sore throat, cough, headache, myalgia, malaise, and rarely fever (Arruda et al., 1997). Overall, the systemic symptoms are much milder than those caused by viruses such as the influenza virus. Computer tomographic scans of individuals infected with colds show: occlusion and abnormalities of the sinus cavities, thickening of nasal passage walls, and engorged turbinates (Gwaltney et al., 1994). The mean duration of the cold is 7-11 days (Arruda et al., 1997) with peak symptoms occurring between the second and third day. In healthy patients RV can be recovered for up to 3 weeks (Jartti et al., 2004). Kling et al. (2005) found that RV RNA persisted in 44% of asthmatic children 6 weeks after infection. Additionally chronic RV infections, persisting for more than 12 months, have been described in some lung transplant recipients (Kaiser et al., 2006).

**Rhinovirus Infection of the Airways**

The underlying histology of RV infection of the airways is not totally understood; however, there is mounting evidence the RV infection does not manifest itself as a
widespread mucosal infection but as localized foci from where inflammatory responses are generated. *In vivo*, this has been demonstrated with nasal biopsies which clearly point to small cellular areas of localized infection, while showing few other histological abnormalities (Douglas et al., 1968; Hamory et al., 1977; Winther et al., 1984). More recent immunohistochemical evidence also supports the finding that RV infection of the airways emerges in a patch-like fashion (Mosser et al., 2005). Mosser *et al.* (2002) demonstrated that only 5-10% of primary airway epithelial cells were susceptible to RV regardless of the infection dose used. Arruda *et al.* (1995) also found that only a very small proportion of nasal epithelial cells were infected with RV in experimentally inoculated volunteers who had indeed developed cold symptoms.

Furthermore, in tissue and cell cultures like: BEAS-2B, A549, or primary tracheal cells there are no cytopathic effects (CPE) such as: cell rounding, wrinkling, rupture, or death observed upon infection with RV (Johnston et al., 1998; Suzuki et al., 2001), although viral replication can easily be detected. The level of RV replication is also considered relatively low with increasingly differentiated cells producing decreased titers of virus (Lopez-Souza *et al.*, 2004). Consequently, there is a growing belief that it is the pro-inflammatory cytokines and chemokines stimulated by RV infection that cause the symptoms and pathogenicity of infection.

**Immune Response to Infection**

In order for RV to infect the airway epithelium several defensive barriers must first be penetrated. Firstly, the virus must breach the mucosal layer of the epithelium, and if successful, the virus may adhere to the epithelial layer, but only if it can out-compete the natural flora of the airways, and evade phagocytes which are especially rich in the lung tissues. Once attached its host receptor, viral entry and replication may occur, and local infection may ensue. Once inside the cell, the virus is not exposed to most elements of the immune system but as viral progeny are released from infected cells, these virions are confronted by the anticipatory immune response.

The tissues defend themselves against potential threats such as physical injury, irritants and pathogens by two types of immunity called: innate and adaptive. The innate response is fast acting, triggered within 0-4 hours and acts over several days. It initiates the same
cascade of events regardless of threat (Janeway, 2005). The adaptive response is slower acting, usually activated after 96 hours of exposure, and results in the production of specific antibodies which resolve infections while ensuring long-term or life-time immunity against specific antigens (Janeway, 2005).

**Innate Immunity**

The innate immune response is the airway cells’ first line of defense once a pathogen has penetrated the epithelial layer. *In vivo*, pathogens are first met by phagocytic macrophages residing in the airway tissues, which both engulf threats and secrete inflammatory mediators to initiate an inflammatory response. These mediators include lipids such as prostaglandins, leukotrienes, and platelet-activating factor, and protein mediators called cytokines and chemokines (Janeway, 2005). The airway epithelial cells themselves are also capable of secreting cytokines and chemokines, and thus this portion of the innate cellular response can be retained *in vitro* (Kim et al., 2000; Sharma et al., 2006; Zhu et al., 1996).

A multitude of different cytokines and chemokines are secreted from various cells interacting to coordinate the initial inflammatory response and prime the impending adaptive response. Inflammation functions to increase microcirculation to the site of infection allowing for increased entry of white blood cells and also increases lymph circulation for initiation of the adaptive response by antibody formation (Janeway, 2005). During inflammation the principal cells recruited to the injured site are neutrophils, which engulf and destroy the invading pathogens. Neutrophil levels are typically elevated during the first day post-infection but quickly return to normal levels (Couch, 1996). The characteristic symptoms associated with this response: swelling, redness, heat and pain are thought to be caused by the inflammatory mediator induced effects on local blood vessels, such as increased dilation and permeability (Janeway, 2005). The inflammatory response is critical in controlling infections while initiating the slower acting adaptive response.

**Cytokines & Chemokines**

Cytokines are soluble intercellular signaling glycoproteins which are secreted by cells and affect the behaviour of other cells bearing receptors for them (Janeway, 2005). To date,
over 100 members of the cytokine family have been identified (Haddad, 2002). In general, cytokines are secreted in response to various injuries and stresses (including viral infection), and act in a paracrine fashion to initiate responses from their neighbours (Janeway, 2005). However, more recently cytokines have been ascribed diverse immunological roles in antigen presentation, adhesion molecule expression, and bone marrow differentiation, thus cytokine roles are much more complex than initially thought (Borish & Steinke, 2003). At the site of injury, which type of immune response initiated (humoral, allergic, cell mediated, or cytotoxic) is dictated by the types and combinations of cytokines secreted (Borish & Steinke, 2003). Generally, cytokines can be divided into two functional groups: pro-inflammatory and anti-inflammatory, where pro-inflammatory cytokines initiate and/or amplify inflammation while anti-inflammatory cytokines negate such effects (Calixto et al., 2004). Cytokines bear specialized receptors which recognize specific patterns present on pathogens thus initiating an immune response (Borish & Steinke, 2003). These receptors activate tyrosine kinase signaling pathways, which include the janus kinases and signal transducer and activators of transcription acting in a hormonal fashion to affect transcription of specific genes (Darnell et al., 1994; Jhle et al., 1994).

Chemokines are small (8-12kD) secreted proteins which attract other chemokine receptor bearing cells (e.g. neutrophils and monocytes) from the bloodstream to the site of injury. The hallmark function of chemokines is the induction of chemotaxis in the various immune cells. Chemokine activity is controlled by G-protein coupled receptors, of which 18 different types have been identified. So far 47 different chemokines have been discovered, thus there is likely some redundancy in receptor binding (Borish & Steinke, 2003). As a group, chemokines are 20-50% homologous, and their differences are largely due to variable positions of cysteine residues. Most chemokines are considered pro-inflammatory, although again more complex roles (e.g. in adaptive immunity and lymphocyte development) are constantly being discovered. Different tissues bear diverse numbers of chemokine receptors from 3000 per cell to 50,000 per cell in some white blood cells (Borish & Steinke, 2003). Both cytokines and chemokines are released by macrophages, which phagocytose pathogens by surface receptor recognition, and are also released by the host airway epithelial cells themselves (Borish & Steinke, 2003). These mediators initiate the process of inflammation. Cytokine and chemokine mediators are
often grouped according to their functions. Distinct categories of cytokines include: lymphokines, tumour necrosis factors (TNFs), and interferons, while chemokines are often divided into: CC chemokines and CXC chemokines, based on their tertiary protein structure (Janeway, 2005). Those mediators thought to directly act on leukocytes (chemokines or cytokines) are termed interleukins (ILs), of which 33 different proteins have been identified (Janeway, 2005). It is important to note however, that the groupings and nomenclatures of the various cytokines and chemokines are not necessarily accurate due to the pleiotropic nature of these proteins.

IL-6 is a cytokine which has many different functions. In general, IL-6 is released from cells in response to stress and injury (Janeway et al., 2005). Excreted IL-6 stimulates neighbouring cells to release more IL-6 themselves helping to activate white blood cells such as T lymphocytes, and Ig production of B lymphocytes (Borish & Steinke, 2003). The primary source of IL-6 is from mononuclear phagocytic cells; however, IL-6 is also secreted from: epithelial cells, endothelial cells, B and T lymphocytes, fibroblasts, keratinocytes, hepatocytes, and bone marrow cells (Akira et al., 1993; Borish & Steinke, 2003). Most of the effects of IL-6 are considered pro-inflammatory, although in some tissues IL-6 also takes on anti-inflammatory roles by decreasing the secretion of other pro-inflammatory cytokines such as IL-1 and TNF (Borish & Steinke, 2003). Scientists have shown that IL-6 is an important mediator of inflammation in the respiratory tract and plays a key role in IgA antibody production (Fraenkel et al., 1995; Gwaltney & Ruckert, 1997; Gwaltney et al., 1966).

IL-8 (systemic name CXCL8) is a chemokine which is also secreted from various cells (mononuclear phagocytes, endothelial and epithelial cells) in response to stress and injury (Janeway, 2005). It has a multitude of functions, the most notable being the chemotactic attraction of neutrophils and adherence to endothelial cells. Two receptors for IL-8 have been identified: CXCR1 and CXCR2 (Borish & Steinke, 2003). Dysregulations in IL-8 levels have been implicated in various airway diseases such as asthma and cystic fibrosis (Damme, 1994). Its secretion is usually triggered by the presence of other cytokines such as IL-1 and TNF, and also by viruses.

RV infection can directly stimulate the release of many cytokines and chemokines, including IL-6 and IL-8, from various pulmonary cells both in vitro and in vivo (Kim et al.,
2000; Papadopoulos et al., 2001; Terajima et al., 1997; Zhu et al., 1996 and 1997). Zhu et al. (1996) showed that IL-6 and IL-8 were released from A549 cells upon stimulation with RV14 and RV1A. IL levels began to increase within 4-8 hours post-infection and peaked at 24 hrs for both RV serotypes. Kim et al. (2000) used BEAS-2B cells infected with RV16 and found that maximum IL-6 and IL-8 protein release occurred at 4-6 hours post-infection and that the maximal mRNA signal for both ILs was detected within 1 hour of infection. Lopez-Souza et al. (2004) infected fully differentiated primary nasal cultures with RV16 and found increases in both IL-6 and IL-8 over 50 hours.

However, other studies report conflicting findings in the pattern of IL release from infected cells. For example, Johnston et al. (1998) infected A549 cell lines with RV9 and found that IL-8 secretion continued to increase over time for the entire 120 hours in which experimental samples were taken. They also found that mRNA for the ILs increased within an hour, but peaked at 3-4 hours post-infection and had disappeared by 96 hours. The cause of these discrepancies is not clear but may be the result of: method used, RV serotype, cell type, cell age, or other factors. For example, the levels of cytokines excreted by cells generally decreases with passage number (Sanders et al., 1998). Also, RV serotypes have similar but not identical properties, and furthermore varying amounts of viral titers and different infection protocols were used in the various experiments.

The mechanism of RV induced cytokine and chemokine secretion is still largely unknown. Both major and minor type RVs have been shown to induce cytokine and chemokine responses despite their differential use of receptors. There is evidence of virus induced inflammatory mediator secretion through nuclear factor kappa-B (NFκB) activation (Zhu et al., 1996; Zhu et al., 1997). The NFκB transcription factor family [NFκB1 (p105/p50), NFκB2 (p100/p52), RelA (p65), RelB, and c-Rel] is known to mediate responses to stress and injury (Caamano & Hunter, 2002). Under normal conditions these transcription factors are bound to inhibitory IκB proteins in the cytoplasm. In response to various stressor stimuli they dissociate from their inhibitors, enter the nucleus and regulate transcription of a variety of genes. Zhu et al. (1996) found evidence of increased transcription of the IL-6 gene upon RV infection which was blocked 90-95% by mutation of the NFκB binding site in the IL-6 promoter region. Furthermore, they demonstrated that RV infection selectively induced NFκB binding in lung cells mediated by p65 and p50.
Similar evidence was also found for IL-8 (Zhu et al., 1997). Other studies have found that the IL-8 gene contains binding sites for several transcription factors including: activating protein-1, activating protein-2, hepatic nuclear factor-1, interferon regulatory factor-1, glucocorticoid response element, NF-IL-6 and NFκB (Oliveira et al., 1994). Clearly cytokine and chemokine transcription is more complicated than simple NFκB binding as inflammatory mediator transcription is affected by various other promoter regions and associated transcription factors are known to interact in complex ways. For example, Sharma et al. (2006) showed increased nuclear content of more than 30 transcription factors, including NFκB, upon RV infection of airway epithelial cells.

Ultraviolet (UV) Inactivation of Rhinovirus

The specific RV trigger for inciting the host inflammatory response is unknown. Traditionally it was thought that virus replication levels were responsible for illness since such a pathophysiology is characteristic of many other viral infections (e.g. human immunodeficiency virus) where systemic viral titers are proportional to disease severity (Clark & Shaw, 1993). However, considering the low levels of RV replication in the airway tissues it is reasonable to suggest that this is not the case for RV. Furthermore, it is unknown whether viral entry or replication is even necessary to induce RV symptoms and illness, or if a mere interaction of RV with its cellular receptor or some other host recognition site may be sufficient to provoke an inflammatory cascade. One method of investigating this potential trigger is by using UV inactivated RV for infection of tissues or cells. Many DNA and RNA viruses, including RV, can be inactivated by UV irradiation at a wavelength of 260nm (UVC) (Hughes et al., 1979). The first site of RV inactivation is the viral nucleic acid. Hughes et al. (1979) found that this site may be destroyed in less than 10 seconds in dilute RV17 and RV40 preparations, and up to 90 seconds for more concentrated stocks. However, RV antigenic specificities were retained for much longer; 7 minutes of UVC exposure resulted in a less than ten-fold decrease in the ability to evoke neutralizing antibodies in guinea pigs, and RVs exposed to UVC for over 13 minutes (the maximum time experimentally considered) were still capable of inducing antibodies. Thus theoretically, if RV is exposed to UVC under optimal conditions it should be possible to create genetically damaged but intact capsids. If this inactivated virus is capable of
inducing an inflammatory response in cells, then the existence of a non-replicative trigger for RV illness can be substantiated. Some evidence of such a phenomenon exists; Johnston et al. (1998) found that 30 minute UV irradiation of RV9 completely halted viral replication in A549 cells, but only reduced IL-8 secretion and mRNA content by about half. However, Griego et al. (2000) observed that BEAS-2B cells challenged with UV inactivated RV39 (20 minute irradiation) only produced slightly elevated levels of IL-6 and IL-8 relative to control. Hence, identifying and defining the trigger for RV-associated illness is a crucial component of understanding RV infection and paramount in the development of appropriate therapeutics.

**Upper Versus Lower Respiratory Tract Infections**

RV infections have traditionally been thought of as upper respiratory tract infections. However, the discovery that RVs may be the cause of exacerbations of pulmonary diseases such as asthma and COPD, which largely involve the lower airways, has lead researchers to the likelihood of lower airway RV infection.

Although lower airway cells have been shown to possess RV receptors, such as ICAM-1, it is not clear whether their receptors are as vulnerable to RV infection, or whether RV virions would routinely reach the receptors residing deep in the airways (Papi, 2002). The greatest debate in terms of lower airway RV infection has been on the subject of temperature. It has been widely accepted that RV replicates and infects optimally at upper airway temperatures, which are typically from 33°C to 35°C (Hayden, 2004). As such, it has been assumed that RV infections were typically confined to these upper regions and not the lower airways which experienced temperatures closer to the core body temperature of 37°C.

However, evidence has suggested that RV viral replication can and does occur in the lower respiratory tracts, and that perhaps temperature is not as limiting as once thought (Papadopolous et al., 1999 and 2000). As previously mentioned, the upper respiratory temperature has traditionally been experimentally set at 33-35°C, while the lower airway temperature is experimentally set at 37°C. Airstream monitoring of airway temperatures by thermistors from the trachea to sub-segmental bronchi showed inspiratory quiet breathing temperatures of 33.2°C in the upper airways to 35.5°C in the lower passages, while
expiratory temperatures were 32.9°C and 36.3°C respectively (McFadden et al., 1985). Furthermore, when test subjects breathed very cold air (-18.6°C) temperatures in the airways declined by 3-4°C, and during increased ventilation at normal ambient temperature lower airway temperatures were very similar to those of the upper airways (McFadden et al., 1985). Thus, due to the constant fluctuation in airway temperature in response to ventilation and ambient temperature, the designation of an upper and lower airway temperature is perhaps quite arbitrary.

Furthermore, it has been demonstrated that RV can replicate efficiently at both 33°C and 37°C. Papadopoulos et al. (1999) demonstrated in HeLa cells that of 8 wild-type RVs, 4 replicated just as well at 37°C, and in fact one serotype replicated more efficiently at 37°C.

Recently evidence, both in vivo and in vitro, has emerged showing the replication of RV in the lower airways. Mosser et al. (2004) experimentally infected volunteers with RV and obtained samples from the upper airways (nasal lavage), sputum, and from bronchoalveolar lavage. They detected RV in all of the upper respiratory tract samples, all sputum samples, and 5 of 19 bronchoalveolar samples 4 days post-infection. Immunohistochemistry of the lower airway samples showed patches of infected cells, which were similar to the infection pattern seen in the upper respiratory tract. It is possible that the lower incidence of RV infection in the bronchoalveolar patches was more the result of RV inocula not reaching the lower airways than the vulnerability of the cells themselves. In vitro, alveolar A549 cells inoculated with RV at 37°C and incubated at 35°C produced significant increases in inflammatory cytokines compared to control, suggesting the successful viral infection of lower airway cells (Zhu et al., 1996).

The inflammatory response, and subsequent release of cytokines, is temperature sensitive. For example, increases in cytokines can be triggered in response to both hypothermia and hyperthermia. Fairchild et al. (2004) showed that monocytic THP-1 cells increase secretion of IL-1 in response to moderate hypothermia (32°C). Bouchama et al. (2005) demonstrated increased plasma IL-6 concentrations after moderate heatstroke temperatures of 42.5°C.

Clearly inflammatory cytokine and chemokine secretion from RV infected airway epithelial cells plays a dominant role in the presentation of cold symptoms. Thus
comprehending the patterns of inflammatory mediator secretion and the mechanisms by which they are secreted are crucial in understanding the pathology of the common cold.

