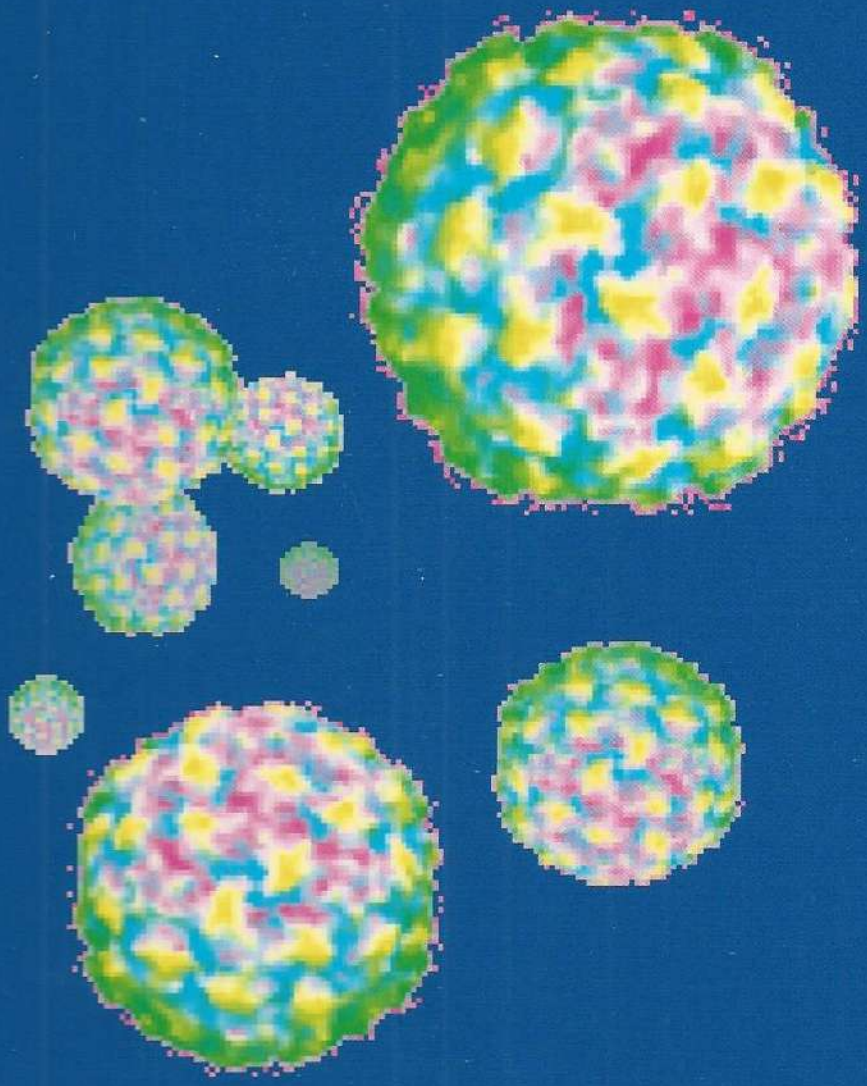
RHINOVIRUSES IN ASTHMA

AN EXPERIMENTAL APPROACH



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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 31 oktober 2000
te klokke 14.15 uur

door

Katrien Grünberg
geboren te Waddinxveen
in 1966

ISBN 90-9014241-X

Druk: Printpartners Ipskamp b.v., Enschede

Promotiecommissie

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The financial support for publication of this thesis by the following sponsors is gratefully acknowledged:

Nederlands Astma Fonds
MSD b.v.

The work presented in this thesis was made possible by the financial support of The Netherlands Asthma Foundation (Astma Fonds, grant 93.17).

The studies were carried out at Department of Pulmonology of the Leiden University Medical Center (Prof. dr P.J. Sterk) in close collaboration with the Departments of, Virology (Prof. dr W.J.M. Spaan) and Pathology (Prof. dr J.H.J.M. van Krieken) of the Leiden University Medical Center, and the Respiratory Virus Research Laboratory of the University of Wisconsin, Madison, Wisconsin, U.S.A. (Prof. dr E.C. Dick and Mrs C.R. Dick).

The financial support for publication of this thesis by the Stichting Longziekten Research, Leiden, AstraZeneca b.v., GlaxoWellcome and Novartis Pharma b.v., is gratefully acknowledged.

Voor Klaas, Julia en Lukas

CONTENTS

1 GENERAL INTRODUCTION AND AIMS OF THE STUDIES

1.1 General introduction

1.1.1 Asthma phenotype

Asthma is a chronic airway disease that affects all age groups. Its phenotype varies, and may change over the years, partly under the influence of lung growth during childhood and decline in lung function at older age (1). A definition of asthma symptoms and pathophysiological characteristics is therefore hard to give, but usually include episodic symptoms of dyspnea, chest tightness, breathlessness, wheezing and cough, with characteristic functional abnormalities such as variable and sometimes excessive airways obstruction, and airways hyperresponsiveness. Both structural and mechanical characteristics of the airways, as well as chronic airway inflammation are key factors in asthma pathogenesis (1). Variable expression is inherent to asthma. Acute symptoms may be incited by various bronchoconstrictive stimuli such as exercise, hyperventilation, breathing cold air, inhalation of irritant gases or fumes, or salicylates (1,2). Usually, inhaled β_2-agonists provide immediate relief for such symptoms. The severity of symptoms, the underlying dysfunction and inflammation may vary with the time of day (worst at night and in the early morning) (3), and may deteriorate from exposure to sensitizing stimuli such as indoor and outdoor allergens (4), ozone (5), certain low molecular weight chemicals (occupational asthma) (6) and respiratory virus infections (7).

1.1.2 Etiology

Asthma is a multi-factorial disease. A pattern compatible with a polygenic or oligogenic inheritance is substantiated by disease-associated polymorphisms or sequence variants on several different chromosomes (5q, 6p, 11p, 12q, 13 and 14q) (8). Interestingly, these genes are often co-located on chromosomes that contain genes for several cytokines that have been implicated in allergic airways inflammation in asthma (e.g. IL-3, IL-4, IL-5, IL-9, IL-12, IL-13) as well as the glucocorticoid receptor and the β_2-adrenergic receptor (8). It has become evident that the prevalence of atopic disease and asthma is vastly increasing worldwide (9,10). The rise in prevalence has been particularly striking in young children. Evidence suggests that asthma prevalence increases under the influence of environmental factors associated with the adoption of western life style (11). However, too little is known about these environmental factors to defy the asthma epidemic. At present, about 10-20% of the Western European population has asthma (12).

The combined contribution of genetic background and environmental factors can be translated into several risk factors for developing asthma (11,13). These include having a parent (particularly mother) with asthma, elevated IgE levels, associated atopic disease (hay fever, eczema), exposure to allergens (14), ranknumber among siblings (risk decreases with ranknumber) (15), lifestyle and nutrition (16). Low initial lung function (particularly in males) appears to be a risk factor for recurrent wheezing with respiratory tract infections in the first 3 years of life, but not thereafter (17). Similarly, passive smoking, by decreasing lung function, is a risk factor for asthma in infants, particularly in those of parents who have asthma (18).

How do genetic predisposition and environment interact? It appears that the Th_2-type response pattern which predisposes to IgE-mediated immune responses is the default pattern present at birth (13,19,20). Exposure to environmental stimuli (for example within the gastrointestinal tract), and repeated infections (either bacterial or viral) (21) from birth onward would, as it were, "educate" the immune system to deviate towards a Th_1-type response pattern (19,22). It is speculated that gene-environment interaction comes into play here. For example, genetic predisposition to enhancement of IL-12 production by monocytes in response to bacterial products (i.e. LPS), or promoting the production or responsiveness to IFN-γ (13) may promote the deviation towards a Th_1 type immune response (22). Alternatively, genetic predisposition to enhanced production of IL-4, or increased responsiveness to IL-4 would represent the other end of the spectrum, promoting the persistence of the Th2 type response pattern and atopic disease (22).

1.1.3 Pathology and pathophysiology

Airway inflammation is a consistent feature of asthma (1,23). The inflammation in asthma is of a chronic nature, and can already be found in the very mild stages of asthma (24). The extent of the airway inflammation varies with disease severity, and is associated with symptoms, variable airways obstruction and airway hyperresponsiveness (25). Pathologic features of asthma typically include epithelial shedding, increased collagen deposition beneath the basement membrane, the presence of a mononuclear infiltrate, mast cells and eosinophils in the bronchial mucosa throughout the bronchial and bronchiolar airway wall (23,25) and the alveolar parenchyma (26).

The mononuclear infiltrate consists predominantly of T lymphocytes. T helper cells (particularly the Th_2 subset), in concert with antigen presenting cells, and their cytokines have been shown to be important in orchestrating the airways inflammation in asthma (27-29), in which inflammatory cells (predominantly eosinophils, mast cells) and residential tissues (e.g. epithelium, airway smooth muscle) participate. T cells orchestrate eosinophilic infiltration, and promote growth of mucosal-type mast cells and immunoglobulin isotype switching towards IgE production (30). In turn, eosinophils and mast cells are also capable of producing cytokines (IL-4, IL-5) (30-32). Once activated, eosinophils are capable of secreting a number of lipid mediators and proteins, (eicosanoids and Platelet Activating Factor) and may release their cytotoxic granules proteins major basis protein, eosinophil-derived neurotoxin, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO)(31,32). Their toxicity to airway epithelium and other cells (33) is in keeping with reports of a correlations between levels of ECP within the airways and airway hyperresponsiveness and the clinical severity of asthma (25,34-36) (chapter 6). Mast cells are the major effector cells of the allergic (type I) hypersensitivity reaction. Classically, cross-linking of their high affinity IgE receptor by IgE-allergen complexes was described to lead to degranulation and secretion of various mediators implicated in the local anaphylactic reaction (37). However, it is now known that such mediator release may occur in response to various chemokines and non-immunological stimuli, implicating mast cells in non-allergic asthma as well as allergic asthma (38). In addition, various chemokines with strong chemotactic activity for eosinophils and basophils (eotaxin, IL-8, RANTES, MCP-1, MCP-3, MIP-1α and MIP-1β) are produced within the airways by several cell types, including epithelial cells and T cells (39). Thus, T cells, other inflammatory cells, antigen presenting cells, residential tissues, and their mediators, enzymes and adhesion molecules form an intricate network of interactions that regulate allergic airways inflammation.

Evidence suggests that the small, unmyelinated airway sensory nerves (C-fibers) are involved in allergic airways inflammation (40,41). Epithelial shedding may expose these fibers

to various endogenous and exogenous stimuli, which may induce cough and chest tightness (42,43), and cause the local release of various neuropeptides, including substance P and neurokinin A (40,42). These neuropeptides may subsequently cause airway smooth muscle contraction, microvascular leakage and vasodilation, leading to airway narrowing, which appear to be more pronounced in asthmatics as compared to normals (42,44-46). The latter phenomenon may in part be explained by an asthma-associated impaired function and/or expression of neutral endopeptidase, the neuropeptide-degrading enzyme, present in various tissues including the epithelium, which may be (partly) reversed by glucocorticoid therapy (47).

The inflammatory processes as summarized above contribute to the histopathological features of asthma, which include smooth muscle hyperplasia/hypertrophy (48-50), vasodilation, vascular leakage (51-53), edema of mucosa and adventitia (54), mucous gland hyperplasia (24), mucus hypersecretion, intraluminal mucus plugging and exudate. These, and possibly other structural changes are considered to be important in the detachment of the bronchioles from the parenchymal structures that provide the retractile force (54,55). Such detachment, together with smooth muscle hyperplasia/hypertrophy (48,49) and altered phenotype and/or functional characteristics of the smooth muscle (56) that may result in decreased relaxation (57,58), are considered to be key factors in airways hyperresponsiveness and (excessive) airway narrowing (54,55,59,60).

1.1.4 Exacerbations of asthma

Hospital admission rates for asthma and asthma mortality (and thereby much of the burden and cost of asthma) hold pace with - or exceed the increase in asthma prevalence, particularly in young children (10,61-63). Lack of accessibility to adequate health care for economically disadvantaged people should be considered as a possible cause (10). When asthma is treated according to current guidelines, therapy with (inhaled) corticosteroids is generally, though not always, effective in reducing asthma symptoms, variable airways obstruction, airway hyperresponsiveness, and airways inflammation (1,64,65). Yet, exacerbations of asthma, which may be severe, intractable, and potentially deadly may still occur, even in patients classified as having mild asthma (66-69), and even during vigorous treatment of asthma (70). This points towards a lack of control over asthma, and its exacerbations in particular (68,69), which suggests that there may be exacerbations of asthma that behave according to different mechanisms (70-72). It can be postulated that such exacerbations might be the ones associated respiratory virus infections.

Virus-associated exacerbations are an important cause for hospitalization for asthma at any age (73-76). Prospective population studies have confirmed what had long been recognized by patients and clinicians, namely that exacerbations of asthma are often associated with common colds: in 44% (in adults) up to 85% (in children) of the exacerbations of asthma a respiratory virus such as influenza -, para-influenza -, respiratory syncytial -, rhino-, or coronaviruses can be detected (7,77,78). Also, infections with chlamydia pneumoniae, an obligate intracellular pathogen, have been associated with exacerbations of asthma (79). Exacerbations of asthma are treated according to guidelines that do not take the cause of the exacerbation into account (1). This is understandable, as evidence on the effectiveness of existing treatment for (rhino)virus-associated exacerbations of asthma is scarce. Yet, the occurrence of severe and potentially deadly exacerbations indicates that there is room for improvement of therapy or prevention of exacerbations. Insight in the pathogenic mechanisms of virus-associated exacerbations might eventually lead to improvement of asthma control at any age, and especially in childhood, when the incidence of infections is highest (80). Improvement of asthma control may reduce the cost of asthma care and improve quality of life of patients with asthma.

Rhinovirus infections generally account for about half of the common colds and also for about half of the exacerbations of asthma associated with a virus infection at any age over one year old (7,77,78,80). Therefore, rhinovirus infections contribute significantly to asthma morbidity. Experimental rhinovirus infections, first in normals, and later in atopic non-asthmatics and mildly asthmatic patients have been shown to be feasible and safe. Its use has opened opportunities for description of the pathophysiologic and pathologic effects and the immunologic mechanisms of rhinovirus infections and the exacerbations of asthma associated with those infections, in well-characterized subjects, and under controlled circumstances. In addition, the use of experimental infections in asthma has allowed doing double blind, placebo-controlled testing of the effectiveness of pharmacological interventions against common colds and rhinovirus-associated exacerbations of asthma. Such experiments have expanded the knowledge of the effects and mechanisms of the common cold (reviewed in chapter 2).

The studies in the present thesis focus on the pathophysiologic and pathologic/immunologic effects of experimental rhinovirus 16 infection in patients with mild persistent asthma, and the effectiveness of prophylaxis with an inhaled corticosteroid. By doing so we aimed to clarify some aspects of monitoring, pathogenetic mechanisms, and treatment of rhinovirus-associated exacerbations of asthma.

1.2 Aims of the Studies

The studies in this thesis aim to address the following issues:

1. Accurate description of the functional changes during rhinovirus infections in asthma form the basis of interpretation of the pathophysiological mechanisms of exacerbations of the disease. Therefore, we aimed to describe the effect of experimental rhinovirus colds on symptoms of asthma, usage of rescue medication, lung function (*chapter 3*) and airway hyperresponsiveness (*chapter 4 and 5*) in patients with asthma.
2. Neurogenic mechanisms, involving small, unmyelinated airway sensory nerves (C-fibers), have been implicated in the development of virus-induced exacerbations of asthma. In order to investigate whether a rhinovirus cold would increase the sensitivity of airway sensory nerves, we examined the effect of rhinovirus colds on airway responsiveness to bradykinin, a sensory nerve stimulus, in patients with asthma (*chapter 5*).
3. In order to gain insight in the inflammatory and immunologic mechanisms of experimental rhinovirus infections in asthma, as a first step, we investigated the effects of rhinovirus colds on production and release of pro-inflammatory mediators in nasal lavage (*chapter 4*) and sputum (*chapter 6*) in relation to changes in lung function and airway hyperresponsiveness.
4. Next, with the same objective as given under aim 3, we examined the effects of rhinovirus colds on infiltration and accumulation of inflammatory cells in the bronchial mucosa of asthmatics, in relation to the accompanying changes in lung function and airway hyperresponsiveness *(chapter 8)*.
5. The adhesion molecule ICAM-1 promotes infiltration and migration of inflammatory cells and provides a costimulatory signal for T cell activation. It is also the receptor for 90% of the rhinovirus serotypes (including RV16). Its expression may be enhanced by various inflammatory conditions. Therefore, we investigated the effect of a rhinovirus cold on the bronchial epithelial ICAM-1 expression in asthmatics *in vivo* (*chapter 7*).

6. Finally, we addressed the issue of prophylaxis of virus-induced exacerbations in asthma, by investigating the effect of placebo-controlled pretreatment with the inhaled steroid, budesonide, on rhinovirus-associated effects on lung function, airway hyperresponsiveness, and accumulation of inflammatory cells in the bronchial mucosa (*chapter 8*), and on bronchial epithelial ICAM-1 expression (*chapter 7*).

The conclusions and implications of this thesis will be summarized in the chapters 9 (in English) and 13 (in Dutch). These chapters include recommendations for new perspectives of research in this area.

2 RHINOVIRUSES AND ASTHMA

Adapted from:
The effects of rhinovirus infections in asthma.
K. Grünberg
Eur Respir Review 1998; 8: 1122-1127.

and:
Rhinovirus infections: induction and modulation of airways inflammation in asthma.
K. Grünberg and P.J. Sterk.
Clin Exp Allergy 1999; 29: 65-73

2.1 INTRODUCTION

At present, about 10-20% of the Western European population has asthma (12), and prevalence increases (10). In spite of improvement of treatment in recent years episodic worsening may still occur, sometimes requiring hospitalization, and potentially leading to near-death or death. Respiratory virus infections, for which there is no cure, have been recognized as an important risk factor, if not cause for such exacerbations of asthma. Rhinovirus infections, the most common cause of colds, account for about half of the virus-associated exacerbations. These intriguing observations have incited a great research effort to unravel the pathogenetic mechanisms of virus-associated exacerbations of asthma, in order to find directions for new treatment strategies. Much progress has been made, but some work remains to be done to fill in the gaps and tie the evidence from all different directions together to a film of the pathogenesis of a rhinovirus-associated exacerbation of asthma. This review describes what is known about the effects of rhinoviruses in humans, with focus on the implications for asthma, thus providing a rough scenario of the events in the course of a rhinovirus-associated exacerbation of asthma.

2.2 RHINOVIRUS INFECTION: THE COMMON COLD

2.2.1 Virology

Rhinoviruses are part of the family of picornaviridae, which also includes enteroviruses. The 2 genera differ in that rhinoviruses, unlike enteroviruses, become instable and non-infectious in acidic environment (pH < 5). A hundred different rhinovirus serotypes have been characterized. Rhinoviruses are small (25 nm diameter) non-enveloped viruses with icosahedral (20-sided) shape, with a genome that consists of single stranded sense RNA (81). The multiplication cycle involves delivery of the RNA to the cytoplasm, mediated by binding to the specific receptor (ICAM-1 for 90% of the serotypes), followed by ribosomal RNA translation and formation of a polyprotein, which includes structural proteins, a proteinase, and an RNA polymerase. The polymerase transcribes the sense RNA, which is then followed by transcription of the antisense RNA in the endoplasmatic reticulum to produce new viral sense RNA strands. Concomitantly, structural viral proteins are being produced in the cytoplasm, which are assembled to capsids. RNA is packaged into these capsids to form new, mature infective virions. These virions are ultimately released by disintegration of the host cell. However, this production line has a large

spill in that it produces many defective, non-infectious particles (80). These particles may retain important functions (e.g. cell binding capacity), and could therefore be immunogenic but not infectious (80).

2.2.2 Rhinovirus epidemiology

Rhinoviruses account for 30% to 50% of all acute respiratory illnesses (80). Incidence gradually declines with age, and varies from 1-2/year in children to 0.25/year in people over 60 years old. Parents (particularly mothers) represent an exception to this rule, probably due to more intense exposure to the respiratory pathogens of their small children. Infections are prevalent throughout the year, but the incidence is highest in early fall and mid-to-late spring. Attack rates vary greatly with serotype, and range from 0 to 71%. Transmission can occur by hand contact (particularly when the hands are contaminated with fresh, infectious mucus) (82) but is most likely to occur by aerosol routes (83). Transmission is facilitated when an infected person has symptoms of a moderate to severe cold, sheds high amount of virus, and in situations where there is a high infected persons/non-infected persons ratio, and population density is high, such as can be found in, for example, a class room.

2.2.3 Host response

"It feels like it sounds:
A rhinovirus is a good indication of how it would be
to have a rhinoceros stuck up your nose"
Hanna Holmes, in: The Skinny On the Rambling Rhinovirus
(www.discovery.com/area/skinnyon/skinnyon980206/)

Most people are familiar with the symptoms of the common cold caused by a rhinovirus infection: sore throat, headache, runny nose, blocked nose, coughing and sneezing, malaise, sometimes low-grade fever (80). The incubation time is usually 2-3 days, but may vary from several hours up to 7 days. A rhinovirus cold in otherwise healthy people is a self-limiting disease, that usually resolves within a 9-11 days. However, in elderly people and nursing home residents the consequences of a rhinovirus cold appear to be more severe, leading to increased morbidity and mortality (84-88). The same holds true for young children (73,89). Rhinovirus infections may be complicated by sinusitis (90,91), elevated middle ear pressure (92) and acute otitis media (93-96), and even pneumonia (73,97-100). Moreover, rhinoviruses may cause interstitial pneumonitis in immunecompromized hosts (101). There is evidence that personality, stress, and life events prior to rhinovirus exposure increase the susceptibility to a symptomatic colds (102-104). Rhinovirus as the underlying viral pathogen during bacterial superinfection increases the risk of failure of antibiotic treatment (105,106). A rhinovirus cold in a carrier of bacteria such as multiple resistant staphylococcus aureus (MRSA) may enhance bacterial spreading (107). Thus, it can be concluded that host factors are important determinants for the severity of a rhinovirus infection.

2.2.4 Therapies and remedies against the common cold

A range of therapies and preventive strategies for the common cold have been studied (108-125)(see also review (80)). Some treatment modalities have been shown to be more or less effective in controlled studies (109,111,112,114-119,121,123-136). However, side effects and relatively poor effectiveness once the infection has manifested itself, together with high price of newly developed compounds, have so far precluded widespread use of such treatments. New treatment strategies for rhinovirus infections aim at blocking the binding to ICAM-1. This may

be accomplished by providing a surplus of pseudoreceptors, such as soluble ICAM (125,137), or by blocking the binding site using so-called pocket factors (138). Until an effective and affordable cure for the common cold becomes available, it is likely that all kinds of drugs to alleviate cold symptoms will remain "big business". In this respect, it is interesting to note that cheap and widely available "old-fashioned" remedies such as the intake old high dose of vitamin C (80,139) applying warmth by inhaling hot steam (111,140) or even drinking hot beverages like chicken soup (141) might not be completely without benefit for patients with common colds.

2.3 EPIDEMIOLOGY OF RHINOVIRUS COLDS AND PULMONARY DISEASE

2.3.1 Asthma

Several clinical and epidemiological studies have described a close temporal association of respiratory virus infections with asthma exacerbations (77,78,142-144) (also reviewed in (7)). Respiratory viruses can be identified in 10 to 44% of the asthma exacerbations in adults (77,144), while in children identification rates vary from 26 to 83% (78,143,145,146). In the two most recent studies the use of polymerase chain reaction technique to detect rhinovirus and coronavirus, rather than the less sensitive techniques that were used previously, has resulted in the highest identification rates so far (77,78). Among the various respiratory viruses identified, rhinovirus predominates in most of these studies, accounting for about 50% of the detected viruses (77,78,143,145,146). The incidence of rhinovirus infections may even be higher in asthmatic patients as compared to non-asthmatic subjects (7,147), although rhinovirus shedding in the absence of cold symptoms does not seem to be associated with clinical worsening of asthma (146,148). Emergency room visits and hospitalization for asthma exacerbations, and even asthma deaths (149) are associated with rhinovirus infection, both in children (73-75) and adults (74,76). This underlines that a common cold is not a harmless condition in patients with asthma.

2.3.2 Other pulmonary diseases

Not only asthmatic patients suffer from rhinovirus-induced exacerbations of their disease. Rhinovirus colds are also associated with worsening of a number of other diseases of the respiratory tract, such as COPD/emphysema (87), broncho-pulmonary dysplasia (150-152) and cystic fibrosis (153), although evidence seems to be inconclusive in the latter disease (154). Taken together, this indicates that rhinovirus infections may have a causal role in exacerbations of pulmonary disease, and asthma in particular. As rhinovirus infections also appear to cause mild lower airways disease in healthy people, the question can be raised which (if any) disease-specific factors are involved in the development of exacerbations of airways diseases.

2.4 RHINOVIRUS INFECTIONS IN ASTHMA: THE EXPERIMENTAL MODEL

As rhinoviruses are largely specific to the human species, animal models (mice, chimpanzee) could only be used to investigate certain aspects of rhinovirus-induced pathology (137,155-159). The introduction of experimental infections, first in normals, then in atopic patients, and later in asthmatic patients filled this gap, and allowed further investigations into mechanisms of rhinovirus-induced exacerbations of asthma, linking them to functional consequences. Such a model allows careful patient selection and monitoring, and intensive assessment of the rhinovirus-induced effects under controlled circumstances and timing. Experimental infections have been shown to be a useful tool for investigating the effects of rhinovirus infections in allergic disease or asthma (35,36,148,160-173). Thus, the effects of a rhinovirus infection can

be assessed at the level of asthma symptoms, use of asthma medication, parameters of airway physiology, such as airways obstruction and hyperresponsiveness, as well as the underlying inflammation. Furthermore, it allows the double blind, placebo-controlled studies of the effects of pharmacological interventions on common colds (see above: therapies and remedies against the common cold) or the exacerbations of asthma associated with those colds.

2.5 Effects on lung function

So far, experimental rhinovirus infections in patients with asthma and/or atopic rhinitis have not shown to induce a significant change in airways obstruction (as reflected by FEV_1) when measured during laboratory visits (148,160,162-164,166,167,170,172). This has been considered to be reassuring in terms of patient-safety in the experimental model. However, frequent home-recordings of FEV_1 (3 times daily), appeared to decrease in atopic asthmatic patients in the acute phase of an experimental RV16 infection (165) *(chapter 3)*. Indeed, the maximal decrease in FEV_1 in the acute phase of the infection, expressed as percentage of the recent personal best, correlated significantly with the observed increase in airway hyperresponsiveness (165). This suggests that there is transient worsening of airways obstruction after rhinovirus infection in asthma, which may improve spontaneously during the day, or as a consequence of repeated deep-breath maneuvers as are being performed in the lung function lab., pointing at either increased sensitivity to bronchoconstrictive stimuli and/or a reduced bronchodilating effect of a deep breath (57).

2.6 Effects on airway hyperresponsiveness

The effects of experimental rhinovirus infection on airway responsiveness vary among different studies, using different rhinovirus serotypes. In normals, experimental rhinovirus infections have not been shown to induce airways obstruction or airways hyperresponsiveness (167,174-176), although a small but consistent increase in the maximal bronchoconstrictive response to methacholine after rhinovirus 16 (RV16) infection was recently reported by de Kluijver *et al.* (177). In non-asthmatic patients with atopic rhinitis Lemanske *et al.* and Gern *et al.* have demonstrated an enhanced hypersensitivity to histamine and allergen challenge after experimental RV16 infection (162,170), which was significantly different from the lack of response in normals (170). However, others have not observed such an effect when using rhinovirus 16 (172) rhinovirus 39 (178) or Hanks strain rhinovirus (171). In asthmatic subjects, Halperin *et al.* has found increased hypersensitivity to histamine in only 4 out of 22 subjects after experimental rhinovirus (serotype 39 and HH strain) infection (160). Moreover, Cheung *et al.* have shown that rhinovirus 16 (RV16) increases asthma symptoms, coinciding an increase in the maximal bronchoconstrictive response to methacholine up to 15 days after infection (163), pointing towards the potential of excessive airway narrowing due to a rhinovirus infection. However, airways sensitivity to methacholine was not shown to be affected by RV16 infection in these patients (163,172). In a similar placebo-controlled design, we have shown a significant increase in airway sensitivity to histamine in asthmatic subjects after RV16 infection, which was most pronounced in those subjects who had severe cold symptoms *(chapter 4)* (148). In addition, experimental RV16 infection was shown to abolish tolerance to a repeated bradykinin challenge in asthma, suggestive of the involvement of airway sensory nerves *(chapter 5)* (166). Evidence suggests that RV16 colds in non-asthmatic patients with allergic rhinitis potentiate the allergen-induced enhancement of both airway responsiveness and airway inflammation (168). In turn, recent allergen exposure may modify the course of infection (169,179). Taken together, these data indicate that patients with asthma and/or atopic rhinitis may suffer from more severe

pathophysiological consequences of a rhinovirus infection as compared to non-atopic non-asthmatic subjects, suggesting an bi-directional interaction of virus-induced airways inflammation and features of the underlying disease, such as altered airway geometry (180) and airways inflammation (24,181).