**Rhinovirus Drugs**

The effective development of preventatives and treatments for RV infection has proved itself a formidable task. Vaccines are generally regarded as impractical because over 100 serotypes of the virus persist with limited cross-neutralization capabilities amongst them. Many different approaches to RV therapy have been investigated, all with limited benefits. From a preventative perspective, a soluble competitive ICAM-1 receptor spray, Tremacamra, has been formulated (Turner et al., 1999), and shows some promise in warding off RV infection, but only when administered just prior to or during inoculation with a major group RV serotype. Specific picornavirus anti-viral agents, which typically disrupt viral replication or attachment to cell membranes, have recently entered clinical trials. Pleconaril is a capsid-binding agent which may interfere with viral entry across the cell membrane. It has been shown to delay viral shedding in enterovirus infection, and to inhibit cell attachment during RV infection in cultured cells (Zhang et al., 2004). The United States Institutes of Health are currently recruiting volunteers for phase II clinical trials evaluating Pleconaril as a treatment for natural colds and asthma exacerbations (United States Food and Drug Administration, [http://www.clinicaltrials.gov/ct/gui/show/NCT00394914]), and Pleconaril is the first anti-RV drug to be evaluated by the United States Food and Drug Administration. Another anti-RV drug, Ruprintrivir (formerly AG7088), is a 3C protease enzyme inhibitor which prevents the cleavage of a newly synthesized RV polyprotein into its functional subunits. Its antiviral effects have been demonstrated *in vitro* by Zalman *et al.* (2000). In clinical trials with experimental RV infection, Ruprintrivir intranasal prophylaxis decreased: proportion of subjects with positive cultures, viral titers, and severity of illness, but had no effect on frequency of colds (Hayden *et al.*, 2003). Bearing in mind the mounting evidence that RV associated illness has little to do with viral replication and is largely associated to host secretion of inflammatory mediators, the development of anti-virals may be futile.

More recently, researchers have become interested in immune-modulating compounds which may be able to mitigate RV-related symptoms through the host response.
Investigation into the use of anti-histamines and corticosteroids has resulted in inconclusive results so far (Doull et al., 1997; Gaffey et al., 1988; Muether & Gwaltney, 2001). These compounds have shown the ability to mitigate secretion of inflammatory mediators (e.g. IL-8) in some cases, but far more research must be conducted. A limited number of chemokine receptor antagonists have been developed but they have not yet been evaluated in an RV infection context (Akahori et al., 2006; Purandare et al., 2006; Tsutsumi et al., 2006).

Two natural treatments associated with RV infection include the use of zinc and the natural herb Echinacea. Zinc has been shown to have an anti-viral effect by inhibiting protease 3C \textit{in vitro}, but clinical trials have produced inconclusive results (Eby et al., 1984; Macknin et al., 1998; Mossad et al., 1996; Turner & Cetnarowski, 2000). Echinacea is a popular natural herb extract (discussed in detail below) with demonstrated immune-modulating activities which may potentially mitigate RV-related illness by directly affecting host inflammatory mediator secretion.

**Echinacea**

Recently in Western culture there has been marked increase in interest in natural and alternative medicines. This interest may in some cases be due to the perceived shortcomings of traditional Western medicine, or perhaps as explorations into complementary therapies. In either case, it seems that these various therapies are becoming more deeply embedded into our culture. For example, most American medical schools, including Harvard and Johns Hopkins, now offer courses in alternative medicine (Harvard Medical School, [http://www.hms.harvard.edu/news/releases/0700compmed.html]; Johns Hopkins School of Medicine, [http://www.hopkinsmedicine.org/CAM/]). According to a survey published in the Journal of the American Medical Association there was a 47% increase per household of visits to alternative practitioners from 1990-1997 (Eisenberg et al., 1998). This new interest has resulted in a boom in the alternative medicine industry which was estimated at 21.2 billion dollars in 1997 (Eisenberg et al., 1998).

One facet of this boom has been a surge in the use of natural medicines such as herbs and dietary supplements. An herbal extract that has gained widespread popularity for the potential treatment and/or prevention of upper respiratory tract infections is Echinacea.
Echinacea sales in the United States are estimated at 300 million dollars annually (Barrett, 2003; Brevoort, 1998).

Echinacea is a natural extract derived from one or a combination from 3 species from the genus Echinacea: *Echinacea purpurea* (common name: purple coneflower), *Echinacea angustifolia*, and *Echinacea pallida*. Echinacea plants are perennial prairie wildflowers native to North America and were first used by Native Americans to treat a variety of infections and illnesses (Barrett, 2003). Extracts can be made from various combinations of species and plant parts (including roots, leaves, petals and seeds). Extractions are performed with diverse solvents, most commonly ethanol or water. Echinacea supplements are sold in various forms such as: capsules, pills, tinctures, lozenges, and teas which are widely available in supermarkets and pharmacies.

Recently, there has been much controversy surrounding Echinacea and its potential health benefits, with numerous contradictory studies being published (Goel et al., 2004; Sperber et al., 2004; Turner et al., 2005; Turner et al., 2000). The challenge that Echinacea faces seems to be two-fold. Firstly, the lack of regulation for this industry puts into question the quality of the extracts available on the market. For example, when Gilroy et al. (2003) examined 59 commercial Echinacea preparations they found huge variations in extract quality and labeling. The daily recommended therapeutic dose by the German Commission E, where Echinacea is approved for the treatment of upper-respiratory tract infections, is 900mg/day. Commercial extracts ranged in recommended doses from 45-1600mg between brands. The quality of the Echinacea extracts itself was also hugely variable. 10% of the extracts had no detectable levels of Echinacea at all, 52% were consistent with their label, while 39% either contained more or less Echinacea than indicated. Also 20% of the products did not have any expiration dates, and there is evidence that many of the active compounds undergo enzymatic degradation, especially in alcoholic extracts (Wolkart, 2004). Hence, improper quality control, dosage, and labeling are key problems in the elucidation of Echinacea’s health benefits.

The second challenge that Echinacea faces is the demonstration of its health benefits *in vitro* and especially *in vivo* using properly standardized and characterized extracts. As mentioned previously, Echinacea extracts are used as preventions and/or treatments for upper-respiratory tract infections. Most cold cases are caused by infection by the RVs and
so the Echinacea-RV model is the most commonly studied (Goel et al., 2005; Koenig & Roehr, 2006; Sharma et al., 2006; Turner et al., 2000).

The mechanism of Echinacea’s effects on RV infected cells is largely unknown. It is thought that the extract may mediate the immune response in such a way that prevents or mitigates RV-associated symptoms. Confusing claims are often made about Echinacea, referring to it as “immune-stimulatory”, “immune-supportive”, or “immune-modulatory”. None of these claims are necessarily untrue; however, one must keep in mind that the immune response is a complex network with no clear “up” or “down”. Echinacea is also sometimes called an “antiviral”; this claim is misleading because although some virucidal effects have been demonstrated in viruses such as herpes simplex virus-1 (Binns et al., 2002), there is no evidence that Echinacea affects viral attachment or replication in any way. Regardless, any potential anti-viral activity may be completely irrelevant, considering the low levels of RV replication during airway infection.

Many biologically active compounds have been identified in Echinacea extracts. The major compounds are grouped as: polysaccharides, alkamides and caffeic acid derivatives. Such compounds are used as markers for standardization which typically include: cichoric acid, 6-O-caffeoylechinacoside, echinacoside, verbascoside, cynarine and chlorogenic acid and 6 defined alkylamides (Sloley et al., 2001). Preparations from different plant species and plant parts often have different profiles of these constituents. For example, *Echinacea purpurea* root extracts are typically rich in alkamides while *Echinacea pallida* aerial parts are richest in cichoric acid (Binns et al., 2002). Further research is needed to investigate the specific effects of the above compounds both individually and in combination (as found in natural crude extracts), as evidence exists that constituents may act in a synergistic fashion (Dalby-Brown et al., 2005).

The actions of these compounds have been demonstrated *in vitro*. Absent of virus, Echinacea extracts tend to stimulate and activate cells such as macrophages (Burger et al., 1997), monocytes, natural killer cells (Gan et al., 2003), and to cause immune and airway epithelial cells to secrete a variety of cytokines and chemokines like IL-6, IL-8, and TNF-alpha (Hwang et al., 2004).

In combination with RV infection these effects may be more complex. For example, Sharma *et al.* (2006) showed increased cytokine and chemokine levels with Echinacea
stimulation, but decreased levels of mediator release in RV infected samples treated with Echinacea than those infected with RV alone. This pattern held true for over 20 of the cytokines and chemokines tested (including IL-6 and IL-8). Although the specific mechanism of Echinacea is still unknown, it has been suggested that Echinacea extracts may affect the cell secretion of cytokines and chemokines through a modulation of the NFκB pathway (Sharma et al., 2006). However, considering the diverse chemical profiles of Echinacea extracts and the unique trends observed when Echinacea interacts with RV infected cells it seems unlikely that one simple mechanism exists. Sharma et al. (2006) showed that Echinacea alone increased the nuclear content of over 30 transcription factors (including NFκB) in airway epithelial cells and in RV infected cells treated with Echinacea those transcription factors were significantly down-regulated nearing control levels. Other research has found that the alkylamides in Echinacea bind the cannabinoid type 2 receptor, and that a subsequent up-regulation TNF-alpha is mediated through cyclic adenosine monophosphate, p38/mitogen activated protein kinase and JNK signaling, as well as NFκB and activating transcription factor-2/cAMP responsive element binding protein-1 activation (Gertsch et al., 2004; Raduner et al., 2006; Woelkart et al., 2005).

How the effects of Echinacea may translate into improved health remains unclear. It has been postulated that the stimulatory effects may enhance a depressed immune system bringing it back to balance (cited in Barrett, 2003). But it seems more likely that an association between RV and Echinacea down-regulates the inflammatory mediators resulting in fewer symptoms of infection.

In vivo, Echinacea extracts have been found to increase white blood cells in mice, and Echinacea treatment resulted in faster recovery of normal cell counts following radiation therapy (Mishima et al., 2004). Furthermore another study found that 74% of mice fed Echinacea from birth survived to 13 months of age, while only 46% of control mice remained alive at 13 months (Brousseau & Miller, 2005). Echinacea administration to aging mice has resulted in the synthesis of natural killer cells de novo in the bone marrow (Currier & Miller, 2000). In humans, administration of Echinacea has also been shown to: elevate white blood cell counts, activate immune cells, increase heat-shock protein expression, and prevent free radical damage of red blood cells (Agnew et al., 2005; Brush et al., 2006).
Clinical studies investigating the potential benefits of Echinacea extracts in treating and preventing RV infections have shown indeterminate results. Some clinical trials have found no health benefits in Echinacea treatment of RV infection (Koenig & Roehr, 2006; Turner et al., 2000), while others have found significant positive effects (Goel et al., 2005). The contradictory findings may be due to many of the issues discussed above (including extract quality and dosage), and also affected by timing of extract administration. It is unclear whether Echinacea is most effective when administered prior to, during, or after RV challenge, and time of administration varies from study to study. Also, if Echinacea does not act on the virus itself, then measuring viral titers as a means of assessing effectiveness, as if often done, may be completely inappropriate. Finally, the use of patient symptom scoring which is entirely subjective may lead to inconclusive results, especially in smaller studies.

In vivo, researchers must also consider whether the biologically active compounds in Echinacea reach their target tissues. Very few studies have been conducted to assess the bioavailability of consumed Echinacea extracts. Matthias et al. (2004) found evidence that alkylamides cross gut cell monolayers quite readily; however, the caffeic acid derivatives in Echinacea diffused poorly across cultured gut cells. Other studies found evidence of bioactive alkylamides in the blood of test subjects after both consuming Echinacea tablets and after the oral administration of 60% ethanolic tinctures (Dietz et al., 2001; Woelkart et al., 2005). However, Matthias et al. (2005) found no evidence of caffeic acid conjugates in any blood samples following Echinacea tablet ingestion. There is also evidence that alkylamides are oxidized by cytochrome P450 enzymes in the liver generating novel metabolites which may have divergent effects (Cech, 2006). On the other hand, the blood bioavailability of Echinacea may not be relevant considering this extract may be most beneficial by direct application to affected airway epithelial tissues, such as in the case of tinctures, sprays, and teas. Consequently, further studies should investigate Echinacea's bioavailability, as well as research the most effective mode of administration for this extract.
RATIONALE AND OBJECTIVES FOR THESIS

Although RVs have been studied for many years, relatively little is known about their mechanism of infection and the central role of the host immune response is still a relatively novel discovery. Furthermore, RV treatments and preventions remain in their primitive stages and no specific RV chemotherapy exists. The mounting evidence suggesting low levels of viral replication and induction of the host immune response requires further support and investigation, as many questions have not yet been fully explained. For example, the patterns of viral replication and cell inflammatory mediator secretion over the course of infection have not been adequately documented. Furthermore, such studies have not compared the effects of RV infection in different airway epithelial cell models, or potential differences in infection between RV serotypes.

It is also unknown what the specific viral trigger for RV illness may be. Considering the low levels of viral replication it is feasible that some other viral trigger (other than RV replication) may be responsible for stimulating some or all of the host inflammatory response. For example, if the inflammatory response can be stimulated by a mere virus-receptor or virus-cell interaction, then it is possible that viral entry and replication are not even necessary to provoke illness. The use of UV irradiated non-replicative RV has generated limited data to support this idea; however, more studies are necessary (Johnston et al., 1998).

Finally, the lack of therapy to treat RV infection has lead scientists to explore many medicinal avenues. A growing enthusiasm for natural therapies has sparked interest in the potential benefits of using Echinacea to prevent and/or treat RV infections. Immune-modulating Echinacea components have been identified; however, the effects of such extracts directly on the host airway epithelial cells have only been investigated by our laboratory. The combined effects of Echinacea in RV infected airway epithelial cells published by our research group (Sharma et al., 2006) suggest a more complex interaction between Echinacea and RV infection which must be further investigated.
General Objectives:

Chapter 2 (In Vitro Characterization of Rhinovirus Infection in Airway Epithelial Cells - Growth Curves): To characterize RV infection in vitro in terms of viral replication, viral RNA, and cell secretion of pro-inflammatory cytokines/chemokines over the typical course of infection using two different receptor-utilizing RV serotypes in two distinct cultured human airway epithelial cell models.

Although in vitro models for RV infection may not mimic in vivo results, they are critical to our understanding of RV infection because they offer highly controlled and standardized conditions under which RV infection may be investigated, with fewer variables than in in vivo models. Accordingly, more subtle and mechanistic differences may be discernable. From a logistic point of view, in vitro experiments are more approachable, less dangerous, and less costly than their in vivo counterparts, and thus play a critical role in viral research.

I chose to study RV infection in two different immortalized human airway epithelial cell models: a bronchial epithelial cell model (BEAS-2B), and a type II alveolar cell model (A549). I selected these models because they are well-characterized and widely used in similar RV experiments. Although both cell lines are derived from the mid to lower region of the airways the BEAS-2B cells represent cells higher in the respiratory tract, while the alveolar cells are the lowest cells of the respiratory epithelium. In the above cell models I investigated the effects of two different receptor-utilizing RV serotypes: RV14 (major group) and RV1A (minor group) in order to observe the extent of differences possible between RV serotypes. In order to address my objectives and characterize RV infection, I chose to measure viral replication, viral RNA levels (for RV14), and cell IL-6 and IL-8 secretion at daily time intervals over the course of a typical infection (about 7 days), and termed these experiments “Growth Curves”.

Viral replication was assessed by plaque assay, which detects the amount of infectious (fully formed replicating units) virus present in a given sample, and thus increases in values from controls indicate viral replication. To assess whether airway epithelial cells allowed only low levels of RV replication, viral titers were compared to those obtained in identical
experiments in the permissive H1 epithelial cell line. H1 cells are considered permissive because they allow for the highest RV titers of any known cells following RV infection (Arruda et al., 1996).

Viral RNA levels were assessed in both cell models for RV14 only, as the entire RV1A genome sequence is not fully sequenced and primer design is problematic. Viral RNA levels are indicative of the number of viral genomes present in the sample, regardless of whether those genomes are present in fully infectious and replicating units. Consequently, increases in viral RNA may not mirror plaque assay results as they represent efficiency in production of viral RNA; functional or not. RNA was detected using quantitative real-time polymerase chain reaction (qRT-PCR) which has been shown to be 10 times more sensitive to detect RV than conventional PCR (Dagher et al., 2004).

Finally inflammatory mediator secretion was measured using enzyme-linked immunosorbent assays (ELISAs) for specific cytokines and/or chemokines. In the BEAS-2B model, IL-6 secretion was determined, while in A549 cells IL-8 secretion was measured based on previous laboratory data (unpublished) which indicated pronounced secretion of those specific proteins in the respective cell models. An increase in inflammatory mediator secretion was used as an indicator for the stimulation of the host inflammatory response as is routine practice for such studies.

**Hypotheses:**

1) RV infection of airway epithelial cells will result in RV replication and stimulation of the host inflammatory response as represented by pro-inflammatory cytokine/chemokine secretion from cells.
2) RV infected airway epithelial cells will not exhibit CPE because RV infection results in little or no cell death or cytotoxicity.
3) RV replication and RNA levels in airway epithelial cells will be relatively low as compared to permissive H1 cells (by at least one order of magnitude).
4) Viral replication, viral RNA, and cell pro-inflammatory IL-6/IL-8 secretions will increase post RV infection and gradually decline to control levels by day 7 as the infection is resolved.
5) Maximum RV replication and RNA levels will occur earlier during the time-course of infection than peak cytokine/chemokine secretion inflammatory mediator secretion, and cytokine/chemokine levels will remain elevated longer than RV replication and RV RNA.

6) RV14 and RV1A serotypes will produce similar infection patterns because they belong to the same virus family.

7) Alveolar A549 cells will be less susceptible to RV infection than bronchial BEAS-2B cells because they are derived from lower regions of the airways.

Chapter 3 (The Effects Ultraviolet Inactivated Rhinovirus on Airway Epithelial Cells): To investigate the potential for a non-replicative viral trigger for initiating the cellular inflammatory response by exposing bronchial epithelial (BEAS-2B) cells to partially and fully UV inactivated virus and quantifying subsequent viral replication, viral RNA and inflammatory mediator secretion.

These experiments were conducted in BEAS-2B cells for both RV14 and RV1A. RV stocks were UV-irradiated for different lengths of time and those samples used during infection procedures in order to observe the effects of UV treated RV on replication, viral RNA (RV14 only), and IL-6 secretion in order to assess whether RV with damaged genetic material could elicit an inflammatory response.

Hypotheses:

1) The viral trigger for the observed host inflammatory response is not related to viral replication, therefore RVs irradiated with UVC sufficiently to damage genomic RNA material but not protein structure, will elicit an IL-6 response when used to infect BEAS-2B cells.

2) A similar IL-6 response from BEAS-2B cells will occur for both RV14 and RV1A because they belong to the same virus family.

Chapter 4 (The Effects of Echinacea Extracts on Rhinovirus Infected and Uninfected Airway Epithelial Cells): To assess the effects of 2 chemically distinct Echinacea extracts
on RV infected and uninfected BEAS-2B cells, in terms of viral replication and pro-inflammatory IL-6 secretion, for RV14 and RV1A.

Experiments were conducted in the BEAS-2B cell model with both RV serotypes. Cells were infected with either RV14 or RV1A and then treated with one of two chemically distinct Echinacea extracts (E1 or E2) immediately post-infection. E1 was an aqueous Echinacea extract and E2 an alcoholic tincture. Both extracts’ chemical profiles were previously determined by high performance liquid chromatography (Binns et al., 2002). Viral replication was assessed at specific time intervals for RV infected samples, and IL-6 secretion was measured in RV infected and uninfected cells treated with the Echinacea extracts.

**Hypotheses:**

1) Treatment of RV-infected BEAS-2B cells with Echinacea will not affect viral replication because Echinacea has no effect on the RV replication cycle.
2) Treatment of uninfected BEAS-2B cells with Echinacea will invoke an inflammatory response and result in increased secretion of IL-6.
3) Treatment of RV-infected BEAS-2B cells with Echinacea will inhibit RV induced IL-6 secretion because of complex interactions between Echinacea and the virus infected cells.
4) E1 and E2 will affect IL-6 secretion differently because they are derived from distinct extract preparations and have different active constituent profiles.
5) Echinacea extracts will have the same effects on RV14 and RV1A because the viruses belong to the same family.

My research offers controlled and standardized experiments which will help to elucidate the effects of RV infection in human airway epithelial cells and to investigate the immune-modulatory effects of Echinacea extracts. Understanding the steps and mechanisms of RV and the roles that natural medicines could play in preventing and/or alleviating infections and symptoms may help to diminish their impact on both our health and the economy.
REFERENCES


Chapter 2: *In Vitro* Characterization of Rhinovirus Infection in Airway Epithelial Cells (Growth Curves)

**BACKGROUND**

Rhinoviruses (RVs) are the leading cause of upper respiratory tract infections in humans (Arruda et al., 1997). For people already affected by other respiratory diseases, such as asthma or chronic obstructive pulmonary disorder (COPD), RV infection can lead to dangerous exacerbations of those conditions (Bardin, 1992; Fraenkel et al., 1995; Gern & Busse, 1999; Grunberg & Sterk, 1999; Greenberg, 2002; Halperin, 1985; Johnston, 1993; Johnston et al., 1995; Johnston, 2005; Message, 2001; Seemungal, 2000; Seemungal, 2001). Furthermore, in the case of asthma RV infections have recently been attributed a causative role in the pathology of the disease (Singh et al., 2006). As of yet, no prevention or cure for RV infection exists and medications only act to alleviate symptoms.

The specific mechanism of RV infection is still largely unknown. RVs generally infect the upper airway epithelia by direct contact with infected individuals, contaminated surfaces, or large particle aerosols (Turner, 2001). However, there is growing evidence that the lower airways are also susceptible to RV infection (Fraenkel et al., 1995; Gern et al., 1997; Gern et al., 2000; Hayden, 2004; Mosser et al., 2002; Mosser et al., 2005; Nicholson et al., 1996; Papadopoulos et al., 2000; Schroth et al., 1999).

Traditionally, it was also thought that the symptoms caused by RV infection: runny nose, cough, sneezing, were directly correlated to the levels of viral replication in the airway tissues. However, recent evidence shows that RV replication occurs at relatively low levels and may not be attributable to the extent of these symptoms (Lopez-Souza et al., 2004). For example, Mosser et al. (2002) showed that only 5-10% of primary airway epithelial cells were susceptible to RV no matter what the infectious dose. Thus, it has been proposed that it is the host immune response to RV that causes the wide-spread symptoms of illness. For example, mounting evidence suggests that RV infection does not manifest itself as a widespread mucosal infection but as small localized foci of infected cells from which widespread inflammatory responses are generated (Arruda et al., 1995; Mosser et al.,

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1 A version of this chapter will be submitted for publication. Machala, A.M., Harris, R.A., Brauner, C.J., Hudson, J.B.
It is this inflammatory response that then causes the cascade of cold symptoms.

At the cellular level, the inflammatory response can be measured by cytokine and chemokine secretion. Cytokines are proteins that are secreted by cells which in turn cause their neighbours to secrete cytokines, thus modulating and attracting various immune cells or mediators (Janeway, 2005). Chemokines are secreted proteins which attract other cells, such as neutrophils and monocytes, from the bloodstream to the site of infection (Janeway, 2005). RV infection of humans can trigger pro-inflammatory cytokine and chemokine secretion from a variety of cells (Gern et al., 2000; Grunberg et al., 1997; Johnston, 1997; Message & Johnston, 2004; Kim et al., 2000; Zhu et al., 1997; Zhu et al., 1996; Subauste et al., 1995; Teran et al., 1997; Turner, 1998). More specifically, airway epithelial cells have demonstrated the ability to secrete various cytokines and chemokines in response to viral infection (Arnold, 1994; Griego et al., 2000; Johnston et al., 1998; Konno et al., 2002; Lopez-Souza et al., 2004; Zhu et al., 1996).

This study aimed to elucidate the biology of RV infection in vitro in cultured human respiratory epithelial cells by relating: cytopathic effects (CPE), viral replication, secretion of pro-inflammatory cytokines/chemokines, and viral RNA over the course of infection for two separate RV serotypes, RV14 (major group) and RV1A (minor group), using two distinct airway epithelial cell lines. These experiments have been termed “Growth Curves” as they follow the effects of RV infection for the duration of a typical infection and address the hypothesis that RV infection is characterized by low levels of viral replication and a pronounced inflammatory response.

MATERIALS AND METHODS

All viral, cell culture and molecular work was conducted under sterile conditions in a type II biosafety cabinet. All protocols were pre-approved by the UBC biosafety committee in certificate H04-0061 (Appendix A).

Growth Curve Experiments (see Appendix B for experimental design): Growth Curves were conducted for the permissive epithelial H1 cell lines, and for the airway epithelial models using bronchial BEAS-2B, and alveolar A549 cell lines (described below), and two
different receptor-utilizing RV serotypes: RV14 a major group intercellular adhesion molecule-1 (ICAM-1) utilizing RV, and RV1A a minor group low density lipoprotein receptor (LDLR) utilizing RV. Cells were infected with RV14 or RV1A at a multiplicity of infection (MOI) of 1, corresponding to 1 infectious viral particle per cell, and parameters such as CPE, viral replication, interleukin (IL)-6/IL-8 secretion, and RV14 RNA were measured over the course of infection, typically between days 0-7 (D0-D7) after RV inoculation. Two separate Growth Curve trials were conducted for each cell type and each RV serotype, and additionally a tandem A549/BEAS-2B Growth Curve was carried out by simultaneously culturing, infecting, and sampling those cell lines, in order to allow for a better direct comparison.

H1 cell Growth Curves (Figure 2.1) were conducted to serve as positive controls for the airway epithelial models. H1 cells are epithelial cells known to be permissive to RV infection allowing for high levels of viral replication. Because RV infection of H1 cells causes cell death and lysis (and therefore secreted cytokine/chemokine cannot be distinguished from lysed cell mediator release) cytokine/chemokine levels were not assayed for H1 cells. Growth Curve samples were collected only up to D4 because complete cell death had occurred by this time.

For BEAS-2B cells (Figures 2.2 and 2.3): CPE, RV14 viral replication, viral RNA, and IL-6 secretion were assessed for infections with both RV serotypes. IL-6 was assayed as an indicatory inflammatory mediator in the BEAS-2B model because previous laboratory data (not shown) indicated a good response for IL-6 secretion from RV infected BEAS-2B cells.

For the A549 cell model (Figures 2.4 and 2.5): CPE, viral replication, viral RNA, and IL-8 secretion were assessed for both RV serotypes. Here, IL-8 was chosen as an inflammatory indicator based on previous data (not shown) which suggested a pronounced IL-8 response in A549 cells compared to a weak IL-6 response.

Finally, for the tandem A549/BEAS-2B (Figures 2.7-2.9) the same parameters were assessed, including both IL-6 and IL-8 secretion and RV14 RNA.

**Cell Culture:** The SV40 adenovirus transformed human bronchial epithelial cell line (BEAS-2B) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and cultured in 75mm² flasks in 50:50 Dulbecco’s Modified Eagle’s Medium (DMEM) and Ham’s F12 with 10% endotoxin free fetal bovine serum (FBS). Culture
reagents were obtained from Invitrogen (Vancouver, Canada). Cells were passaged weekly and incubated at 35-37°C with 5% carbon dioxide in 95% air. A549 cells, a transformed alveolar epithelial cell line, were also cultured under similar conditions but in DMEM and 5% FBS. Finally, RV-sensitive H1 cells (from ATCC) were cultured under the same conditions with DMEM and 5% FBS.

**Viruses:** Both RV14 and RV1A were obtained from the ATCC. RVs were propagated by infecting H1 cells grown to confluence in 75mm² flasks containing DMEM, allowing for full CPE. Cell-free culture fluid was harvested when CPE were at a maximum by centrifugation at 10,000 x g for 20 minutes at 4°C. This centrifugation clarified the virus suspension from bacteria and cell debris. The stock virus suspension was aliquoted into cryovials and stored at -80°C for experimental use. Titer of viral stock was determined by viral plaque assay (see below) of serially diluted stock, and expressed in plaque forming units (pfu) per mL. Pfu represent the number of infectious RV particles present in a known volume of sample. For experiments, aliquoted stock virus was rapidly thawed at 37°C and vortexed prior to use. Because such clarified viral stocks contain H1 cell remnants (e.g. soluble proteins, organelles) control experiments were conducted to confirm that any observed changes in IL-6 and IL-8 secretion were due to virus, and not some other cellular component present in the inocula (Appendix C).

**Viral Infections and Growth Curves:** H1, BEAS-2B, or A549 cells were grown until freshly confluent under standardized conditions in sterile 6-well plates. Once cells reached confluence it was assumed that cell number did not change significantly over the course of experiments for either control of RV infected cultures. This assumption was confirmed experimentally (Appendix D). Cell number per well (6-well plate) at confluence was predetermined for cell lines (Appendix E) and used to calculate viral dose. During infection, cells were inoculated with RV14 or RV1A at an MOI=1, or mock infected with medium, and incubated at 35°C for 1 hour. After infection, cells were washed 3 times with 1mL of medium to remove any exogenous virus, and incubated at 35°C in 3mL of fresh medium and 1% FBS. Washes were assayed to confirm virus removal (not shown). Samples were collected at the same time daily between D0 (immediately post-infection and washes) and
D7. Sampling consisted of first removing 1mL of supernatant from appropriate wells, centrifuging at 1000 x g to remove cellular debris, and freezing samples at -20°C for future cytokine/chemokine assays. Cells from RV infected samples were scraped into the remainder of the medium (2mL), pipetted into cryovials, and stored at -80°C for plaque assays. Finally, for quantitative real-time polymerase chain reaction (qRT-PCR) designated samples, medium was removed, cell monolayers washed twice with sterile phosphate buffered saline, and 1mL of TRIZOL reagent was added to each well, allowing for cell lysis to occur. This suspension was stored in Eppendorf tubes at -80°C for RNA extraction. Control experiments were conducted in order to assess the stability of the RVs and IL-6/IL-8 under experimental conditions (Appendices F and G).

**Plaque Assays** (See Appendix H for image): Viral infectivity and replication were measured by plaque assays in permissive H1 cells. Previously frozen RV infected cell samples from Growth Curves were rapidly frozen and thawed twice at 37°C to rupture cells and release virus. These samples were then serially diluted and used to infect permissive H1 cells in duplicate where 0.75mL of sample was added onto freshly confluent H1 cells grown in 6-well trays, and allowed incubated for 1 hour at 35°C. After infection, the inocula were aspirated and replaced with a 50:50 liquid mixture of 2x MEM (with 5% FBS) and 1% sterile agarose in dH2O. The agarose was allowed to solidify at room temperature, and plates were incubated at 35°C for 4 days. As the virus replicated in infected cells, lysis occurred in the H1 cells forming round areas of cell death called “plaques”. Once incubation was complete plates were fixed with 3% formaldehyde in phosphate buffered saline, agarose was removed, and H1 cells were stained with 1% crystal violet in dH2O to reveal the clear unstained plaques. Plaques were counted and reported as pfu/mL (infectious virions/mL). An increase in infectious virus over time (relative to D0) indicated viral replication.

**Cytokines and Chemokines:** IL-6 and IL-8 were assayed in duplicate from thawed Growth Curve supernatant samples according to standard protocols provided by enzyme-linked immunosorbent assay (ELISA) commercial kits from Immunotools (Friesoythe, Germany). Absorbencies were determined by an ELISA plate reader (Pasteur Diagnostics
LP400) at a 450nm wavelength. Highly concentrated samples were diluted with medium and re-assayed to fall within the standard assay range which was from 0-450 pg/mL for both IL-6 and IL-8. Sensitivity of the ELISAs was 4 pg/mL.

**RNA Extraction:** RNA was extracted following standard TRIZOL reagent protocol and reconstituted in 50μL of RNAse/DNase free water and stored at -80°C for qRT-PCR. RNA for RV14 standard curves was extracted from stock RV14 suspensions using the QIAGEN RNeasy kit (Mississauga, ON).

**qRT-PCR:** qRT-PCR was conducted for RV14 samples. Primers were designed using the known RV14 genetic sequence from the National Center for Biotechnology Information Database and purchased from Operon (Huntsville, AL). Primers were designed with the following sequences: forward 5’ GACATGGTGTGAAGACTCGC 3’ and reverse 5’ TCTGTGTAGAAACCTGAGCGC 3’ creating a 238 base pair product. Primers were tested by conventional two-step PCR of known RV infected samples, and PCR products confirmed by gel electrophoresis (not shown).

For RV14 RNA samples a one-step qRT-PCR kit from QIAGEN (Mississauga, ON) was used. The PCR mixture contained: 25μL MasterMix (HotStarTaq DNA Polymerase, QuantiTect SYBR Green Buffer, SYBR Green I dye, 1.5mM MgCl2, 200μM each dNTP), 2.5μL of each primer, 0.5 μL RT mix (Omniscript and Sensiscript Reverse Transcriptases), and 10μL of the RNA template to a final volume of 50μL. Standard curve RV concentrations were determined spectrophotometrically and serially diluted at known concentrations. Duplicate reactions were carried out in an MJ Research DNA Engine Opticon Continuous Fluorescence Detector (OP000537) programmed to: incubate at 42°C for 55min, 94°C for 5min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1min, repeat for 35cycles, 72°C for 10min, melting curve from 55°C to 95°C read every 0.5°C, incubate 72°C for 10min, and 4°C forever. Data were analyzed using Opticon Monitor analysis software version 1.07.

**Statistics:** Growth Curve infectious virus (replication) data (n=4 for each day per trial) were subjected to Kruskal-Wallis ranks analysis of variance (ANOVA) with day as a
factor, followed by post hoc multiple comparisons Dunnet’s tests in order to compare daily data relative to D0 controls. Non-parametric testing was employed because data did not satisfy parametric assumptions (normality and/or equal variances) and could not be transformed. A significant increase in infectious virus over time (relative to D0) indicated viral replication. ELISA cytokine/chemokine data (n=3 per day for each treatment) were analyzed using two-way ANOVAs with day and treatment (control uninfected and RV infected cells) as factors and post-hoc multiple comparisons Dunnet’s tests with daily control treatments as controls. In the case of BEAS-2B/A549 tandem Growth Curves, a 2-way ANOVA was performed for infectious virus data using day and treatment as factors followed by a post-hoc Dunnet’s test using D0 as a control. For IL-6 and IL-8 data a 3-way ANOVA was performed with: day, treatment, and cell type as factors and a post-hoc Dunnet’s test with daily control treatments as controls. RNA data (n=2 per cell type for each day) were analyzed by 2-way ANOVA with day and cell type as factors and post-hoc Tukey tests. All data were analyzed using Sigma Stat 3.0 software and statistical significance was set at α=0.05. Significant differences correspond to p<0.05, and highly significant differences correspond to p<0.001. Results are presented as mean ± standard error of the mean.

RESULTS
For all Growth Curve experiments trials were significantly different from each other (data could not be pooled).

H1 Cells (Figure 2.1):
CPE: For H1 cells infected with RV14 CPE began to occur on D1, and manifested themselves as rounded cells which eventually became detached from the substratum. Full CPE and cell death was observed for all samples and usually complete by D4.
H1 cells infected with RV1A also showed CPE resulting in complete cell death by the D3 or D4. Anecdotally, the occurrence of CPE seemed to occur faster with RV1A than RV14.
RV14 Replication (Figure 2.1a): For both trials, significant viral replication (relative to D0) occurred on D1 and D2. For trial 1, maximum infectious virus was observed on D1 (at
1.8 x 10^7 ± 2.8 x 10^6 pfu/mL), and for trial 2 infectious virus also peaked significantly on D1 at 4.4 x 10^6 ± 1.1 x 10^5 pfu/mL.

**RV1A Replication** (Figure 2.1b): Again, for both trials, significant RV replication (relative to D0) was observed on D1 and D2. Peak viral titers were measured on D1 (3.7 x 10^7 ± 2.4 x 10^6 pfu/mL and 1.9 x 10^7 ± 1.2 x 10^6 pfu/mL respectively).

**BEAS-2B Cells** (Figures 2.2 and 2.3):

**CPE:** Upon infection of BEAS-2B cells with RV14 or RV1A, no CPE were observed at any point during Growth Curves.

**RV14 Replication** (Figure 2.2a): For trial 1, no significant replication was observed relative to D0. Peak viral titers occurred on D1 (4.4 x 10^3 ± 1.3 x 10^2 pfu/mL), declining to 1.7 x 10^2 ± 2.9 x 10^1 pfu/mL by D7.

In trial 2, significant viral replication (relative to D0) occurred on D1 at 2.2 x 10^4 ± 1.2 x 10^3 pfu/mL.