2.7 Antigen presentation and T cell-mediated immune response

2.7.1 T cell activation and proliferation

Rhinovirus does appear to replicate inside monocytes and airway macrophages (182). However, the uptake of infectious rhinovirus particles by antigen presenting cells (either or not mediated by ICAM-1) may increase non-specific T cells activation (183). This is reflected by the increased expression of the activation marker CD69, but not CD25 on the cell membrane (183), and spontaneous (184,185) or mitogen-induced secretion of IL-2 and IFN-γ (183,186). In addition, from three weeks after infection onward, antigen-specific lymphocyte proliferation could be demonstrated (186-188), indicative of a T cell-mediated immune response, generated against T cell epitopes that are present within the viral capsid (158). Such CD4+ cells may be either specific to serotype-restricted or shared viral epitopes (156,158,184,189), which is likely to increase the chance of rapid and potent recall immune responses, and might explain the resistance to a rhinovirus infection during an ongoing rhinovirus infection (36,80).

2.7.2 Monocytes modulate T cell response

Incubation of either infectious or inactivated rhinovirus particles with a mixture of antigen presenting cells and lymphocytes induces a proliferative response to rhinovirus (184), while concomitantly hampering the monocytes-induced, ICAM-1 mediated specific T cell proliferation and cytotoxicity to other antigens (190,191). Natural killer cell activation and cytotoxicity, which is less dependent on ICAM-1/LFA-1 interaction, was not shown to be affected, or even increased after *in vitro* inoculation (186,190,192). As the ICAM-1 binding sites for LFA-1 and rhinovirus overlap partially (193), it has been postulated that binding of rhinovirus to ICAM-1 may interfere with ICAM-1/LFA-1 interaction (190,193,194). Dendritic cell- or B-LCL-induced allogeneic T cell response critically depend on ICAM-1/LFA-1 binding, yet, rhinovirus alone did not inhibit such a response (191), whereas this could be achieved in dendritic cells by the culture supernatant of rhinovirus stimulated monocytes (191). Indeed, rhinovirus binding to monocytes induces the production of high levels of IL-10, which may account for inhibition of the accessory function of both monocytes and dendritic cells, and inhibition of IL-12 production, although the latter effect is in part mediated by rhinovirus stimulation itself (191). It has been postulated that dampening of allogeneic cellular immune responses in the course of infection might predispose to secondary infections (e.g. otitis media) and failure of antibacterial treatment (105,106).

2.7.3 Non-specialized antigen presentation cells

MHC class I and II molecules can be expressed on respiratory epithelial cells (195). Recent evidence suggests that rhinovirus infection upregulates the expression of MHC class I molecules and costimulatory molecules, implying a role for the epithelium as antigen presenting cells during rhinovirus infection (196-198). Moreover, eosinophils, when expressing ICAM-1, may bypass specialized antigen presenting cells by acting as antigen presenting cells, inducing rhinovirus-specific CD4+ cell proliferation and enhancing IFN-γ production (185). This may contribute to viral clearence, but on the other hand, it has been postulated that such T cell activation might enhance pre-existing airway inflammation (183).

2.7.4 *Disease-specific differences*

In a comparative study, using *in vivo* experimental RV39 infections, patients with allergic rhinitis appeared to have lower numbers of T helper cells (either or not activated), and less RV39-induced peripheral blood mononuclear cell proliferation as compared to normals (188), while only in the rhinitis patients an increase in proliferation to RV39 was noted as early as during the acute phase of the infection. Furthermore, the decrease in NK-activity was less marked in the rhinitis patients as compared to the normals (188). This suggests that there may be disease-related differences in T cell-mediated immunity. It can be speculated that, like in mice, such differences are associated with the major histocompatiblity haplotype of the host (158). However, it is presently unclear as to how these differences might be interpreted in terms of mechanisms of rhinovirus-induced exacerbations of asthma.

2.8 HUMORAL IMMUNE RESPONSE

Immunoglobulins, in contrast to T cell mediated immunity, are by definition highly specific for rhinovirus serotypes (81). The Fab fragment fits only partly into the rhinovirus canyon, but binds to the VP2 region in upper part of the canyon (81,199-202), thus preventing binding to ICAM-1 (199,201). In normals, the level of protection against infection, or at least cold symptoms is related to levels of nasally secreted IgA and circulating virus-specific antibodies (80,80,203,203-205), although the immune system can be overwhelmed by high viral doses (80). There is evidence that atopic, and/or asthmatic subjects may be more susceptible to repeated infection with the same serotype, even in the presence of circulating antibodies (148,161).

2.9 ICAM-1 INTERACTION

2.9.1 *Role of ICAM-1*

ICAM-1, in its dual role as an intercellular adhesion molecule on antigen presenting cells, lymphocytes, eosinophils, mast cells, submucosal glands, airway smooth muscle and epithelial cells (185,206-209) and as the major rhinovirus receptor (210,211) which catalyzes rhinovirus uptake (212), has been the focus of much attention in rhinovirus-induced inflammation. Asthmatic patients have enhanced expression of ICAM-1 in their bronchial epithelium (213,214). Moreover, experimental rhinovirus colds have been shown to increase ICAM-1 expression on the basal cells of the bronchial epithelium, both in normals (177) and asthmatics (173) *(chapter 7)*.

2.9.2 *ICAM-1 binding and susceptibility to infection*

ICAM-1 binding to its ligands, lymphocyte function associated antigen (LFA-1) and macrophage-1 antigen (MAC-1, CD11b/CD18) is involved in the process of leukocyte adherence and migration, and in addition, may provide costimulatory signals for CD4+ cell and lymphokine-activated killer cell activation, T cell-mediated cytotoxicity, and T cell dependent B cell activation. It has been postulated that cytokines such as TNF-α, IL-1β, IFN-γ, and IL-4 may alter the expression, function or configuration of ICAM-1, thereby specifically promoting (TNF-α, IFN-γ, IL-4) or impeding (IL-6, IL-1) lymphocyte adhesion to, for example, cytokine-pretreated fibroblasts or primary tracheal epithelial cells, without affecting binding of rhinovirus particles (194,215,216), thus leaving the uptake of rhinovirus particles unaffected (212).

ICAM-1 expression is upregulated upon stimulation with rhinovirus in primary cultures of human nasal epithelial cells, pulmonary epithelial cell lines, submucosal gland cells and airway

smooth muscle cells *in vitro* (197,208,209,217-220). Upregulation of ICAM-1 by secondarily released cytokines such as IL-1α, IL-1β, and TNF-α facilitates rhinovirus infection in susceptible cell types (209,212,215,218). In contrast, IFN-γ increases ICAM-1 expression and rhinovirus binding in uninfected cells (194,215), while decreasing the susceptibility to infection (215,219) by downregulating ICAM-1 expression in infected cells (219).

Taken together, it seems likely that not only the amount of ICAM-1 expression, but also the local cytokine milieu, and the virus-induced production of cytokines are determinants for the susceptibility to rhinovirus infection.

2.10 EFFECT OF RHINOVIRUS ON AIRWAY EPITHELIUM

2.10.1 *Epithelial integrity*

In vitro rhinovirus infection of pulmonary or nasal epithelial cell lines does not cause a cytolytic infection or decrease cell viability (215,218,221-223), although cytopathic alterations and decreased viability may occur in primary bronchial epithelial cells (223). This is in keeping with observations in nasal epithelium *in vitro* (224) and *in vivo* (148), where infection may cause increased shedding of alive epithelial cells (225), ciliary defects (164), and impair mucosal clearence in the nose (226) (but apparently not in the trachea (227,228), in conjunction with mucus hypersecretion (229).

2.10.2 *Adhesion molecules*

Rhinovirus has various immunological effects on epithelial cells, fibroblasts and submucosal glands. As discussed, rhinovirus infection upregulates expression of MHC class I molecules and costimulatory molecules B7- and B7-1 (198), as well as the MHC class II costimulatory molecules ICAM-1 and VCAM-1 (196,197) on epithelial cells, thereby promoting antigen presentation as well as infiltration of inflammatory cells.

2.10.3 *Cytokines*

There is ample evidence that rhinovirus binding and/or infection of epithelial cells, fibroblasts and submucosal glands *in vitro* induces synthesis of a number of pro-inflammatory cytokines, such as GM-CSF, IL-11, IL-6, IL-1β, IL-1RA, TNF-α and the chemokines IL-8 and RANTES (209,215,218,221-223,230-233). Evidence suggests that the increased transcription is mediated in part by transcription factors such as NF-κB (223,231,234), which may be activated by oxidative species that are produced in response to rhinovirus infection (235). Interestingly, rhinovirus attachment to ICAM-1 or infection does not seem to be required for production of reactive oxygen species (236). Alternatively, double-strand (ds) RNA may stimulate secretion of chemokines such as RANTES, implicating the involvement of dsRNA-sensitive enzymes in the signaling process (237). Release of such pro-inflammatory cytokines provides strong stimuli for chemotaxis and migration of inflammatory cells towards the bronchial mucosa and the airway lumen, and may enhance cell activation and survival, thereby promoting the release of more cytokines and granule proteins from various inflammatory cells.

2.10.4 *Nitric oxide*

Levels of NO in exhaled air have been shown to rise after rhinovirus 16 infection in asthmatic subjects (238), possibly due to upregulation of iNOS (239). *In vitro*, NO has been shown to inhibit RV16 replication and cytokine protein synthesis in airway epithelial cells, thus providing a defense mechanism against infection (232,240), although this could not be confirmed when using RV serotype 39 (241). Indeed, during infection the elevated levels of exhaled NO were associated

with protection against the RV16-associated enhancement of airway hyperresponsiveness (238). It has been postulated that the role of NO (protective or harmful) may depend on its site of action, and thus on the NOS isoform present at that site (239,242). It is presently unclear which (if any) NO synthase isoforms (constitutively expressed *versus* inducible NOS) are affected (defected or induced) by rhinovirus infection.

In conclusion, the airway epithelial layer as the primary target of infection appears to provide a defense against rhinovirus contamination or infection, which, at the same time, may increase airways, inflammation.

2.11 Effect of rhinovirus on airway smooth muscle

Recent evidence suggests that *in vitro* incubation of airway smooth muscle with rhinovirus results in increased constrictor responsiveness to acetylcholine and attenuated β–receptor-mediated relaxation (208,243,244), which is associated with attenuated cAMP accumulation in response to a β-agonist, and upregulated expression and action of $G_{i\alpha 3}$ protein (208). These effects are similar to the effect of passive sensitization of the smooth muscle with serum of atopic asthmatic humans (245), and can be attributed to autocrine signaling, involving upregulated IL-5-mediated production and release of IL-1β (243-245). The above effects require at least rhinovirus binding to ICAM-1, as the effects could be prevented by blocking ICAM-1 (208,243,244). Moreover, ICAM-1 expression on smooth muscle increases after exposure to rhinovirus (208). These findings point towards airway smooth muscle as a potential player in increasing airway responsiveness and infiltration of inflammatory cells after a rhinovirus infection. However, it is uncertain as to whether viral penetration of the epithelium and submucosa required to reach the airway smooth muscle actually occurs in the *in vivo* situation.

2.12 Mediator release and cellular infiltration in vivo

2.12.1 Pro-inflammatory mediators

In humans, *in vivo* observations show elevated levels of various pro-inflammatory mediators in airways secretions during common colds, and are therefore in keeping with data from *in vitro* studies. These mediators include the cytokines GM-CSF, IL-11, IL-6, IL-1β, IL-1RA, TNF-α, and the chemokines IL-8 and RANTES eotaxin and MIP-1α in nasal secretions (172,223,229,230,246-253) *(chapter 4)* and IFN-γ, IL-5, IL-8 and IL-6 in sputum (35,172,254,255). In addition, a rhinovirus cold increases the levels of major basic protein and histamine in nasal lavage (247,253), albeit that the latter mediator was found to be elevated only in atopics (247). Moreover, sputum levels of the eosinophil granule protein ECP rise during an RV16 cold in asthmatic subjects (35).

2.12.2 Cellular infiltration

Fitting in with the observed release of chemoattractant mediators and increased expression of for example ICAM-1, rhinovirus infections have been shown to lead to infiltration of leucocytes, particularly neutrophils and mononuclear cells, into nasal secretions (248,256-258), rather than the nasal mucosa (256,259). Both in normals and asthmatic subjects this occurs in conjunction with an increase in the number of neutrophils in peripheral blood, whereas the number of circulating lymphocytes fall in the acute phase of infection (148,163,164,260). These effects, as well as the increase in airway sensitivity to histamine in asthmatic subjects are significantly related to the severity of the cold, as reflected by the cold

score (148,260) and increase in IL-8 in nasal lavage (148), underlining the relationship between the severity of the cold and the pathophysiological consequences (146,148).

Studies of bronchial biopsies show an elevation of T lymphocytes numbers in the bronchial mucosa of atopic asthmatic subjects following RV16 infection (164), which was marginal (164) or absent in normal subjects (36,261,262). Nonetheless, the more accumulation of CD3+ cells in the lamina propria, the bigger the increase in the maximal bronchoconstrictive response in normals, indicating that induction of airway inflammation may lead to abnormal airway function (261,262). However, in the asthmatic patients the increase in epithelial CD8+ cell number was associated with improvement of airway sensitivity to histamine after rhinovirus infection (36). Based on these two observations one could postulate that a rhinovirus infection causes airway inflammation that may interfere with the pre-existing airways inflammation in asthma.

With respect to eosinophils, different studies have now shown an increase of sputum ECP at 2 days after RV16 inoculation, correlating significantly with the increase in airway sensitivity to histamine *(chapter 6)* (35). Next, a small increase in the numbers of activated eosinophils (EG2+ cells) in the bronchial epithelium has been observed at 4 days after RV16 inoculation (164), and finally, we observed a trend towards a decrease in the number of EG2+ cells in both the epithelium and lamina propria at day 6 after inoculation *(chapter 8)*.

In view of the chemotactic properties of chemokines such as IL-8, RANTES and eotaxin on (memory/Th2) T lymphocytes, eosinophils, basophils, and neutrophils one can postulate that recruitment of T cells and migration of eosinophils towards the airway lumen is driven by such chemokines.

2.13 Rhinovirus infection of the lower airways

In the nose, rhinovirus replicates in the epithelium and the lymphepithelium of the adenoid (263), and can readily be detected by tissue culture or RT-PCR (264-269), or even *in situ* hybridization (270,271). The apparent lack of sensitivity of *in situ* techniques may in part be explained by the patchy distribution of the infection (272,273). Evidence of rhinovirus infection of the lungs has been more difficult to obtain. Although rhinoviruses can occasionally be cultured from sputum (274), tracheal brushings (167), and bronchoalveolar lavage (BAL) fluid (169,275), possible nasopharyngeal contamination precludes definite conclusions as to virus infection of the lower airways. The use of RT-PCR on BAL cells rather than BAL fluid increased the likelihood of detecting lower airways infection, and has led to a detection rate of 80%, while the detection rate in nasal lavage fluid in the same subjects was 100% after experimental RV16 infection (276). Only recently, the application of *in situ* techniques to detect the viral genome and its replicative strand in brochial tissue specimens (277,278) has strengthened the evidence for lower airways infection. In view of the difficulties in detecting rhinoviruses in the lungs so far, and their relative fastidiousness for culture conditions, particularly the usually relatively low optimal culture temperature of 33° C (279), a high grade infection of the lower airways may not be very likely. However, one could speculate that host factors such as increased ICAM-1 expression in the nasal (280) and pulmonary epithelium (206) or inclination towards a Th_2-type immune response (254) might increase the susceptibility of asthmatic and or atopic patients to symptomatic or prolonged rhinovirus colds (147), with (more severe) lower airways infection of the upper and lower airways, and its consequences.

2.14 Summary

In summary, experimental infections induce a mild exacerbation of asthma in atopic patients with mild persistent asthma, as indicated by increased variable airways obstruction *(chapter 3)* and airways hyperresponsiveness *(chapters 4, 5)*, which suggests an increase of airways inflammation. The upper airways infection of epithelial cells and lymphepithelium is followed by a local and a systemic cellular immune response *(chapter 4)*. In asthma, neither the infection nor the inflammatory response to the common cold was found to be limited to the upper airways, as enhancement of airways inflammation was observed in sputum *(chapter 6)* and bronchial tissue *(chapters 7, 8)*. The evidence points towards inflammatory cells (eosinophils, neutrophils, lymphocytes), antigen presenting cells, and residential tissues such as epithelium, airway sensory nerves *(chapter 5)* and airway smooth muscle as players that interact in the immune response. This response involves the release of nitric oxide and proinflammatory mediators such as cytokines, chemokines and granule proteins, both in the upper and lower airways *(chapter 4, 6)*. In addition, rhinovirus infections induce upregulation of adhesion molecules and co-stimulatory molecules, notably on epithelium *(chapter 7)*. The upregulation of ICAM-1 is of particular interest in view of its role as the major rhinovirus receptor and the potential for augmenting infection.

An important issue that remains to be resolved is the disease-specificity of virus-associated exacerbations in asthma with respect to both immunological/neurogenic effects and pathophysiological effects. In other words: which host factors predispose to – or protect against a (severe) virus-associated exacerbation of asthma? Next, more detailed insight in the involvement of various pulmonary tissues in the infection, the subsequent immune response, and the extra-cellular and intracellular signaling pathways might be helpful to generate hypotheses on specific new therapeutic interventions or tailoring existing treatment to the cause of the exacerbation. Rhinovirus infections in asthmatic patients in the experimental setting allow double blind placebo-controlled testing of such pharmacological interventions, thereby providing a valuable tool to investigate the usefulness of both existing anti-asthma drugs as well as newly developed compounds against rhinovirus infection or asthma.

3 EXPERIMENTAL RHINOVIRUS 16 INFECTION CAUSES VARIABLE AIRWAYS OBSTRUCTION IN ATOPIC ASTHMATIC SUBJECTS

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Am J Respir Crit Care Med 1999;160:1375-1380.

3.1 Abstract

Exacerbations of asthma are often associated with rhinovirus infections. However, it has not been investigated whether rhinovirus infection can induce variable airways obstruction in asthma. We examined the effect of experimental rhinovirus 16 (RV16) infection on daily home-recordings of FEV_1 in 27 non-smoking atopic, mildly asthma subjects, who participated in a parallel placebo-controlled study. The subjects recorded FEV_1 3 times daily from 4 days before- until 10 days after RV16 (n = 19) or placebo (n = 8) inoculation, using a microspirometer. In addition, symptoms of asthma and symptoms of common cold were scored. Airway hyperresponsiveness to histamine was measured 3 days before -, and on days 4 and 11 after RV16/placebo administration. Home-recordings of FEV_1 decreased significantly after RV16 infection, reaching a minimum at 2 days after inoculation (ANOVA: $p \leq 0.005$), which was significantly different from placebo ($p \leq 0.004$). In the RV16 group the lowest FEV_1 (expressed as % personal best) during days 0-3 after infection (mean ± SEM: 78.7 ± 2.6% vs. baseline: 85.6 ± 1.2, $p = 0.008$) correlated significantly with the cold score ($r = -0.47$, $p = 0.04$), asthma score ($r = -0.47$, $p = 0.04$), and with the decrease in airway hyperresponsiveness at day 4 as compared to baseline ($r = 0.50$, $p = 0.03$). We conclude that experimental RV16 infection augments variable airways obstruction in asthmatics. This favors a causative role of rhinovirus colds in asthma exacerbations, and is in keeping with rhinovirus-induced worsening of airway inflammation.

3.2 INTRODUCTION

Asthma is a chronic airway disease, characterized by episodic exacerbations of symptoms, often accompanied by transient airway obstruction and an increase in airway hyperresponsiveness to specific or non-specific stimuli (1). Prospective epidemiological studies in children as well as adults have shown that episodic falls in peak flow are often associated with respiratory virus infections, and rhinovirus infection in particular (77,78). This fits in with data obtained by experimental rhinovirus infections in asthmatic subjects, demonstrating an increase of both asthma symptoms and non-specific airway hyperresponsiveness, thereby suggesting that rhinovirus infections can indeed induce mild exacerbations of asthma (148,163). However, the development of variable airways obstruction has not yet been investigated following laboratory inoculation with rhinovirus. So far, several experimental studies have failed to demonstrate a significant increase in airways obstruction in asthmatics, as measured by spirometry under standardized laboratory conditions (148,163,166).

It can be argued that frequent (daily) measurements of peak flow or FEV_1 are more sensitive measures of variable airways obstruction than less frequent lab recordings of lung function (1). In asthma, such daily variability of airways obstruction is associated with the inflammatory changes within the airway wall (281), whilst it improves in response to anti-inflammatory treatment (282,283). There is evidence that experimental rhinovirus infections are associated with an increase in airways inflammation (35,164,169), characterized by cellular infiltration of the airway wall (164). Such cellular infiltration may be accompanied by release of proinflammatory mediators, potentially promoting vasodilation and vascular leakage, and airway wall swelling, which enhances airways obstruction (284,285). Therefore, in the present study we hypothesized that experimental rhinovirus infection augments variable airways obstruction in atopic, mildly asthmatic subjects.

To test this hypothesis, we examined the effect of experimental RV16 infection on home-recordings of FEV_1, in conjunction with cold- and asthma symptom scores in a placebo-controlled parallel study in subjects with atopic asthma. In addition, we also measured lung function and airway hyperresponsiveness to histamine under standardized conditions in the lung function laboratory.

3.3 METHODS

3.3.1 Subjects

Twenty-seven adult, non-smoking asthmatic subjects participated in the study. The patients' characteristics have been published previously (35,148), and are summarized in table 1. The subjects had not used inhaled or oral corticosteroids for at least 3 months preceding the study, nor had they used any other medication other than inhaled short-acting b_2-agonists on demand. They were atopic as determined by skin prick test to 12 common aeroallergens (at least 1 positive wheal, > 3 mm). The LUMC Medical Ethics Committee gave its approval for this study, and informed consent was obtained from all the subjects.

3.3.2 Design

The study had a parallel placebo-controlled design. Nineteen patients were inoculated with RV16 on two consecutive days (days 0 and 1), and 8 patients received placebo. Prior to the study the patients were screened for in- and exclusion criteria. Home-recordings of FEV_1, symptoms of common cold, and symptoms of asthma were noted by the subjects from 4 days before inoculation until 11 days afterwards. Airway sensitivity was measured by histamine challenge tests at 3 days before inoculation (day -3) and at days 4 and 11 after inoculation. Nasal lavage was performed and a blood sample was taken immediately before inoculation. Nasal washings were repeated at days 2 and 9, and a second blood sample was taken 4 weeks after inoculation.

Table 3.1: Subject characteristics

subject number	sex	age (yr.)	FEV_1[A] (% pred.)	PC_{20}[B] (mg/ml)	antibody titer[C] (before/day 28)	RV culture day 2/day 9
placebo						
1	M	25	76	0.28	<1 / <1	neg/neg
2	M	26	68	0.60	<1 / <1	neg/neg
3	M	18	71	0.64	32 / 32	neg/neg
4	F	26	75	0.78	8 / 8	neg/neg
5	M	25	73	0.80	16 / 8	neg/neg
6	M	24	80	1.37	128 / 128	neg/neg
7	M	24	89	1.85	1 / <1	neg/neg
8	M	25	87	1.93	1 / <1	neg/neg
mean±SEM			77.4±2.6	0.87±0.4		
RV16						
9	F	25	71	0.12	<1 / 4	pos/neg
10	F	23	77	0.25	<1 / 4	pos/neg
11	M	21	103	0.32	<1 / 4	pos/neg
12	M	18	80	0.34	<1 / 16	pos/pos
13	M	19	81	0.37	1 / 128	pos/pos
14	M	22	85	0.41	<1 / 32	pos/neg
15	M	20	87	0.71	<1 / 128	pos/pos
16	F	25	88	0.86	<1 / 1	pos/pos
17	M	26	108	0.87	<1 / 4	pos/neg
18	F	21	92	1.19	2 / 32	neg/neg
19	M	20	101	1.33	16 / 256	pos/pos
20	M	21	80	1.39	<1 / 64	pos/neg
21	F	24	89	1.76	8 / 512	pos/pos
22	M	22	89	2.03	4 / 512	pos/neg
23	F	23	77	2.12	<1 / 128	pos/pos
24	M	26	69	2.39	4 / 8	pos/neg
25	M	24	77	2.69	<1 / 32	pos/neg
26	M	21	90	2.98	<1 / 32	pos/pos
27	F	22	105	4.17	<1 / 16	pos/pos
mean±SEM			86.4±2.6	0.90±0.2		

A: Baseline FEV_1 recorded at day -3

B: Airway hyperresponsiveness to histamine (provocative concentration of histamine causing a 20% fall in FEV_1) measured at entry of study.

C: Titer of neutralizing antibodies against RV16 measured on screening day and at day 28 after inoculation (1 = ≤ 1:1).

3.3.3 RV16 inoculation

The RV16 strain and stock were the same as used in previous experiments by others (162) and ourselves (148,163,166). The RV16 was cultured according to standards of good laboratory practice and the inoculum was tested to be safe for human *in vivo* usage (286). RV16 was inoculated according to a previously described protocol (148,163,166). Briefly, 0.25 - 1.45 x

10^4 times the 50% tissue culture infective dose ($TCID_{50}$) was administered by nasal inhalation of nebulized virus suspension (DeVilbiss 646, DeVilbiss Co., Somerset, PA), then by spraying the suspension into both nostrils (DeVilbiss 286, powered by a compressor), and finally by instilling droplets of the suspension into both nostrils using a pipette. This procedure was repeated on the next day. In 8 patients the diluent was administered in the same fashion.

One or more RV16-positive viral cultures of nasal lavages, and/or a 4-fold or greater increase in RV16 specific serum neutralizing antibodies confirmed the infection (148). In addition, all lavages were cultured in rhesus monkey kidney cells (LLC-MK2), Hep2 cell cultures, and HEL cell cultures at 37° C in order to exclude other intercurrent virus infections.

3.3.4 Home recording of symptoms and FEV_1

Throughout the study period, from day -4 to day 11, symptoms of common cold or asthma were evaluated with a questionnaire that was completed by the participants 3 times a day. Cold symptoms included sore throat, headache, nasal discharge, sneezing, stuffy nose, malaise, cough and chills or fever, which were graded from 0 (absent) to 3 (severe) and added up to a total symptom score for each recording time (maximum 24). The maximal score after infection ("cold score") was used for correlation analysis (35,148,162,163,286). Asthma symptoms

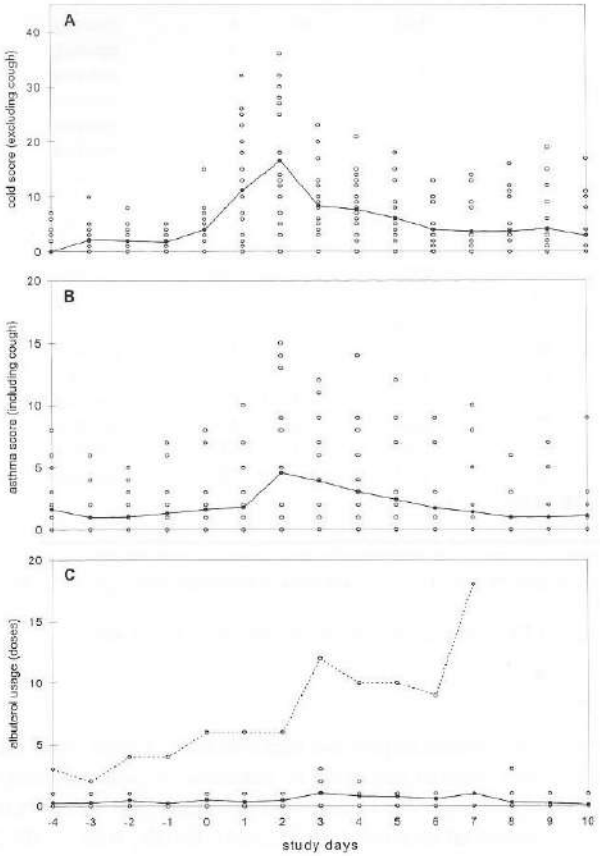


Figure 3.1. The closed symbols (•) indicate the mean value.
A. Cold symptoms (excluding cough score) in RV16 group, expressed as total of 3 daily recordings.