**RV14 and IL-6 Secretion** (Figures 2.2b and c): For trial 1 (Figure 2.2b), no significant differences were observed between uninfected (control) and RV14 infected cells at any time post-infection.

For trial 2 (Figure 2.2c), IL-6 secretion in RV14 infected cells was highly significant when compared to uninfected controls on D3, D5, and D7 post-infection. Peak IL-6 concentration was observed on D7 at 3517.9 ± 993.8, and on D3 IL-6 concentration in RV infected cells was nearly 1375 times its control.

**RV1A Replication** (Figure 2.3a): For trial 1, viral replication was significantly different from D0 on D2 and D3. Peak viral titers of 7.3 x 10^4 ± 2.6 x 10^3 pfu/mL were observed on D2.

In trial 2, significant viral replication occurred on D1 relative to D0, which represented the peak RV titer at 2.9 x 10^4 ± 1.4 x 10^3 pfu/mL.

**RV1A and IL-6 secretion** (Figures 2.3b and c): In trial 1, IL-6 concentration (Figure 2.3b) was significantly different between treatments on D3, D5, and D7. Peak IL-6 concentration occurred on D7 at 2434.3 ± 215.3 pg/mL which represented an 8-fold increase over its control.
For trial 2 (Figure 2.3c), there was a significant difference between uninfected and RV1A infected cells on D3, D5, and D7. Peak IL-6 concentrations were measured on D7 at $4224.3 \pm 530.0$ pg/mL, which was 570 times control levels.

**A549 Cells** (Figures 2.4 and 2.5):

**CPE:** No CPE or cell death was observed at any point, D0-D7, for A549 cells infected with RV14 or RV1A.

**RV14 Replication** (Figure 2.4a): For both trials, no significant RV14 replication occurred relative to D0.

**RV14 and IL-8 Secretion** (Figures 2.4b and c): In the first trial (Figure 2.4b), no significant differences were observed in IL-8 secretion between treatments.

For trial 2 (Figure 2.4c), there were statistically significant differences between treatments on D1, D2, D3, and D5. Peak IL-8 concentration was observed on D5 at $302.3 \pm 39.5$ pg/mL, while on D2 the largest increase relative to control was measured (5-fold).

**RV1A Replication** (Figure 2.5a): For the first trial, a significant difference in infectious virus was observed on D1 and D2 relative to D0. Peak viral replication was measured on D1 with $3.7 \times 10^5 \pm 6.9 \times 10^3$ pfu/mL.

In trial 2, a significant difference in viral replication was also observed on D1 relative to D0. Peak RV1A titers were measured on this day at $5.6 \times 10^4 \pm 1.3 \times 10^4$ pfu/mL.

**RV1A and IL-8 Secretion** (Figures 2.5b and c): For trial 1 (Figure 2.5b), IL-8 concentrations were highly significant between treatments on D1, D2, D3, and D6. Peak IL-8 concentration was observed on D6 at $769.0 \pm 57.4$ pg/mL; however, the largest increase in IL-8 relative to control was measured on D1 (3.5-fold).

In trial 2 (Figure 2.5c), there was a highly significant increase in IL-8 secretion between treatments for D2, D3, and D6. Peak IL-8 concentration was $1864.9 \pm 25.3$ pg/mL on D6, and the largest increase in IL-8 secretion relative to its control was observed on D2 (5.2 times).

**Growth Curve RV14 RNA Levels** (Figure 2.6):

There is a statistically significant difference in RNA levels between H1 cells and both BEAS-2B and A549 cells (all days combined). H1 cells produced increasing RNA levels to
a peak of $2.8 \times 10^5 \pm 1.4 \times 10^4$ pg/well at D3, and all time-points after D0 were in the $10^3$-5 pg range. For BEAS-2B cells peak RV14 RNA levels occurred on D3 measuring $17.7 \pm 7.2$ pg/well and then decreasing to $0.005 \pm 0.001$ pg/well by D7. For A549 cells, peak RV14 RNA was measured on D0 at $1.7 \times 10^{-6} \pm 6.7 \times 10^{-7}$ pg/well, and then declined to $2.3 \times 10^{-10} \pm 7.8 \times 10^{-11}$ pg/well on D1, after which RV14 RNA was undetectable for D2, D3 and D6. Within the H1 cells, RV14 RNA is significantly higher on D2 relative to D0. For BEAS-2B and A549 cells there were no significant differences observed within the respective cell types when compared to D0.

**BEAS-2B/A549 Tandem** (Figures 2.7-2.9):

**CPE:** No CPE or cell death was observed in any of the samples infected with either RV14 or RV1A for BEAS-2B or A549 cells.

**RV14 Replication** (Figure 2.7a): Replication levels for BEAS-2B cells were significantly different from A549 cells on D1, D2 and D3. Within BEAS-2B cells there was a significant difference observed on D1 and D2 relative to D0. Peak viral titer was measured on D1 at $1.6 \times 10^4 \pm 9.6 \times 10^2$ pfu/mL. Within A549 cells there were no statistically significant differences in RV14 replication.

**IL-8 Secretion** (Figure 2.7b): IL-8 concentrations were highly significant between BEAS-2B and A549 cells (all days combined); however, no significant differences between infected and uninfected cells were observed in either cell line for IL-8 secretion. The maximum IL-6 concentration measured was $327.1 \pm 42.3$ pg/mL in the BEAS-2B cells on D7.

**IL-6 Secretion** (Figure 2.7c): Again mediator secretion was highly significant between BEAS-2B and A549 cells (all days combined), but there were no differences observed between treatments. Maximum IL-6 concentration in BEAS-2B cells was measured on D1 at $27.4 \pm 2.8$ pg/mL.

**RV1A Viral Replication** (Figure 2.8a): Replication levels in BEAS-2B cells were highly significant compared to A549 cells as observed on D1 and D2. Within BEAS-2B cells highly significant increases in viral replication were measured on D1 and D2. Peak viral replication (relative to D0) occurred on D1 at $3.4 \times 10^4 \pm 3.6 \times 10^3$ pfu/mL. Within A549 cells no significant differences in RV1A replication were observed, although a typical...
Growth Curve trend was observed with peak viral titer of $4.5 \times 10^3 \pm 1.4 \times 10^2$ pfu/mL on D1.

**IL-8 Secretion** (Figure 2.8b): IL-8 concentrations were significantly different between BEAS-2B and A549 cells (all days combined). Within the BEAS-2B cells those infected with RV were highly significant from their respective controls for D2, D3, D5, and D7. Peak IL-8 concentration was measured on D5 at $2236.7 \pm 60.4$ pg/mL. The same trend was observed for A549 cells where there was a highly significant difference between treatments for D2, D3, D5, and D7 and maximum IL-8 concentration was measured on D3 ($1966.7 \pm 83.3$ pg/mL).

**IL-6 Secretion** (Figure 2.8c): Differences between BEAS-2B and A549 cells were highly significant (all days combined). Within BEAS-2B cells, treatments were significantly different on D3 and highly significant on D7. Peak IL-6 concentration for BEAS-2B was $2615 \pm 124.1$ pg/mL which was 53 times its control. In A549 cells both D3 and D7 were highly significant between treatments. Peak IL-6 concentration for A549 cells was measured on D7 at $1114.7 \pm 105.5$ pg/mL which represented the maximum increase relative to control at 124 times.

**BEAS-2B/A549 Tandem RV14 RNA** (Figure 2.9): There was a statistically significant difference in RV14 RNA levels between BEAS-2B and A549 cells as observed on D1 and D2. For BEAS-2B peak RNA levels were measured on D2 at $11.6 \pm 5.8$ pg/well, while peak RV14 RNA in A549 cells was observed on D0 at $4.0 \times 10^{-2} \pm 9.4 \times 10^{-3}$ pg/well. However, within cell lines no significantly differences were observed relative to D0.

**DISCUSSION**

RV infection of the airway epithelial cells (BEAS-2B and A549) resulted in significant viral replication and stimulation of cytokine/chemokine secretion in the overwhelming majority of experiments, certainly supporting the growing consensus that RV infection does provoke an inflammatory response in its host cells.

**CPE:** Previous studies of *in vitro* cell line experiments, cultured primary cells, and biopsy derived airway epithelial tissues indicated that no CPE or cell death was observed during
RV infection (Mosser et al., 2002; Griego et al 2000, Lopez-Souza et al., 2004). For example, Jang et al. (2005) found that nasal turbinate mucosa infected with RV16 did not cause any observable damage to the pseudostratified columnar epithelium, basement membrane, or cilia. My results also support these findings since no CPE or cell death were observed for either the BEAS-2B or A549 cell lines at any point during Growth Curve experiments for either RV serotype. In contrast, all permissive H1 cells infected with RV exhibited CPE by D1 and complete cell death by D4 (a typical lytic cycle). These observations suggest that RV has some unknown mechanism of crossing out the plasma membrane without rupturing the cell, or alternatively, a minute fraction of cells lyse, and these low localized levels of RV are sufficient to induce a pronounced immune response. It may be that both the above mechanisms do occur, and act synergistically to elicit the observed inflammatory response.

**Low levels of Replication Compared to H1 Cells:** There is growing consensus that RV replicates at very low levels in the airway epithelial tissues. When comparing my Growth Curve data between H1 cells and the airway cell models this hypothesis certainly seems to be supported. H1 cells produced peak RV titers at 3-4 orders of magnitude higher than BEAS-2B cells, and this trend was generally more exaggerated for the A549 cell model. Even taking into account the 1.4-fold greater H1 cell number at confluence (see Appendix E) the levels of RV replication remain impressive. What makes the airway cells less susceptible to RV infection than the permissive cells remains unknown. It is feasible that the airway cells allow less RV-receptor binding and entry than H1 cells because of fewer surface ICAM-1 or LDLR receptors. Since RV receptor expression is a highly dynamic process this hypothesis is difficult to test. There is evidence that RV infection causes a rapid up-regulation in the surface expression of ICAM-1 (Grunberg, 2000; Papi & Johnston, 1999; Whiteman et al., 2003; Winther et al., 2002), but perhaps this process is not as efficient in the airway cells when compared to permissive cells. Taking the Growth Curve data into consideration, it seems that H1 cells may allow for increased viral passage across the cell membrane. D0 levels in H1 cells are on the order of $10^4$, while the airway cells D0 values are lower, even bearing in mind initial cell numbers and viral dose. Furthermore, regardless of infection susceptibility, airway epithelial cells may only allow
for limited RV replication. This could potentially occur by an active mechanism, or passively due to slower and/or less efficient replication machinery.

**Growth Curve Replication Trends:** In the vast majority of the Growth Curve replication data a significant level of viral replication was observed between D1 and D2 (relative to DO), with the majority of peak titers being measured on D1. Following the increases in RV replication titers tended to gradually decrease to starting levels and were not significantly different from D0 after D2. How these cultured cells manage to resolve RV infection is still largely a mystery. *In vivo*, inflammatory mediators recruit and activate white blood cells to engulf pathogens, and antibody formation is thought to ultimately rid the body of infection (Janeway, 2005). However the airway cell models also seem to be capable of mitigating RV infection without the aid of immune cells. It is possible that the epithelial cell is able to recognize viral replication by some intra-cellular immune process and down-regulate its genomic machinery in response to RV. There is emerging evidence of intra-cellular immunity against retroviruses, and furthermore, some eukaryotic cells are capable of blocking polycistronic mRNA translation (Fire, 2005; Zheng et al., 2005). RV induced cytokine/chemokine secretion from affected cells may also alert neighbouring cells to decrease susceptibility to RV infection. This could potentially be accomplished by affecting the transcriptional cascades needed by RV.

The question remains whether RV infects many cells in the monolayer all producing low RV titers, or if only a few cells become infected each replicating RV at high levels. Mosser *et al.* (2005) found that in biopsy derived tissues only 5-10% of the cells were infected regardless of viral dose, and histologically RV infection manifested itself as small localized clusters of infected cells. However, it remains unclear if viral entry is even necessary to induce an inflammatory response, arguably a mere RV-receptor or cell interaction is sufficient to trigger cytokine/chemokine secretion. For example, one study found evidence of cytokine/chemokine stimulation by ultraviolet (UV) inactivated RV (Johnston *et al*., 1998). In such a case, RV replication levels and the number of infected cells may be completely irrelevant considering that RV could stimulate epithelial cells to secrete inflammatory cytokines/chemokines irrespective of RV crossing the plasma membrane. At the same time, it is clear that RV does infect at least some cells and significantly replicate in the airway epithelium. For a majority of patients presenting with
self-diagnosed colds RV can be consistently isolated from airway lavages and tissues (Arruda et al., 1997; Johnston et al., 1995; van Gageldonk-Lafeber et al., 2005). Perhaps RV replication is not the cause of cold symptoms but plays a crucial role in the propagation of the infection by causing the release of new viral progeny onto yet unaffected cells and generating an additive inflammatory response.

**Immune Response Hypothesis:** Because of the observed low levels of RV replication, there is a growing hypothesis that the illness associated with RV infection is initiated by the virus, but propagated and amplified by the host immune system through the secretion of pro-inflammatory cytokines and chemokines like IL-6 and IL-8. Such a mechanism is very different from many viruses where cell death caused by viral replication is the source of virus pathology. Johnston *et al.* (1998) found a prolonged release of IL-8 up to 120 hours in the pulmonary epithelium to low doses of RV9 even though replication peaked at 24 hours. Gern *et al.* (2000) also found increased IL-8 secretion in response to RV16 *in vivo* with no correlation to quantities of viral shedding. In experimental colds rhinorrhea symptoms usually peaked on D2 while in natural colds the maximum occurred on D3, but throat and cough symptoms peaked closer to D4, indicating increasing symptoms well beyond maximum viral replication (Gwaltney *et al.*, 2003). In an *in vitro* context, my cytokine/chemokine data also support these findings. As previously described, peak viral titers were consistently measured between D1 and D2 for all cell models with no significant viral replication from D3 onward. However, elevated cytokine/chemokine secretions and maximum increases when compared to respective controls were typically observed later in infection (often D2-7). Also cytokine and chemokine secretion remained elevated well beyond detectable RV replication.

The mechanism leading from RV interaction/infection with its host cell to the secretion of pro-inflammatory cytokines/chemokines (such as IL-6 and IL-8) is not well described. IL-6 and IL-8 transcription is controlled by various transcription factors including nuclear factor kappa-B (NFκB) (Caamano & Hunter, 2002; Oliveira *et al.*, 1994). NFκB is a cytosolic protein, normally bound by inhibitors, which becomes unbound in response to various stressors and binds its promoter sites within the nucleus resulting in the increased transcription of various cytokines/chemokines (Caamano & Hunter, 2002). There is some evidence that RV infection is mediated through an NFκB mechanism resulting in the up-
regulation of inflammatory mediator secretion (Spurrell et al., 2005; Zhu et al., 1997). However, Sharma et al. (2006) showed elevated expression of over 30 transcription factors (including NFκB) upon RV infection, thus clearly the RV mechanism remains poorly understood.

**Cell Model and RV Serotype Comparisons:** A direct comparison of the two airway epithelial models showed significantly more RV replication, RNA, and cytokine/chemokine secretion in BEAS-2B cells than observed in A549 cells regardless of RV serotype. A cause for this increased susceptibility of BEAS-2B cells to infection is not known but may be attributable to increased expression of RV receptors, and/or a more efficient or faster replication cycle. Whether this increased BEAS-2B susceptibility is representative of cells occurring higher in the airways is difficult to say considering that cultured cells may not represent the native airway epithelium adequately, and overall both cell lines are derived from the mid to lower respiratory tract. Furthermore, A549 cells represent surfactant secreting lung cells which are intrinsically different from bronchial epithelial tissues. However, it seems that at least in vitro both bronchial and alveolar-derived cells can be infected by RV and initiate inflammatory responses. This evidence further supports growing evidence that lower airway cells are vulnerable to RV infection (Gern et al., 1997; Hayden, 2004), and is important in the pathology of diseases such as asthma and COPD which are considered lower airway diseases.

RV1A produced more pronounced effects than RV14 in terms of viral replication and IL-6/IL-8 secretion for both BEAS-2B and A549 cells, and additionally RV14 failed to replicate in the A549 cells. This may be due to differential expression of LDLR receptor versus ICAM-1 receptors but could also be caused by intrinsic differences between the two serotypes. The RV14 genome has been fully sequenced (Stanway et al., 1984); however, only certain portions of the RV1A genome are known thus sequence homology between the two is undetermined. RV studies may utilize any of 100+ serotypes and although serotypes are documented the potential differences amongst them are largely ignored. My data suggest that serotype selection may be an important factor for RV research. RV14 experiments were also much more variable in their IL-6 and IL-8 responses than RV1A. For some Growth Curves RV14 failed to stimulate a pro-inflammatory response (Figure 2.2b), while in other experimentally identical experiments IL-6/IL-8 stimulation was highly
significant (Figure 2.2c). The source of this variability is unknown, but perhaps the RV14 viral dose was near some threshold level for triggering a cytokine/chemokine response in airway epithelial cells. Overall, the BEAS-2B/RV1A is the most consistent model for RV infection of the airways. RV infection of cultured airway epithelial cells results in relatively low levels of viral replication accompanied by a prolonged secretion of cytokines and chemokines such as IL-6 and IL-8. Further research should focus on the specific links between RV infection and the elicited airway epithelial inflammatory response in order to further define the mechanisms involved.
Figure 2.1: Effect of a) RV14 and b) RV1A on viral replication over time in H1 cells. Each trial represents n=4. An asterisk (*) indicates a significant difference (α=0.05) in infectious virus relative to D0 within a given trial.
Figure 2.2: Effect of RV14 on: a) viral replication and b) and c) IL-6 secretion over time in BEAS-2B cells. In a) two trials (n=4 each) are depicted, and an asterisk (*) indicates a significant difference (α=0.05) in infectious virus relative to D0 within a given trial. In b) IL-6 secretions correspond to trial 1. All measurements (control and RV for all days) are plotted but some are not visible because they are so close to 0. In c) IL-6 secretion corresponds to trial 2. An asterisk (*) indicates a significant difference in IL-6 concentration between RV infected and uninfected controls (n=3) at a given time (see b for further details).
Figure 2.3: Effect of RV1A on: a) viral replication and b) and c) IL-6 secretion over time in BEAS-2B cells. See Fig. 2.2 legend for further details.
Figure 2.4: Effect of RV14 on: a) viral replication and b) and c) IL-8 secretion over time in A549 cells. See Fig. 2.2 legend for further details.
Figure 2.5: Effect of RV1A on: a) viral replication and b) and c) IL-8 secretion over time in A549 cells. See Fig. 2.2 legend for further details.
Figure 2.6: RV14 RNA for H1, BEAS-2B, and A549 cells over time. Daily RV14 RNA levels were measured for each cell line (n=2 each). An asterisk (*) indicates a significant difference (α=0.05) in RV14 RNA within a given cell type relative to its D0. There is a significant difference in RNA levels (all days combined) between H1 cells and both BEAS-2B and A549 cells. RV14 RNA in A549 cells was undetectable after D1.
Figure 2.7: Effect of RV14 on: a) viral replication and b) IL-8 secretion and c) IL-6 secretion in simultaneously cultured (tandem) BEAS-2B and A549 cells over time. In a) an asterisk (*) indicates a significant difference ($\alpha=0.05$) in infectious virus relative to D0 within a cell type ($n=3$). A plus (+) denotes a significant difference in infectious virus between cell types on a given day. In b) IL-8 secretion from BEAS-2B and A549 cells corresponds to RV infections shown in a. A significant difference ($\alpha=0.05$) was found in secretion patterns between cell types (all days combined). All measurements (control and RV for all days) are plotted but some are not visible because they are so close to 0. In c) IL-6 secretion for BEAS-2B and A549 cells is shown (see b for further information).
Figure 2.8: Effect of RV1A on: a) viral replication b) IL-8 secretion and c) IL-6 secretion in simultaneously cultured (tandem) BEAS-2B and A549 cells over time. In b) and c) an asterisk (*) indicates a significant difference in IL-6 or IL-8 concentration between RV infected and uninfected controls (n=3 each) at a given time. See Fig. 2.7 legend for further details.
Figure 2.9: RV14 RNA levels over time in tandem cultures of BEAS-2B and A549 cells. No significant differences ($\alpha=0.05$) were found in RV14 RNA levels within the same cell type over time (n=2 each). A plus (+) indicates a significant difference in RV14 RNA levels between cell types on a given day.
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CHAPTER 3- The Effects Ultraviolet Inactivated Rhinovirus on Airway Epithelial Cells

BACKGROUND

Despite the fact that rhinovirus (RV) colds are the most prevalent illness in humans, very little is known about RV pathogenesis. Since evidence suggests that RVs cause little or no damage to their host tissues and replicate at relatively low levels within the host, it has been postulated that the symptoms of common colds are the result of a host inflammatory response characterized by the secretion of pro-inflammatory cytokines (and chemokines) such as interleukin (IL)-6, and not RV replication per se (Lopez-Souza et al., 2004; Mosser et al., 2005; Mosser et al., 2002). Furthermore, a link has been demonstrated between the severity of cold symptoms (e.g. rhinorrhea, sore throat, coughing, malaise) and increases in inflammatory mediator secretion (Gwaltney et al., 2003). If replication is not the cause of RV pathology then it is conceivable that RV triggers an inflammatory response in its host airway epithelial cells by some virus-cell interaction without the necessity of RV replication or passage across the plasma membrane.

One method for investigating this hypothesis is by using ultraviolet (UV) inactivated RV for experimental infections. There is evidence that UVC (260nm) irradiation of RV first affects the viral nucleic acid site before impacting protein structures. Consequently UV exposure causes infectivity to be lost before observed changes in antigen specificity (Hughes et al., 1979). UV primarily causes damage in the form of cyclobutylpyrimidine dimers and photoproducts which inhibit the replication of genetic material (Myatt et al., 2003). If noninfectious RV is capable of stimulating an inflammatory response in host cells, then this supports the idea of a non-replicative trigger for RV pathology. Very few studies have investigated such a hypothesis; however, there is limited evidence that inactivated RV may retain some of its cytokine stimulating abilities. For example, Johnston et al. (1998) found that 30 minute UVC irradiation of RV9 completely inhibited viral replication in A549 cells, but only reduced IL-8 secretion by about one half. On the other

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2 A version of this chapter will be submitted for publication. Machala, A.M., Harris, R.A., Brauner, C.J., Hudson, J.B.
Hand, Griego et al. (2000) found that UV treated RV did not induce much IL-6 or IL-8 secretion in BEAS-2B cells beyond control values.

It is unclear whether noninfectious RV is capable of eliciting an immune response in airway epithelial cells. This study investigated the ability of UV-treated RV to stimulate IL-6 secretion in the bronchial BEAS-2B cell line.

MATERIALS AND METHODS

All viral, cell culture and molecular work was conducted under sterile conditions in a type II biosafety cabinet. All protocols were pre-approved by the UBC biosafety committee in certificate H04-0061 (Appendix A).

Experiments: Cultured BEAS-2B cells were inoculated with either RV14 or RV1A previously treated with UVC for exposure times of 2, 5, 10, 15 and 30 minutes (UV2, UV5, UV10, UV15, and UV30 respectively), or with untreated RV (NO UV0, NO UV30). Infectious virus, IL-6 secretion, and viral RNA were measured at time=0 (0hrs) immediately post-infection, and 48 hours later (48hrs). Although the nucleic acid site is thought to be affected by UV first, the exact timing of nucleic acid and protein disruption is largely unknown thus RVs were exposed to UV for different lengths of time to produce noninfectious virus which retained as many of its other characteristics (e.g. antigenic activity) as possible. Two separate trials were conducted for each RV serotype (n=3 each).

Cell Culture: The SV40 adenovirus transformed human bronchial epithelial cell line (BEAS-2B) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and cultured in 75mm² flasks in 50:50 Dulbecco’s Modified Eagle’s Medium (DMEM) and Ham’s F12 with 10% endotoxin free fetal bovine serum (FBS). Culture reagents were obtained from Invitrogen (Vancouver, Canada). Cells were trypsinized and passaged weekly and incubated at 35-37°C with 5% carbon dioxide in 95% air. RV-sensitive H1 cells (from ATCC) were cultured under the same conditions with DMEM and 5% FBS.
Viruses: Both RV14 and RV1A were obtained from the ATCC. RVs were propagated by infecting H1 cells grown to confluence in 75mm² flasks containing DMEM, allowing for full cytopathic effects (CPE). Cell-free culture fluid was harvested when CPE were at a maximum by centrifugation at 10,000 x g for 20 minutes at 4°C. The stock virus suspension was aliquoted into cryovials and stored at -80°C for experimental use. Titer of viral stock was determined by viral plaque assay (see below) of serially diluted stock, and expressed in plaque forming units (pfu) per mL. Pfu represent the number of infectious RV particles present in a known volume of sample. For experiments, aliquoted stock virus was rapidly thawed at 37°C and vortexed prior to use. Because such clarified viral stocks contain H1 cell remnants (e.g. soluble proteins, organelles) control experiments were conducted to confirm that any observed changes in IL-6 and IL-8 secretion were due to virus and not some other component present in the inocula (Appendix C).

Preparation of UV inactivated virus: Stock suspensions of either RV14 or RV1A with known viral titers (10⁸ pfu/mL) and equal volumes were placed in single-well culture plates (lids removed) and irradiated by a 260nm UVC light source placed 10 cm away in a dark room. Plates were gently agitated throughout UVC exposure. At specific durations of UV treatment: 2, 5, 10, 15 and 30 minutes (UV2, UV5, UV10, UV15, and UV30 respectively), equal volumes of RV stocks were removed, placed in cryovials, and immediately stored at -80°C. Sufficient stock RV volume was used to avoid drying of samples during UV exposure. Control RV stocks were prepared simultaneously under the same conditions except shielded from light exposure in order to control for RV stability over the 30 minute sampling period. The control samples were collected just prior to UV treatment (NO UV0), and at the 30 minute endpoints (NO UV30).

Infections with UV inactivated RV: BEAS-2B cells were plated into 6-well trays and grown until freshly confluent. Once cells reached confluence it was assumed that cell number did not change significantly over the course of experiments for either control or RV infected cultures. This assumption was confirmed experimentally (Appendix D). Cell number per well (6-well plate) at confluence was pre-determined for cell lines (Appendix E) and used to calculate viral dose. RV inocula were prepared in culture medium (no FBS)
from the above described UV treated stocks (NO UV0, NO UV30, UV2, UV5, UV10, UV15, UV30) with equivalent amounts of virus, based on a pre-UV treatment viral dose of MOI=1. At infection, medium was aspirated from cells and replaced with 1mL of the appropriate UV-treated RV inocula, or with medium alone (control), and cells were incubated for 1 hour at 35°C. After infection, inocula were aspirated and cells washed 3 times with 1mL culture medium to remove exogenous virus (virus removal experimentally confirmed, not shown). Following this, 3mL of fresh medium (1% FBS) was added to the cells and one subset of the plates was sampled at 0hrs while the remainder were incubated at 35°C and sampled at 48hrs. Sampling consisted of first removing 1mL of supernatant from appropriate wells, centrifuging at 1000 x g to remove cellular debris, and freezing samples at -20°C for future IL-6 assays. Cells from RV infected samples were scraped into the remainder of the medium (2mL), pipetted into cryovials, and stored at -80°C for plaque assays. Finally, for quantitative real-time polymerase chain reaction (qRT-PCR) designated samples, medium was removed, cell monolayers washed twice with sterile phosphate buffered saline, and 1mL of TRIZOL reagent was added to each well allowing for cell lysis to occur. This suspension was stored in Eppendorf tubes at -80°C for subsequent RNA extraction.

**Plaque Assays** (See Appendix H for image): Viral infectivity and replication were measured by plaque assay in permissive HI cells. Once all the cell scraping samples were collected, they were rapidly frozen and thawed at 37°C twice, to rupture cells and release virus. These samples were used at serial dilutions to infect the permissive H1 cells in duplicate. 0.75mL of sample was added onto freshly confluent H1 cells grown in 6-well trays and allowed to infect for 1 hour at 35°C. After infection, the inocula were aspirated and replaced with a 50:50 liquid mixture of 2x MEM (with 5% FBS) and 1% agarose gel. The agarose gel was allowed to solidify at room temperature, and then the plates were incubated at 35°C for 4 days. As the virus replicated in infected cells, lysis occurred in the H1 cells forming round areas of cell death called “plaques”. Once incubation was complete, plates were fixed with 3% formaldehyde, agarose was removed, and cells stained with crystal violet to reveal the clear unstained plaques. These plaques were then counted and
reported as pfu/mL (infectious virions) present in the sample. An increase in infectious virus over time (relative to D0) indicated viral replication.

**Cytokines:** IL-6 was assayed in duplicate from supernatant samples using standard protocol provided by commercially available enzyme-linked immunosorbent assay (ELISA) kits from Immunotools (Friesoythe, Germany). Absorbencies were read on an ELISA plate reader (Pasteur Diagnostics LP400) at a 450nm wavelength. Highly concentrated samples were diluted with medium and re-assayed to fall within the standard assay range which was from 0-450 pg/mL. Sensitivity of the assay was 4 pg/mL.

**RNA Extraction:** RNA was extracted following standard TRIZOL reagent protocol and reconstituted in 50µL of RNAse/DNase free water and stored at -80°C for qRT-PCR. RNA for RV14 standard curves was extracted from stock RV14 suspensions using the QIAGEN RNeasy kit (Mississauga, ON).

**qRT-PCR:** qRT-PCR was conducted for RV14 samples. Primers were designed using the known RV14 genetic sequence from the National Center for Biotechnology Information database and purchased from Operon (Huntsville, AL). Primers were designed with the following sequences: forward 5' GACATGGTGTGAAGACTCGC 3' and reverse 5' TCTGTGTAGAAACCTGAGCGC 3' creating a 238 base pair product. Primers were tested by conventional two-step PCR of known RV infected samples, and PCR products confirmed by gel electrophoresis (not shown).

For RV14 RNA samples a one-step qRT-PCR kit from QIAGEN (Mississauga, ON) was used. The PCR mixture contained: 25µL MasterMix (HotStarTaq DNA Polymerase, QuantiTect SYBR Green Buffer, SYBR Green I dye, 1.5mM MgCl2, 200µM each dNTP), 2.5µL of each primer, 0.5 µL RT mix (Omniscript and Sensiscript Reverse Transcriptases), and 10µL of the RNA template to a final volume of 50µL. Standard curve RV concentrations were determined spectrophotometrically and serially diluted at known concentrations. For standards and samples, duplicate reactions were carried out in a MJ Research DNA Engine Opticon Continuous Fluorescence Detector (OP000537) programmed to: incubate at 42°C for 55min, 94°C for 5min, 94°C for 30 sec, 55°C for 30
sec, 72°C for 1min, repeat for 35cycles, 72°C for 10min, melting curve from 55°C to 95°C
read every 0.5°C, incubate 72°C for 10min, and 4°C forever. Data were analyzed using
Opticon Monitor analysis software version 1.07.

Statistics: Infectious virus (replication) was analyzed by two-way analysis of variance (ANOVA) with time and treatment as factors, and post-hoc Dunnet’s test with 0hrs as a control. IL-6 data for each serotype were subjected to a 1-way ANOVA with treatment as a factor and post-hoc Dunnet’s test to compare treatments with control (C). RNA data were analyzed by one-way ANOVA with treatment as a factor and post-hoc Dunnet’s test with untreated RV (NO UV0) as a control. All data were analyzed using Sigma Stat 3.0 software and statistical significance was set at α=0.05. Results are presented as mean ± standard error of the mean. All statistical differences were considered significant when p < 0.05; however, in most cases, when differences were found they were highly significant (p<0.001).

RESULTS
CPE: No CPE were observed in the BEAS-2B cells for any of the control, RV infected, or UV-treated RV infected samples.

RV14 Replication (Figures 3.1a and 3.2a): There was a highly significant difference between trial 1 and 2; however, the trends observed were the same. For both experiments, highly significant viral replication was measured from 0hrs to 48hrs for both the NO UV0 and NO UV30 samples. However, no significant differences were found between NO UV0 and NO UV30 samples for either sample time (i.e. 0hrs or 48hrs), indicating that no measurable (non-UV) RV14 degradation had occurred during preparation of UV irradiated virus. Furthermore, no significant RV replication was observed in BEAS-2B cells infected with any of the UV treated (UV2-UV30) samples demonstrating a complete inhibition of RV14 replication by UV.

RV14 and IL-6 Secretion (Figures 3.1b and 3.2b): The differences between trial 1 and 2 were highly significant, and trends observed were not similar. In the first trial, RV14 failed to stimulate an IL-6 response from BEAS-2B cells when compared to control (C). Although there was some statistical differences found (NO UV0 and UV5), considering the
very low concentrations of IL-6 (maximum 40.0 ± 8.0 pg/mL), it is highly doubtful that this was of any biological relevance. However, in trial 2, RV14 induced highly significant IL-6 secretion in the cells for NO UV0 and NO UV30 treatments, but none of the UV-irradiated RV14 treatments stimulated IL-6 secretion in BEAS-2B cells.

**RV1A Replication** (Figures 3.3a and 3.4a): There was also a highly significant statistical difference between trials 1 and 2; however, the trends observed were again the same between experiments and the same as seen for RV1A replication. There was a highly significant difference from 0hrs to 48hrs for NO UV0 and NO UV30, indicating RV replication. However, no significant differences were found between NO UV0 and NO UV30 samples at either 0hrs or 48hrs, indicating that no measurable (non-UV) RV1A degradation had occurred during preparation of UV irradiated virus. Furthermore, no significant RV replication was observed in BEAS-2B cells infected with any of the UV treated (UV2-UV30) samples, demonstrating a complete inhibition of RV1A replication by UV.

**RV1A and IL-6 Secretion** (Figures 3.3b and 3.4b): A highly significant difference was found between trials 1 and 2, but with the same trends as previously described. Again the NO UV0 and NO UV30 treatments produced highly significant differences when compared to control (C). None of the UV-irradiated RV1A treatments stimulated significant IL-6 secretion from cells when compared to controls.

**RV14 RNA** (Figure 3.5): There was a highly significant difference between the UV untreated (NO UV0) RV14 RNA treatment, which contained 14 pg/well of viral RNA, and all other treatments. This indicated damage to the viral genetic material for all of the UV treated samples.

**DISCUSSION**

The postulate that RVs may provoke the secretion of pro-inflammatory cytokines such as IL-6 without actually infecting airway epithelial cells is certainly provocative. Although cytokine secretion has been linked more closely to cold symptoms than RV replication (Gwaltney et al., 2003), there is no evidence that noninfectious virus is actually capable of causing illness. Very few studies have investigated the effects of UV inactivated RV on airway epithelial cells, and those published have yielded inconclusive results. For example,
Johnston et al. (1998) found that 30 minute UV inactivation of RV9 inhibited viral replication completely but only reduced IL-8 secretion by half in A549 cells. However, both Griego et al. (2000) and Papadopolous et al. (2001) demonstrated that UV inactivation of RV1b and RV39 halted both RV replication and IL-8, IL-6, and RANTES secretion from BEAS-2B cells. My results support the latter two studies, as no increased IL-6 secretion was observed for UV inactivated RV14 or RV1A for any UV exposure time. Even 2 minutes of UVC exposure was sufficient to arrest both viral replication and IL-6 secretion from the BEAS-2B cells. Hughes et al. (1979) showed that UVC treatment of RV17 and RV40 inactivated the viral nucleic acid in less than 10 seconds for dilute virus preparations and up to 90 seconds for more concentrated samples. However, the same study demonstrated that antigenic activity (provocation of antibody formation) of RV could be retained with at least 13 minutes of UV exposure. It is possible that the capacity to induce antibody formation is retained longer than the ability to stimulate an inflammatory response since white blood cells may recognize RV more effectively than the airway epithelial cells.

My experiments also showed a significant difference in RV14 RNA for UV treated samples when compared to untreated samples. This result is interesting because there have been conflicting reports suggesting that UV treatment of poliovirus and RV may or may not affect PCR assays (Ma et al., 1994; Myatt et al., 2003).

The reason for the discrepancy between the Johnston et al. (1998) study and the other studies (including this one) is not clear. The choice of cell line could be the source of the difference since only Johnston et al. (1998) used A549 cells (versus BEAS-2B). Moreover, RV serotype differences could also contribute to the observed differences, for example the RV9 capsid may be more resistant to UV damage than other RVs. Since UV inactivation of RV is also affected by initial virus concentration and UV intensity, it is possible that even the UV2 virus treatment had both non-functional genetic material and protein structure which could not provoke an IL-6 response. Finally, the choice of the cytokine or chemokine measured may affect results, bearing in mind that numerous interacting inflammatory mediators are released from airway cells in response to RV infection.