B. Asthma symptoms in the RV16 group (including cough score), expressed as total of 3 daily recordings.

C. Albuterol use in the RV16 group, expressed as total daily use in number of 200 µg doses. The open symbols (O) connected by a dotted line represent the albuterol use of subject #9, who dropped out at day 7 due to an exacerbation of asthma.

included breathlessness, wheeze, chest tightness, cough, and nocturnal symptoms. The cumulative asthma score of the first 5 days after infection, corrected for the baseline symptom score was used for correlation analysis ("asthma score"; maximum 156). In addition, the daily consumption of albuterol (number of 200 µg doses by metered dose inhaler -MDI-) was noted (148).

Each time after evaluation of symptoms, the patients performed 3 maximal forced expirations on a portable spirometer (Micro Spirometer, Micro Medical Ltd. Rochester, England), in order to record FEV_1. The highest FEV_1 of 3 attempts was noted in the diary card. For statistical analysis, occasionally missing values in the diary cards were replaced by interpolation of the 2 adjacent data points.

3.3.5 Lab assessment of lung function, airway hyperresponsiveness

Histamine inhalation challenge tests were performed using histamine-di-phosphate in serial doubling concentrations ranging from 0.03 to 8.0 mg/ml as described previously (35,148). The response was measured as FEV_1, by dry rolling spirometer (Morgan spiroflow, Rainham, United Kingdom). The tests were discontinued when FEV_1 decreased by more than 20% from baseline value.

3.3.6 Statistical analysis

Home recorded FEV_1 was expressed as % of the predicted value. In addition, the maximal degree of worsening in each individual was expressed as the lowest FEV_1 (% personal best) (287) during the period before RV16 inoculation (period 0), and during 3 periods after inoculation: days 0-3 (period 1), days 4-7 (period 2) and days 8-10 (period 3). The response to histamine was expressed as provocative concentration causing 20% fall in FEV_1 (PC_{20}). Log-transformed values of PC_{20} were used in the analysis and changes in PC_{20} were expressed as doubling doses (DD).

The effects of RV16 or placebo on symptoms, FEV_1 and PC_{20} were explored using repeated measures analysis of variance (ANOVA) for each group separately, and for all subjects with RV16 or placebo as between group factors, and with time as a within-group factor. This analysis was repeated for morning, afternoon, and evening recordings separately, in order to exclude the effect of variability during the day. In the case of significant ANOVA effects, paired and unpaired Student's T-tests were applied to analyze within-group and between-group effects, respectively. Pearson's correlation test was used for evaluation of associations between different parameters. P values < 0.05 were considered statistically significant. The summary statistics were expressed as mean ± SEM.

3.4 RESULTS

One of the RV16-treated subjects (#9) dropped out of the study at day 7 due to a moderate exacerbation of asthma that required oral prednisone treatment, to which the subject responded well. RV16 infection was confirmed in all RV16-treated subjects, whereas in the placebo group all the nasal lavages remained negative for respiratory viruses, without a rise in RV16 neutralizing antibodies (table 1).

3.4.1 Symptoms

Before RV16 infection common cold symptoms were not different between the groups (ANOVA: $p = 0.92$). In the RV16 group symptoms of common cold started to increase from

day 0 onwards, peaking at day 2 and returning to baseline within one week in most infected subjects (ANOVA: $p < 0.001$) (figure 1A). This increase was significantly different from the placebo group (ANOVA: $p < 0.001$). The asthma score was also not different between the groups at baseline (ANOVA: $p = 0.43$). Asthma symptoms gradually increased from day 0 onward, peaking at days 2 and 3, and returning to baseline in most RV16-treated subjects within a week (ANOVA: $p < 0.001$) (figure 1B). This increase was significantly different from the placebo group (ANOVA: $p = 0.04$). Before RV16/placebo inoculation, the albuterol use (mean ± SEM) was 0.35 ± 0.13 doses/day. The RV16 group showed a non-significant increase in the use of albuterol (ANOVA: $p = 0.13$), which peaked at day 3 (figure 1C). This change was not significantly different from placebo (ANOVA: $p = 0.09$). The patient who dropped out due to an exacerbation of asthma recorded the highest albuterol use.

3.4.2 Lung function and airway hyperresponsiveness: laboratory recordings

As described previously, there were no significant changes in FEV_1 either within the group (ANOVA: $p \geq 0.13$) or between the groups (ANOVA, $p = 0.40$) (148). In the placebo group there were no significant changes in PC_{20} during the study (ANOVA, $p = 0.67$). In the RV16 group PC_{20} decreased significantly at day 4 as compared to baseline ($p = 0.02$), but not at day 11 ($p = 0.19$). These changes were not significantly different from the changes in the placebo group ($p \geq 0.10$) (148).

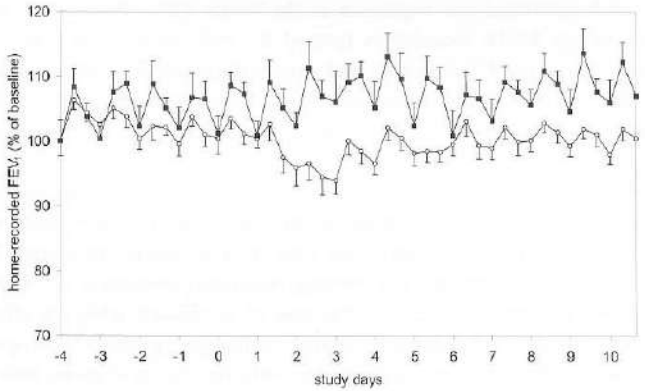


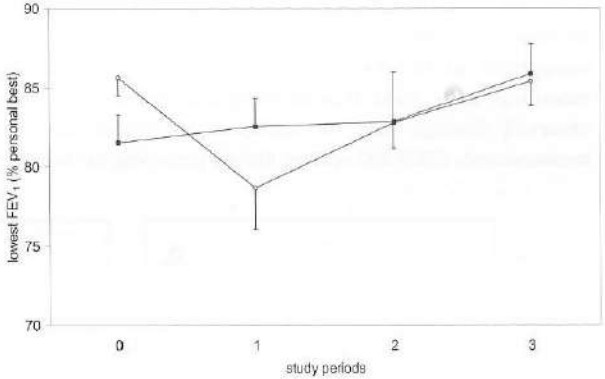
Figure 3.2. Home-recorded FEV_1 in the RV16 group (o) and the placebo group (■), expressed as percentage of baseline (mean ± SEM). RV16 or placebo was inoculated at days 0 and 1.

3.4.3 Home recordings of FEV_1

During the days before inoculation the home-recorded FEV_1 was higher in the RV16 group as compared to the placebo group (mean ± SEM: RV16: 94.0 ± 2.4 % pred. placebo: 82.4 ± 2.8, ANOVA, $p = 0.009$). During the study, FEV_1 showed variability during the day (ANOVA, $p < 0.001$) (figure 2). When considering morning, afternoon and evening FEV_1 separately, there was only a slight increase in afternoon FEV_1 in the placebo group (ANOVA, morning: $p = 0.16$, afternoon: $p = 0.01$, evening: $p = 0.14$) (figure 2). In the RV16 group FEV_1 decreased significantly (ANOVA, morning: $p = 0.005$, afternoon: $p = 0.003$, evening: $p = < 0.001$), reaching a minimum at day 2 after inoculation, returning to baseline value during the following 3 days (figure 2). These effects were significantly different between the groups (ANOVA, morning: $p = 0.004$, afternoon: $p = 0.001$, evening: $p = 0.001$). The lowest FEV_1 in period 0

was not significantly different between the groups (RV16: 85.6 ± 1.2 % best, placebo: 81.5 ± 1.8, $p = 0.07$). In the RV16 group the lowest FEV_1 decreased in period 1 (78.7 ± 2.6 % best, $p = 0.008$), still tended to be decreased in period 2 (82.8 ± 1.6, $p = 0.06$), and returned to baseline in period 3 (85.4 ± 1.5, $p = 0.77$), whereas in the placebo group the lowest FEV_1 did not change significantly (ANOVA: $p = 0.14$) (figure 3). The change in period 1 as compared to period 0 was significantly different between the groups ($p = 0.04$). In the RV16 group the maximal fall in home-recorded FEV_1 (% best) in period 1, correlated significantly with the cold score ($r = -0.47$, $p = 0.04$) (figure 4A), asthma score ($r = -0.47$, $p = 0.04$), and the change in PC_{20} at day 4 as compared to baseline ($r = 0.50$, $p = 0.03$) (figure 4B). In the placebo-group, only the maximal fall in home-recorded FEV_1 (% best) in period 1 and asthma score were significantly correlated ($r = -0.74$, $p = 0.04$).

Figure 3.3. Lowest home-recorded FEV_1 in the RV16 group (o) and the placebo group (■), expressed as percentage of personal best, during period 0 (days -4 to -1), period 1 (days 0 to 3), period 2 (days 4 to 7) and period 3 (days 8 to 10). RV16 or placebo was inoculated at days 0 and 1.



3.5 Discussion

This study demonstrates that experimental RV16 infection leads to a transient fall in daily home recordings of FEV_1 in asthmatic subjects. This virus-induced decrease in FEV_1 appears to be significantly related to the accompanying cold symptoms, asthma symptoms and to the increase in airway hyperresponsiveness to histamine. These data indicate that experimental RV16 infection leads to variable airways obstruction, suggesting that a rhinovirus-induced exacerbation of asthma may be associated with airways inflammation and airway wall swelling.

This is the first study demonstrating worsening of airways obstruction by experimental rhinovirus infection in asthma. This finding is in keeping with epidemiological data, showing that the detection of rhinovirus in the nasal lavage is associated in time with a drop in peak flow in asthmatic patients (77,78). The experimental design of the present study allows assessment of the time course of this phenomenon, and the relationship with symptoms and changes in airway hyperresponsiveness. Previous experimental studies in asthmatics, including the present one, have failed to demonstrate an effect of rhinovirus infection on laboratory recordings of FEV_1 (148,163,164,166). This suggests that the timing or the procedure of the spirometric measurements in the lung function lab may mask the occurrence of variable airways obstruction, which is associated with asthma symptoms in everyday life.

The data in the present study were obtained by applying a controlled design, and carefully validated methods for RV16 inoculation (148,163) and standardized laboratory measurement of lung function (288) and airway hyperresponsiveness (289). We chose to study home recordings

of FEV_1 rather than peak flow, since FEV_1 may be a slightly more sensitive and more specific measure of airways obstruction, being less effort-dependent than peak flow (288). It can be argued that the home recordings of FEV_1 may have been influenced by on-demand use of albuterol. However, albuterol usage was generally low in these mildly asthmatic subjects, and did not increase significantly during the study in either group. Moreover, the use of albuterol would have led to an underestimation of airways obstruction, and can therefore not explain the fall in FEV_1 together with a rise in asthma symptoms in the RV16 group.

We observed a decrease in home-recorded FEV_1, whereas such a change could not be detected using lab-recordings of FEV_1. Studies on exacerbations of asthma which were either virus-induced (290), or due to experimental tapering of steroid treatment (291) have shown that home-recordings are more sensitive in picking up changes in airways obstruction than lab.-recordings in asthmatic patients. We speculate that this is also the case in our study. In the before mentioned studies PEFR rather than FEV_1 was recorded in the home setting. However, frequent recordings of FEV_1 show a pattern of variable airways obstruction that is qualitatively comparable to PEFR-recordings in asthmatic patients (292). Therefore, we consider the recordings to be valid, thus allowing assessment of effects of RV16 infection on FEV_1. The observed findings may be attributed to factors such as frequency and timing of the measurements (292) and volume history preceding the measurements (293).

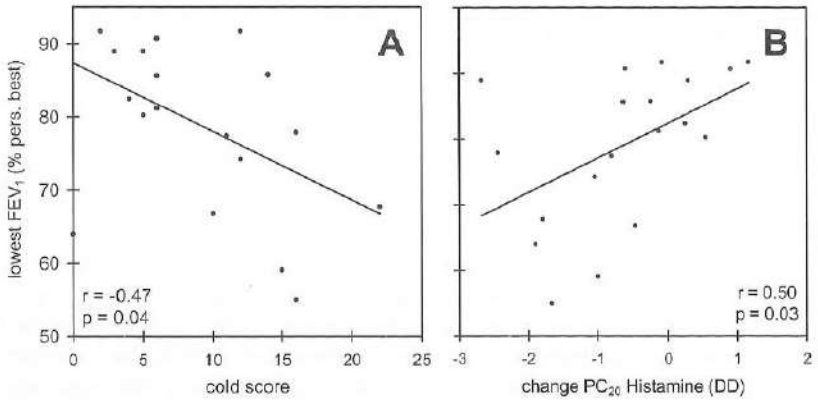


Figure 3.4. Correlations of cold score and the maximal fall in home-recorded FEV_1 (% personal best) during the acute phase after infection (period 1), (A), and of the maximal fall in home-recorded FEV_1 (% personal best) and the change in PC_{20} to histamine between days -3 and 4 (expressed as doubling doses -DD-) (B) in the RV16 group.

How can a rhinovirus cold lead to variable airways obstruction? There is evidence to suggest that experimental rhinovirus 16 infections are accompanied by an increase in airway inflammation (35,164,169), characterized by cellular infiltration of the airway wall, particularly with lymphocytes and eosinophils (164). Such inflammation appears to be accompanied by release of proinflammatory mediators from these cells (35) and airway tissues such as the epithelium (215,218), which subsequently may cause airway smooth muscle contraction, vasodilation and vascular leakage, leading to edema and airway wall swelling (284). It is highly likely that increased airway wall thickness, both internal and external from the airway smooth muscle layer, augments airway narrowing during smooth muscle contraction (55,285,294). This

has been difficult to confirm in humans, since it probably occurs predominantly in the small airways (55), where asthmatic airways inflammation has indeed been demonstrated (26,295,296). It has been postulated that respiratory virus infections may induce small airways inflammation (297), but this remains to be established for rhinovirus colds in humans.

Our data implicate that the use of home-recorded FEV_1 (even when FEV_1 is measured only once daily) appears to be a sensitive parameter for early detection of virus-induced exacerbations of asthma, thereby allowing timely adjustment of bronchodilator and/or anti-inflammatory therapy. As part of an adequate self-management plan, this could lead to prevention of severe airways obstruction or perhaps even the development of severe virus-associated exacerbations in asthma.

In conclusion, this study shows that experimental rhinovirus 16 infection induces the clinical and functional features of a mild exacerbation of asthma. This resembles exacerbations observed after natural rhinovirus infection, thereby allowing further experimental studies on both immunological mechanisms and therapeutic interventions, in order to optimize treatment of virus-induced exacerbations of asthma.

4 EFFECT OF EXPERIMENTAL RHINOVIRUS 16 COLDS ON AIRWAY HYPERRESPONSIVENESS TO HISTAMINE AND INTERLEUKIN-8 IN NASAL LAVAGE IN ASTHMATIC SUBJECTS IN VIVO.

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Clin Exp Allergy 1997;27:36-45

4.1 ABSTRACT

Asthma exacerbations are closely associated with respiratory virus infections. However, the pathophysiological consequences of such infections in asthma are largely unclear. The objective of the present study was to examine the effect of rhinovirus 16 (RV16) infection on airway hypersensitivity to histamine, and on interleukin-8 (IL-8) in nasal lavage. Twenty-seven non-smoking atopic, mildly asthmatic subjects participated in a placebo-controlled, parallel study. A dose of 0.5 - 2.9 x 10^4 $TCID_{50}$ RV16 or placebo was nasally administered. Cold symptoms were recorded by questionnaire throughout the study. Histamine challenges were performed at entry and on days 4 and 11 after inoculation. Nasal lavages were obtained at entry, and on days 2 and 9. The response to histamine was measured by PC_{20} (changes expressed as doubling doses: DD). IL-8 levels were obtained by ELISA, and were expressed in ng/ml. RV infection was confirmed by culture of nasal lavage and/or by antibody titer rise in each of the RV16-treated subjects. Among the 19 RV16-treated subjects, 8 developed severe cold symptoms. Baseline FEV_1 did not change significantly during the study in either treatment group ($p = 0.99$). However, in the RV16-treated subjects there was a decrease in PC_{20} at day 4, which was most pronounced in those with a severe cold (mean change ± SEM: -1.14 ± 0.28 DD, $p = 0.01$). In addition, IL-8 levels increased in the RV16 group at days 2 and 9 ($p < 0.001$). The increase in nasal IL-8 at day 2 correlated significantly with the change in PC_{20} at day 4 ($r = -0.48$, $p = 0.04$). We conclude that the severity of cold, as induced by experimental RV16 infection, is a determinant of the increase in airway hypersensitivity to histamine in patients with asthma. Our results suggest that this may be mediated by an inflammatory mechanism, involving the release of chemokines such as IL-8.

4.2 Introduction

Asthma is a chronic disease of the airways, characterized by episodic chest tightness and wheezing, and by airway hyperresponsiveness to non-sensitizing stimuli, as measured by the sensitivity to inhaled histamine or methacholine (298). Airway inflammation underlies both stable asthma and exacerbations of the disease, as appears from a predominantly eosinophilic and mononuclear cell infiltrate in bronchial biopsy specimens (181). It is generally considered that the exacerbations are due to exposure to respiratory virus infections and/or to airborne allergens, potentially leading to a flare-up of airway inflammation (181,298).

Several clinical and epidemiological studies have described a close temporal association of respiratory virus infections with asthma exacerbations (7). Respiratory viruses can be identified in 10 to 44% of the asthma exacerbations in adults (77,144), whilst in children identification rates vary from 26 to 83% (78,143,145,146). The use of sensitive techniques to detect rhinovirus and coronavirus in the two most recent studies have resulted in the highest identification rates so far (77,78). Among the various respiratory viruses identified, rhinovirus predominates in most of these studies (77,78,146). Interestingly, rhinovirus shedding in the absence of cold symptoms does not seem to be associated with clinical worsening of asthma (146).

The effects of experimental rhinovirus infection on airway responsiveness to inhaled histamine are somewhat controversial. Lemanske *et al.* demonstrated an induction of hypersensitivity to histamine after experimental rhinovirus 16 (RV16) infection in nonasthmatic patients with atopic rhinitis (162), whereas others have not observed such an effect when using other rhinovirus serotypes (176,178). In asthmatic subjects Halperin *et al.* found increased hypersensitivity to histamine in only 4 out of 22 subjects after experimental rhinovirus (serotype 39 and HH strain) infection (160), whilst in the most recent study by Fraenkel *et al.* a rather small, but significant increase in sensitivity to histamine could be detected in 6 asthmatic subjects after infection with RV16 (164). Since none of these studies was placebo-controlled, it seems mandatory to examine the effect of experimental rhinovirus infection on airway sensitivity to histamine in asthmatic subjects by using such a design. This has successfully been employed in our previous study, showing excessive airway narrowing to methacholine after RV16 infection in atopic asthmatic subjects (163).

Rhinovirus infection has been shown to lead to infiltration of inflammatory cells into nasal secretions and mucosa (256-258), as well as into the bronchial mucosa (164) in normal and/or atopic subjects. *In vitro*, there is evidence that bronchial epithelial cell lines, fibroblasts and mononuclear cells produce pro-inflammatory cytokines in response to infection with rhinovirus (182,215,299). *In vivo*, the levels of chemokines such as interleukin-8, RANTES and MIP-1α were found to be elevated in nasal secretions of asthmatic children during naturally acquired colds (251,253). Consequently, one can postulate that chemokines such as IL-8 drive recruitment of inflammatory cells (300), thus promoting airway inflammation, and thereby airway sensitivity to histamine.

In the present study, we hypothesized that experimental RV16 colds in atopic asthmatic patients increase airway sensitivity to histamine, particularly in those with severe cold symptoms. In addition, we postulated that this effect is associated with a rise of IL-8 in nasal secretion. To that end, we measured dose-response curves to inhaled histamine and levels of IL-8 in nasal washings before and after placebo-controlled nasal inhalation of wild type RV16 in atopic, mildly asthmatic patients.

4.3 Materials and methods

4.3.1 Subjects

Twenty-seven non-smoking, atopic asthmatic subjects participated in this study. The subjects had not used inhaled or oral corticosteroids for at least 3 months, nor had they used theophyllines, antihistamines, sodium cromoglycate, or nedocromyl sodium for at least 6 weeks preceding the study. Symptoms of asthma were stable and controlled by on demand usage of inhaled salbutamol alone, which was withheld for at least 8 hours before the measurements. There was no history of relevant exposure to allergens from 2 weeks before until the end of the study. The patients were not selected on basis of a history of virus-induced exacerbations. Among the eight subjects who received placebo-inoculation, four did not have neutralizing antibodies in their undiluted sera against 20-25 tissue culture infective dose ($\leq$ 1:1), and four had titers between 1:2 and 1:128 serum dilution. Fourteen out of the nineteen subjects who received virus did not have neutralizing antibodies, and five had titers in the range of 1:2 to 1:16 serum dilution. The study was conducted from July to December 1994. The Hospital's Medical Ethics Committee approved the study, and informed consent was obtained from all participants. The subjects' characteristics are listed in table 1.

4.3.2 Design

The study had a double-blind, placebo-controlled parallel design. Prior to the study, each subject was screened for inclusion and exclusion criteria. Three days before the experimental inoculation of virus or placebo, a histamine inhalation test was carried out. Subsequently, virus or placebo (diluent) was administered on two successive days. Histamine challenges were repeated at days 4 and 11 after the first inoculation of virus or placebo. Nasal washing was performed and a blood sample was taken immediately before the first inoculation of virus or placebo, and then at days 2 and 9. Four weeks after inoculation all subjects returned to the laboratory for a final nasal washing and a blood sample to determine the convalescent antibody titer.

4.3.3 RV16 inoculation

The RV16 virus strain and stock was the same as used in previous experiments in humans *in vivo* by others (162) and by ourselves (163). The virus was cultured according to standards of good laboratory practice and the inoculum was tested to be safe for human *in vivo* usage (286). Nasal inoculation of the rhinovirus was performed following a previously described method (162), that was slightly modified by adding nasal virus inhalation (163). A total dose of 0.5 - 2.9 x 10^4 $TCID_{50}$ diluted in 3 ml Hanks' balanced salt solution (HBSS) with 0.5 % (w/v) gelatin was administered to each subject. This dose was divided over two days. On each day the same procedure for virus inoculation was followed. First, 0.5 ml of the inoculum was inhaled through the nose by using a nebulizer (DeVilbiss 646: median mass aerosol diameter (MMAD) 2.4 µm) connected to a face mask. Second, 0.5 ml was sprayed by atomizer (DeVilbiss 286, powered by a compressor, MMAD: >10 µm) into the nostrils. And finally, 0.5 ml was instilled into the nostrils by pipette.

4.3.4 Laboratory confirmation of infection

We considered a fourfold or greater increase in virus-specific neutralizing antibody in the serum and/or recovery of the virus from nasal washes as confirmation of RV16 infection (162,163). Before and 28 days after virus or placebo inoculation, levels of neutralizing

antibodies were determined by a neutralization assay using homologous virus (162,163). Nasal lavages were obtained before the first virus or placebo administration, and subsequently on days 2, 9 and 28. Human embryonic lung fibroblast (HEL) cultures were inoculated with these lavages and incubated at 32° C for 14 days. If the culture showed the characteristic rhinovirus-induced cytopathic effects, RV16 was identified by a neutralization assay, using RV16 specific guinea pig immune serum (1126AS/GP-VR; American Type Culture Collection, Rockville, MD). All nasal washes were also inoculated into rhesus monkey kidney (LLC-MK2) cells, HEp-2 cell cultures and HEL cells, and cultured at 37° C, in order to exclude any intercurrent respiratory virus infection.

Table 4.1: Characteristics of study subjects

subject	sex (M/F)	age (yr)	FEV_1 (% pred.)	$PC_{20}FEV_1$* (mg/ml)	cold score	asthma score	titer† pre/post	culture days 2/ 9	β_2	allergy*
placebo										
1	M	25	76	0.28	1	0	<1/ <1	neg/neg	-	H
2	M	26	68	0.60	4	2	<1/ <1	neg/neg	β_2	H, C
3	M	18	71	0.64	2	1	32/ 32	neg/neg	β_2	H, C
4	F	26	75	0.78	2	0	8/ 8	neg/neg	β_2	H, C, P
5	M	25	73	0.80	1	0	16/ 8	neg/neg	β_2	H, C
6	M	24	80	1.37	2	2	128/ 128	neg/neg	-	H, C, P
7	M	24	89	1.85	11	5	<1/ <1	neg/neg	-	C
8	M	25	87	1.93	4	0	1/ <1	neg/neg	β_2	H, C, P
			77.4±2.6	0.87±0.4						
RV16										
9	F	25	71	0.12	15	32	<1/ 4	pos/neg	β_2	H, P
10	F	23	77	0.25	10	3	<1/ 4	pos/neg	β_2	H, P
11	M	21	103	0.32	4	0	<1/ 4	pos/neg	-	H, P
12	M	18	80	0.34	6	6	<1/ 16	pos/pos	β_2	H, C
13	M	19	81	0.37	5	-10	1/ 128	pos/pos	-	C
14	M	22	85	0.41	0	0	<1/ 32	pos/neg	β_2	H, C, P
15	M	20	87	0.71	16	23	<1/ 128	pos/pos	β_2	H, P
16	F	25	88	0.86	11	23	<1/ <1	pos/pos	-	H, C, P
17	M	26	108	0.87	2	2	<1/ 4	pos/neg	β_2	H
18	F	21	92	1.19	12	23	2/ 32	neg/neg	β_2	C
19	M	20	101	1.33	12	15	16/ 256	pos/pos	β_2	H, C, P
20	M	21	80	1.39	16	28	<1/ 64	pos/neg	-	H, C, P
21	F	24	89	1.76	22	49	8/ 512	pos/pos	-	H, C, P
22	M	22	89	2.03	6	8	4/ 512	pos/neg	β_2	H, C
23	F	23	77	2.12	14	29	<1/ 128	pos/pos	-	H, C, P
24	M	26	69	2.39	6	4	4/ 8	pos/neg	β_2	H, C
25	M	24	77	2.69	5	2	<1/ 32	pos/neg	β_2	H, C, P
26	M	21	90	2.98	3	0	<1/ 32	pos/pos	-	C
27	F	22	105	4.17	6	0	<1/ 16	pos/pos	-	H, C
			86.4±2.6	0.90±0.2						

* Airway hyperresponsiveness to histamine (provocative concentration of histamine causing a 20% fall in FEV_1) measured at entry of study. † Titer of neutralizing antibodies against RV16 (serum dilution) measured on screening day and at day 28 after inoculation. * Allergy C = cat, H = house dust mite, P = pollen (grasses and/or trees), determined by skin prick test (Vivodiagnost, ALK, Benelux)

4.3.5 *Diary cards*

Throughout the study period, symptoms of common cold and asthma were evaluated with a questionnaire that was completed by the participants three times daily. Cold symptoms included sneezing, sore throat, nasal discharge, stuffy nose, headache, cough, malaise, chills, or fever, which were graded: 0 = absent, 1 = mild, 2 = moderate, 3 = severe and added up to a total cold symptom score. In subjects in whom infection was confirmed, those who recorded a total cold symptom score ≥ 11 at any time-point after inoculation were defined as having a severe cold, whereas subjects with scores < 11 were considered having a mild cold (162,163,286).