Interestingly, for one of the RV14 experiments no IL-6 response was observed for untreated RV even though significant viral replication was apparent. This same phenomenon was observed in previous experiments (see Chapter 2); however, the
explanation remains unclear. Perhaps the RV14 dosage is near some threshold value for eliciting a cytokine response from the cells.

In conclusion, these experiments demonstrated that UV inactivated RV was not capable of eliciting IL-6 secretion from BEAS-2B cells for either RV14 or RV1A. This suggests that RV infection and/or replication may be necessary to the pathology of the common cold.
Figure 3.1: Trial 1. Effect of UV treated and untreated RV14 on a) viral replication and b) IL-6 secretion in BEAS-2B cells after 48 hours. RV14 was pre-treated with UVC (260nm) for exposure times of 2, 5, 10, 15 or 30 minutes (UV2, UV5, UV10, UV15 and UV30 respectively). Simultaneously, control RV14 samples, which were shielded from light, were prepared at time=0 (NO UV0) and after 30 minutes (NO UV30). In a) an asterisk (*) indicates a significant (α=0.05) difference in infectious virus from 0 to 48hrs within a treatment (n=3). Symbols that differ (a for 0hrs; x,y for 48hrs) indicate significant differences in infectious virus between treatments at a given time (i.e. 0 or 48hrs). All measurements are plotted but some are not visible because they are so close to 0. In b) an asterisk (*) indicates a significant difference in IL-6 concentration between control (C) cells and other treatments (n=3).
Figure 3.2: Trial 2. Effect of UV treated and untreated RV14 on a) viral replication and b) IL-6 secretion in BEAS-2B cells after 48 hours. See legend Fig. 3.1 for further details.
Figure 3.3: Trial 1. Effect of UV treated and untreated RV1A on a) viral replication and b) IL-6 secretion in BEAS-2B cells after 48 hours. See legend Fig. 3.1 for further details.
Figure 3.4: Trial 2. Effect of UV treated and untreated RV1A on a) viral replication and b) IL-6 secretion in BEAS-2B cells after 48 hours. See legend Fig. 3.1 for further details.
Figure 3.5: Effect of UV treated and untreated RV14 inocula on RV14 RNA levels in BEAS-2B cells after 48 hours. Cells were inoculated with untreated RV14 (NO UV), and RV14 pre-treated with UVC for 2, 5, 15 or 30 minutes (UV2, UV5, UV15, and UV30 respectively). An asterisk (*) indicates a significant difference (α=0.05) in RV14 RNA levels relative to untreated RV14 (NO UV0) control (n=2).
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CHAPTER 4: The Effects of Echinacea Extracts on RV Infected and Uninfected Airway Epithelial Cells

BACKGROUND

Rhinovirus (RV) infections of airway epithelial cells are the most important cause of the common cold (Monto, 2002). For people already affected by respiratory diseases such as asthma and chronic obstructive pulmonary disorder (COPD), RV infection may cause dangerous exacerbations of those conditions (Bardin, 1992; Gern & Busse, 1999; Gern, 2002; Grunberg et al., 1997; Grunberg & Sterk, 1999; Halperin, 1985; Johnston et al., 1995; Singh et al., 2006). Surprisingly, RVs replicate at relatively low levels in the airway epithelial tissues, and mounting evidence suggests that cold symptoms are the result of a pronounced host inflammatory response to infection characterized by the secretion of pro-inflammatory cytokines such as interleukin (IL)-6, and not RV replication (Gwaltney, 2002; Grunberg et al., 1997; Hendley & Gwaltney, 2004; Lopez-Souza et al., 2004). Furthermore, in vivo studies have linked increases in pro-inflammatory cytokine secretion to increases in severity of cold symptoms (Gwaltney et al., 2003). Airway epithelial cells, both in vivo and in vitro, have demonstrated the ability to secrete various cytokines (including IL-6) in response to environmental stresses including RV infection (Arnold, 1994; Berg et al., 2004; Johnston et al., 1998; Lopez-Souza et al., 2004; Sharma et al., 2006; Spannhake et al., 2002; Takizawa et al., 2000; et al., 1998; Zhu et al., 1997; Zhu et al., 1996).

No cure or prevention for RV infection exists and most drugs only act to alleviate symptoms. Vaccines have been difficult to develop because over 100 poorly cross-neutralizing serotypes of RV persist. Scientists have developed various RV anti-viral compounds, such as Pleconaril and Rupintrivir, which prevent RV replication or entry across the plasma membrane (Hayden et al., 2003; Zhang et al., 2004). However, considering the possibility that RV may stimulate cytokine secretion from cells without the necessity of RV replication or cell entry, anti-viral development may be futile.

3 A version of this chapter will be submitted for publication. Machala, A.M., Harris, R.A., Brauner, C.J., Hudson, J.B.
A growing number of researchers are investigating the ability of immune-modulating compounds to mitigate RV-associated symptoms. For example, compounds capable of down-regulating pro-inflammatory cytokine secretion could conceivably reduce cold symptoms. Echinacea is a popular natural herb extract which is thought to have immune-modulating effects. There is evidence that Echinacea has stimulatory effects on macrophages, monocytes, T lymphocytes, natural killer cells, and epithelial cells, resulting in increased pro-inflammatory cytokine release (Brousseau & Miller, 2005; Brush et al., 2006; Currier & Miller, 2000; Goel, 2005; Mozzaroni et al., 2005; Sasagawa et al., 2006; Sharma et al., 2006). However, Sharma et al. (2006) showed that when Echinacea was administered to RV infected airway epithelial cells, RV-induced cytokine secretion was inhibited, suggesting a more complex interaction between the herb extract and virus infected cells than that observed in uninfected cells.

Clinical trials investigating Echinacea have yielded inconclusive results (Goel et al., 2004; Sperber et al., 2004; Turner et al., 2005; Turner et al., 2000), and the quality of many commercial formulations is questionable (Gilroy et al., 2003; Krochmal et al., 2004). Standardized in vitro studies are needed to further elucidate the effects of Echinacea in RV infected airway epithelial cells.

This study aimed to assess the effect of two chemically distinct Echinacea extracts on cultured bronchial epithelial cells (BEAS-2B) infected with RV14 or RV1A. Viral replication and IL-6 secretion were measured in order to address the hypothesis that Echinacea is immune-modulatory and stimulates IL-6 in uninfected cells, but inhibits RV-induced cytokine secretion in cultured airway epithelial cells.

**MATERIALS AND METHODS**

All viral, cell culture and molecular work was conducted under sterile conditions in a type II biosafety cabinet. All protocols were pre-approved by the UBC biosafety committee in certificate H04-0061 (Appendix A).

**Cell Culture:** The SV40 adenovirus transformed human bronchial epithelial cell line (BEAS-2B) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in 75mm² flasks in 50:50 Dulbecco’s Modified Eagle’s Medium
(DMEM) and Ham’s F12 with 10% endotoxin free fetal bovine serum (FBS). Culture reagents were obtained from Invitrogen (Vancouver, Canada). Cells were passaged weekly and incubated at 35-37°C with 5% carbon dioxide in 95% air. RV-sensitive H1 cells (from ATCC) were cultured under the same conditions with DMEM and 5% FBS.

**Echinacea Extracts:** Two commercial preparations (E1 and E2) were analyzed for their major constituents. E1 was a spray dried expressed juice extract of the aerial parts of *E. purpurea* (accession number UO19180). E1 was rich in water extractable polysaccharides, with a total extractable polysaccharide content of 23.7% w/w (Sharma et al., 2006). E2 was a 55% ethanolic tincture from *E. purpurea* roots (1:9 w/v). High performance liquid chromatography analysis of the E2 tincture showed the presence of alkamides and caffeic acid derivatives (caftaric acid 59.5μg/mL, chlorogenic acid 19.3μg/mL, caffeic acid 2.4μg/mL, cyanarins 0μg/mL, echinacoside 0μg/mL, cichoric acid 37μg/mL and tetraene alkylamides 80.5μg/mL). Both of these extracts were analyzed previously by Binns *et al.* (2002) and provided to our laboratory. Extracts were filtered at 0.2μm (pre-characterization), diluted in culture medium, and stored at -20°C.

**Viruses:** Both RV14 and RV1A were obtained from the ATCC. RVs were propagated by infecting H1 cells grown to confluence in 75mm² flasks containing DMEM, allowing for full cytopathic effects (CPE). Cell-free culture fluid was harvested when CPE were at a maximum by centrifugation at 10,000 x g for 20 minutes at 4°C. The stock virus suspension was aliquoted into cryovials and stored at -80°C for experimental use. Titer of viral stock was determined by viral plaque assay (see below) of serially diluted stock, and expressed in plaque forming units (pfu) per mL. Pfu represent the number of infectious RV particles present in a known volume of sample. For experiments, aliquoted stock virus was rapidly thawed at 37°C and vortexed prior to use. Because such clarified viral stocks contain H1 cell remnants (e.g. soluble proteins, organelles) control experiments were conducted to confirm that any observed changes in IL-6 and IL-8 secretion were due to virus and not some other cellular component present in the inocula (Appendix C).
**Viral Infections:** BEAS-2B cells were cultured in 6-well plates until freshly confluent in 50:50 DMEM: Ham’s F12 mix with 10% FBS. Once cells reached confluence it was assumed that cell number did not change significantly over the course of experiments for either control of RV infected cultures. This assumption was confirmed experimentally (Appendix D). Cell number per well (6-well plate) at confluence was pre-determined for cell lines (Appendix E) and used to calculate viral dose. Prior to infections media was aspirated and replaced with 0.75mL of RV1A or RV14 inocula at a multiplicity of infection (MOI)=1, or mock infected with medium. Cells were incubated at 35°C for 1 hour. Following infection inocula were aspirated and cells were washed 3 times with 1mL of medium in order to remove exogenous virus (virus removal experimentally confirmed, not shown). Following this, fresh culture medium containing either: 50µg/mL of E1, 1:50 E2/medium dilution, 0.9% ethanol (E2 vehicle control), or medium alone (control) was added to wells, and cells were incubated at 35°C in a 5% carbon dioxide incubator. Samples were collected at the same time immediately post-infection and washes (0hrs), and 48 hours (48hrs), and 96 hours (96hrs) later. Sampling consisted of first removing 1mL of supernatant from appropriate wells, centrifuging at 1000 x g to remove cellular debris, and freezing samples at -20°C for future cytokine/chemokine assays. Cells from RV infected samples were scraped into the remainder of the medium (2mL), pipetted into cryovials, and stored at -80°C for plaque assays.

**Plaque Assays:** Viral infectivity and replication were measured by plaque assays in permissive H1 cells. Previously frozen RV infected cell samples from Growth Curves were rapidly frozen and thawed twice at 37°C to rupture cells and release virus. These samples were then serially diluted and used to infect permissive H1 cells in duplicate where 0.75mL of sample was added onto freshly confluent H1 cells grown in 6-well trays and allowed incubated for 1 hour at 35°C. After infection the inocula were aspirated and replaced with a 50:50 liquid mixture of 2x MEM (with 5% FBS) and 1% sterile agarose in dH2O. The agarose was allowed to solidify at room temperature, and plates were incubated at 35°C for 4 days. As the virus replicated in infected cells, lysis occurred in the H1 cells forming round areas of cell death called “plaques”. Once incubation was complete, plates were fixed with 3% formaldehyde in phosphate buffered saline, agarose was removed, and H1
cells were stained with 1% crystal violet in dH2O to reveal the clear unstained plaques. Plaques were counted and reported as pfu/mL. An increase in infectious virus over time (relative to D0) indicated viral replication.

**Cytokines**

IL-6 was assayed from supernatant samples using standard protocol provided by commercially available enzyme-linked immunosorbent assay (ELISA) kits from Immunotools (Friesoythe, Germany). Absorbencies were read on an ELISA plate reader (Pasteur Diagnostics LP400) at a 450nm wavelength. Highly concentrated samples were diluted with medium and re-assayed to fall within the standard assay range which was from 0-450 pg/mL. Sensitivity of the assay was 4 pg/mL.

**Statistics:** Infectious virus (replication) data (n=3 per treatment for each sampling time) were analyzed by two-way analysis of variance (ANOVA) with day and treatment as factors, followed by post-hoc Tukey tests. For IL-6 secretion (n=3 for each treatment) a one-way ANOVA with treatment as a factor was performed with post-hoc Tukey tests in order to compare all treatments. All data were analyzed using Sigma Stat 3.0 software and statistical significance was set at α=0.05. Significant statistical differences correspond to p<0.05, and highly significant statistical differences correspond to p<0.001. Results are presented as mean ± standard error of the mean.

**RESULTS**

**CPE:** No CPE were observed for any of the samples including all RV infected cells, E1 and E2 treated cells, and RV+Echinacea treated combinations.

**RV14 Replication** (Figure 4.1a): There was a highly significant difference in infectious virus between 0hrs and 48hrs (all treatments combined) indicating viral replication, but no viral replication was detected at 96hrs (relative to 0hrs). Average of 0hrs treatment pfu/mL values (all treatments combined) was $3.6 \times 10^3 \pm 9.3 \times 10^1$ pfu/mL, increasing to $3.5 \times 10^4 \pm 7.9 \times 10^2$ pfu/mL at 48hrs. In comparing treatments, only RV and RV+Etoh were significantly different, and this difference was only observed at 48hrs, but was highly significant.
**RV14 and IL-6 Secretion** (Figure 4.1b): There is a highly significant difference between treatment groups. None of the uninfected cell treatments (C, E1, E2, Etoh) were significantly different from each other. All RV infected treatments: RV, RV+E1, RV+E2, and RV+Etoh were found to be significantly different from uninfected cells. Within RV infected cells there were no significant differences between RV, RV+E1 or RV+Etoh; however, there was a significant difference between RV+E2 and all other treatments. RV+E2 represented peak IL-6 secretion at 243.4 ± 9.3 pg/mL.

**RV1A Replication** (Figure 4.2a): Significant viral replication relative to 0hrs was observed at 48hrs but not 96hrs (all treatments combined). Average pfu/mL of 0hrs treatments (all treatments combined) was $3.6 \times 10^3 \pm 9.3 \times 10^1$ pfu/mL increasing to $3.5 \times 10^4 \pm 7.9 \times 10^2$ pfu/mL at 48hrs. No significant differences were observed between treatments.

**RV1A and IL-6 Secretion** (Figure 4.2b): A highly significant difference was found between treatment groups. Only RV and RV+Etoh treatments were significantly different from all other treatments (83.0 ± 10.2 pg/mL), but they were not significantly different from each other. Peak IL-6 concentration (RV treatment) was 349.3 ± 49.7 pg/mL.

**DISCUSSION**

Echinacea is the most popular natural extract used to treat common upper respiratory tract infections typically caused by RV infection. Historically, crude Echinacea extracts in various forms have been used to treat and/or prevent a variety of infections (Barrett, 2003). However, no modern consensus on the potential health benefits of Echinacea for the treatment of RV infection exists, and a possible mechanism of action is largely unknown.

**RV Replication:** Depending on the source consulted, the effects of Echinacea have been described as immune-stimulatory, immune-protective and/or anti-viral. Although growing research supports the idea that Echinacea’s effects are largely immune-mediated the above claims are somewhat ambiguous and contribute to the controversy surrounding this natural medicine. For example, although there is some evidence that Echinacea has a virucidal effect on viruses such as herpes simplex virus-1 (Binns et al., 2002), there is no evidence of any effect on viral replication thus broad “anti-viral” claims may be misleading. In my experiments significant RV replication was detected at 48hrs relative to 0hrs, but there was no differences observed between Echinacea treated and untreated cells for either extract...
formulation in RV14 or RV1A infected BEAS-2B cells. These data suggest that Echinacea most likely confers its physiological effects through an interaction with the host cells rather than by affecting viral replication.

**IL-6 Secretion:** IL-6 is a pro-inflammatory cytokine secreted from a variety of cells, including airway epithelial cells, in response to injury and stress such as viral infection (Janeway, 2005). There is evidence that RV infection can stimulate IL-6 secretion in airway epithelial cells (Sharma et al., 2006), and increases in pro-inflammatory cytokines have been linked to intensified cold symptoms (Gwaltney et al., 2003). This evidence is also supported by my findings, as RV and RV+Etoh treated cells showed significantly increased IL-6 concentrations compared to control. Several hypotheses have been postulated about how the immune-modulating effects of Echinacea may translate into health benefits. Some scientists have suggested that Echinacea stimulates an inflammatory response, thus protectively “heightening” the immune system (cited in Barrett, 2003); however, bearing in mind that pro-inflammatory mediator release is thought to cause cold symptoms further exaggerating this process seems counterproductive. A more plausible hypothesis may be that Echinacea down-regulates RV induced inflammatory responses, such as IL-6 secretion, thereby reducing cold symptoms.

Interestingly, Echinacea treatment of uninfected cells for both experiments did not yield any significant IL-6 secretion relative to control. These results differ from some published studies indicating an increase of pro-inflammatory cytokine release after treatment with Echinacea (Hwang et al., 2004; Sharma et al., 2006). It may be that the concentration of the extracts administered was not sufficient to stimulate cellular IL-6 secretion, or perhaps some of the active compounds had degraded during extract storage. On the other hand, significant differences in IL-6 secretion were observed for both RV14 and RV1A infected cells treated with Echinacea, indicating that the extracts were biologically active. Considering that crude Echinacea preparations contain many immunologically active compounds (e.g. alkamides and caffeic acid derivatives), it is possible that different constituents (or combinations thereof) are responsible for Echinacea’s contrasting effects on RV infected and uninfected cells.

A divergent trend was observed for IL-6 secretion between RV14 and RV1A. In RV14 infected cells, IL-6 secretion was not significantly different for RV+E1, but was
significantly elevated from other RV infected treatments for RV+E2. However, in RV1A infected cells, Echinacea treatment resulted in IL-6 concentrations that were similar to control levels, even though RV and RV+Etoh treatments stimulated significant IL-6 secretion. The differences observed between serotypes are difficult to explain because the physiological mechanism of Echinacea is largely unknown. Some studies propose an nuclear factor kappa-B (NFKB) dependent pathway for the action of this herbal extract, and overall such a mechanism is plausible, considering that NFKB is implicated in both pro-inflammatory cytokine secretion and RV pathology (Zhu et al., 1997). However, one study conducted by our laboratory (Sharma et al., 2006) showed that Echinacea treatment of BEAS-2B cells resulted in changes of over 30 transcription factors (including NFKB), demonstrating the involvement of complex biochemical pathways. Recent research has found that alkamides in Echinacea extracts bind the cannabinoid type-2 receptor and may affect NFKB transcription by this pathway (Gertsch et al., 2004; Raduner et al., 2006). RV14 and RV1A also utilize different receptors to infect cells (intercellular adhesion molecule-1 and low density lipoprotein receptors respectively); therefore, the cascades signaled by the binding of these receptors may lead to differences in cytokine secretion. In another study, Sharma et al. (2006) showed increased IL-6 secretion from BEAS-2B cells upon Echinacea treatment alone and decreased secretion when Echinacea was administered to RV14 infected cells. Although the same trend was not observed for my RV14 experiments, RV1A did support the Sharma et al. (2006) findings. Considering that these are the only studies investigating the effects of Echinacea on RV infected airway epithelial cells, more evidence is needed before definite conclusions can be drawn.

**E1 and E2 Echinacea Extracts:** The quality of commercially available Echinacea extracts is questionable (Gilroy et al., 2003; Krochmal et al., 2004). One study found that 39% of Echinacea extracts contained more or less Echinacea than indicated while 10% of the products contained no Echinacea at all (Gilroy et al., 2003). Furthermore, the best mode of administration (e.g. caplet, tincture, tea) or the proper dosage is ill-defined. Even in standardized preparations of Echinacea, the number and concentrations of biologically active constituents differ greatly due to factors such as species (E. purpurea, E. pallida, E. angustifolia), part of plant utilized (e.g. root, leaf, aerial) and method of extraction (e.g. alcoholic, aqueous) (Adinolfi et al., 2006; Sloley et al., 2001). This lack of extract
homology certainly contributes to the controversy surrounding the therapeutic benefits of Echinacea, especially in clinical studies where so many additional confounding factors may exist (Gagnier et al., 2006; Sperber et al., 2004; Turner et al., 2000; Turner et al., 2005; Wolsko et al., 2005).

As previously mentioned, no effects of either E1 or E2 were observed for either RV14 or RV1A replication in the BEAS-2B cells. In my RV1A experiments both E1 and E2 suppressed RV stimulation of IL-6 secretion in a similar manner, while in RV14 infected cells E1 failed to suppress RV induced IL-6 secretion and E2 seemed to exaggerate the IL-6 response. Again, these data suggest that distinctive Echinacea extracts may interact in complex ways with varying RV serotypes.

An ethanolic vehicle control was included to account for the potential non-Echinacea based differences between the aqueous and ethanolic extracts. No significant ethanolic effects were observed for IL-6 secretion in uninfected cells regardless of RV serotype, and in RV infected cells no IL-6 secretion beyond that induced by the virus could be observed for RV+Etoh treatments. In the RV14 replication data, a significant difference was found between RV and RV+Etoh treatments; however, considering the inherent variability in plaque assays, it is doubtful that this difference is biologically relevant (see Figure 4.1a). Overall little or no ethanolic effects were observed.

It is becoming increasingly clear that Echinacea extracts are capable of modulating inflammatory cytokine secretion in a variety of cells. Moreover, previous studies conducted by our laboratory and my experiments suggest that Echinacea interacts with RV infected cells differently than with uninfected cells, at least in the case of the BEAS-2B cell line (Sharma et al., 2006). Future studies should examine the effects of standardized Echinacea extracts on various airway cells infected by different RV serotypes in order to determine if the observed effects can be conserved under various experimental conditions.
Figure 4.1: Effects of Echinacea extracts (E1, E2) and ethanol on a) RV14 replication and b) IL-6 secretion in RV14 infected and uninfected BEAS-2B cells. BEAS-2B cells were infected with RV14 and known concentrations of either Echinacea extract: aqueous E1 (RV+E1), or alcoholic E2 (RV+E2), 1% Ethanol (RV+Etoh) as an E2 vehicle control, or medium alone (RV). In a) an asterisk (*) indicates a significant difference (α=0.05) in infectious virus (all treatments combined) at 48hrs or 96hrs relative to 0hrs control. Symbols that differ (a,b) indicate significant differences between treatments at 48hrs; no significant differences were found between treatments at 0hrs or 96hrs. In b) IL-6 secretion after 48 hours is shown. Symbols that differ (a,b,c) indicate a significant difference in IL-6 concentrations between treatments (n=3).
**Figure 4.2:** Effects of Echinacea extracts (E1, E2) and ethanol on a) RV1A replication and b) IL-6 secretion in RV1A infected and uninfected BEAS-2B cells. In a) no significant differences were found between treatments at any time (i.e. 0, 48, or 96 hours). In b) symbols that differ (a, b) indicate a significant difference in IL-6 concentrations between treatments (n=3). See Fig. 4.2 legend for further details.
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CHAPTER 5: General Discussion and Conclusions

Biological Relevance of Studies: Rhinovirus (RV) infection is the most frequent acute illness in humans, although surprisingly little is known about its pathogenesis, and its role in the exacerbations of serious respiratory diseases has only recently been reported. Even in healthy individuals, RV infection impacts not only our sense of well-being but often results in missed days from work. For example, Fendrick et al. (2003) reported 500 million incidences of non-influenza viral respiratory infections per year in the United States with an annual estimated cost burden of over 40 billion dollars. Moreover, the emerging role of RV infection in the morbidity and mortality of diseases such as asthma and chronic obstructive pulmonary disorder (COPD) illustrates the clinical significance of an infection which may have been previously considered more nuisance than danger. Johnston et al. (1995) demonstrated that up to 85% of asthma exacerbations in 292 children were associated with viral infection, and picornaviruses (RVs and enteroviruses) accounted for close to 66% of those infections. A study by Khetsuriani et al. (2005) found that over 63% of asthma exacerbations were associated with viruses, of which 60% were identified specifically as RVs. Furthermore, evidence suggests that patients with moderate to severe COPD are more susceptible to RV infection than their healthy counterparts, and RV infection causes more severe symptoms in COPD patients than in healthy people (Greenberg et al., 2000). Additionally, acute respiratory infections are the leading cause of infant mortality, which was initially attributed to the influenza and respiratory syncytial viruses; however, a recent study of 263 children under the age of 12 months found that almost 50% of the viruses isolated during upper and lower acute respiratory tract infections were RVs (Kusel et al., 2006). Finally, RV infection has also been implicated in cases of viral pneumonia, co-infections with bacterial pneumonia, and complications in lung transplant recipients (Falsey & Walsh, 2006; Kaiser et al., 2006; Lehtinen et al., 2006). Understanding RV infection is becoming increasingly important to scientists especially considering the growing research indicating that RV plays an important role in the pathology of many other serious conditions.

The emerging evidence that RV illness may be largely caused by a host inflammatory response and not necessarily by viral replication is important from both a basic virological
and clinical therapeutic perspective. The RV mechanism may illustrate a unique viral "strategy" where RV is able to incite illness while causing little or no cell damage or death. To date, it is unknown whether RV replication is necessary in order to cause illness. Although RV symptoms have been linked to increases in pro-inflammatory mediator secretion, some scientists suggest that viral replication may be the trigger for this inflammatory response (Gwaltney et al., 2003), while conversely, limited evidence has shown that noninfectious virus may be capable of provoking pro-inflammatory cytokine secretion in vitro (Johnston et al., 1998). Furthermore, the emerging implication of the host immune response as the cause of RV-associated illness has inspired scientists to investigate RV treatments, such as Echinacea, which are thought to mediate symptoms largely through host immunity.

General Discussion: My studies investigated the relationships between RV replication and pro-inflammatory cytokine/chemokine secretion for two different receptor-utilizing RV serotypes (RV14 and RV1A) using two distinct airway epithelial cell models (BEAS-2B and A549). I also investigated the necessity of RV replication in stimulating an inflammatory response by assessing the ability of noninfectious virus to stimulate an interleukin (IL)-6 response in the BEAS-2B model. Finally, I studied the effects of two chemically characterized Echinacea extracts on both viral replication and pro-inflammatory IL-6 secretion.

For the Growth Curve experiments (Chapter 2), I found that significant RV replication occurred in both BEAS-2B and A549 cells between day 1 (D1) and D2 post-infection; however, replication was no longer detectable after D3 in all cases. Furthermore, the levels of viral replication were relatively low when compared to the permissive H1 cells which produced RV titers 3-4 orders of magnitude higher than either of the airway epithelial cell models. For example, H1 cell peak titers reached $10^{7.8}$ pfu/mL which corresponds to approximately 10-100 infectious RV particles present per cell. However, peak RV titers for BEAS-2B and A549 cells typically represented 0-0.1 infectious viral particles present per cell. Furthermore, RV14 did not appear to replicate in the A549 cells. RV14 RNA levels also supported the replication data, where H1 cells produced levels of viral RNA much higher than in either of the airway models. This evidence supports the hypothesis that RV
replicates at low levels in the airway epithelial cells, and these trends were supported for both airway models and RV serotypes. However it is unclear if the majority of the cells were infected with RV each producing low levels of virus, or whether a small number of cells replicated the virus at very high levels. Additionally, as hypothesized, no cytopathic effects (CPE) were observed in any of the experiments involving airway epithelial cells, suggesting that viral progeny either have some unknown mechanism of crossing the plasma membrane without lysing cells, or perhaps a very small number of cells ruptured releasing their viral progeny. In vivo, Mosser et al. (2002 and 2005) found immunohistochemical evidence of patchy RV infection affecting 5-10% of the cells with few or no abnormalities of the airway epithelial tissues, suggesting that perhaps only a small proportion of cells are affected by RV.

For the majority of experiments RV was also found to stimulate pro-inflammatory IL-6 and/or IL-8 secretion and these elevated secretions were typically observed after the second day post-infection and persisted well beyond significant RV replication. This evidence supports the hypotheses that RV would stimulate pro-inflammatory cytokine/chemokine secretion and that peak secretions would occur later and persist longer than peak replication and viral RNA levels. These data also demonstrate that RV triggers a pronounced inflammatory response from cells and mirrors in vivo research where RV induced a prolonged inflammatory response from airway cells which was linked to cold symptoms independent of viral shedding (Gwaltney et al., 2003).

In contrast to what was hypothesized, ultraviolet (UV) inactivation (Chapter 3) of RV14 and RV1A completely inhibited both viral replication and the ability to stimulate an IL-6 response suggesting that genetically intact virus is necessary to trigger IL-6 secretion, at least in the BEAS-2B model. Thus it may be the case that viral replication is the indirect cause of RV symptoms by stimulating the host cell to secrete inflammatory cytokines/chemokines, although the mechanism is not yet known. Furthermore in Appendix C, I confirmed that infectious virus was necessary and responsible for the observed IL-6 and IL-8 stimulation, and that this response could not be induced by other cellular components (e.g. soluble proteins and organelles) present in the RV inocula.

In comparing the cell models, BEAS-2B cells were found to be more susceptible to RV infection than the A549 cells. They produced significantly higher titers of both RV14 and
RV1A observed between D1 and D3 post-infection and higher levels of RV14 RNA were detectable in BEAS-2B on D1 and D2 in tandem BEAS-2B/A549 cultures.

Furthermore, RV14 failed to replicate in A549 cells although in one case an increased IL-8 response was evident. Although these results did not support the initial hypothesis, the differences observed between cell models may not be surprising considering that type-II surfactant secreting alveolar cells (A549) are intrinsically different from bronchial cells (BEAS-2B). These cultured cell lines most likely reflect undifferentiated basal-type cells; however, the A549 may retain physiological differences such as a decreased number of ICAM-1 receptors relative to BEAS-2B cells. In general, considering that these cell lines are derived from the mid to lower airways, my studies support the growing evidence that both upper and lower airway epithelial cells are susceptible to RV infection (Mosser et al., 2005; Papadopoulos et al., 2000; Zhu et al., 1996).

Although both RV14 and RV1A produced similar trends in replication and pro-inflammatory mediator release, some differences were observed between serotypes. As previously mentioned, RV14 failed to replicate significantly in the A549 cells. Additionally, in some cases there was an apparent uncoupling between RV14 replication and stimulation of IL-6 and/or IL-8 release. For example, in a few instances, although RV14 replication was evident there was no difference observed between control and RV infected IL-6 or IL-8 cell secretions (Figures 2.7b and c). In one A549 case, a significant increase in IL-8 secretion was observed although no significant replication was detectable (Figure 2.4c). Finally, during the first trial of the UV experiments, RV14 failed to stimulate any IL-6 response from BEAS-2B cells even though significant viral replication was measured (Figures 3.1a and b). The cause of this discrepancy remains unclear; it may be that in some cases elevated control cytokine/chemokine secretions may have masked observable differences in RV infected cells. These elevated control levels may be attributable to changes in cell physiology with passage number, or possibly some other confounding factor. However, it seems curious that this discrepancy could only be observed in RV14 experiments. During tandem experiments (where both RV14 and RV1A designated cells were plated simultaneously) RV1A infected cells showed clear increases in IL-6 and IL-8 secretion relative to control while RV14 did not, even though control levels for A549 cells were similar between RV serotypes. It may be that the viral dose for RV14...
may be near some threshold for eliciting a cytokine/chemokine response which could be
influenced by other factors such as cell age. Or perhaps RV replication and IL-6/IL-8
responses are controlled by different mechanisms which may or may not be related. For
RV1A a consistent trend was observed for both serotypes and cell lines resulting in
significant viral replication and IL-6/IL-8 release in all cases. Regardless of this observed
discrepancy, it is clear that RV infection of the airway cells is capable of inducing viral
replication and the secretion of pro-inflammatory cytokines and chemokines for both
intercellular adhesion molecule-1 (ICAM-1) and low density lipoprotein receptor (LDLR)
utilizing RV serotypes.

The treatment of BEAS-2B cells with Echinacea extracts also yielded interesting results
(Chapter 4). Neither the aqueous polysaccharide-rich (E1) nor the alcoholic alkamide-rich
(E2) Echinacea extracts had any observable effect on RV14 or RV1A replication. Although
the presence of cichoric acid in Echinacea has been shown to have some virucidal effects
on viruses such as herpes simplex virus-1 (Binns et al., 2002), the lack of impact on RV
replication further supports the notion that Echinacea exerts most of its effect through the
host immune response. No effect of Echinacea on IL-6 secretion was observed in
uninfected BEAS-2B cells. This result is surprising considering that many studies have
reported stimulatory effects of Echinacea on various immune cells and airway epithelial
cells (Brush et al., 2006; Brousseau & Miller, 2005; Currier & Miller, 2000; Goel et al.,
2005; Mozzaroni et al., 2005; Sasagawa et al., 2006; Sharma et al., 2006). It may be that
the extracts used were not concentrated enough to produce such stimulatory effects, or
some of the active constituents had degraded during storage. However, there was a clear
effect of Echinacea when administered to RV infected cells, although the results were
markedly different between RV serotypes. For RV14, E1 did not have any significant effect
on RV infected cells, while E2 treatment stimulated further IL-6 secretion from RV14
infected cells. However, both E1 and E2 treatment of RV1A infected cells completely
inhibited IL-6 secretion. If Echinacea confers its health benefit by inhibiting pro-
inflammatory cytokine secretion (and therefore cold symptoms) then in this case a health
benefit may be observed during RV1A infection, but not for RV14, where E2 could
arguably exaggerate the inflammatory response. Bearing this in mind, the evaluation of
Echinacea treatment of RV infected cells should consider not only the active constituent profile of the herb extracts but also the RV serotype responsible for infection.

The strength of my research is the characterization of RV infection for two different receptor-utilizing serotypes in two distinct airway epithelial cell models over the course of a typical infection. I have demonstrated that RV infection results in significant viral replication and pro-inflammatory cytokine/chemokine stimulation through both the ICAM-1 and LDLR pathways; however, the specific trends observed are dependent on both cell model and RV serotype choice. Most prior RV studies of this nature have been carried out by choosing one specific cell type and one RV serotype and sampling only at limited time intervals. Moreover, in comparing RV studies, many researchers treat RV serotype and cell choice as largely redundant factors, but my findings suggest that such broad comparisons may be of questionable validity. I have also supported the findings of Sharma et al. (2006), where IL-6 secretion from airway epithelial cells stimulated by Echinacea treatment was different between RV infected and uninfected cells, suggesting the involvement of largely unknown but complex biochemical pathways.

**Overall Significance and Future Studies**

My Growth Curve experiments strengthen the growing consensus that RV infection causes an increase in pro-inflammatory cytokine secretion that can be observed beyond peak viral replication. I demonstrated this effect under controlled conditions for two different airway epithelial cell models and for two different receptor-utilizing RV serotypes. These results offer a more comprehensive experimental examination than previously demonstrated and show that RV replication and stimulation of cell pro-inflammatory cytokine/chemokine secretion occurs for both the ICAM-1 and LDLR pathways. Future studies should consider utilizing other airway epithelial cell models and primary cultures, as well as additional RV serotypes. The UV experiments supported previous findings in BEAS-2B cells that genetically intact virus is necessary to induce a pro-inflammatory cytokine response for both RV14 and RV1A (Griego et al., 2000; Papadopoulos et al., 2001). Furthermore, my results demonstrated that quantitative real-time polymerase chain reaction (qRT-PCR) is capable of differentiating between UV treated and untreated RV14, an issue which is in dispute in the literature (Ma et al., 1994;
Myatt et al., 2003). Further studies should consider the effect of UV treated RV on the secretion of other chemokines and cytokines, and should also be conducted in the A549 cell model where some evidence of noninfectious RV stimulating IL-8 secretion exists (Johnston et al., 1998). My Echinacea experiments support the evidence of Sharma et al. (2006) that the effects of Echinacea on cytokine/chemokine secretion in airway epithelial cells differ between RV infected and uninfected cells. Therefore, future research should concentrate on interactions between RV (and possibly other pathogen) infected cells and Echinacea extracts in addition to investigating this herbal extract’s effects on uninfected “healthy” cells. Furthermore, considering the different trends observed between the two RV serotypes, it would be important to study the effects of Echinacea on cells infected with various RV serotypes, as it is possible that this extract may only confer protection against some RVs.