In the same manner, asthma symptoms including cough, breathlessness, wheeze, chest tightness, and nocturnal symptoms were recorded (163). The daily consumption of inhaled salbutamol as rescue medication was also noted.

4.3.6 *Histamine challenge*

Standardized histamine challenge tests (289) were performed using histamine-di-phosphate in phosphate buffered saline. Histamine was stored at 4° C, and nebulized at room temperature in serial doubling concentrations ranging from 0.03 to 8 mg/ml. A DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, PA)(output 0.13 ml/min.) was used, in connection to the central chamber of an in- and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T). The aerosol was inhaled by tidal breathing for 2 min at 5 min intervals with the nose clipped. The lung function response was measured as FEV_1, obtained by a dry rolling spirometer (Morgan spiroflow, Rainham, United Kingdom). First, baseline FEV_1 was determined as the mean of three reproducible values (within 5 %). Subsequently, single measurements of FEV_1 were made 90 seconds after each histamine dose. The tests were discontinued if FEV_1 decreased by more than 20% from baseline. Afterwards the subjects inhaled 200 µg salbutamol to provide immediate bronchodilation.

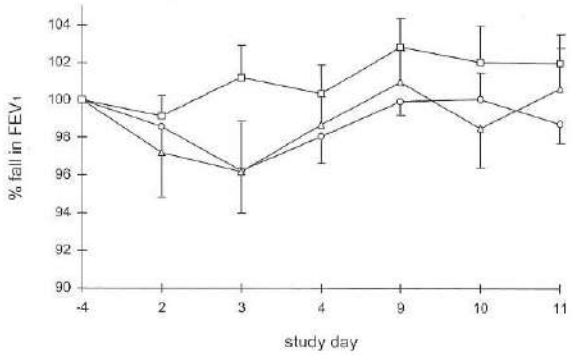

Figure 4.1. Change in baseline FEV_1 as compared to the value at entry, expressed in percentage fall in FEV_1 from individual mean baseline value (100%) in the placebo group (squares), the mild cold group (triangles) and the severe cold group (circles). There was no significant effect on FEV_1 during the study in any group (MANOVA: p = 0.99).

4.3.7 *Nasal lavage*

A modified "nasal pool device" (301) was used for nasal lavage. This consists of a syringe, connected to a rubber tube, equipped with an inflatable balloon, serving as a nasal adapter. The balloon was inserted into the *vestibulum nasi*, and gently inflated. While the patient was sitting in writing position, 10 ml of pre-warmed Hanks' balanced salt solution (HBSS) (one nostril with and the other without 0.5 % gelatin) was instilled by gently compressing the syringe. After

5 min the fluid was recovered by retracting the syringe. This procedure was carried out in each nostril. The lavage recovered from the first (with 0.5% gelatin) and second nostril (without gelatin) was used for confirmation of RV infection and to determine the IL-8 level, respectively.

4.3.8 IL-8 in nasal washing

Immediately after recovery, nasal washings were centrifuged at 250 x g for 10 min. Supernatant was removed and stored at -70° C until further analysis. The IL-8 levels were determined by ELISA (CLB, Amsterdam, The Netherlands), according to the manufacturer's directions. The detection limit of this assay was 40 pg/ml.

4.3.9 Leukocyte counts in peripheral blood

Before, and on days 2 and 9 after placebo or virus administration, absolute and differential leukocyte counts were assessed by automated blood count analysis (Technicon H1, Technicon, Tarrytown, NY).

4.3.10 Statistical analysis

The highest individual total cold symptom score (referred to as cold score), and the cumulative asthma score recorded from day 0 to 5 after inoculation minus the cumulative scores from 4 to 1 day before inoculation (referred to as asthma score) were used for correlation testing (163). The response of FEV_1 to histamine was expressed as percentage fall from baseline value (289), and was plotted against log nebulized concentration of histamine in mg/ml. The concentration-response curves were characterized by their position, expressed as the provocative concentration causing 20% fall in FEV_1 from baseline value (PC_{20}), which was calculated by log-linear interpolation between the last two adjacent data-points (289). The logarithm of PC_{20} was used in the analysis, and changes in PC_{20} were expressed in doubling doses (DD). IL-8 levels were expressed in ng/ml, peripheral blood leukocyte numbers were expressed in cells/L.

Changes in the variables were analyzed by repeated-measure analysis of variance (MANOVA), with placebo, RV16 treatment or severe cold and mild cold as a between-group factors and time as a within-group factor. Significant MANOVA effects were explored with Student *t*-tests. Differences in PC_{20}, IL-8 levels and leukocyte numbers within the groups between the study days were examined using two-tailed paired *t*-tests, and differences between the groups were analyzed using unpaired *t*-tests. The summary statistics were expressed as means ± SEM. For evaluation of associations between the variables, the Pearson's correlation test was used. P values less than 0.05 were considered statistically significant.

4.4 RESULTS

One of the RV16-treated subjects (subject 9) dropped out of the study at 7 days after the first inoculation because of a moderate exacerbation of asthma, requiring treatment with oral prednisone, to which she responded well. One nasal washing sample was excluded from the analysis (subject 7, day 9), because of a recent bleeding nose.

4.4.1 Confirmation of infection

In the placebo group all cultures of nasal washes remained negative for RV16 during each visit. In the virus-treated group RV16 could not be detected in the nasal lavage before inoculation, whilst at day 2 RV was detected in the nasal lavage of all but one subject (subject 18). At day 9, RV16 was identified in 10 out of 19 subjects, whereas at day 28 all nasal washings were

negative. No other respiratory viruses were identified in any of the nasal washings (table 1).

In the placebo group none of the subjects showed an increase in RV16 neutralizing antibodies. In the RV16 group all subjects but two (subjects 16 and 24) showed at least a fourfold increase in neutralizing antibodies in the convalescent sera (range: 4-fold to 128-fold increase) (table 1).

4.4.2 Cold - and asthma scores

In the placebo group, there was no significant change in cold score or asthma score (MANOVA, $p \geq 0.52$). In the RV16 group, there was a significant increase in cold score (MANOVA, $p < 0.001$), that peaked 1 day after the first inoculation, gradually returning to baseline within 5 days. The highest cold scores were significantly different between the groups ($p < 0.001$). Eight of the RV16-infected subjects had a severe cold as shown by a symptom score ≥ 11 (table 1).

In the RV16-treated subjects there was a significant increase in asthma symptoms (MANOVA, $p < 0.001$) that peaked on the second and third day after the first inoculation, and returned to baseline within 5 days. The asthma score in the subjects with a severe cold was significantly higher as compared to the asthma score in those with a mild cold and the placebo-treated subjects ($p < 0.001$) (table 1). Cold score and asthma score were significantly correlated in the RV16 group ($r = 0.92$, $p < 0.001$). The use of salbutamol did not change significantly within the groups at any time point (MANOVA, $p = 1.00$).

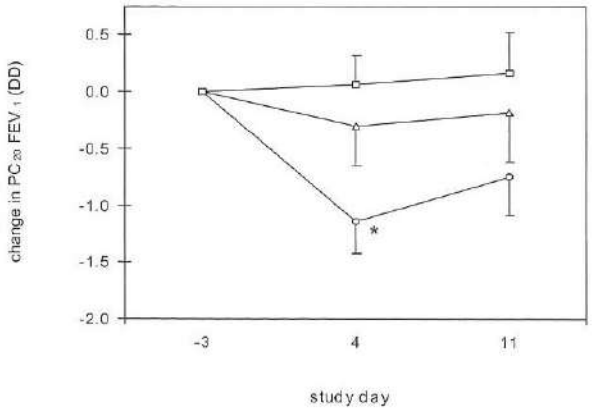

Figure 4.2. Change in sensitivity to inhaled histamine ($PC_{20}FEV_1$) as compared to the value at entry, expressed in doubling doses (DD, geometric mean ± SEM) in the placebo group (squares), the mild cold group (triangles) and the severe cold group (circles). In the severe cold group the changes were significantly different from placebo at day 4 ($p = 0.01$), but not at day 11 ($p = 0.09$).

4.4.3 Baseline lung function

Before RV16 or placebo inoculation, FEV_1 % predicted was slightly higher in the RV16 group as compared to the placebo group ($p = 0.04$) (table 1). During the course of the study, there were no significant effects on baseline FEV_1 within either the placebo group or in the RV16-treated subjects with a mild or severe cold (MANOVA, $p = 0.99$) (figure 1). The maximal change in FEV_1 after infection did not correlate significantly with the asthma score ($p = 0.98$).

4.4.4 *Sensitivity to histamine*

Before inoculation of RV16 or placebo, the mean $PC_{20}FEV_1$ was not different between the 2 treatment groups ($p = 0.93$). In the placebo group, there was no significant change in PC_{20} during the study (MANOVA, $p = 0.67$) (figure 2). In the RV16 group, there was a significant decrease in PC_{20} at day 4 (mean difference ± SEM: -0.65 ± 0.25 DD, $p = 0.02$), which was no longer significant at day 11 (mean difference ± SEM: -0.40 ± 0.30 DD, $p = 0.19$). These changes were not significantly different from placebo ($p = 0.10$ and $p = 0.27$, respectively). However, in the subjects with a severe cold, this decrease was more pronounced: mean difference ± SEM: -1.14 ± 0.28 DD, $p = 0.005$ at day 4, with a trend towards a decrease at day 11 (mean difference ± SEM: -0.75 ± 0.34 DD, $p = 0.07$) (figure 2). This change was significantly different from placebo at day four, but not at day 11 ($p = 0.01$ and $p = 0.09$, respectively). In the mild cold group there was neither a change in PC_{20} at day 4 (mean difference ± SEM: -0.30 ± 0.35 DD, $p = 0.42$), nor at day 11 (mean difference ± SEM: -0.18 ± 0.44 DD, $p = 0.68$) (figure 2).

The changes in PC_{20} in the 5 subjects with pre-existing neutralizing antibodies against RV16 were not statistically different from the changes in those without such antibodies (MANOVA, $p = 0.33$) (day 4: mean change ± SEM: -0.53 ± 0.46 DD, $p = 0.31$, and -0.69 ± 0.31 DD, $p = 0.04$, respectively. Day 11: -1.10 ± 0.28 DD, $p = 0.02$, and -0.13 ± 0.38 DD, $p = 0.73$, respectively).

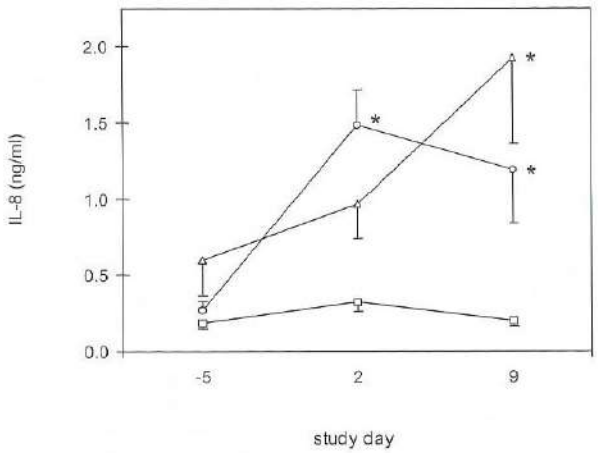

Figure 4.3. Levels of IL-8 in nasal lavage fluid (ng/ml, mean ± SEM) in the placebo group (squares), the mild cold group (triangles) and the severe cold group (circles). In the severe cold group, the changes in the levels of IL-8 as compared to the values at entry were significantly different from placebo both at days 2 and 9 ($p = 0.002$ and $p = 0.04$, respectively), whilst in the mild cold group there was only a significant change at day 9 ($p = 0.01$).

4.4.5 *IL-8 in nasal washing*

IL-8 in the nasal washings did not change significantly in the placebo group (MANOVA, $p = 0.06$) (figure 3). In the RV16 group, IL-8 increased both at days 2 and 9 ($p < 0.001$) as compared to day -5. In subjects with a severe cold this increase was significant at both time-points (within group, $p \leq 0.04$), and also as compared to placebo ($p < 0.05$). In those with a mild cold the increase in IL-8 was only significant at day 9, both within the group ($p = 0.008$) and as compared to placebo ($p < 0.01$) (figure 3). The increase in IL-8 at day 2 in the RV16 group correlated significantly with the cold score ($r = 0.80$, $p < 0.001$), asthma score ($r = 0.68$, $p = 0.001$), and with the change in PC_{20} at day 4 ($r = -0.48$, $p = 0.04$) (figure 4A, 4B).

4.4.6 *Leukocyte counts in peripheral blood*

In the RV16 treated subjects differential leukocyte counts showed a significant rise in the number of neutrophils ($p < 0.001$) and a concomitant decrease in lymphocyte number at day 2 after inoculation ($p < 0.001$) (figure 5). Only in subjects with a severe cold the cell numbers at day 2 were significantly different from placebo ($p \leq 0.01$). Neutrophil and lymphocyte numbers had returned to baseline at day 9 ($p > 0.17$).

The number of neutrophils at day 2 correlated significantly with cold score ($r = 0.59$, $p = 0.008$), asthma score ($r = 0.65$, $p = 0.002$), the change in PC_{20} at day 4 ($r = -0.49$, $p = 0.03$), whilst there was a trend towards a significant correlation with the change in IL-8 levels at day 2 ($r = 0.39$, $p = 0.10$) (figure 4C). Furthermore, the number of lymphocytes at day 2 was also significantly related to the cold scores ($r = -0.69$, $p = 0.001$), asthma score ($r = -0.62$, $p = 0.004$) and the change in IL-8 at day 2 ($r = -0.73$, $p < 0.001$) (figure 4D), with a trend towards a correlation with the change in PC_{20} at day 4 ($r = 0.42$, $p = 0.07$).

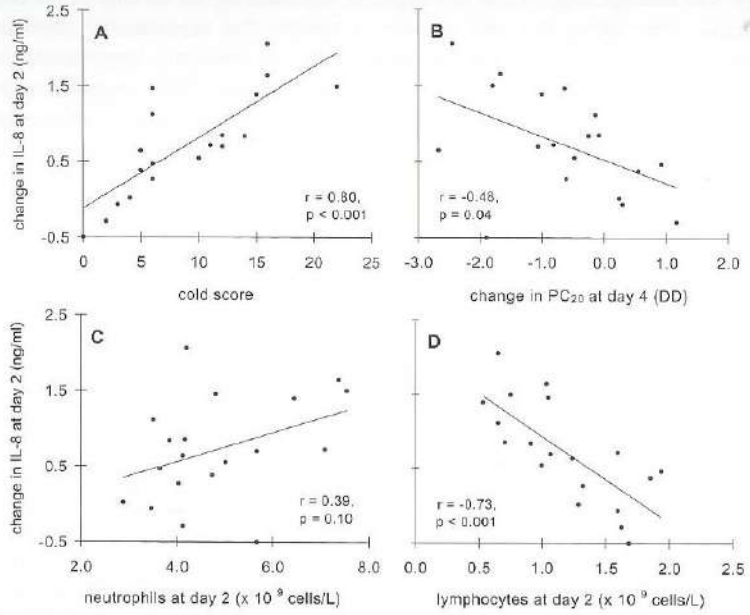

Figure 4.3. Relationship (Pearson's correlation coefficient, r) between the changes in IL-8 levels in nasal washing at day 2 as compared to the values at entry, and cold score (A), changes in PC_{20} at day 4 as compared to the values at entry (B), and the number of neutrophils (C) and lymphocytes (D) in peripheral blood at day 2.

4.5 DISCUSSION

This study shows that experimental rhinovirus 16 infection in atopic asthmatic subjects induces airway hypersensitivity to histamine, particularly in those patients who develop a severe cold. In addition, we have demonstrated that the levels of the pro-inflammatory chemokine IL-8 in nasal secretions rise after infection. This rise is associated with cold score, change in airway hyperresponsiveness, and numbers of neutrophils and lymphocytes in peripheral blood after

infection. These findings suggest that the severity of the cold is a major determinant of rhinovirus-induced airway hyperresponsiveness in asthma. Our results fit in with the hypothesis that this is mediated through an inflammatory mechanism involving locally produced chemokines.

This is the first placebo-controlled study showing the development of airway hypersensitivity to histamine after experimental infection with wild type rhinovirus. The change in airway hypersensitivity in the asthmatic subjects with severe colds was about 1 doubling dose, which is similar to what is usually observed after allergen challenge (302). Rhinovirus-induced hypersensitivity of such magnitude has also been demonstrated in patients with atopic rhinitis by Lemanske *et al.* (162). However, in the latter subjects the histamine hypersensitivity lasted up to 4 weeks after infection, whereas in our study the effect was no longer significant at day 11. One could speculate that the prolonged effect in atopic rhinitis might have been due to the additional allergen challenges during that study. Two previous studies on experimental rhinovirus infection in asthma (160,178) showed small and variable changes in airway hypersensitivity. Our findings suggest that this might be explained by the severity of the colds that were induced. After taking this into account, it appears that experimental rhinovirus infection in asthma does lead to substantial worsening of PC_{20} to histamine. Interestingly, this also occurred in the small number of subjects who had pre-existing RV16 neutralizing, but possibly cross-reactive circulating antibodies. The latter may not be surprising, since atopic subjects with low titers of neutralizing antibodies, as opposed to normal subjects with such titers, have been shown to develop severe cold symptoms after experimental RV16 inoculation (161).

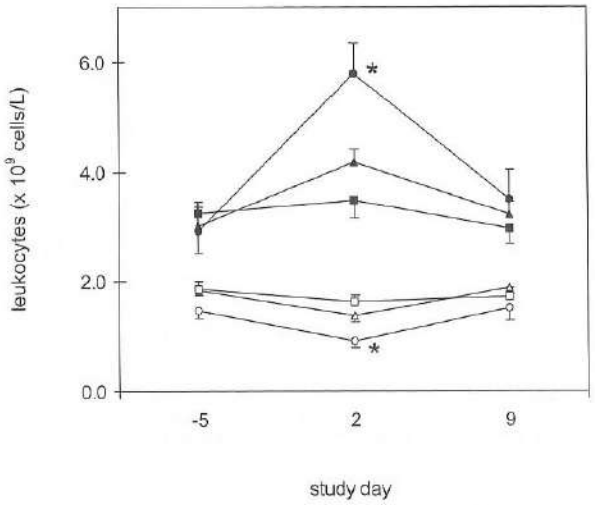

Figure 4.4. Number of neutrophils (closed symbols) and lymphocytes (open symbols) in peripheral blood during the study in the placebo group (squares), the mild cold group (triangles), and the severe cold group (circles). Only in the severe cold group, the number of neutrophils at day 2 was significantly elevated ($p = 0.01$), whilst the number of lymphocytes was significantly reduced as compared to placebo ($p = 0.003$).

The design of the present study allowed us to differentiate the responses to rhinovirus inoculation from normal fluctuations in symptoms and airway physiology that are characteristic to asthma. The study was performed in the months July to December, but no attempt was made to exclude coinciding allergen exposure (pollen, house dust mite), since this would have been hard to accomplish. However, the present circumstances can be considered as those

encountered during naturally occurring infections. Despite the fact that the subjects were clinically stable as assessed by history, and by symptom control with *p.r.n.* β_2 adrenergic medication alone, a moderate exacerbation of asthma developed in the subject who had the lowest PC_{20} at entry into the study. This underlines the potential of exacerbations after rhinovirus infection in patients with asthma (7,78,149), despite the usually small accompanying changes in lung function (160,163,164).

In this study, we applied validated procedures for inoculation and measuring the responses to rhinovirus infection. First, by using a combination of three methods of virus administration, including nasal inhalation, the natural ways of transmission were mimicked (80). In this way, the virus may even have reached the intrapulmonary airways (303). Second, commonly used and well standardized methods for lung function testing and histamine challenges were used (289). And third, we applied a validated method for nasal lavage (301), which allowed IL-8 to diffuse into the lavage fluid during a 5 min exposure period of the nasal epithelium, resulting in IL-8 levels well above the detection limit of the IL-8 ELISA.

How can the present results be interpreted? The increase in airway hypersensitivity, in the absence of a significant decrease in lung function, during the acute phase of infection might be explained by physiological phenomena such as airway wall swelling, potentiating the airway narrowing effect of smooth muscle shortening (297). Such an explanation would be in keeping with the observations by Cheung *et al.* (163), who showed that experimental RV16 infection leads to excessive airway narrowing in response to inhaled methacholine in subjects with asthma. Airway wall swelling in asthma is generally considered to be a consequence of inflammation (181). Indeed, Fraenkel *et al.* (164) recently described the infiltration of inflammatory cells, particularly lymphocytes and eosinophils into the bronchial mucosa in patients with asthma after experimental RV16 infection. The presently observed correlation between the numbers of neutrophils and lymphocytes in peripheral blood after infection and the change in airway hypersensitivity indirectly supports an active role of these cells in the virus-induced airway inflammation.

We found a marked rise in IL-8 in nasal secretions after RV16 infection. In general, this confirms the ability of rhinovirus to increase the release of a number of pro-inflammatory mediators and/or cytokines within the airways, such as e.g. kinins (246,258) and interleukin-1 (248) in nasal secretions, and histamine in broncho-alveolar lavage fluid (169). The present results obtained by experimental rhinovirus infection are in keeping with the preliminary data of Teran *et al.* (251), who showed that levels of IL-8 in nasal secretions were elevated in nasal secretions during a natural acquired cold in children with asthma. Our results extend these previous observations by showing an association between the increase in IL-8 in nasal washings and cold- or asthma symptoms, as well as the degree of worsening of airway hyperresponsiveness. IL-8 is a CXC-chemokine, produced by tissue cells (epithelial cells, fibroblast and endothelial cells), leukocytes, macrophages and mast cells (300,304). IL-8 displays various activities, such as chemotactic activity for neutrophils, lymphocytes and basophils (305). In addition, IL-8 may be involved in the recruitment of primed eosinophils, implicating its involvement in allergic inflammation (306). Since rhinovirus *in vitro* induces the production of IL-8 in epithelium, fibroblasts and peripheral blood mononuclear cells (215,223,299), our findings support the hypothesis that the release of mediators such as the chemokine IL-8 can drive the airway inflammation, and thereby the hypersensitivity to histamine after rhinovirus infection in allergic asthma.

What are the clinical implications of this study? First, a common cold aggravates airway hypersensitivity in patients with asthma, fitting in with the close epidemiological association

between rhinovirus infections and exacerbations of asthma (77,78). And second, it appears that atopic asthmatic patients with low titers of neutralizing antibodies may not be fully protected against experimental RV16 infection, and its detrimental effects on their asthma. This observation in a small number of subjects in the present study first needs confirmation in larger series of experimental or naturally occurring rhinovirus infections in patients with allergic asthma.

In conclusion, experimental RV16 infection can be employed as a useful laboratory model for the development of airway hypersensitivity during an asthma exacerbation. One of the potential mechanisms for this might be the rhinovirus-induced release of pro-inflammatory chemokines. This hypothesis needs further testing in models of rhinovirus infection *in vitro* and *in vivo*, focusing on the pathological mechanisms in the intrapulmonary airways in patients with asthma.

5 EFFECTS OF EXPERIMENTAL RHINOVIRUS 16 (RV16) INFECTION ON AIRWAY HYPER-RESPONSIVENESS TO BRADYKININ IN ASTHMATIC SUBJECTS *IN VIVO*.

Katrien Grünberg, Eugenie A.P. Kuijpers, Erik P.A. de Klerk, Heidi W.F.M. de Gouw, Aloys C.M. Kroes, Elliot C. Dick, Peter J. Sterk.
Am J Respir Crit Care Med 1997;155:833-838.

5.1 Abstract

Disturbance of the balance between excitatory and inhibitory activity of the airway sensory nerves has been implicated in asthma pathogenesis, particularly during exacerbations of the disease. The objective of this study was to examine the effect of experimental rhinovirus 16 (RV16) infection on airway responsiveness to bradykinin, a potent sensory nerve stimulus, in asthma. Thirteen atopic, mildly asthmatic subjects participated in a parallel, placebo-controlled study. A total dose of 2.6 - 5.6 x 10^4 $TCID_{50}$ RV16 (n = 7) or its diluent (n = 6) was inoculated on 2 consecutive days (days 0, 1). Histamine and bradykinin challenges were performed before (days -7, -6) and after inoculation (days 3, 4). The response was measured by FEV_1 and partial flow-volume curves, and was expressed as $PC_{20}FEV_1$ and $PC_{40}V_{40p}$, respectively (changes expressed in doubling dose: DD). Before inoculation, $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ to histamine were not significantly different between the groups ($p \geq 0.22$), while $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ to bradykinin tended to be higher in the RV16 group ($p = 0.11$ and $p = 0.06$, respectively). $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ to histamine decreased significantly in the RV16 group (mean change ± SEM: -0.65 ± 0.20 DD, $p = 0.02$ and -0.98 ± 0.28 DD, $p = 0.01$, respectively), but not in the placebo group ($p \geq 0.26$). $PC_{40}V_{40p}$ to bradykinin increased significantly in the placebo group (+2.46 ± 0.92 DD, $p = 0.04$), with a similar trend for $PC_{20}FEV_1$ (+1.50 ± 0.62 DD, $p = 0.06$), whilst there were no significant changes in the RV16 group ($p \geq 0.77$). These changes in $PC_{40}V_{40p}$ to histamine and bradykinin were significantly different between the groups ($p = 0.02$). We conclude that repeated bradykinin challenge over a 10 days interval induces tachyphylaxis in asthmatic subjects *in vivo*, and that experimental RV16 infection abolishes such tachyphylaxis to bradykinin, while it enhances airway responsiveness to histamine. These results do not favor a predominant role of airway sensory nerves in rhinovirus-induced exacerbations of asthma.

5.2 Introduction

Asthma is a chronic inflammatory disease of the airways characterized by episodic worsening of symptoms such as chest tightness, dyspnea and cough, accompanied by variable airways obstruction (1). In prospective epidemiological studies, it appears that exacerbations of asthma are closely associated in time with respiratory virus infections (78). A respiratory virus can be found in 44 to 83 % of all asthma exacerbations, and about half of these viruses can be identified as rhinovirus (78).

Experimental studies have shown that rhinovirus 16 (RV16) colds induce airway hyperresponsiveness to inhaled histamine and methacholine, and an increase in maximal bronchoconstrictive response to methacholine in subjects with atopic rhinitis (162) or asthma (148,163,164). One could postulate a virus-induced increase in airway wall inflammation (297). Indeed, infiltration of lymphocytes and eosinophils into the mucosa has been described in bronchial biopsies after experimental RV16 infection in six atopic asthmatic subjects (164). This finding is in keeping with the hypothesis that inflammatory changes are involved in rhinovirus-induced exacerbations of asthma, even though the involvement of cholinergic mechanisms within the airways cannot be excluded (43).

It has been postulated that non-adrenergic non-cholinergic (NANC) sensory nerves can drive airway inflammation in asthma, particularly during exacerbations of the disease (307). Triggering of such unmyelinated sensory nerve endings (C-fibers) in the airway mucosa by endogenous or inhaled substances may cause an axon reflex, leading to the release of neuropeptides (*e.g.* substance P and neurokinin A), and/or a bronchoconstrictive cholinergic reflex (42). Neuropeptides potentially contribute to airway smooth muscle contraction, microvascular leakage, mucus hypersecretion, tracheo-bronchial vasodilatation and recruitment and activation of inflammatory cells, which might contribute to worsening of non-specific bronchial hyperresponsiveness in asthma (42). In asthma, inhalation of bradykinin, one of the most potent stimuli of airway sensory nerves (308), closely mimics the symptoms of the disease such as airway narrowing, cough, and chest tightness, whereas normal subjects do not respond (309). Moreover, levels of bradykinin in bronchoalveolar lavage fluid in asthmatic subjects have been found to be elevated as compared to normals (310).

From animal studies there is evidence that mechanisms opposing the sensory nerve-mediated bronchoconstriction, such as the production of nitric oxide (NO) by NO-synthase or degradation of neuropeptides and bradykinin by neutral endopeptidase (NEP) may become dysfunctional after parainfluenza-3 infection (242,311). Interestingly, in normal humans the level of bradykinin in nasal lavages has been shown to increase during rhinovirus colds (258). In addition, C-fiber nerve endings may become more exposed to inhaled stimuli secondary to epithelial shedding, as has been observed in the nose during rhinovirus infections in normals (225). One could postulate that rhinovirus infection induces comparable effects in the lower airways, thereby promoting the airway hyperresponsiveness to bradykinin in asthma.

In the present study, we hypothesized that RV16 infection increases airway hyperresponsiveness to bradykinin in asthmatic patients. To that end, we performed bradykinin inhalation challenges before, and four days after placebo-controlled experimental RV16 infection in atopic subjects with mild asthma *in vivo*.

5.3 Methods

5.3.1 *Subjects*

Fourteen nonsmoking adults with atopic, mild persistent asthma (1) (mean age, 22.6 yr.; range 20 to 26 yr.) participated in this study. Their asthma was characterized by episodic symptoms of chest tightness and wheezing, with $FEV_1 \geq 70$ % predicted and $PC_{20}FEV_1$ to histamine < 4 mg/ml (289). Atopy was defined by one or more positive skin prick tests (wheal > 3 mm) to 12 common allergens. The subjects had not used oral or inhaled corticosteroids for at least 3 months before entry into the study, and they had not suffered from common cold symptoms during the 4 weeks preceding the study. Symptoms of asthma had been stable during that period, and were controlled by on demand usage of inhaled salbutamol alone, which was withheld for at least 8 hours before each study visit. The subjects did not use any other medication. Before entry into the study, none of the 7 subjects who received RV16 had circulating neutralizing antibodies against RV16 (titer ≤ 1:2 against 20-25 x 50% tissue culture infective dose -$TCID_{50}$-). The study was conducted from September to November 1995. The protocol was approved by the Hospital's Medical Ethics Committee, and informed consent was obtained from all participants. Patients' characteristics are summarized in table 1.

Table 5.1: Patients' characteristics

subjects	sex	age (yr)	FEV_1 (%pred.)	PC_{20} H* (mg/ml)	culture days 2&10	titer pre-post	cold score	allergy
placebo								
1	m	22	102.8	0.27	neg/neg	4-4	1	H, P
2	f	24	86.9	0.31	neg/neg	32-16	4	P, C
3	f	21	90.7	0.41	neg/neg	16-16	3	H, C
4	f	23	86.4	0.58	neg/neg	256-256	4	H, C
5	m	21	87.4	0.74	neg/neg	8-4	2	H, P
6	f	20	90.9	1.30	neg/RV	4-8	0	H, P, C
mean±SEM			90.8±2.5	0.52±0.35†			2.3 ± 1.5	
RV16								
7	m	26	77.5	0.15	RV16/neg	1-16	9	H
8	f	22	85.5	0.22	RV16/neg	1-4	1	H, P, C
9	m	20	93.2	0.62	RV16/neg	2-16	8	H
10	m	25	70.0	0.80	RV16/neg	1-8	14	H
11	m	20	91.5	1.08	RV16/RV16	1-128	8	P, C
12	m	25	95.8	2.98	RV16/RV16	1-2	4	C
13	f	24	89.2	4.00	neg/neg	2-32	12	H, P, C
mean±SEM			86.1±3.5	0.80±0.66†			8.0 ± 1.7	

M: male, F: female. Allergy: positive skin prick test (Vivodiagnost, ALK, Benelux). H: house dust mite, C: cat, P: grass pollen. *: $PC_{20}FEV_1$ to histamine before inoculation of RV16 or placebo. Titer: serum dilution in RV16 neutralization assay pre- and post RV16 or placebo inoculation. †: geometric mean ± SEM (doubling dose).

5.3.2 *Design*

The study had a placebo-controlled, parallel design. Each subject was screened for inclusion and exclusion criteria, including a bradykinin challenge at least 10 days before entering the study (mean: 20.9 days, range: 10 - 40 days). On the first 2 study visits a histamine challenge (day -7) and a bradykinin challenge (day -6) were performed. Subsequently, RV16 (n = 7) or diluent (n = 6) was administered on two successive days (days 0 and 1). Histamine challenge

and bradykinin challenge was repeated at 3 and 4 days after the first inoculation, respectively. Nasal lavages for viral culture were taken immediately before the first inoculation, and 2 and 10 days later. A blood sample was drawn at the first study visit and 3 weeks after inoculation, to determine the RV16 neutralizing antibody titer in serum. Bronchial challenges were performed at the same time of the day (± 1 h) in each subject.

5.3.3 RV16 inoculation

The RV16 strain and stock was the same as used in previous experiments in humans *in vivo* by others (162) and by ourselves (148,163). The virus was cultured according to standards of good laboratory practice and the inoculum was tested to be safe for human *in vivo* usage (286). Nasal inoculation was performed according to a previously described protocol (148,163). Briefly, a dose of 1.3 - 2.8 x10^4 $TCID_{50}$ diluted in 1.5 ml Hanks' balanced salt solution with 0.5% gelatin (w/v) was nasally administered to 7 subjects by inhalation of 0.5 ml from a nebulizer (DeVilbiss 646, DeVilbiss Co., Somerset, PA) connected to a face mask, spraying of 0.5 ml by atomizer (DeVilbiss 286) powered by a compressor, and instillation of 0.5 ml by pipette into the nostrils. This procedure was repeated after a 24 h interval. In 6 subjects the diluent was administered in the same fashion.

5.3.4 Laboratory confirmation of infection

We considered a fourfold or greater increase in RV16-specific neutralizing antibody in the serum, and/or recovery of RV16 from the nasal lavages being confirmative of infection (163). The levels of neutralizing antibodies were determined by a neutralization assay using homologous virus. Nasal lavages were inoculated into human embryonic fibroblast (HEL) cultures, and incubated at 32^0 C for 14 days. In the positive cultures RV16 was identified by neutralization assay, using RV16 specific guinea pig immune serum (1126AS/GP-VR; American Type Tissue Culture Collection, Rockville, MD). All nasal lavages were also inoculated into rhesus monkey kidney cells (LLC-MK2), HEp2 cell cultures and HEL cell cultures, and cultured at 37^0 C, in order to exclude other intercurrent respiratory virus infections.

In addition, the subjects scored their cold symptoms including sneezing, sore throat, nasal discharge, stuffy nose, headache, cough, malaise and chills on a 4-point scale, ranging from none (0) to severe (3). These scores were added up to a maximum of 24, and are referred to as cold score. Cold scores were recorded 3 times daily throughout the study period (148). The highest cold score recorded after inoculation is presented in table 1.

5.3.5 Inhalation challenges

The histamine challenges tests were performed using histamine-bi-phosphate (Bufa, Uitgeest, The Netherlands) in phosphate buffered saline, according to a standardized procedure (289). Serial doubling concentrations ranging from 0.03 to 8 mg/ml were nebulized using a DeVilbiss 646 nebulizer (output 0.13 ml/min), in connection to an in- and expiratory valve box with an expiratory filter (Pall Ultipor BB50T). The aerosol was inhaled by tidal breathing for 2 min at 5 min intervals, with the nose clipped.

The bradykinin challenges were performed using bradykinin (Bachem, Switzerland) in phosphate buffered saline. Bradykinin, which was derived from a single batch and lot number, was stored in a stock solution (1.1 ml portions) of 8 mg/ml, at -20^0 C. On the day of the challenge test, serial doubling concentrations, ranging from 0.03 to 8 mg/ml were prepared from the stock solution in siliconized glass tubes by the hospital pharmacy, and were kept on

melting ice until use. Bradykinin was aerosolized by an efficient and previously validated jet nebulizer (Mallinckrodt Diagnostica, Petten, The Netherlands) (46). The nebulizer was filled with 0.5 ml of each concentration. The whole fluid content of the nebulizer was sprayed by compressed nitrogen during 1 min into a drying chamber (30 L collapsible bag), in which the droplets evaporate to dry particles. Subsequently, the whole content of the collapsible bag was inhaled through a three-way valve box and a mouth piece by tidal breathing for 3-4 min, while 100% oxygen (4 L/min) was supplied into the valve box. First, the diluent of bradykinin was inhaled and baseline measurements were made. Then, increasing doses of bradykinin were given at 8 min intervals.

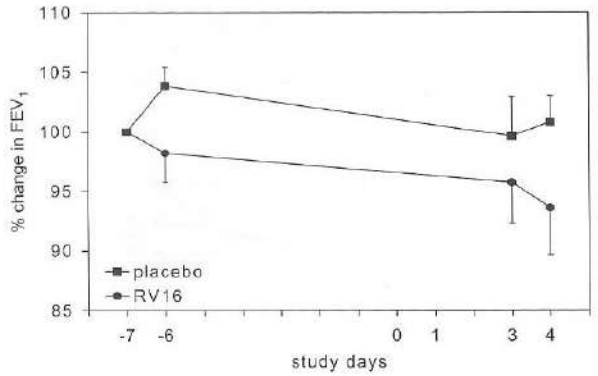

Figure 5.1. Change in FEV_1 during the study, expressed as the percentage change as compared to the FEV_1 measured at day -7 (100%) in the placebo group (squares) and in the RV16 group (circles). There was no significant change in FEV_1 in either treatment group (MANOVA: p = 0.37).

5.3.6 Response to inhalation challenge

The response to the inhaled drugs was measured by the FEV_1 and V_{40p}, obtained from maximal and partial expiratory flow-volume curves, respectively (46,312). The expiratory flow-volume curves were standardized for volume and volume history. First, a maximal inhalation to TLC was carried out, followed by tidal breathing for 45 sec. Then, a partial flow-volume curve was initiated at a standardized lung volume, being 60% of the largest FVC, marked of from TLC. Immediately thereafter, a maximal flow-volume maneuver was carried out. The measurements were performed using a dry rolling-seal spirometer (Morgan spiroflow, Rainham, United Kingdom) and an analogue recorder (X-Y recorder BD 90; Kipp, Delft, The Netherlands). Baseline FEV_1 and V_{40p} values were determined as the mean of three reproducible measurements (FEV_1 within 5%). Subsequently, single measurements of FEV_1 and V_{40p} were made, starting 45 s after each histamine dose, and 1 and 3 min after each bradykinin dose (46). The measurements accompanying the lowest FEV_1 were used in the analysis (46). The test was discontinued when FEV_1 decreased by more than 20% from baseline, after which 200 µg salbutamol was inhaled to provide immediate bronchodilation.

5.3.7 Analysis

The response in FEV_1 or V_{40p} was expressed as the percent fall from mean baseline value, and was plotted against the log concentration of the drug (289). The position of the curve was expressed as the concentration leading to 20% fall in FEV_1 ($PC_{20}FEV_1$) or 40% fall in V_{40p} ($PC_{40}V_{40p}$), which was calculated by linear interpolation of the last two adjacent data points (289).

Two-tailed paired Student's *t*-test was applied in testing the changes in $\ln(PC_{20}FEV_1)$ and $\ln(PC_{40}V_{40p})$ within each treatment group, whilst unpaired Student's t-test was used to test the differences in the changes between the groups. Changes in baseline FEV_1 (measured before each challenge test) were analyzed by repeated measures analysis of variance (MANOVA), with RV16 or placebo as between-group factors, and time as a within-group factor. $P < 0.05$ was considered statistically significant (313). Mean values of the log-transformed data were expressed as geometric mean ± SEM in doubling dose (DD). Changes in $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ were expressed as DD.

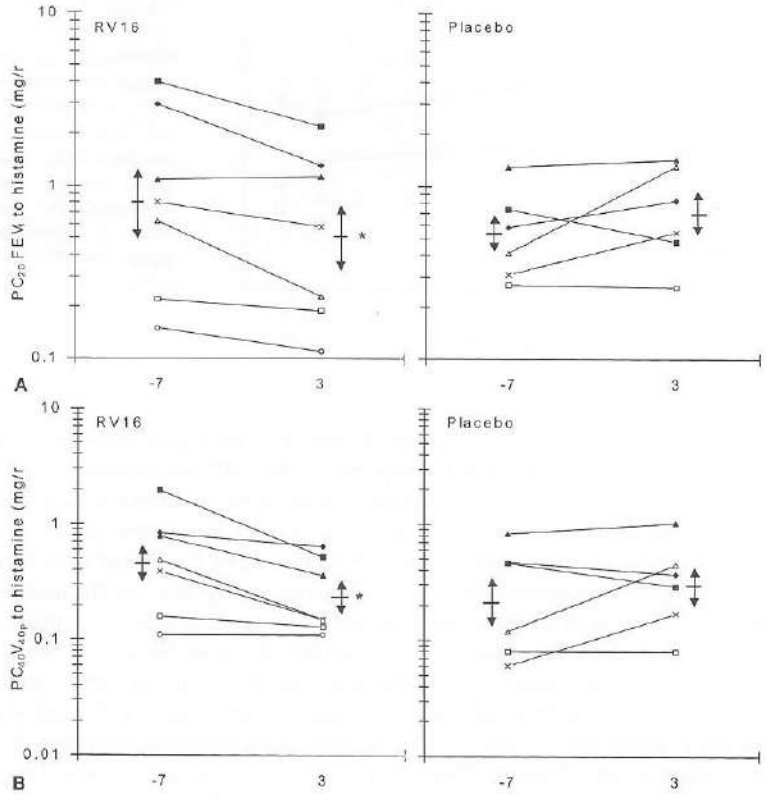

Figure 5.2. The $PC_{20}FEV_1$ (A) and $PC_{40}V_{40p}$ (B) to histamine before (day -7) and after (day 3) inoculation in the RV16 group (left panel) and the placebo group (right panel). In the RV16 group PC_{20} and PC_{40} decreased significantly ($p \leq 0.02$). These changes were significantly different from placebo ($p = 0.02$). The bars and arrows indicate geometric mean ± SEM (in doubling dose). Each symbol represents an individual subject. ✱: $p \leq 0.02$ (paired t-test).

5.4 Results

5.4.1 Confirmation of infection

In the placebo group, all cultures except one (positive for rhinovirus, not RV16, in patient 6, on day 10 after inoculation) remained negative for RV16 during the study. None of the placebo-treated subjects showed an increase in RV16 neutralizing antibodies. In the RV16-treated group RV16 infection was confirmed in all subjects. No other viruses were detected in this group (table 1).

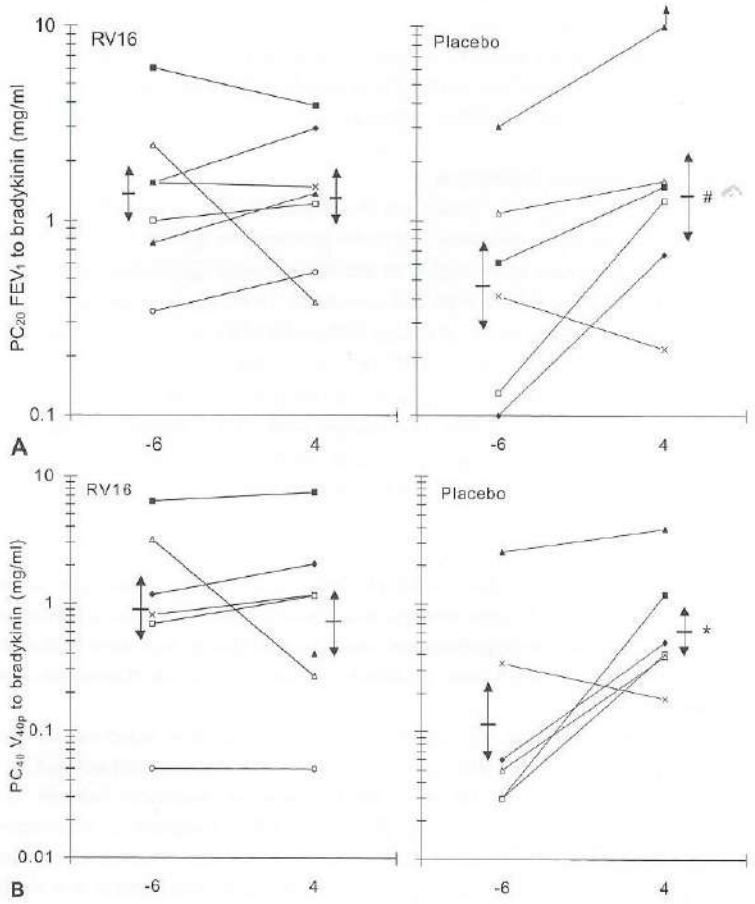

Figure 5.3. The $PC_{20}FEV_1$ (A) and $PC_{40}V_{40p}$ (B) to bradykinin before (day -6) and after (day 4) inoculation in the RV16 group (left panel) and the placebo group (right panel). In the placebo group PC_{20} tended to increase ($p = 0.06$), while PC_{40} increased significantly ($p = 0.04$). The change in PC_{20} tended to be different from RV16 ($p = 0.07$), while the change in PC_{40} was significantly different from RV16 ($p = 0.02$). The bars and arrows indicate geometric mean ± SEM (in doubling dose). Each symbol represents an individual subject. #: $p = 0.06$ (paired t-test), ✱: $p = 0.04$ (paired t-test).

5.4.2 *Baseline lung function*

Before inoculation with RV16 or placebo, FEV_1 % predicted was not different between the treatment groups (MANOVA, p = 0.31) (table 1). During the study, there were no significant changes in FEV_1 in either treatment group (MANOVA, p = 0.37) (figure 1).

5.4.3 *Airway responsiveness to histamine*

At baseline, mean $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ to histamine were not different between the groups (p = 0.44 and p = 0.22, respectively) (table 1). In the RV16 group there was a significant decrease in $PC_{20}FEV_1$ (mean change ± SEM: -0.65 ± 0.20 DD, p = 0.02) and in $PC_{40}V_{40p}$ (-0.98 ± 0.28 DD, p = 0.01) at day 3 as compared to baseline values, whereas $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ did not change significantly in the placebo group (0.41 ± 0.32 DD, p = 0.26 and 0.45 ± 0.42 DD, p = 0.34, respectively). The changes in $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ were significantly different between the groups (p = 0.02, both variables) (figure 2).

5.4.4 *Airway responsiveness to bradykinin*

At one visit (subject 11, day -6) V_{40p} could not be measured due to a technical problem. $PC_{20}FEV_1$ to bradykinin was not significantly different between the groups before inoculation (p = 0.11), while $PC_{40}V_{40p}$ tended to be higher in the RV16 group (geometric mean ± SEM: placebo: 0.12 ± 1.04 DD, RV16: 0.93 ± 0.98 DD, p = 0.06). In the placebo group there was a trend towards a significant increase in $PC_{20}FEV_1$ (+1.50 ± 0.62 DD, p = 0.06) together with a significant increase in $PC_{40}V_{40p}$ (+2.46 ± 0.92 DD, p = 0.04). However, there were no significant changes in $PC_{20}FEV_1$ and $PC_{40}V_{40p}$ in the RV16 group (-0.09 ± 0.48 DD, p = 0.86 and -0.36 ± 0.60 DD, p = 0.77, respectively). The changes in $PC_{20}FEV_1$ showed a trend towards a significant difference between the groups (p = 0.07), whilst the changes in $PC_{40}V_{40p}$ were significantly different between the groups (p = 0.02) (figure 3).

5.5 DISCUSSION

This study shows that bradykinin induces tachyphylaxis to a repeated challenge in atopic asthmatic subjects, even over a 10 days time period. Experimental infection abolishes such tachyphylaxis, but does not enhance hyperresponsiveness to bradykinin, as it does to histamine. These findings do not favor a major role of airway sensory nerves in rhinovirus-induced exacerbations of asthma.

This is the first study addressing the effects of a respiratory virus infection on airway responsiveness to bradykinin in asthmatic subjects *in vivo*. Our results confirm and extend those obtained by Summers *et al.* (176), who did not observe rhinovirus-induced airway responsiveness to bradykinin after experimental RV-EL or RV 2 infection in non-asthmatic volunteers. However, interpretation of those data is hampered by the absence of a placebo-inoculated control group. Since no increase in airway responsiveness to histamine was observed in these normal subjects, factors such as subject selection and rhinovirus serotype seem to preclude further comparison with the present findings.

In this study, we applied validated methods for inoculation of the rhinovirus (148,162,163), and for measuring airway hyperresponsiveness (289). The degree of enhancement of airway sensitivity to histamine as observed in the present study is similar to that reported in previous studies by others (164) and by ourselves (148), and fits in with the findings of Cheung *et al.* (163), describing an increase in the maximal bronchoconstrictive response to methacholine up to 2 weeks after infection. This indicates that experimental RV16 infection in asthma is a

reproducible model for virus-induced exacerbations of asthma. Special care was taken to ensure optimal delivery of bradykinin into the lungs. First, by using freshly prepared serial dilutions of constant peptide content, and secondly by using a highly efficient jet nebulizer system (46). Therefore, we believe that the absence of virus-induced increase in hyperresponsiveness to bradykinin is not due to technical factors. This is further supported by the consistent decrease in responsiveness to bradykinin in the placebo group. Tolerance to repeated bradykinin challenges is a well known phenomenon (314,315), which, together with the characteristic fluctuations of lung function and airway hyperresponsiveness in asthmatic patients, warrants a placebo-controlled design. The placebo-controlled design, in combination with the use of a very sensitive index of bronchoconstriction such as V_{40p} (163,312) allowed us to demonstrate differences in changes in bradykinin responsiveness between the RV16 and the placebo group.

The effects of bradykinin are various. In guinea pigs *in vivo*, mainly three mechanisms have been shown to be involved in bradykinin-induced bronchoconstriction: the secondary release of prostanoids, evocation of cholinergic reflexes, and release of neuropeptides (316). In humans *in vitro*, these effects appear to be predominantly mediated by the bradykinin B_2 receptor (317). In asthmatic subjects *in vivo*, pretreatment with the cycloxygenase inhibitor flurbiprofen had only a small protective effect against inhaled bradykinin (318), whereas inhalation of the anti-cholinergic agent ipratroprium bromide and the tachykinin receptor antagonist FK-224 had considerable protective effects (309,319). The bronchoconstrictive response to bradykinin can be potentiated by inhalation of the NEP inhibitor phosphoramidon (320), and by inhalation of L-NMMA, a nitric oxide (NO) synthase pseudo-substrate (321). This suggests that in humans *in vivo*, the bradykinin-induced bronchoconstriction is mediated primarily by its effect on afferent nerve fibers, leading to tachykinin release and cholinergic reflex mechanisms. However, functional antagonists (such as NO) may counteract this response.

Does this provide an explanation for the development of tolerance? In asthmatics *in vivo*, it has been demonstrated that tolerance to bradykinin over a period of several hours can not be prevented by inhibition of cycloxygenase (314). We can only speculate on other potential explanations for this phenomenon, such as: increased production of functional antagonists, like NO, upregulation of kinases such as neutral endopeptidase, downregulation or decreased expression of bradykinin receptors, or relative depletion of tachykinins from the sensory nerve endings, as has been demonstrated for capsaicin (42). The time span of 10 days of tolerance to inhaled bradykinin in the present study was remarkable, and deserves further study in view of the above mechanisms.

RV16 infection abolished the tolerance to bradykinin, but did not induce hyperresponsiveness to this stimulus as compared to baseline. The latter contrasts to the recently described enhancement of responsiveness to bradykinin 2 days after an allergen challenge in atopic asthmatic subjects (315). Apparently, sensory nerve activity does not predominate after rhinovirus infection in asthma, as it does *e.g.* after Sendai virus infection in guinea pigs and rats *in vivo* (311,322). The loss of tolerance to bradykinin in the RV16 group might be due to the interaction of RV16 with any of the possible mechanisms listed above. If a rhinovirus cold leads to epithelial damage or dysfunction in the bronchi, a number of endogenous bradykinin inhibitor systems might be impaired. Human *in vitro* studies have shown that the removal of the epithelium markedly potentiated the bronchoconstrictive effect of bradykinin (317), fitting in with loss of NEP (311), or reduced functional antagonism by relaxant cycloxygenase metabolites (317) and NO (242). In view of the increased levels of kinins found in the nasal lavage during rhinovirus infection, such impairment in counteracting this potent mediator might be clinically relevant. However, the present lack of worsening in

bradykinin responsiveness following RV16 infection strongly suggests that sensory nerve activation is not a major pathway of rhinovirus-induced exacerbations of asthma.

In conclusion, RV16 infection induces an increase in airway hyperresponsiveness to histamine, and prevents the development of tolerance to repeated bradykinin challenges in asthmatic subjects *in vivo*. These findings do not favor a major involvement of sensory nerves in virus-induced exacerbations of asthma. The development of specific receptor antagonists (*e.g.* for bradykinin or neuropeptides) for human *in vivo* usage will give new opportunities to study the role of neurogenic mechanisms in virus-induced exacerbations of asthma, and may eventually lead to new therapeutic strategies.

6 EXPERIMENTAL RHINOVIRUS 16 INFECTION: EFFECTS ON CELL DIFFERENTIALS AND SOLUBLE MARKERS IN SPUTUM IN ASTHMATIC SUBJECTS

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Am J Respir Crit Care Med 1997;156:609-616

6.1 ABSTRACT

Asthma exacerbations are often associated with respiratory virus infections, particularly with rhinovirus. In the present study we investigated the effect of experimental rhinovirus 16 (RV16) infection on airway inflammation as assessed by analysis of hypertonic saline induced sputum. Twenty-seven non-smoking atopic, mildly asthmatic subjects participated in a placebo-controlled parallel study. RV16 ($n = 19$) or its diluent ($n = 8$) was nasally administered. Sputum inductions were performed at entry, and on days 2 and 9 after inoculation, while airway responsiveness to histamine (PC_{20}) was measured on days 4 and 11. Cell differentials and levels of albumin, ECP, IL-8 and IL-6 were determined. The cellular origin of IL-8 was investigated by intracellular staining. RV infection was confirmed by culture and/or by antibody titer rise in each of the RV16-treated subjects. There were no significant changes in the sputum differentials of non-squamous cells (MANOVA, $p \geq 0.40$). In the RV16 group, there was a significant increase in the levels of ECP, IL-8 and IL-6 at day 2 after infection ($p < 0.05$) whereas the albumin levels did not change ($p = 0.82$). The levels of IL-8 and IL-6 remained elevated up to 9 days after infection ($p < 0.05$). The increase in the percentage IL-8 positive cells at day 2 after infection could be attributed to the increase in IL-8 positive neutrophils ($p < 0.02$). There was a significant decrease in PC_{20} at day 4 ($p = 0.02$), which was no longer significant at day 11 ($p = 0.19$). The decrease in PC_{20} correlated significantly with the increase in ECP in the first week ($r = -0.60$), and with the change in the percentage eosinophils in the second week after inoculation ($r = -0.58$).

We conclude that experimental RV16 infection in atopic asthmatic subjects increases airway hyperresponsiveness, in conjunction with augmented airway inflammation, as reflected by an increase in ECP, IL-8 and IL-6 in sputum. Our results suggest that the RV16-enhanced airway hyperresponsiveness is associated with eosinophilic inflammation.

6.2 INTRODUCTION

Asthma is a chronic disease, that is characterized by episodic chest tightness and wheezing, associated with variable airways obstruction (1). At the level of the bronchi, chronic inflammation and epithelial shedding are regarded to be characteristic to airway pathology in asthma (181). The inflammatory process is considered to be mediated by the selective release of cytokines and other mediators by infiltrating leukocytes, but also by resident cells within the airways (323), bringing about the characteristic accumulation and activation of eosinophilic granulocytes and other accompanying features such as airway wall edema and luminal exudation (324).

Asthma exacerbations appear to be associated in time with respiratory virus infections (78). The detection rate of such viruses during wheezing episodes has been reported to be as high as 83% in 9 - 11 year old children, rhinovirus accounting for approximately 50% of the identified viruses (78). Furthermore, experimental rhinovirus infections in asthma and atopic rhinitis have been shown to transiently enhance airway hyperresponsiveness to histamine (148,164), and to increase the maximal bronchoconstrictive response to methacholine in mildly asthmatic subjects (163).

These functional changes in asthma suggest a rhinovirus-induced increase in airway inflammation (297). Indeed, Fraenkel *et al.* (164) recently described an increase in numbers of submucosal T-lymphocytes, without a change in the CD4/CD8 ratio in bronchial biopsies after experimental RV16 infection in a group of 11 normals and 6 atopic asthmatics. Moreover, the numbers of activated eosinophils in the epithelium increased in the acute phase of infection in both normals and asthmatics, whilst interestingly, in the latter group the eosinophils counts remained elevated even after recovery. These findings are suggestive of rhinovirus-induced eosinophilic airway inflammation in asthma.