The development of cold preventions and therapeutics has proved itself a difficult task for scientists. Since RV cold symptoms have been linked to pro-inflammatory cytokine and chemokine secretion, immune-modulating compounds such as Echinacea may offer relief from symptoms (at least for some RV serotype infections) by inhibiting the release of these mediators. Although there is growing consensus that RV replication is not the cause of cold symptoms and that the virus causes little or no damage to the airway epithelium, it remains unclear whether RV replication is necessary to trigger the inflammatory response. Some evidence (including my own) indicates that genetically intact infectious RV is necessary to stimulate pro-inflammatory cytokine/chemokine secretion. Furthermore, in studies where noninfectious RV was capable of eliciting cytokine/chemokine secretion, those observed secretions were still not as pronounced as measured in their infectious RV control counterparts. For example, Johnston et al. (1998) found that UV treated (noninfectious) RV9 only stimulated 50% of the IL-8 secretion measured in infectious RV9 controls, thus even in this case infectious virus may have been necessary to induce at least part of the IL-8 response. Considering the above, the best RV drug formulation could potentially be a combination of immune-modulating compounds, such as Echinacea in addition to anti-viral compounds such as Tremecamra and Pleconaril (Turner et al., 1999; Zhang et al., 2004). The timing and mode of administration of such a drug would also be important in view of the relatively rapid onset of RV infection. Pills and capsules, which must first be
metabolized and biologically available in the bloodstream, may be of lesser benefit than preparations that could be applied directly to the affected tissues (e.g. nasal sprays). Overall, the development of an effective cure for the common cold would universally benefit all people, improving the well-being of the healthy, and potentially saving the lives of the vulnerable.

**Conclusions**

Described below are the general conclusions that can be drawn from this thesis in relation to the objectives and hypotheses proposed in Chapter 1.

**Chapter 2 (In Vitro Characterization of Rhinovirus Infection in Airway Epithelial Cells - Growth Curves):**

1. As hypothesized, RV infection did not cause any observable cell death or CPE in BEAS-2B or A549 cells supporting evidence that RV is not cytotoxic to airway epithelial cells.

2. As hypothesized, RV replicated at relatively low levels in airway epithelial cells (0 to 0.1 infectious virus particles per cell) when compared to permissive H1 cells (1-100 infectious virus particles per cell). Furthermore, RV14 failed to replicate in the alveolar A549 cells.

3. Significant RV replication was observed between D1 and D2 post-infection for all cell lines (H1, BEAS-2B and A549). No significant replication was detected from D3 onward. The RV replication trends observed supported the hypothesis that viral replication would peak post-infection and gradually decline to control levels over time.

4. RV infection stimulated pro-inflammatory IL-6 and IL-8 secretion from airway epithelial cell lines. Maximum cytokine/chemokine levels were typically measured between D2 and D7 and, once stimulated, usually remained elevated (relative to control) over the course of infection. IL-6 and IL-8 levels did not typically decline by D7 as was originally hypothesized.

5. Considering that RV replication was no longer detectable after D2, my results support the hypothesis that it is the pro-inflammatory cytokines/chemokines that are responsible for cold symptoms which typically last for at least one week.
6. As hypothesized, the bronchial BEAS-2B cells were more susceptible to RV infection than the alveolar A549 cells. This may suggest that bronchial cells derived from higher in the airway epithelium are more susceptible to infection than alveolar cells.

7. In contrast to what was hypothesized, RV1A produced more pronounced effects than RV14 in terms of viral replication and IL-6/IL-8 secretion in the airway epithelial cells suggesting substantial differences between RV serotypes.

Chapter 3 (The Effects Ultraviolet Inactivated Rhinovirus on Airway Epithelial Cells):

8. Contrary to what was hypothesized, UV treatment of RV14 and RV1A completely inhibited viral replication and IL-6 secretion in BEAS-2B cells, indicating that genetically intact (infectious) virus was necessary to stimulate this response.

Chapter 4 (The Effects of Echinacea Extracts on Rhinovirus Infected and Uninfected Airway Epithelial Cells):

9. As hypothesized, Echinacea treatment had no effect on RV replication suggesting that Echinacea does not affect the RV replication cycle.

10. In contrast to what was hypothesized, Echinacea did not stimulate IL-6 secretion from uninfected BEAS-2B cells; however, as hypothesized Echinacea did affect IL-6 secretion in RV infected cells suggesting complex interactions between Echinacea extracts and RV infected cells.

11. Unlike the original hypothesis, different effects were observed for RV14 and RV1A; therefore, Echinacea may not confer benefit against all RVs.

12. As hypothesized, the two distinct Echinacea extracts produced different IL-6 results indicating that the effects of Echinacea may depend on their chemical profiles.

Understanding the pathogenicity of RV infection is crucial in treating the common cold and further elucidating its role in respiratory disease. Furthermore, the evaluation of cold treatments, such as Echinacea, helps the public and health practitioners make more
informed treatment decisions. My *in vitro* characterization of RV infection strengthens the current scientific knowledge of this viral infection, and such studies offer a backbone from which further *in vivo* studies and clinical trials may be developed.
REFERENCES


APPENDIX B: Growth Curves Experimental Design

Cells grown to confluence

Cell media aspirated and replaced with RV inocula or medium alone (control):

Infection: 1 hour at 35°C

Washes (to remove exogenous RV)

Replace medium (1% FBS) and incubate (35°C)

At given time-point (D0-D7) take samples:

1. IL-6/IL-8: Supernatants removed, centrifuged, and stored at -20°C for ELISAs (n=3 controls, n=3 RV)

2. Infectious Virus: RV infected cells scraped into medium and stored at -80°C for plaque assays (n=4)

3. RV14 RNA: Media aspirated, cells washed with phosphate buffered saline, TRIZOL reagent added, stored at -80°C for RNA extraction (n=2)
APPENDIX C: Effect of Purified and Unpurified Rhinovirus Inocula on Viral Replication and Cytokine/Chemokine Secretion from BEAS-2B and A549 Cells.

RV stock solutions used in all experiments were harvested from lysed H1 cells which were clarified by centrifugation at 10,000 x g in order to remove cellular debris and produce concentrated RV containing stocks suspended culture medium. However, these RV stocks were not purified from other small cellular components (e.g. soluble proteins, organelles) which could have potentially affected cytokine/chemokine secretion, independent from virus, when administered in RV inocula during experiments. Therefore, the effects of purified and unpurified RV on IL-6 and IL-8 secretion were compared in order to confirm that the use of unpurified RV stocks in experiments was valid, and that any observed effects on cytokine and/or chemokine secretion were a result of virus only (and not other H1 residual cellular components).

Methods: RV14 and RV1A stocks (harvested from H1 cells) were aliquoted into sterile tubes (0.5mL each) and ultracentrifuged under vacuum at 100,000 x g in order to pellet the virus. These virus pellets were then either re-suspended in their original culture medium (unpurified) or fresh medium (purified). The purified and unpurified RV stocks were then used to infect BEAS-2B and A549 cells (MOI=1) using standard infection procedures (n=6). The effects of unpurified/purified RV on viral replication and IL-6 (for BEAS-2B) or IL-8 (for A549) secretion after 48 hours were ascertained by plaque assays and ELISAs.

Results: There were no statistically significant (α=0.05, 1-way ANOVAs and post-hoc Tukey tests) differences found between purified and unpurified RV inocula on infectious virus in BEAS-2B or A549 cells 48 hours post-infection (Figures C1 and C2). Furthermore, there were no significant differences (α=0.05, 1-way ANOVAs and post-hoc Tukey tests) found in IL-6 or IL-8 stimulation in the BEAS-2B and A549 cells respectively between purified and unpurified RV inocula 48 hours post-infection (Figures C3 and C4).
**Figure C1:** Effect of purified and unpurified RV14 and RV1A on infectious rhinovirus in BEAS-2B cells 48 hours post-infection. RV stocks were pelleted by ultracentrifugation and virus was either re-suspended in fresh medium (pure) or in its original medium (unpure). No statistical differences ($\alpha=0.05$) in infectious virus were observed between purified and unpurified RV inocula for either RV14 or RV1A (n=6).

**Figure C2:** Effect of purified and unpurified RV14 and RV1A on infectious rhinovirus in A549 cells 48 hours post-infection. See Fig. C2 legend for further details.
**Figure C3:** Effect of purified and unpurified RV14 and RV1A on IL-6 secretion from BEAS-2B cells 48 hours post-infection. RV stocks were pelleted by ultracentrifugation and virus was either re-suspended in fresh medium (pure) or in its original medium (unpure). No significant differences ($\alpha=0.05$) in IL-6 secretion were observed between purified and unpurified RV inocula for either RV14 or RV1A ($n=6$).

**Figure C4:** Effect of purified and unpurified RV14 and RV1A on IL-8 secretion from A549 cells 48 hours post-infection. See Fig. C3 legend for further details.

**Conclusion:** There is no difference observed in: infectious virus (replication), IL-6 secretion from BEAS-2B cells, or IL-8 secretion from A549 cells between unpurified or purified RV14 and RV1A inocula. Therefore, the effects on cytokine/chemokine secretion observed in both cell lines are attributable to the virus and not other H1 cell proteins and/or remnants derived from RV harvesting protocols. Overall, the use of unpurified (clarified) RV stocks as inocula is experimentally valid.
APPENDIX D: Cell Counts for Rhinovirus Infected and Uninfected BEAS-2B and A549 Cells.

Cell counts were conducted for uninfected and RV infected A549 and BEAS-2B cells on D0, D2, and D7 post-infection in order to assess whether RV infection had any effect on cell growth and number, and to confirm that cell numbers remained relatively unchanged once cultures had reached confluence. This experiment was carried out to confirm that changes in cell number were not contributing factors in RV replication, RV14 RNA, and cytokine/chemokine data.

Methods: BEAS-2B and A549 cells were grown to confluence under standardized conditions (see Chapter 2) in 24 well plates. Supernatants were then aspirated and inocula of: RV14, RV1A or medium alone were added and allowed to infect for 1 hour at 35°C. After infection, cells were washed with DMEM and fresh culture medium was added to the wells (1% FBS). At D0, D2, and D7 post-infection cells were trypsinized, suspended in 1mL of fresh medium, and counted by hemacytometer (with trypan blue exclusion for dead cells) with a 10μL loading volume (n=6).

Results: There were no statistical differences (α=0.05, 2 way ANOVAs, Dunnet’s Test) found in number of cells between any of the treatments (Control, RV14, or RV1A) at any of the sampling time-points for either the BEAS-2B or A549 cell lines (Figures D1 and D2). Furthermore, cell number remained fairly constant from D0 to D7 in RV infected and control cells. There were very few dead cells present and supernatants contained virtually no cells. Anecdotally, the culture medium for RV14 and RV1A infected cells did appear more acidic than that of the uninfected BEAS-2B and A549 cells.
Figure D1: Cell counts for rhinovirus infected and uninfected BEAS-2B cells over time. No significant differences (α=0.05) in cell number were found between uninfected and RV infected cells at any time (n=6).

Figure D2: Cell counts for rhinovirus infected and uninfected alveolar epithelial A549 cells over time. See Fig. D1 legend for further details.

Conclusion: Infection of airway epithelial cells with RV14 or RV1A had no effect on cell number when compared to controls and cell number remained quite constant for the duration of the sampling period for both BEAS-2B and A549 cell lines. Therefore, changes in cell number did not affect to RV replication, RV14 RNA, or cytokine/chemokine secretion data in my experimental designs.
APPENDIX E: H1, BEAS-2B, and A549 Cell Counts at Confluence

Cell counts in 6-well plates at confluence for H1, A549, and BEAS-2B cell lines were conducted in order to determine calculation of viral infection dose (MOI=1) for RV infection experiments.

Methods: Cells were grown under standardized conditions (3mL DMEM or 50:50 DMEM/F12 with 5-10% FBS) at 35°C for until freshly confluent in 6-well plates. After 48 hours, supernatants were aspirated, cells trypsinized, diluted 5:1 in culture medium and counted by hemacytometer (n=5) with trypan blue exclusion to stain dead cells with a loading volume of 10μL.

Results (Figure E1): Cell count at confluence was significantly (α=0.05) higher in H1 cells by 1.4-fold compared to BEAS-2B and A549 cells but no difference in cell number was observed between the airway cell lines (1-way ANOVA and post-hoc Tukey test). Cell counts were (in number of cells/well): H1=1.9 \times 10^6 \pm 3.3 \times 10^4, BEAS-2B=1.3 \times 10^6 \pm 8.2 \times 10^4 and A549=1.4 \times 10^6 \pm 8.5 \times 10^4. All cell counts were at an order of magnitude of 10^6 cells per well. RV infection doses of 1 infectious viral particle per cell (MOI=1) in all RV infection experiments were based on these cell counts.

Figure E1: Cell counts for uninfected H1, BEAS-2B and A549 cells at confluence. An asterisk (*) indicates that H1 cell counts at confluence were significantly higher (α=0.05) than BEAS-2B and A549 cell types, although all cell numbers were at a 10^6 order of magnitude.
APPENDIX F: Rhinovirus Stability

The stability of RV14 and RV1A under typical experimental conditions was ascertained, in order to determine whether infectious virus assayed at given Growth Curve sampling times (D0-D7) represented daily secretions or accumulated RV secretions up to sampling day.

Methods: RV14 and RV1A stocks diluted with culture medium (DMEM) and 1% FBS to an initial starting concentration of $10^{4.5}$ pfu/mL and aliquoted into sterile 6-well trays (3mL of RV solution per well) in order to mock typical Growth Curve conditions (n=4). Samples were taken daily, frozen at -80°C, and assayed for infectious virus (pfu/mL).

Results: For both RV14 (Figure F1) and RV1A (Figure F2) infectious virus decreased significantly ($\alpha=0.05$, 1-way ANOVAs and post-hoc Tukey tests) from day to day until it was no longer detectable on D4 for RV14, and somewhere between D4 and D7 for RV1A. However, the initial concentration of RV1A (D0) was higher than for RV14 probably accounting for time difference in degradation. Overall, infectious virus decreased by 1-2 orders of magnitude daily for both RV14 and RV1A.

Figure F1: RV14 Stability at 35°C under mock Growth Curve conditions over 7 days. Symbols that differ (a, b, c, d) indicate significant differences ($\alpha=0.05$) in infectious virus between sampling times (n=4). Infectious virus was undetectable at D4 and D7.
Figure F2: RV1A Stability at 35°C under typical experimental conditions over 7 days. Symbols that differ (a, b, c, d, e) indicate significant differences (α=0.05) in infectious virus between sampling times (n=4). Infectious virus was undetectable on D7.

Conclusion: In culture medium, RV14 and RV1A stock solutions undergo daily degradation of 1-2 orders of magnitude. However, RV remains detectable for at least 3 days under these conditions depending on initial RV concentrations. Therefore, Growth Curve replication data following the initial peak in infectious virus (usually around D1) could represent gradually degrading virus synthesized on D1, or a combination of accumulated and newly synthesized RV. Furthermore, this experiment does not take into account the possibility that RV contained within cells is protected from the degradation observed in the extracellular environment. Additionally, in culture systems cells may secrete factors which facilitate the degradation of RV beyond what can be observed in culture medium alone.
APPENDIX G: Interleukin-6 and Interleukin-8 Stability

The stability of IL-6 and IL-8 under typical experimental conditions was assessed in order to determine whether cytokine/chemokine assayed at given time-points (D0-D7) in experiments represented daily secretions or accumulated IL-6/IL-8 secretions up to sampling day.

Methods: Cytokine secretion was stimulated in BEAS-2B cells by infection with RV1A. After 72 hours supernatants were collected, combined, centrifuged at 1000 x g to remove cellular debris, re-distributed to sterile 6-well plates (3mL per plate), and incubated at 35°C. Samples were collected immediately (D0) and seven days after re-distribution (D7), stored at -20°C, and assayed by ELISA (n=3).

Results: Both IL-6 (Figure G1) and IL-8 (Figure G2) were stable under the given experimental conditions up to D7 post-incubation at 35°C (α=0.05, 1-way ANOVA and post-hoc Dunnet’s test).

![Figure G1: IL-6 stability at 35°C under typical experimental conditions over 7 days. No significant differences (α=0.05) in IL-6 concentration were observed between D0 and D7 (n=3).](image)
Figure G2: IL-8 stability at 35°C under typical experimental conditions over 7 days. See Fig. G1 legend for further details.

**Conclusion:** Experimentally measured IL-6 and IL-8 secretions most likely represent accumulated cytokine/chemokine levels up to the specified day of sampling (unless the cells secrete factors which degrade IL-6 and IL-8). This model is the typical model used for cytokine/chemokine secretion. Alternatively, one could remove and replace the medium in cell cultures daily; however, this is not usually done because of the problems that it may cause. For example, daily removal of supernatant would also remove any secreted virus in the medium thus interfering with replication data samples. Furthermore, the addition of fresh daily medium may disturb cells, and addition of new nutrients may encourage cell growth and other cellular changes.

Modification of the data to represent “daily” secretions by subtracting values from the previous sampling day was done; however, for the most part this did not affect overall trends. Figure G3 shows an example of such a data modification for trial 1 IL-6 secretion of the RV1A/BEAS-2B Growth Curve (originally Figure 2.3b). The overall trend is similar with maximum IL-6 secretion occurring on D5 and D7; however, considering that these sampling times may represent accumulated IL-6 over 2 days the actual daily secretion would likely be lower (possibly half this value). This could result in a much less pronounced effect on D5 and D7; however, these values would remain the maximum increases in IL-6 secretion relative to their controls.
Figure G3: Modification of Trial 1 BEAS-2B Growth Curve IL-6 data to show “daily” (day - previous day) secretion. RV and control IL-6 data from the trial 1 BEAS-2B Growth Curve with RV1A (originally Figure 2.3b) were modified by subtracting each daily value from its corresponding previous sampling time in order to show secretions for a particular day (versus accumulated IL-6 up to sampling point). An asterisk (*) indicates a significant difference (α=0.05) in IL-6 secretion between treatments at a particular time.
APPENDIX H: Image of Plaque Assay

Figure H1: Image of representative plaque assay. This image depicts a completed plaque assay which was treated with cell-staining 1% crystal violet in dH2O. Viral plaques appear as clear unstained circular areas while intact cells appear dark. One plaque corresponds to one infectious virus particle present in the assayed sample. Plaques per well were counted (multiplied by a dilution factor if applicable) and reported as pfu/mL.