One of the potential explanations for enhanced cellular infiltration into the airways after infection may be virus-induced production of chemoattractant mediators, such as chemokines or other cytokines within the airway mucosa. *In vitro* studies have shown that bronchial epithelial cell lines and primary epithelial cell subcultures produce several cytokines in response to incubation with rhinovirus (215,218,221-223,231). This is in keeping with results in humans *in vivo*, showing an increase in the levels of IL-8 in the nasal lavage after experimental rhinovirus infections (148). Hence, it can be postulated that epithelial-derived chemokines are involved in the cellular and physiological responses of the airways to a rhinovirus infection.

In the present study we hypothesized that experimental RV16 infection in asthmatic subjects induces changes in the cellular constituents and the production of cytokines within the airways in atopic, mildly asthmatic subjects. To that end, we applied the recently validated, non--invasive technique of sputum induction by means of inhalation nebulized hypertonic saline. We performed sputum inductions and measured lung function and airway responsiveness to histamine before and twice after experimental RV16 infection in atopic, mildly asthmatic subjects *in vivo*. Sputum was examined for cell differentials and for albumin, ECP, IL-8, and IL-6 in sputum supernatant. The cellular origin of one of the cytokines was investigated by intracellular IL-8 protein staining.

6.3 METHODS

6.3.1 Subjects

Twenty-seven non-smoking, atopic subjects with mild persistent asthma participated in this study (1). The subjects' characteristics are listed in table 1. Their FEV_1 was greater than 70% of the predicted value, and the provocative concentration of histamine causing a 20% fall in FEV_1 (PC_{20}) was smaller than 8 mg/ml (1). Atopy was examined by skin prick test, using a panel of 10 common aeroallergens (Vivodiagnost, ALK, The Netherlands). The subjects did not report symptoms of a common cold in the month preceding the study, and they had not used inhaled or oral corticosteroids for at least 3 months, nor had they used theophyllines, antihistamines, sodium cromoglycate, or nedocromyl sodium for at least 6 weeks preceding the study. Symptoms of asthma were stable and controlled by on demand usage of inhaled salbutamol alone, which was withheld for at least 8 hours before the measurements. Before entry into the study, 4 out of 8 placebo-treated subjects did not have detectable levels of circulating neutralizing antibodies against RV16 (titer: $\leq$ 1:1) against 25 times the 50% tissue culture infective dose ($TCID_{50}$), while 4 subjects had titers in the range of 1:2 to 1:128. In the RV16 group 14 out of 19 subjects had no detectable neutralizing antibodies, while 5 subjects had low titers, between 1:2 and 1:16. The study was conducted during the months of July to December. The study was approved by the Medical Ethics Committee, and informed consent was obtained from all participants.

6.3.2 Design

The study had a double-blind parallel placebo-controlled design. Each subject was screened for the in- and exclusion criteria before entry into the study. Before inoculation with RV16 or placebo a sputum induction (day -5) and a histamine challenge were performed (day -3). Subsequently, RV16 or placebo was inoculated on two consecutive days (days 0 and 1). Thereafter, sputum induction was repeated at days 2 and 9 after the first inoculation, while the histamine challenge was repeated at days 4 and 11. The sputum inductions were preceded by nasal lavage.

6.3.3 RV16 inoculation

The RV16 strain and stock was the same as used in previous experiments in humans *in vivo* by others (162) and by ourselves (148,163). The virus was cultured according to standards of good laboratory practice and the inoculum was tested to be safe for human *in vivo* usage (286). Nasal inoculation of the RV16 was performed following a previously described protocol (163). Briefly, a dose of 0.5 - 2.9 x 10^4 was nasally administered by inhalation form a nebulizer (DeVilbiss 646, DeVilbiss Co. Somerset, PA), spraying by atomizer (DeVilbiss 286), and instillation by pipette into both nostrils. This procedure was repeated after 24 hr.

6.3.4 Confirmation of infection

A four-fold or greater increase in RV16-specific neutralizing antibody in serum, and/or recovery of RV16 from the nasal lavages was considered to confirm infection. Serum titers of neutralizing antibodies were determined by a neutralization assay, using 25 $TCID_{50}$ RV16. Nasal lavages were inoculated onto human embryonic lung fibroblasts (HEL) cultures, and incubated at 32° for 14 days. In the positive cultures RV16 was identified by neutralization assay, using RV16 specific guinea pig immune serum (1126AS/GP-VR; American Type Tissue Culture Collection, Rockville, MD). All nasal lavages were also inoculated onto LLC-MK2 cell cultures, Hep2 cell cultures and HEL cell cultures, and cultured at 37° C, in order to exclude

other intercurrent respiratory virus infections. In addition, the subjects recorded their cold symptoms including sneezing, sore throat, nasal discharge, stuffy nose, headache, cough, malaise and chills on a 4-point scale, ranging from none (0) to severe (3). These scores were added up to a maximum of 24, and are referred to as cold score. Cold scores were recorded 3 times daily throughout the study period (148). The highest cold score recorded after inoculation is presented in table 1.

Table 6.1: subject's characteristics

subject	sex	age (yr)	FEV_1 (% pred.)	PC_{20} (mg/ml)*	culture** (day 2/9)	antibody titer† (before/after)	cold score	β_2	allergy††
RV16									
1	F	25	71	0.12	+ / –	1 / 4	15	β_2	H, P
2	F	23	77	0.25	+ / –	1 / 4	10	β_2	H, P
3	M	21	103	0.32	+ / –	1 / 4	4	–	H, P
4	M	18	80	0.34	+ / +	1 / 16	6	β_2	H, C
5	M	19	81	0.37	+ / +	1 / 128	5	–	C
6	M	22	85	0.41	+ / –	1 / 32	0	β_2	H, C, P
7	M	20	87	0.71	+ / +	1 / 128	16	β_2	H, P
8	F	25	88	0.86	+ / +	1 / 1	11	–	H, C, P
9	M	26	108	0.87	+ / –	1 / 4	2	β_2	H
10	F	21	92	1.19	– / –	2 / 32	12	β_2	C
11	M	20	101	1.33	+ / +	16 / 256	12	β_2	H, C, P
12	M	21	80	1.39	+ / –	1 / 64	16	–	H, C, P
13	F	24	89	1.76	+ / +	8 / 512	22	–	H, C, P
14	M	22	89	2.03	+ / –	4 / 512	6	β_2	H, C
15	F	23	77	2.12	+ / +	1 / 128	14	–	H, C, P
16	M	26	69	2.39	+ / –	4 / 8	6	β_2	H, C
17	M	24	77	2.69	+ / –	1 / 32	5	β_2	H, C, P
18	M	21	90	2.98	+ / +	1 / 32	3	–	C
19	F	22	105	4.17	+ / +	1 / 16	6	–	H, C
mean±SEM			86.4±2.6	0.90±0.2					
placebo									
20	M	25	76	0.28	– / –	1 / 1	1	–	H
21	M	26	68	0.60	– / –	1 / 1	4	β_2	H, C
22	M	18	71	0.64	– / –	32 / 32	2	β_2	H, C
23	F	26	75	0.78	– / –	8 / 8	2	β_2	H, C, P
24	M	25	73	0.80	– / –	16 / 8	1	β_2	H, C
25	M	24	80	1.37	– / –	128 / 128	2	–	H, C, P
26	M	24	89	1.85	– / –	1 / 1	11	–	C
27	M	25	87	1.93	– / –	1 / 1	4	β_2	H, C, P
mean±SEM			77.4±2.6	0.87±0.4					

★ : Geometric mean ± SEM in doubling dose. ★★ : Rhinovirus culture of nasal lavage fluid.
† : RV16 neutralizing antibody titer in serum, expressed as serum dilution (1 = ≤ 1:1 dilution). ††: Allergy, as determined by skin prick test (wheal ≥ 3 mm). H = house dust mite, C = cat, P = grass and tree pollen mixture.

6.3.5 *Histamine challenge*

The histamine challenge was performed using histamine-bi-phosphate (Bufa, Uitgeest, The Netherlands), according to a standardized procedure (289). Serial doubling concentrations of nebulized histamine (0.03 to 8 mg/ml, DeVilbiss 646 nebulizer, output 0.13 ml/min) were inhaled by tidal breathing for 2 min at 5 min intervals, with the nose clipped. The response was measured by the FEV_1, using a dry rolling-seal spirometer (Morgan spiroflow, Rainham, United Kingdom) and an analogue recorder (X-Y recorder BD 90; Kipp, Delft, The Netherlands). Baseline FEV_1 was determined as the mean of three reproducible measurements. Subsequently, FEV_1 was measured after each dose. The test was discontinued when FEV_1 decreased by more than 20% from baseline. The provocative concentration causing 20% fall in FEV_1 (PC_{20}) was calculated by log-linear interpolation of the last two data points.

6.3.6 *Sputum induction*

Sputum was induced and processed according to the method described by Fahy *et al.* (325), which was slightly modified (326). Sodium chloride (4.5% w/v) was aerosolized using an ultrasonic nebulizer (DeVilbiss Ultraneb 2000, Somerset, PA) with a calibrated mass median aerodynamic diameter of 4.5 µm, and the output set at 2.5 ml/min. The aerosol was inhaled through a 100 cm long tube with an internal diameter of 22 mm, connected to a two-way valve (Hans-Rudolph, Kansas City, MO) equipped with a mouth piece, while the subjects' nose was clipped.

The aerosol was inhaled for serial doubling time periods (0.5 to 8 min), and subsequently for repeated 5 min periods, for a maximum of 30 min. After each inhalation period FEV_1 was measured. Then, the subjects were instructed to rinse their mouth and throat and gargle thoroughly with water, and if necessary the nose was blown. Subsequently, the subjects were encouraged to cough up and expectorate sputum into a plastic container. A sputum weight of at least 2 g was considered sufficient. If FEV_1 dropped by more than 20% from baseline value, 200 µg salbutamol was administered.

6.3.7 *Sputum processing and cell differentials*

In order to homogenize the sputum, the whole sputum samples were gently mixed with an equal weight of dithiothreitol 0.1% (w/v) (Sputolysin, Calbiochem, Lajolla, CA), using a wide bore plastic pipette, and placed in a shaking water bath at 37° C for 15 min. The samples were then centrifuged at 350 x *g* for 10 min. The supernatants were collected and stored at -70° C until further analysis. The pellets were re-suspended in phosphate buffered saline, filtered through a gauze, and cytospins were made (200 x g, 3 min) (Shandon 3 cytocentrifuge, Shandon Southern Instruments, Seickley, PA). Of each sputum sample 2 cytospins were Diff-quik stained and coded. All cell differentials were made by one observer (H.H.S.), counting at least 500 cells per cytospin. In addition, at least 4 cytospins were dried and stored at -70° C pending intracellular IL-8 staining.

6.3.8 *Biochemical analysis*

In sputum supernatant albumin levels were assessed by nephelometric assay (Beckman, Brea, CA), IL-8 levels by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (mAb) against IL-8 (CLB, Amsterdam, The Netherlands), IL-6 levels by mAb ELISA (CLB) and ECP by fluoro-enzyme immuno-assay (FEIA) (Pharmacia, Uppsala, Sweden) according to the manufacturers' instructions. The detection limits of the ELISA for IL-8 and IL-6 were 4

pg/ml and 1.5 pg/ml, respectively, and 4 pg/ml for the ECP FEIA. Addition of DTT to the recombinant cytokines resulted in >90% recovery of the proteins in the ELISA.

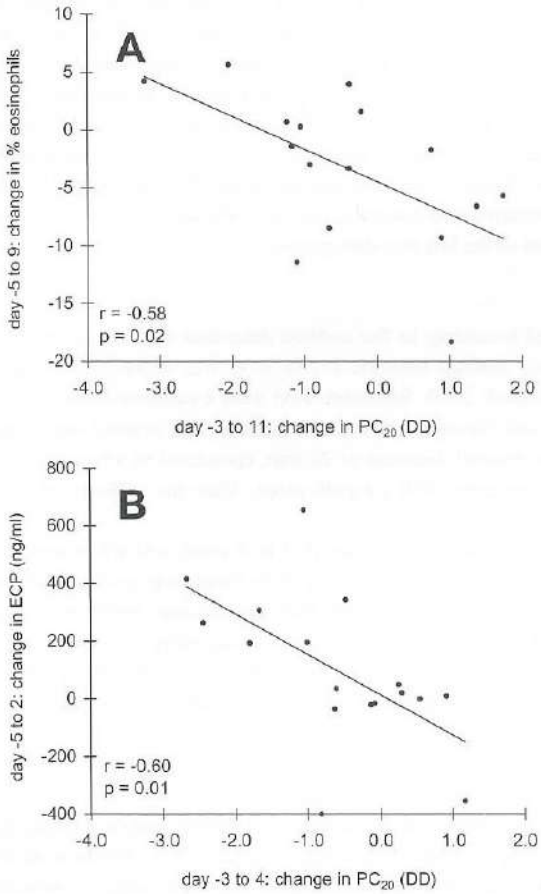


Figure 6.1.
A: Relationship between the change in the percentage eosinophils in sputum and the change in PC_{20} in the RV16 group in the second week after infection as compared to baseline.

B: Relationship between the change in ECP in sputum supernatant and the change in PC_{20} in the RV16 group in the first week after infection as compared to baseline.

6.3.9 Intracellular IL-8 staining

The intracellular IL-8 staining procedure (327) was performed on thawed, para-formaldehyde fixed cytospins, according to Dolhain *et al.* (328). In order to obtain and maintain permeabilized cells all washing and staining solutions contained saponin 0.1% (w/v) (Riedel-de Häen, Seelze, Germany). First, endogenous peroxidase (H_2O_2 0.3% (v/v), azide 0.3% (w/v)) and non-specific binding sites were blocked (fetal calf serum 5%, Gibco, Breda, The Netherlands). The actual staining procedure consisted of a monoclonal IgG_1 antibody against IL-8 (CLB), followed by horse-radish peroxidase (HRP)-linked rabbit-anti-mouse and swine-anti-rabbit antibody amplifying steps (DAKO, Glostrup, Denmark). Then, the cytospins were incubated in the dark with 0.1% H_2O_2 and diamino-benzidine (DAB) (0.5 mg/ml) for 30 min. Finally, the cytospins were counterstained with Mayer's Lösung and eosin. In pilot experiments it was shown that stimulation with LPS of peripheral blood polymorphonuclear cells and macrophages isolated from bronchoalveolar lavage greatly increased the intracellular staining for IL-8. Likewise, cells of the pulmonary epithelial cell line A549 cells stimulated with TNF-α stained positively for IL-8, while unstimulated cells were always negative. TNF-α stimulated A549 cells were therefore used as a positive control for the staining procedure. As a negative control, a second cytospin of all samples was stained with control mouse IgG_1 (CLB) instead of mAb anti-IL-8. All cytospins were counted twice, counting at least 500 cells. Cell were characterised by cell type and presence or absence of the typical perinuclear IL-8 staining pattern (327,328).

6.3.10 Statistical analysis

The squamous cells in sputum cell differentials were expressed as a percentage of all cells, while all other cell types were expressed as a percentage of all non-squamous cells. IL-8-positive staining cells were expressed as a percentage of all non-squamous cells, or as a percentage of a particular cell type. Eosinophils and lymphocytes only occasionally stained positively for IL-8, and were therefore also lumped as one "cell type" in the statistical analysis. The mean data of the 2 differential cell counts were used in the analysis. Regrettably, data on sputum volume were lost. Therefore, cell concentrations could not be calculated. PC_{20} and levels of ECP, IL-8, IL-6 and albumin were log-transformed before analysis, in order to obtain normally distributed data. Changes in PC_{20} were expressed as doubling dose (DD), and changes in levels of soluble markers were expressed in doubling concentrations (DC).

Multivariate analysis of variance (MANOVA) was applied, either with factor "time" for analysis of the separate groups, or with "RV16" or "placebo" as a between-group factor, and "time" as a within-group factor for analysis of all subjects (313). Significant effects were explored using paired Student's t-tests for within-group effects, and unpaired Student's t-tests for between-group effects. Values of $p < 0.05$ were considered statistically significant (313). The summary statistics were expressed as mean ± SEM, or geometric mean ± SEM in DC or DD for the log-transformed data.

6.4 RESULTS

One of the RV16-treated subjects (# 1, table 1) dropped out at 7 days after the first RV16 inoculation due to an asthma exacerbation, requiring treatment with oral prednisone. Two subjects (# 6 and 15) did not produce sputum at all three time points, one subject (# 22) did not produce sputum at the baseline visit (day -5).

In all RV16-treated subjects the infection was confirmed by a rise in RV16 neutralizing antibody titer in serum and/or at least one rhinovirus-positive culture of the nasal lavage, while in none of the placebo-treated subjects a viral infection could be demonstrated (table 1).

6.4.1 Lung function and histamine challenge

Baseline lung function was slightly higher in the RV16 group as compared to the placebo group ($p = 0.04$). During the study, there were no significant changes in FEV_1 either within the group (MANOVA: $p \geq 0.34$) or between the groups (MANOVA, $p = 0.61$).

PC_{20} to histamine was not significantly different between the groups at baseline ($p = 0.93$). In the placebo group there were no significant changes in PC_{20} during the study (MANOVA, $p = 0.67$). In the RV16 group PC_{20} decreased significantly at day 4 as compared to baseline (mean difference: -0.65 ± 0.25 DD, $p = 0.02$), but not at day 11 (-0.40 ± 0.30 DD, $p = 0.19$). These changes were not significantly different from the changes in the placebo group ($p \geq 0.10$).

6.4.2 *Sputum cell differentials*

At baseline sputum cell differentials were not significantly different between the groups ($p \geq 0.32$). There were no significant changes in cell differentials within the placebo group (MANOVA, $p \geq 0.68$). In the RV16 group there was only a significant decrease in the percentage squamous cells at days 2 and 9 after inoculation as compared to baseline (mean change: -13.2 ± 5.2, $p = 0.02$, and -17.3 ± 5.5, $p = 0.006$, respectively) (table 2). Changes in cell differentials were not significantly different between the placebo and RV16 group (MANOVA: $p \geq 0.25$) (table 2). However, in the RV16-group the change in the percentage eosinophils between days -5 and 9 correlated significantly with the change in PC_{20} ($r = -0.58$, $p = 0.02$) (figure 1A).

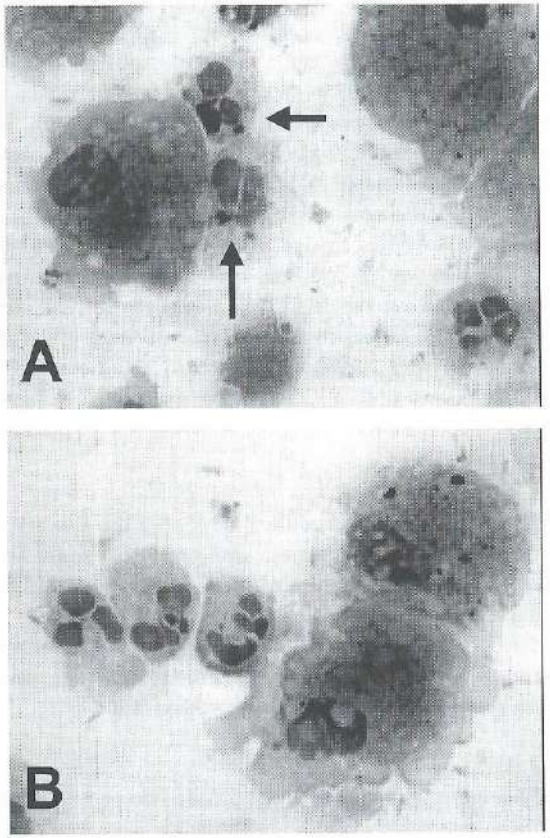


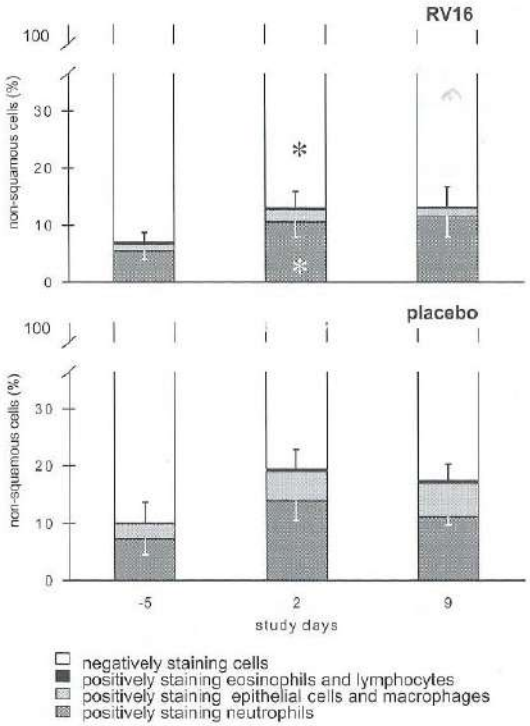
Figure 6.2.
Example of typical intracellular staining pattern for IL-8 **(A)** and the negative control **(B)** in sputum cells. Arrows indicate positively staining neutrophils.

6.4.3 *Sputum soluble mediators*

At baseline, albumin levels were higher in the RV16 group as compared to placebo (mean difference: 2.35 doubling concentrations (DC), $p = 0.045$). However, within each group there were no significant changes in albumin levels during the study (MANOVA: $p \geq 0.10$) (table 3).

The groups were not statistically different at baseline with respect to the levels of IL-8, IL-6 and ECP ($p \geq 0.34$). In the RV16 group the levels of IL-8 increased significantly at days 2 and 9 after inoculation as compared to baseline (mean change: 1.18 ± 0.38 DC, $p = 0.007$, and 1.01 ± 0.48 DC, $p = 0.049$, respectively). In addition, IL-6 levels increased significantly during the study (mean change, day 2: 2.89 ± 0.74 DC, $p = 0.001$, day 9: 2.87 ± 0.78 DC, $p = 0.002$) while ECP levels increased only significantly at day 2 (mean change: 0.68 ± 0.32 DC, $p = 0.047$). There were no significant effects on the biochemical markers in the placebo group (MANOVA: $p \geq 0.17$), and the effects were not significantly different between the placebo and the RV16 group (MANOVA: $p \geq 0.11$) (table 3). In the RV16 group, only the increase in ECP between days -5 and 2 correlated significantly with the decrease in PC_{20} between days -3 and 4 ($r = -0.60$, $p = 0.01$) (figure 1B).

Figure 6.3. Intracellular IL-8 staining: negatively or positively staining cells, expressed as a percentage of non-squamous cells in the RV16 group **(upper panel)** and the placebo group **(lower panel)**. The upward and downward error bars represent the SEM for the negatively staining cells and the positively staining neutrophils, respectively. In the RV16 group the percentage negatively staining cells decreased significantly at day 2 after inoculation (mean change ± SEM: -6.1 ± 2.4, $p = 0.02$), whilst the percentage positively staining neutrophils increased at this time point (mean change ± SEM: 5.4 ± 2.1, $p = 0.02$).



6.4.4 *Intracellular IL-8 staining*

Three samples were rejected because the quality of the cytospins was not adequate for interpreting the intracellular staining (#14 visit 1, #22 visit 3, and #26 visit 3). A representative example of a cytospin stained for IL-8 and its negative control are shown in figure 2. At baseline, the groups were not significantly different with respect to the percentage IL-8 positive non-squamous cells (mean ± SEM: RV16: $7.0 \pm 1.6\%$, Placebo: $8.7 \pm 3.6\%$, $p = 0.64$), or the percentages of the positively staining individual cell types ($p \geq 0.24$). The IL-8 positive cells appeared to be predominantly neutrophils (figure 3), which at baseline accounted for 77.9% of

all positively staining non-squamous cells. Within the individual cell types, 22.5 ± 4.1% of all neutrophils stained positively, and 2.6 ± 0.7% of the epithelial cells and macrophages stained positively. Lymphocytes and eosinophils only occasionally stained positively, while squamous cells were never observed to stain positively for IL-8.

In the RV16 group the percentage IL-8 positive non-squamous cells increased at day 2 (mean change: 6.1 ± 2.4, p = 0.02), but this increase was no longer significant at day 9 (mean change: 6.3 ± 4.0, p = 0.13) (figure 3). The increase at day 2 was due to an increase in the percentage IL-8 positive neutrophils (mean change: 5.4 ± 2.1, p = 0.02), with a trend towards an increase in the percentage positively staining epithelial cells and macrophages (mean change: 0.8 ± 0.4%, p = 0.07) (figure 3). There was no significant change in the percentage IL-8 positive non-squamous cells in the placebo group (MANOVA, p = 0.23), and the changes were not significantly different between the groups (MANOVA, p = 0.89). In the RV16-group, the change in the percentage IL-8 positive neutrophils between days 2 and 9 correlated significantly with the change in IL-8 levels in sputum supernatant (r = 0.64, p = 0.008), while there was no significant correlation between changes IL-8 positive staining cells and changes in airway hyperresponsiveness (p > 0.19).

Table 6.2. Sputum cell differentials: descriptive and statistical analysis

		RV16 (n = 17)		Placebo (n = 8)		MANOVA (p value) within group		between groups
		mean ± SEM	(range)	mean ± SEM	(range)	RV16	Plac	
squamous c's*	day -5	40.0 ± 4.4	(8.1 - 85.5)	42.6 ± 10.3	(8.4 - 83.2)			
	day 2	26.7 ± 5.0	(4.6 - 65.2)	40.7 ± 9.7	(12.5 - 83.7)	0.03	0.88	0.25
	day 9	22.4 ± 3.4	(5.3 - 48.1)	38.9 ± 7.6	(17.0 - 80.4)			
eosinophils†	day -5	9.3 ± 1.9	(0 - 30.3)	8.6 ± 3.1	(0 - 25.0)			
	day 2	7.5 ± 2.1	(0.1 - 34.4)	8.8 ± 4.2	(0 - 29.4)	0.17	0.90	0.79
	day 9	5.5 ± 1.4	(0.4 - 23.3)	7.3 ± 1.8	(0.7 - 16.0)			
neutrophils†	day -5	22.4 ± 3.3	(2.6 - 47.3)	27.6 ± 5.8	(10.8 - 57.6)			
	day 2	28.5 ± 4.9	(3.9 - 61.6)	32.6 ± 5.6	(12.7 - 55.9)	0.42	0.80	0.93
	day 9	27.3 ± 5.3	(6.2 - 67.2)	29.4 ± 5.2	(6.0 - 48.8)			
lymphocytes†	day -5	3.9 ± 0.5	(1.5 - 8.5)	3.2 ± 0.8	(0.9 - 6.9)			
	day 2	2.8 ± 0.6	(0.3 - 11.9)	3.7 ± 0.4	(2.0 - 5.8)	0.23	0.71	0.40
	day 9	2.8 ± 0.4	(1.1 - 8.2)	3.1 ± 0.3	(1.8 - 4.6)			
macrophages†	day -5	54.6 ± 3.2	(24.9 - 73.1)	48.8 ± 4.4	(32.0 - 61.0)			
	day 2	51.1 ± 3.7	(30.3 - 85.6)	44.3 ± 5.9	(15.7 - 66.7)	0.68	0.71	0.97
	day 9	53.8 ± 4.2	(17.4 - 75.7)	48.7 ± 5.3	(20.9 - 70.9)			
bron. epith.c's†	day -5	9.7 ± 1.4	(2.1 - 21.5)	9.0 ± 2.0	(1.5 - 15.5)			
	day 2	10.1 ± 2.1	(1.3 - 31.4)	8.1 ± 1.9	(2.2 - 17.1)	0.84	0.68	0.83
	day 9	10.9 ± 2.1	(1.9 - 32.6)	11.3 ± 4.8	(3.7 - 43.0)			

* squamous cells as percentage of all sputum cells
† cell type expressed as percentage of non-squamous sputum cells

6.5 DISCUSSION

In this study we have demonstrated that RV16 infection in atopic asthmatic subjects does not change the cellular composition of the sputum, or levels of albumin in sputum supernatant. However, an increase in ECP levels was shown in the first week after infection. The increase in airway hyperresponsiveness to histamine correlated positively and significantly with the increase in ECP in the first week, and with the change in the percentage eosinophils in the second week after infection. Furthermore, RV16 infection induced an increase in the levels of IL-8 and IL-6 up to 9 days after infection. When staining IL-8 in permeabilized sputum cells it appeared that IL-8 is predominantly present in neutrophils, and that the percentage IL-8 positive neutrophils increased at day 2 after infection. These results suggest that experimental rhinovirus infections increase lower airway inflammation, involving the release of proinflammatory cytokines into the airway lumen.

Table 6.3. Soluble sputum markers: descriptive and statistical analysis

		RV16 (n = 17)			Placebo (n = 8)			MANOVA (p value) within group		between groups
		g. mean	[cv]	(range)	g. mean	[cv]	(range)	RV16	plac.	
albumin (ng/ml)	day -5	124.0	[1.3]	(4.6 - 809)	24.3	[2.5]	(2.0 - 868)			
	day 2	125.2	[1.4]	(4.4 - 1290)	55.7	[2.2]	(2.0 - 589)	0.82	0.10	0.03
	day 9	108.9	[0.9]	(17.7 - 527)	87.4	[1.1]	(16.8 - 437)			
IL-8 (ng/ml)	day -5	2.6	[1.0]	(0.8 - 32.6)	4.0	[1.0]	(1.6 - 24.7)			
	day 2	5.8	[1.0]	(0.7 - 36.5)	5.4	[0.8]	(1.8 - 22.2)	0.009	0.50	0.47
	day 9	5.4	[1.0]	(0.8 - 23.7)	5.5	[0.6]	(2.4 - 13.0)			
IL-6 (pg/ml)	day -5	11.6	[2.2]	(0.0 - 192)	4.6	[2.6]	(0.0 - 228)			
	day 2	85.6	[2.7]	(0.0 - 6200)	6.2	[2.6]	(0.0 - 250)	0.001	0.56	0.11
	day 9	79.0	[1.5]	(0.0 - 431)	8.2	[2.3]	(0.0 - 181)			
ECP (ng/ml)	day -5	119.1	[1.3]	(8.9 - 1146)	70.1	[1.3]	(15.4 - 800)			
	day 2	190.6	[1.4]	(17.2 - 800)	107.8	[1.4]	(12.1 - 800)	0.07	0.17	0.73
	day 9	157.6	[1.0]	(27.8 - 800)	72.2	[1.1]	(21.8 - 800)			

g. mean = geometric mean
cv = coefficient of variation

This is the first study showing changes in inflammatory mediators in the airways after rhinovirus infection in asthmatic subjects. The relationship between RV16-induced changes eosinophil markers and changes in airway hyperresponsiveness are in keeping with cross-sectional studies, demonstrating a correlation of sputum eosinophilia with lung function (329,330) during exacerbations of asthma of unknown cause. The present study extends the observation by Fraenkel and coworkers (164), showing an increase in activated (EG_2-positive) eosinophils in the bronchial epithelium after experimental RV16 infection in a group of normal and asthmatic subjects. Hence, the correlation between the increase in ECP, the change in the percentage eosinophils, and the change in airway hyperresponsiveness support a role for the eosinophil in rhinovirus-induced exacerbations of asthma.

The presently described increase in IL-8 levels after RV16 infection, together with intracellular staining for IL-8 predominantly in neutrophils is in keeping with a relatively high percentage of neutrophils and high levels of IL-8 in sputum found during acute exacerbations of asthma (255). Based on the chemotactic properties of IL-8 for neutrophils, T-lymphocytes, basophils and primed eosinophils (300), and the pleiotrophic effects of IL-6, including B-cell activation and proliferation of cytotoxic T-cells (331) one could postulate the involvement of these cytokines in the immune response against a rhinovirus cold. This is further supported by an increase of neutrophil numbers in the nasal lavage (248) and peripheral blood (148), and the

increase of T-lymphocytes in the bronchial submucosa during a rhinovirus cold (164). However, the role of IL-8 and IL-6 in the development of rhinovirus-induced changes in airway physiology remains to be elucidated, since we did not find a correlation between changes in the percentages neutrophils and lymphocytes, IL-8, IL-6 levels or IL-8 positive staining cells and changes in airway hyperresponsiveness.

In the present study, we applied a placebo-controlled design in order to differentiate the responses to rhinovirus inoculation from fluctuations in airway pathophysiology that are characteristic to asthma. Despite evident changes in the soluble mediators in the RV16 group, there were no significant differences between the RV16 and placebo group. One could speculate that this is due to the smaller sample size of the placebo-group, resulting in low statistical power to detect such between-group differences (326).

Validated methods were used to inoculate the rhinovirus and measure the physiologic responses (148,162,163,289). The natural way of transmission was mimicked by using a combination of three methods of virus administration, including nasal inhalation. In this way, virus particles may even have reached the lungs. However, we have not attempted to assess lower airway infection by viral culture of the sputum, since oropharyngeal contamination would have biased a positive result.

Sputum induction was performed according to a validated technique (326), which included careful avoidance of oropharyngeal contamination during the sputum induction, thereby reducing, (but not excluding) possible bias by mixing the sputum sample with upper airway secretions and saliva (332). In cross-sectional studies the sputum eosinophil numbers and ECP levels have been shown to correlate significantly with those in bronchoalveolar lavage (BAL) and bronchial wash (333-335), whilst a trend towards a significant correlation has been shown between eosinophils in sputum and the submucosa of bronchial biopsies (334,335). Moreover, sputum analysis has revealed a decrease in eosinophil numbers and ECP levels after glucocorticoid treatment in asthma (282), in accordance with findings in bronchial biopsies (336). Therefore, the cellular and biochemical constituents of hypertonic saline-induced sputum seem to be an adequate reflection of rhinovirus-induced airway inflammation in asthma.

The decrease in the percentage of squamous cells in the RV16-treated subjects might have been caused by an increase in sputum volume and/or sputum cell concentration after infection. Unfortunately, the data on sputum volume were lost in the present study, which precluded calculations of cell concentrations. However, the cell differentials by themselves seem to provide the most useful information, since these appear to be rather stable under conditions in which absolute cell counts are variable due to volume dilution effects (332).

How do we interpret the results? The increase in airway hyperresponsiveness to histamine, in the absence of a significant decrease in lung function may be explained by inflammatory changes, such as airway wall swelling or thickening, potentiating the airway narrowing effect of smooth muscle shortening (297). Such inflammation may be characterized by an infiltrate of inflammatory cells, typically eosinophils and lymphocytes, and the release of pro-inflammatory mediators, which may bring about features such as vasodilation and vascular hyperpermeability, oxidative stress, cellular activation and tissue damage. The current association between changes in PC_{20} and changes in sputum ECP and the percentage eosinophils suggest that eosinophilic inflammation is involved in rhinovirus-induced enhancement of airway hyperresponsiveness.

There are several mechanisms potentially driving the cell infiltration and activation after RV16 infection. First, airway inflammation may be driven by T-lymphocytes, infiltrating the airways (164,337), and producing several cytokines such as IL-2, IFN-γ, IL-4, and IL-5 (323),

thereby orchestrating the augmentation of the immune response. Second, there is increasing evidence that cultured epithelial cells and fibroblasts produce pro-inflammatory cytokines, such as IL-8, IL-6, GM-CSF and RANTES in response to infection with several rhinovirus serotypes, including RV16 (215,218,221-223,231). Epithelium and airway cells, such as macrophages release TNF-α and IL-1β upon uptake of rhinovirus (182,218), which may in turn induce and potentiate cytokine production in *e.g.* epithelial cells and neutrophils (215). It is presently uncertain as to whether rhinovirus actually infects lower airway tissues in humans *in vivo*, although there is evidence to suggest that rhinovirus is present in the lower airways during a cold (276). Thus, one could envision the interaction between airway resident cells in the induction of an immune response, involving the release of chemotactic mediators such as chemokines, which may lead to infiltration of inflammatory cells and subsequent pathophysiological changes.

What are the clinical implications of this study? The present results suggest that a common cold increases airway hyperresponsiveness, together with an increase in lower airways inflammation. This fits in with the epidemiological association between respiratory virus infections and exacerbations of asthma (78). Glucocorticoids are the treatment of choice for exacerbations of asthma (1). In view of their effectiveness against eosinophilic inflammation (282,336) one could postulate that our data support a rationale for such therapeutic use of glucocorticoids during rhinovirus-induced exacerbation of asthma. However, it remains to be established whether steroids can actually prevent the resulting pathological and functional changes in the airways.

We conclude that experimental RV16 infection in atopic asthmatic subjects enhances airway hyperresponsiveness to histamine in conjunction with a rise in sputum levels of IL-8, IL-6 and ECP, which suggests an increase in lower airways inflammation. The relationship between changes in ECP, the percentage eosinophils and changes in airway responsiveness suggests that eosinophilic inflammation is involved in rhinovirus-induced enhancement of airway hyperresponsiveness. In order to further investigate the mechanisms of rhinovirus-induced exacerbations of asthma, the state of activation and cytokine production of resident cells and infiltrating cells within the airway wall/mucosa need to be examined.

As part of the study on the effects of placebo-controlled experimental RV16 infection on levels of pro-inflammatory mediators in airway secretions we analyzed nasal lavage for levels of total protein, ECP and Antileukoproteinase (ALP, also known as secretory leukocyte proteinase inhibitor, SLPI).

For methods for obtaining and processing nasal lavage, see chapter 3. Biochemical assessment ECP levels was done by fluoro-enzyme immuno-assay (Pharmacia, Uppsala, Sweden). ALP was assessed by a double-antibody sandwich ELISA (338). The detection limit of the ALP ELISA was 0.5 ng/ml (338).

Table A1. Descriptive and statistical analysis of total protein, ECP and ALP in nasal lavage.

product	group	day -5			day 2			day 9			-5/2	-5/9
		g.mean	±	SEM	mean	±	SEM	mean	±	SEM	p†	p†
protein*	placebo	0.27	±	0.02	0.29	±	0.01	0.28	±	0.02	0.21	0.61
	RV16	0.36	±	0.03	0.41	±	0.02	0.58	±	0.07	0.12	0.004
p°		0.054			0.002			0.02			0.55‡	0.03‡
ECP**	placebo	1.23	±	0.391	1.57	±	0.690	1.14	±	0.56	0.47	0.68
	RV16	2.15	±	0.378	2.14	±	0.363	2.84	±	0.52	0.97	0.12
p°		0.443			0.253			0.021			0.53‡	0.24‡
ALP	placebo	11.01	±	2.80	11.16	±	2.96	8.91	±	1.52	0.91	0.33
	RV16	11.97	±	1.71	10.71	±	1.33	27.01	±	4.69	0.51	0.006
p°		0.77			0.87			0.03			0.64‡	0.02‡

* : total protein

** : analysis of log-transformed data, presented as geometric mean ± SEM (Doubling Concentrations).

° : p value of between-group comparisons (unpaired t-tests) at days –5, 2 and 9

† : p value of within-group comparisons (paired t-test) between days –5 and 2 and days –5 and 9.

‡ : p value of between-group comparisons (unpaired t-tests) of the changes between days –5 and 2, and days –5 and 9.

7 EXPERIMENTAL RHINOVIRUS 16 INFECTION INCREASES INTERCELLULAR ADHESION MOLECULE-1 EXPRESSION IN BRONCHIAL EPITHELIUM OF ASTHMATICS, REGARDLESS OF INHALED STEROID TREATMENT

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Clin Exp Allergy 2000;30:1015-23

7.1 ABSTRACT

Rhinovirus infections in airway epithelial cells *in vitro* have been shown to upregulate intercellular adhesion molecule-1 (ICAM-1) expression. Epithelial ICAM-1, in its dual role as the major rhinovirus receptor and as adhesion molecule for inflammatory cells may be involved in the pathogenesis of rhinovirus-induced exacerbations of asthma.

In the present study we aimed to investigate the effect of experimental rhinovirus 16 (RV16) infection on ICAM-1 expression in bronchial mucosal biopsies in asthma. In addition, the effect of 2 weeks pre-treatment with inhaled budesonide (800 µg b.i.d.) on RV16-associated changes in ICAM-1 expression was studied.

The study had a parallel, placebo-controlled design in 25 steroid-naï ve non-smoking atopic asthmatic subjects. After 2 weeks budesonide or placebo pre-treatment bronchoscopy was performed 2 days before (day -2) and 6 days after (day 6) RV16 inoculation (on days 0&1). Immunohistochemical staining for ICAM-1 was performed on snap-frozen bronchial biopsies. ICAM-1 staining intensity on the basal epithelial cells was scored semi-quantitatively from 1 (weak) to 3 (intense). Similarly, epithelial intactness was noted (1 = basal cells only, 2 = basal and parabasal cells, 3 = intact epithelium).

At baseline, ICAM-1 scores were not significantly different between the groups at day -2 ($p \geq 0.08$). Subsequent RV16 infection was associated with a trend towards an increase in ICAM-1 expression in the BUD-group ($p = 0.07$), whereas the increase was significant in the PLAC-group ($p = 0.03$). However, the increase was not significantly different between the groups ($p = 0.74$). Epithelial intactness score was not different between the groups before RV16 infection ($p \geq 0.07$), and no significant changes were observed in either group ($p \geq 0.59$). Moreover, ICAM-1 score did not correlate significantly with epithelium score in either group, at any time-point ($p \geq 0.27$).

We conclude that an RV16 common cold in atopic asthmatic subjects is associated with increased ICAM-1 expression in the bronchial epithelium, which is not related to epithelial intactness. Glucocorticoid treatment does not appear to prevent the RV16-associated increased ICAM-1 expression. This suggests that other treatment modalities are required to protect against the spreading of infection during rhinovirus-induced exacerbations in asthma.

7.2 Introduction

Asthma is a chronic airway disease that is characterised by episodic worsening of symptoms such as chest tightness, dyspnea and cough, associated with variable airways obstruction and airway hyperresponsiveness (1). This is associated with airway mucosal inflammation, as reflected by epithelial shedding (339), cellular infiltration and increased expression of proinflammatory cytokines and adhesion molecules (213,340). The long recognized temporal relationship between asthma exacerbations and respiratory viral infections has been reconfirmed in recent studies, showing that a respiratory virus can be found in up to 83% of all asthma exacerbations in children, about half of these viruses being identified as rhinovirus (78). In experimental studies it has been shown that rhinovirus 16 (RV16) colds increase airway hyperresponsiveness to inhaled histamine in patients with atopic rhinitis (162) or asthma (148). In addition, elevated numbers of submucosal lymphocytes and epithelial eosinophils have been reported in bronchial mucosal biopsies after experimental RV16 infection in a sample of both normal subjects and asthmatic subjects (164).

Adhesion molecules are involved in leucocyte infiltration in asthma (213,341). Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin supergene family, is the major ligand for the β_2 integrins lymphocyte function associated antigen (LFA-1, CD11a/CD18) which is expressed on most leukocytes, and macrophage-1 antigen (Mac-1, CD11b/CD18) which is expressed on neutrophils and mononuclear cells (342). Interestingly, 90% of rhinoviruses use ICAM-1 as their cellular receptor (210). There is evidence to suggest that respiratory viruses, including rhinoviruses can upregulate ICAM-1 transcription and expression in pulmonary epithelial cells *in vitro* (197,218,343-345), which is associated with enhanced adherence of polymorph mononuclear cells (343) and migration of T cells (346). Such upregulated expression may in part be mediated by cytokines such as IL-1 (218,341,344), which is an inducer of ICAM-1 expression (218). Thus, one could envisage an interactive mechanism, in which a increased ICAM-1 promotes both rhinoviral spreading (218) and influx of inflammatory cells (164), thereby potentially contributing to the development of an exacerbation of asthma.

Patients with asthma have been reported to demonstrate elevated ICAM-1 expression in their bronchial epithelium as compared to normal controls (213,214). It its presently unclear as to whether such expression is dependent on the clinical control of the disease. Glucocorticoids, a commonly used class of drug in asthma, appear to down-regulate both basal- and cytokine-induced epithelial ICAM-1 expression *in vitro* (347). However, in steady state asthma regular treatment with glucocorticoids did not seem to have an effect on epithelial ICAM-1 expression (340). The influence of an asthma exacerbation on epithelial ICAM-1 expression has not been studied.

In the present study we hypothesized that a rhinovirus-induced cold in patients with asthma is accompanied by elevated ICAM-1 expression in the bronchial epithelium. In addition, we hypothesized that glucocorticoids are able to prevent such elevated ICAM-1 expression. To that end, we investigated first, the effect of experimental RV16 infection on the expression of ICAM-1 in bronchial mucosal biopsies. Secondly, we examined the effect of 2-weeks pre-treatment with inhaled budesonide (a glucocorticoid) on RV16-associated changes in ICAM-1 expression in atopic, mildly asthmatic subjects.

7.3 Methods

7.3.1 Subjects

Twenty-five non-smoking or ex-smoking (>12 months, <5 pack years) adult asthmatics (13 female, 12 male; age 19-25 yr.) were recruited. The subjects' characteristics are shown in table 1. The subjects had low titers of circulating antibodies specific to rhinovirus 16 using a RV16 serum neutralization assay (≤ 1:4 serum dilution against 20-25 x 50% tissue culture infective dose - $TCID_{50}$-), and were atopic, as reflected by one or more wheal (> 3 mm) and flare response to skin prick tests to 10 common aero-allergen extracts (Soluprick, ALK, Benelux). In the 3 months preceding the study the subjects had not used oral or inhaled glucocorticoids or any other medication for their asthma or allergies except for inhaled short-acting β_2 agonists on demand. All subjects were healthy, apart from their asthma during the 6 weeks preceding the study. The characteristics of the 7 control patients (PLAC/PLAC group) (3 female, 4 male; age 19-34 yr.) are presented in table 2.

Table 7.1: Characteristics of rhinovirus-inoculated patients

Patient	Sex (M/F)	Age (yr)	Baseline FEV_1 (% predicted)	Baseline PC_{20} histamine (mg/ml)	titer pre/post-inoculation (1:...)[B]	culture nasal lavage[C]
BUD/RV16						
1	M	24	73.5	0.14	1 / 1	pos/pos
2	F	21	86.4	0.17	1 / 8	neg/neg
3	M	23	82.5	0.29	1 / 16	pos/pos
4	F	24	86.1	0.30	1 / 1	pos/neg
5	M	23	95.2	0.54	1 / 8	pos/neg
6	M	24	76.4	0.54	1 / 32	pos/neg
7	M	23	81.7	0.65	4 / 128	pos/pos
8	F	23	91.0	1.28	2 / 2	neg/neg
9	M	24	76.8	1.34	1 / 256	pos/neg
10	M	25	82.6	2.13	1 / 8	pos/pos
11	F	24	80.8	3.32	1 / 16	neg/neg
12	F	25	103	5.92	1 / 2	pos/pos
mean ± SEM			**84.6 ± 2.4**	**0.74 ± 0.49[A]**		
PLAC/RV16						
13	M	22	80.9	0.06	1 / 16	pos/neg
14	F	19	80.5	0.14	1 / 4	pos/pos
15	F	19	84.2	0.30	1 / 1	pos/pos
16	F	20	95.7	0.31	1 / 8	pos/pos
17	M	19	101	0.46	1 / 1	neg/neg
18	M	23	74.6	0.52	1 / 32	pos/neg
19	F	20	83.9	0.53	1 / 1	pos/neg
20	F	23	84.0	0.65	1 / 32	pos/pos
21	F	24	94.2	1.44	1 / 16	neg/neg
22	F	20	96.6	1.81	1 / 1	pos/pos
23	M	23	103	2.18	2 / 2	neg/neg
24	F	20	95.9	2.40	1 / 8	pos/pos
25	M	22	84.1	4.34	1 / 256	pos/pos
mean ± SEM			**89.2 ± 2.5**	**0.65 ± 0.49[A]**		

A : geometric mean ± doubling dose
B : Serum RV16 neutralizing antibody titer: obtained at days -2 and 28
C : Nasal lavage: obtained at days 3 and 6

The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and the subjects gave their written informed consent before entering the study.

7.3.2 Design

The study had a randomized double-blind, parallel, placebo-controlled design, including two groups of asthmatics receiving RV16 inoculation during treatment with either budesonide (BUD/RV16 group) or placebo (PLAC/RV16 group). Budesonide by dry powder inhaler (Turbohaler®, 800 µg, bid) or placebo was given for a period of 4 weeks, starting 16 days before RV16 inoculation (day -16). After two weeks of treatment (day -2), bronchoscopy was performed and bronchial biopsies were taken in all patients. Two days later this was followed by experimental RV16 infection on two consecutive days (day 0 and day 1). The bronchoscopy was repeated 6 days after infection (day 6).

To ascertain that the bronchoscopy procedure itself would not interfere with ICAM-1 expression, bronchial biopsy specimens were obtained at an 8 days interval without intervention or treatment in 7 asthmatic subjects, thereby serving as controls. This third group of patients (PLAC/PLAC group) had the same inclusion criteria as the other subjects.

Table 7.2. Characteristics of control patients

Patient number	Sex (M/F)	Age (yr)	Baseline FEV_1 (% predicted)	Baseline PC_{20} methacholine[A] (mg/ml)
PLAC/PLAC				
26	M	20	111	0.31
27	M	23	115	0.68
28	M	34	94	1.41
29	F	22	99	1.45
30	F	19	115	1.95
31	F	23	115	4.79
32	M	20	101	6.02
mean ± SEM			**107.0 ± 3.4**	**1.57 ± 0.56[B]**

A : methacholine chloride

B : geometric mean ± doubling dose

7.3.3 Rhinovirus-16 inoculation

The RV16 inoculum was obtained from the same strain and stock as used in previous experiments in humans *in vivo* (148,162). The virus was cultured according to standards of good laboratory practice and the inoculum was tested to be safe for human *in vivo* usage (286). A total dose of 0.6 - 2.1 x 10^4 $TCID_{50}$ RV16 was administered to each subject according to a previously described procedure (148). Briefly, the total RV16 dose, suspended in Hanks' Balanced Salt Solution (HBSS) containing 0.5% (w/v) gelatin, in a volume of 3 ml, was divided over two days. On each day, 0.5 ml of the inoculum was administered by nasal inhalation (DeVilbiss 646 nebulizer, connected to a face mask). Then, 0.5 ml was administered by spraying equal portions into each nostril (DeVilbiss 286 atomizer, powered by a compressor). Finally, 0.5 ml of the inoculum was instilled in equal portions into each nostril by pipette.

The confirmation of RV16 infection was established by a fourfold or greater increase in virus-specific neutralizing antibody titer in serum and/or by recovery of the virus from the nasal washes (148). The nasal washes were inoculated onto Human Embryonic Lung fibroblast (HEL) cultures and incubated at 32° C for 14 days. The identity of rhinovirus in a positive culture was confirmed by neutralization assay, using RV16-specific guinea-pig immune serum (1126AS/GP-VR; American Type Culture Collection, Rockville, MD). In order to exclude any intercurrent respiratory infection, all nasal washes were inoculated into rhesus monkey kidney cells (LCC-MK2), Hep-2 and HEL cell cultures and cultured at 37°C (148).

7.3.4 Bronchoscopy

Fiberoptic bronchoscopy was carried out by experienced investigators (JJB, JV, LW, EB), using a standardized protocol based on international guidelines, as has been used in previous studies in our department (47). After 6 hr of fasting, pre-medication consisting of atropine 0.5 mg subcutaneously, codeine 20 mg orally and salbutamol 400 µg by metered dose inhaler was administered to the subjects. Local anesthesia was performed with 10% (w/v) lignocaine aerosol in the oropharynx, and with 2% (w/v) lignocaine solution in the lower airways. If necessary, additional lignocaine 2% was administered through the bronchoscope during the procedure. Fiberoptic bronchoscopy was performed using a Pentax bronchoscope (outer diameter 6 mm; Pentax Optical Co., Japan). The bronchoscope was introduced through the mouth, with the patient in supine position. Six bronchial biopsies were taken at (sub)segmental level from either the right lung (right lower and middle lobe) or the left lung (lingula and left lower lobe), using a pair of cup forceps (Olympus FB-20C, Tokyo, Japan). Alternate biopsy sites (right or left lung) were randomized over the 2 bronchoscopy visits.

Throughout the procedure oxygen was delivered through the nasal canula at a rate of 4 L/min while oxyhemoglobin saturation was monitored continuously in all subjects using a transcutaneous oximeter (N-180, Nellcor Inc., Hayward, CA, USA) with a finger probe placed on a finger. Afterwards, the subjects were observed for one hour for any adverse events.

7.3.5 Processing of the biopsies

After bronchoscopy, the biopsy samples were immediately embedded in OCT medium (Miles Inc. Diagnostics Division, Elkhart, USA), and snap-frozen in isopentane cooled by iced CO_2. Thereafter, the samples were stored in airtight containers at -70° C, pending further processing (47).

Table 7.3: Epithelium intactness scores (median [range]).

Group	Day -2	Day 6
BUD/RV16	2.0 [1 – 3]	2.0 [1 – 3]
PLAC/RV16	1.0 [1 – 3]	1.5 [1 – 3]
PLAC/PLAC	1.0 [1 – 2]	1.0 [1 – 3]

There were no significant differences in epithelium intactness score between the groups at baseline ($p \geq 0.08$), nor within the groups between the days ($p \geq 0.23$).

7.3.6 Immunohistochemistry

Four micrometer thick cryostat sections of frozen biopsies were air dried for one hour, and fixed in acetone for 10 minutes. Immunohistochemical staining was performed using monoclonal antibodies against the markers of interest, and the avidin-biotin complex (ABC) as visualizing method. In short, the sections were incubated with an optimal dilution of monoclonal antibodies in 1% BSA/PBS at room temperature for 60 min. After washing with PBS (3 x 5 min), the slides were incubated with mouse primary antibodies in appropriate dilution for 30 min (1:500 for ICAM-1, CD54-clone MEM-112, MONOSAN, Uden, The Netherlands; 1:100 for platelet-endothelial cell adhesion molecule-1 or PECAM-1, NCl-CD31, NOVOCASTRA Laboratories,

Newcastle upon Tyne, UK). The slides were washed with PBS (3 x 5 min) and incubated with biotinylated rabbit-anti-mouse antibody (dilution 1:200) for 30 min. Thereafter, the slides were again washed with PBS (3 x 5 min) incubated for another 30 min with the avidin-biotin complex. Then, the slides were washed with PBS, rinsed in Na-acetate buffer 0.1 M (pH 5.0) for 5 minutes and the reaction was revealed by putting the slides in 5% AEC (3-amino-9-ethylcarbazol) for 7 minutes. The reaction was stopped in demineralized water and the sections were counterstained with Mayer's Haematoxyline. For negative controls, the primary antibody was omitted from this procedure.

7.3.7 Analysis of stained sections

All coded biopsy specimens examined at a 250x magnification by one observer (KG), who was blinded to the patient's characteristics, treatment, and the study day on which the biopsy was taken. Biopsy sections were analyzed semi-quantitatively, with regard to intensity and distribution pattern of the staining of ICAM-1 positive basal epithelial cells on a three-point scale (from 1 = weak, 2 = medium, focal 3 = intense, continuous staining of basal cell layer) (47,214). Areas with non-adjacent epithelial cells, or layers of epithelial cells not connected to the basement membrane were not taken into account. Since ICAM-1 staining was most intense on basal cells, less intense on parabasal cells, and not present on ciliated and goblet cells, we focused on ICAM-1 staining in basal epithelial cells only, in order to avoid bias by epithelial damage. The association between epithelial damage and ICAM-1 staining was further investigated by scoring epithelial intactness in a semiquantitative way, based on a qualitative description of the bronchial epithelium in asthma by Jeffery *et al.* (339): 1 = predominantly (≥50%) adjacent basal cells, 2 = predominantly basal and parabasal cells, 3 = predominantly intact epithelium. Each biopsy specimen was scored twice and the mean value of the 2 scores was used in the analysis. Following this procedure, the intra-observer repeatability of ICAM-1 score and epithelium score of the same slides over a 2-days interval was satisfactory ($\kappa > 0.69$). Platelet endothelial cell adhesion molecule-1 (PECAM-1), which has been described to be constitutively expressed on the endothelium of all vessel types (348), was used as a technical internal staining control.

Non-parametric statistical analysis was applied in order to examine the effect of RV16 or bronchoscopy on ICAM-1 immunostaining within the groups (Wilcoxon rank test), and the effect of inhaled steroids in the BUD/RV16 group as compared to the PLAC/RV16 group (Mann-Whitney U-test). Spearman rank test was used to test the relationship between ICAM-1 score and epithelium score. P-values less than 0.05 were considered to be statistically significant.

7.4 Results

Thirty-two subjects completed the study. One subject (# 3) did not undergo the bronchoscopies due to strong subjective discomfort. All the other subjects underwent bronchoscopy twice. The quality and size of the biopsies obtained during the first bronchoscopy of subjects # 1, 19, 26, 27 and 32, and during the second bronchoscopy of subjects # 1, 10 and 20 were inadequate for staining or analysis. RV16 infection was confirmed in all RV16 inoculated subjects, except subjects #2 and 17, who were only included in the between-group statistical analysis at day -2. All RV16-treated subjects had an anti-RV16 titer serum ≤ 1:1 before entering the study. However, reassessment just before inoculation of RV16 revealed slightly elevated titers in subjects # 7, 8 and 23, coinciding with symptoms of a common cold in subjects # 8 and # 23, which was confirmed in subject # 8 by rhinovirus-positive (RV16-negative) culture of the nasal lavage. Subjects 8 and 23 were therefore excluded from the analysis. Since low levels of neutralizing

antibodies *per se* have been shown not to preclude a symptomatic common cold (148), subject # 7 was not excluded from the analysis. Hence, 7 paired samples were available for analysis in the BUD/RV16 group, 9 paired samples were available in the PLAC/RV16 group, and 4 paired samples were available in the PLAC/PLAC group.

7.4.1 PECAM-1 and ICAM-1 staining

Examples of ICAM-1 staining in biopsy specimens before and after rhinovirus inoculation are shown in figure 1A and 1B, respectively. Epithelial ICAM-1 staining was most intense on basal cells, less intense on parabasal cells, whereas ciliated cells and goblet cells did not stain in any of the biopsy sections. Positive staining varied from focal staining of light to medium intensity, to continuous, intense staining of the basal cell layer. The control staining of PECAM-1 showed endothelial staining of similar intensity throughout all biopsy specimens (data not shown).

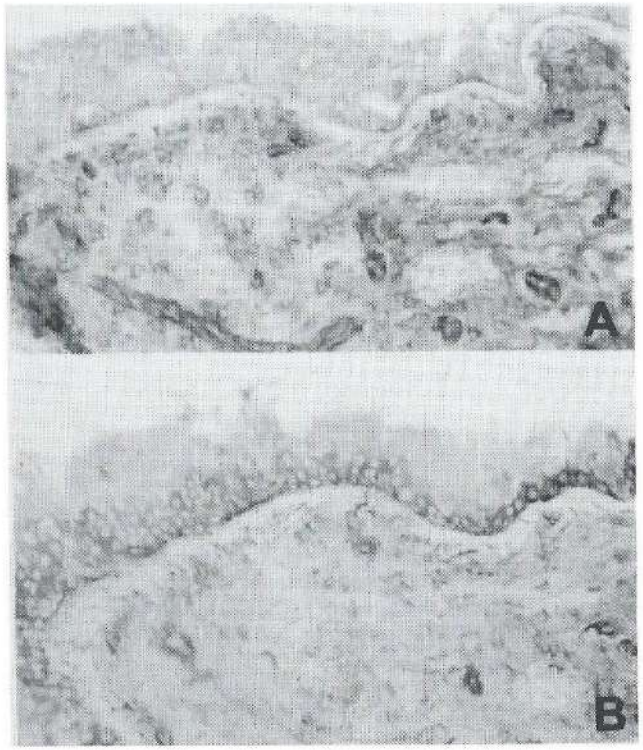

Figure 7.1. Immunohistochemical staining for ICAM-1 of frozen tissue of bronchial biopsy specimens. **A** = 2 days before virus inoculation, **B** = 6 days after virus inoculation in the same patient. Within the lamina propria leukocytes and endothelial cells stain similarly in both biopsies. However, staining of intact epithelium is almost absent before virus inoculation (**A**) and clearly present in basal and parabasal epithelial cells after virus inoculation (**B**).

Before RV16 infection, the ICAM-1 scores were not significantly different between the groups (MWU test: $p \geq 0.08$). The ICAM-1 score increased significantly after RV16 infection within the PLAC/RV16 group ($p = 0.03$), whilst there was a similar trend within the BUD/RV16 group ($p = 0.07$). These increases in ICAM-1 staining were not significantly different between the 2 groups ($p = 0.74$) (figure 2). Consequently, at the time of the second bronchoscopy, ICAM-1 intensity levels were no longer significantly different between the BUD/RV16 group and the PLAC/RV16

group (p = 0.53). Repeated bronchoscopy alone did not affect the intensity of ICAM-1 staining (PLAC/PLAC: p = 0.32) (figure 2).

At both time-points, the epithelial intactness score was not different between the BUD/RV16 group and the PLAC/RV16 group ($p \geq 0.16$) (table 3). However, the epithelial intactness score tended to be higher in the BUD/RV16 group as compared to the PLAC/PLAC group (p = 0.07). There was no significant effect of RV16 infection or bronchoscopy on epithelial quality either group ($p \geq 0.32$). There was no significant correlation between epithelium score and ICAM score within the treatment groups for each study visits ($p \geq 0.27$). Likewise, there was no significant correlation between the change in epithelium score and the change in ICAM score within the treatment groups ($p \geq 0.79$). Hence, we did not find evidence that epithelial intactness affected the ICAM-1 scores in our study.

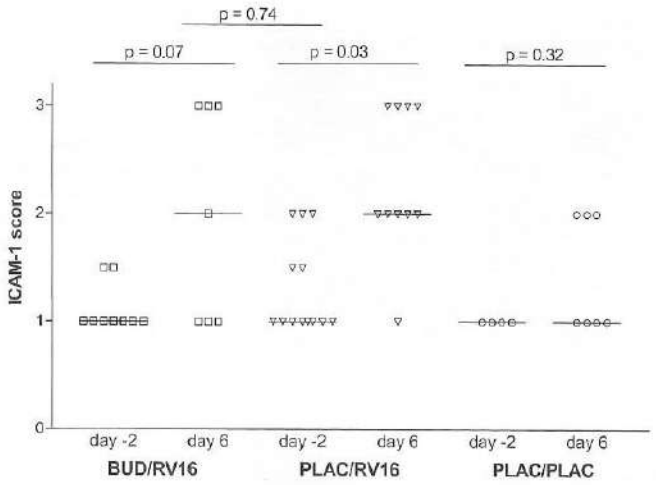

Figure 7.2. ICAM-1 scores before (day -2) and after (day 6) experimental RV16 infection. Within-group analysis (Wilcoxon) showed a trend towards an increase in ICAM-1 score in the BUD/RV16 group ( ), and a significant increase in the PLAC/RV16 group (∇), whereas ICAM-1 scores did not change in the PLAC/PLAC group (O). The increase in ICAM-1 scores within the BUD/RV16 group not significantly different from the PLAC/RV16 group (MWU-test).

7.5 Discussion

This study has demonstrated an increased expression of ICAM-1 in the epithelium of bronchial biopsy specimens after experimental infection with rhinovirus 16 in patients with atopic asthma. Regular treatment with inhaled budesonide did not prevent the RV16-induced upregulation of ICAM-1. These data indicate that RV16 infection triggers elevated expression of its cellular receptor within the intrapulmonary airways in asthmatics *in vivo*, irrespective of inhaled glucocorticoid treatment. This suggests that by upregulating its own receptor, rhinovirus may potentially facilitate its own bronchial epithelial infection and leukocyte infiltration, which might contribute to the development of rhinovirus-induced exacerbations of asthma.

This is the first study examining the effect of a common cold on the expression of ICAM-1 within the lower airways in asthmatic subjects *in vivo*. Such rhinovirus-associated upregulation of ICAM-1 expression fits in with reports of increased ICAM-1 expression *in vivo* after several other pro-inflammatory stimuli such as hyperoxia (349), ozone exposure (350) and allergen inhalation challenges (213,341). In addition, evidence of *in vitro* studies suggests that respiratory viruses, including rhinoviruses can upregulate ICAM-1 transcription and subsequent expression in epithelial cells (197,218,343-345). Hence, our results in asthmatics *in vivo* confirm previous observations *in vitro*, and extend these by providing evidence of a potential mechanism for the development of rhinovirus-induced exacerbations of asthma.

The present findings have been obtained after careful patient selection and study design, and by applying validated methods for RV16 experimental infection. RV16 inoculation was performed as previously described (148), by using a combination of three methods of virus administration, including nasal inhalation, thus mimicking the natural ways of transmission (83). By choosing the dosage of inhaled glucocorticoids (800 µg b.i.d.) which was higher than the recommended dose for mild persistent asthma (1), we intended to affect inflammatory mechanisms within the airways (336). We purposely added a third group of 7 patients who did not receive an experimental RV16-infection, nor any other intervention. This control group did not show a significant change in ICAM-1 expression, indicating that there was no confounding effect of a bronchoscopy or any of the other procedures on ICAM-1 expression. Although these asthmatic subjects were selected according to the same inclusion criteria, baseline FEV_1 was slightly higher as compared to the other 25 asthmatics, indicating that their asthma may have been slightly milder. However, since this group was added to investigate the effect of the bronchoscopy procedure on ICAM-1 expression only, we feel that such a minor difference is not relevant to the outcome of this study. The present results may be negatively affected by the loss of statistical power caused by missing data. However, data in large samples on the repeatability of assessment of ICAM-1 expression in bronchial biopsies over time are presently not available. Since the reasons for loss of data points were various, and the success rate of RV16 infection was equally distributed over the two RV16-treated groups, one can argue that the trend towards an increase in ICAM-1 expression in the BUD/RV16 group is comparable to the significant increase in the PLAC/RV16 group.

The present data critically depend on the immunohistochemical analysis. We used the CD54-clone MEM112 antibody because of its ability to detect ICAM-1 (351). We focused on the expression of ICAM-1 in the bronchial epithelium because the available evidence indicates that the epithelium is the primary target of rhinovirus infection in the nose (273). Furthermore, primary tracheal epithelial cell cultures as well as a bronchial epithelial cell line appear to be susceptible to rhinovirus infection (197,218). The stained sections were analyzed by semi-quantitative analysis, as has been previously described others (213), and by ourselves (47) for measuring the expression of molecular markers in bronchial biopsies. A potential bias by epithelial damage in the ICAM-1 score was avoided by focusing on staining in the basal cell layer. The lack of effect of budesonide treatment and the RV16 intervention on epithelial intactness and the lack of a significant correlation between ICAM-1 staining and epithelial intactness support the absence of such a bias. Finally, the validity of the semi-quantitative analysis of both ICAM-1 staining and epithelial quality is supported by a satisfactory intra-observer repeatability ($\kappa > 0.69$). As a technical control for staining of the biopsies we chose PECAM-1, because it is equally and constitutively expressed in the endothelium and it has not been reported to be affected by steroids (348).

How can the present results be interpreted? A possible pathway of the rhinovirus-induced ICAM-1 upregulation is mediation by cytokines such as IL-1β (218). In addition, one could

speculate that ICAM-1 cross-linking by rhinovirus particles, which contain multiple ICAM-1 binding sites, could induce AP-1 mediated IL-1β transcription and expression (352). Indeed, elevated IL-1β levels have been demonstrated in nasal lavage after rhinovirus infection (248). Alternatively, the rhinovirus infection itself could lead to enhanced transcription of the ICAM-1 gene (197) and other genes (223). This may occur through activation of transcription factors such as NF-κB, subsequent increased DNA binding (223,231), and activation of the ICAM-1 promoter region (197). The latter two mechanisms require the presence of rhinovirus particles within the intrapulmonary airways, which seems likely (276), although conclusive evidence is presently lacking.

We did not observe a difference in epithelial ICAM-1 expression in the budesonide-pre-treated group as compared to the placebo group. The present study was not designed to examine the effect of inhaled steroids on ICAM-1 expression *per sé*. Nevertheless, the budesonide pre-treatment did not appear to influence the RV16-associated increase in ICAM-1 expression. Few studies are available on the effect of glucocorticoids on pulmonary epithelial ICAM-1 expression. *In vitro*, glucocorticoids appear to decrease basal and IFN-γ-induced ICAM-1 expression (347). In asthmatic patients *in vivo*, 6 weeks pre-treatment with inhaled glucocorticoids had no effect on bronchial epithelial ICAM-1 staining (340), fitting in with our data, whereas it was recently demonstrated that such a treatment did decrease the ICAM-1 expression on BAL cells (mainly macrophages) (353). The lack of protection against RV-associated enhanced expression of ICAM-1 may not be surprising, since a combination of systemic and nasal glucocorticoid treatment of experimental rhinovirus infection in normal volunteers did not affect the virus-induced increase in IL-1β in nasal lavage (248). However, it can be speculated that the pretreatment period might have been too short and/or the steroid dose too low for some antiinflammatory effect to take place.

What are the clinical implications of this study? The observed rhinovirus-associated increase in its own cellular receptor in the bronchial epithelium might facilitate the spreading of the infection within the intrapulmonary airways in asthmatics (218), thereby potentially increasing the patients susceptibility to a severe immune response and its consequences, such as an asthma exacerbation. It is remarkable that the widely used inhaled steroids in a clinically relevant dose do not protect against this apparent positive feedback mechanism. It can be speculated that other treatment modalities such as cromolyns (354) or soluble ICAM-1 (137) will appear to be more useful in breaking this vicious circle, thus potentially providing effective protection against rhinovirus-induced asthma exacerbations.

In conclusion, experimental RV16 infection upregulates ICAM-1 expression in the bronchial epithelium of asthmatics *in vivo*. Inhaled steroids do not affect baseline ICAM-1 expression, and do not appear to have a protective effect against the RV16-associated upregulation. This suggests that other treatment modalities are required to treat or prevent rhinovirus-induced exacerbations in patients with asthma.

8 EFFECT OF PRETREATMENT WITH INHALED CORTICOSTEROIDS ON RHINOVIRUS 16-INDUCED AIRWAYS INFLAMMATION IN ASTHMA

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8.1 ABSTRACT

Asthma exacerbations are often associated with rhinovirus infections. However, the inflammatory pathways are poorly understood, whilst treatment is often unsatisfactory. Therefore, we selected 25 atopic, mildly asthmatic patients who underwent experimental rhinovirus 16 (RV16) infection, whilst receiving double-blind, placebo-controlled pretreatment with the inhaled corticosteroid budesonide (800 μg b.i.d.), starting 2 weeks before infection. We assessed inflammatory cell numbers in bronchial biopsies 2 days before - and 6 days after RV16 infection, and analyzed those in relation to cold symptoms, changes in blood leukocyte counts, airways obstruction and airway hyperresponsiveness. RV16 colds induced an increase in tissue CD3+ cells and tended to decrease the numbers of eosinophils. The T cell accumulation was positively associated with cold symptoms. Greater epithelial accumulation of either CD8+ cells (in the placebo group) or CD4+ cells (in the budesonide group) was associated with less worsening of airways hyperresponsiveness. Similarly, the larger the decrease in eosinophil counts in the lamina propria, the less worsening of airways hyperresponsiveness, whilst epithelial accumulation of neutrophils was significantly correlated with worsening of airways obstruction. Budesonide pretreatment improved airway hyperresponsiveness and eosinophilic airways inflammation. Yet, it did not significantly affect the RV16-associated change in numbers of any of the inflammatory cell types. We conclude that the physiological worsening of asthma after RV16 infection is inversely associated with accumulation of T cells and dissipation of eosinophils in the bronchial mucosa, whilst being positively associated with accumulation of neutrophils. Prophylaxis with inhaled corticosteroids does not appear to affect the RV16-induced airways inflammation, which is in keeping with its limited protection against virus-induced exacerbations of asthma.

8.2 Introduction

Patients with asthma frequently suffer from transient worsening of their disease during respiratory virus infections. Rhinoviruses are most commonly associated with such exacerbations (77,78). Indeed, experimental rhinovirus infection in asthmatics worsens asthma symptoms (148,163) variable airways obstruction (165), and airways hyperresponsiveness to various bronchoconstrictor stimuli (148,163,170). This suggests that rhinovirus infection is able to promote airways inflammation in pre-existing asthma.

Evidence indeed suggests that rhinovirus infections enhance airways inflammation in asthma, as reflected by an increase in local production of inflammatory mediators such as IL-1β, IL-8, IL-6 and ECP, detectable in nasal lavage (148,258) and/or in induced sputum (35). In addition, rhinovirus 16 (RV16) infections enhance ICAM-1 expression in the bronchial epithelial layer (173), and promote the infiltration of T cells into the bronchial lamina propria, and of eosinophils into the bronchial epithelium during the acute phase of infection in a sample that included both normal and asthmatic subjects (164). Yet, the relevance of these observations for development of rhinovirus-induced exacerbations of asthma is still unclear.

Inhaled or oral glucocorticosteroids are the most commonly used anti-inflammatory drugs for regular asthma therapy (1). However, their clinical effectiveness in preventing (355) or treating (356) rhinovirus-induced exacerbations of asthma could not be confirmed in clinical studies. Glucocorticoids have various anti-inflammatory effects, including lowering the numbers of eosinophils and lymphocytes in the mucosa of asthmatics (336), and inhibition of the effects of pro-inflammatory cytokines such as IL-1 (357,358), a cytokine that has been implicated in rhinovirus-induced airways inflammation (248). On the other hand, there is some evidence that glucocorticoids, by suppressing the immune response (359), may hamper viral clearence (119), thereby potentially worsening or protracting the disease. Hence, there is inconclusive evidence on the effects of glucocorticoids within the airways in rhinovirus-associated exacerbations of asthma.

In the present study we hypothesized first, that RV16 colds in asthmatic subjects induce worsening of the underlying airways inflammation. Second, we tested whether this can be prevented (in part) by pretreatment with inhaled glucocorticoids. Therefore, we conducted a trial in atopic asthmatic patients who all underwent experimental RV16 infection, and received double-blind, placebo-controlled pretreatment with inhaled budesonide, starting 2 weeks before infection. The primary outcome parameters of the study were the numbers of inflammatory cells in bronchial lamina propria and epithelium before and after RV16 infection, as obtained from bronchial biopsy specimens. The observed changes in these outcome parameters in either treatment group were analyzed in relation to the severity of the cold (as reflected by cold score, changes in peripheral blood leukocyte counts) and the physiological severity of asthma (as reflected by airways obstruction and airway hyperresponsiveness).

8.3 Methods

8.3.1 Subjects

Twenty-five non-smoking or ex-smoking (>12 months, <5 pack years) adult asthmatics (13 female, 12 male; age 19-25 yr.) were recruited. The subject characteristics are presented in table 1. The patients had low titers of circulating antibodies specific to rhinovirus 16 using a RV16 serum neutralization assay (≤ 1:4 serum dilution against 20-25 x 50% tissue culture infective dose -$TCID_{50}$-), and were atopic, as indicated by one or more wheal (> 3 mm) and flare response to skin prick tests to 10 common aero-allergen extracts (Soluprick, ALK, Benelux). In the 3 months preceding the study the subjects had not used oral or inhaled glucocorticoids or any other medication for their asthma or allergies except for inhaled short-acting β_2 agonists on demand. All subjects were healthy, apart from their asthma, during the 6 weeks preceding the study. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and the subjects gave their written informed consent before entering the study.

8.3.2 Design

The study had a randomized double-blind, parallel, placebo-controlled design, which included two groups of asthmatics receiving RV16 inoculation during treatment with either budesonide (BUD) or placebo (PLAC). Budesonide by dry powder inhaler (Turbohaler®, 800 µg, b.i.d.) or placebo was given for a period of 4 weeks, starting 16 days before RV16 inoculation (day -16). Experimental RV16 infection took place on two consecutive days (day 0 and day 1). The maximal forced expiratory volume in one second (FEV_1) and the provocative concentration of histamine causing a 20% fall in FEV_1 (PC_{20}) were recorded at days -17, and repeated at day -4, day 4 and finally at day 13. Blood samples were drawn at days -17, -2, 3, 6 and 28. Bronchial biopsies were by fiberoptic bronchoscopy taken at days -2 and 6.

8.3.3 RV16 inoculation, confirmation of infection

The RV16 inoculum was obtained from the same strain and stock as used in previous experiments in humans *in vivo* (148,162). The virus was cultured according to standards of good laboratory practice and the inoculum was tested to be safe for human *in vivo* usage (286). A total dose of 0.6-2.1 x 10^4 50% tissue culture infective dose ($TCID_{50}$) RV16 was administered to each subject according to a previously described procedure (148). Briefly, the total RV16 dose was divided over two days. On each day, equal portions of the inoculum were administered by nasal inhalation (DeVilbiss 646 nebulizer, connected to a face mask), nasal spraying (DeVilbiss 286 atomizer, powered by a compressor), and finally by instilling the fluid into each nostril by pipette.

The confirmation of RV16 infection was established by a fourfold or greater increase in RV16-specific neutralizing antibody titer in serum and/or by recovery of the virus from the nasal washes (148). The nasal washes were inoculated onto Human Embryonic Lung fibroblast (HEL) cultures and incubated at 32° C for 14 days. The identity of rhinovirus in a positive culture was confirmed by neutralization assay, using RV16-specific guinea-pig immune serum (1126AS/GP-VR; American Type Culture Collection, Rockville, MD). In order to exclude any intercurrent respiratory infection, all nasal washes were inoculated into rhesus monkey kidney cells (LCC-MK2), Hep-2 and HEL cell cultures and cultured at 37°C (148). In addition, the subjects scored their cold symptoms three times daily, and recorded those on diary cards as described previously (148).

8.3.4 Peripheral blood leukocyte count and differentiation

Unclotted venous whole blood samples (EDTA) were used for absolute and differential leukocyte counts by automated blood count analysis (Technicon H1, Technicon, Tarrytown, NY).

Table 8.1: Subjects' characteristics

Patients[A]	Sex	Age	Baseline	Baseline	Cold	titer pre/post-	culture
Budesonide							
1	M	24	73.5	0.14	4	1:1 / 1:1	pos/pos
2*	F	21	86.4	0.17	2	1:1 / 1:8	neg/neg
3	M	23	82.5	0.29	9	1:1 / 1:16	pos/pos
4	F	24	86.1	0.30	8	1:1 / 1:1	pos/neg
5	M	23	95.2	0.54	3	1:1 / 1:8	pos/neg
6	M	24	76.4	0.54	14	1:1 / 1:32	pos/neg
7	M	23	81.7	0.65	12	1:4 / 1:128	pos/pos
8*	F	23	91.0	1.28	11	1:2 / 1:2	pos/neg
9	M	24	76.8	1.34	8	1:1 / 1:256	pos/neg
10	M	25	82.6	2.13	8	1:1 / 1:8	pos/pos
11	F	24	80.8	3.32	4	1:1 / 1:16	neg/neg
12	F	25	103	5.92	1	1:1 / 1:2	pos/pos
mean±SEM			84.6±2.4	0.74±0.49	7±1.2		
Placebo							
13	M	22	80.9	0.06	8	1:1 / 1:16	pos/neg
14	F	19	80.5	0.14	8	1:1 / 1:4	pos/pos
15	F	19	84.2	0.30	3	1:1 / 1:1	pos/pos
16	F	20	95.7	0.31	17	1:1 / 1:8	pos/pos
17*	M	19	101	0.46	10	1:1 / 1:1	neg/neg
18	M	23	74.6	0.52	5	1:1 / 1:32	pos/neg
19	F	20	83.9	0.53	10	1:1 / 1:1	pos/neg
20	F	23	84.0	0.65	4	1:1 / 1:32	pos/pos
21	F	24	94.2	1.44	2	1:1 / 1:16	neg/neg
22	F	20	96.6	1.81	5	1:1 / 1:1	pos/pos
23*	M	23	103	2.18	6	1:2 / 1:2	neg/neg
24	F	20	95.9	2.40	10	1:1 / 1:8	pos/pos
25	M	22	84.1	4.34	3	1:1 / 1:256	pos/pos
mean±SEM			89.2±2.5[A]	0.65±0.49[B]	7±1.1		

Asterixes indicate the subjects who were excluded from statistical analysis either because RV16 infection could not be confirmed (#2, 17, 23) or because a rhinovirus other than RV16 was detected (#8). **A**: FEV_1 between-group comparison: p = 0.21. **B**: Geometric mean PC_{20} ± SEM (doubling dose) at day –4. Between-group comparison: p = 0.79. **C**: M: male; F: female. **D**: Titer of neutralizing antibodies against RV16 (serum dilution, 1: ..) at day –2 / day 28. **E**: Tissue culture of RV16 at day 3 / day 6.

8.3.5 Lung function and airway hyperresponsiveness

Standardized histamine challenge tests (289) were performed using doubling concentrations of histamine-di-phosphate in phosphate buffered saline (0.03-8 mg/ml). A DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, PA, output 0.13 ml/min.) was used, in connection to the central chamber of an in- and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T). The aerosol was inhaled by tidal breathing for 2 min, at 5 min intervals, with the nose clipped. The lung function response was measured as FEV_1, obtained by a dry rolling spirometer (Morgan spiroflow, Rainham, United Kingdom). First, baseline FEV_1 was determined as the mean of three reproducible values (within 5 %). Subsequently, single measurements of FEV_1 were made 90 seconds after each histamine dose. The tests were

discontinued if FEV_1 decreased by more than 20% from baseline. Afterwards the subjects inhaled 200 µg salbutamol to provide immediate bronchodilation.

8.3.6 Bronchoscopy

Fiberoptic bronchoscopy was carried out by experienced investigators, using a standardized protocol based on international guidelines, as has been used in previous studies in our department (360). After 6 hr of fasting, pre-medication consisting of atropine 0.5 mg subcutaneously, codeine 20 mg orally and salbutamol 400 µg by metered dose inhaler was administered to the subjects. Local anesthesia was performed with 10% (w/v) lignocaine aerosol in the oropharynx, and with 2% (w/v) lignocaine solution in the lower airways. Throughout the procedure oxygen was delivered through the nasal canula, while oxyhemoglobin saturation was monitored continuously using a transcutaneous oximeter (N-180, Nellcor Inc., Hayward, CA, USA). Bronchoscopy was performed using a Pentax bronchoscope (outer diameter 6 mm; Pentax Optical Co., Japan), that was introduced through the mouth, with the patient in supine position. Bronchial biopsies were taken at (sub)segmental level from either the right lung (right lower and middle lobe) or the left lung (lingula and left lower lobe), using a pair of cup forceps (Olympus FB-20C, Tokyo, Japan). Alternate biopsy sites (right or left lung) were randomized over the 2 bronchoscopy visits. Afterwards, the subjects were observed for one hour for any adverse events.

8.3.7 Biopsy processing and staining

The biopsies were immediately fixed in buffered formalin 10% (v/v). Then, the biopsies were embedded in paraffin and stored until further processing. Four micrometer thick sections were used. If required, immunohistochemical staining was preceded by antigen retrieval (table 2). Immunohistochemical staining was performed using the monoclonal (EG2, elastase, CD4, CD8, AA1) and polyclonal (CD3) mouse primary antibodies, and visualized using the streptavidin-biotin-complex (SABC). In short, the sections were incubated with an optimal dilution of antibodies in 1% BSA/PBS at room temperature for 60 min. Then, the sections were incubated with biotinylated rabbit-anti-mouse antibodies (for monoclonal antibodies) or swine-anti-rabbit antibodies (for CD3 polyclonal antibodies) for 30 min and the streptavidin-peroxidase complex was added. Finally, the reaction was revealed by putting the slides in 5% AEC (3-amino-9-ethylcarbazol) for 7 minutes. The sections were counterstained with Mayer's haematoxyline. For negative controls, the primary antibody was omitted from this procedure.

8.3.8 Analysis of bronchial biopsies

All analyses were performed in a blinded fashion, on coded material. The numbers of the different inflammatory cells in lamina propria and epithelium were counted by fully automated cell counting (361,362). First, images were digitized using a 3-chip color camera ($433 \cdot 10^3$ pixels, 660x496µm^2, 3x256 gray values) (KS-400 system, Kontron/Zeiss). Then, the area of lamina propria or epithelium was determined in these images by manually delineating the epithelial basement membrane. Lamina propria, defined by the widest possible 125 µm deep zone beneath the basement membrane of at least 86,000 µm^2 (excluding BALT and airway smooth muscle) was automatically determined and presented on the screen. Likewise, epithelial area, defined by the area above the basement membrane enclosed by the airway lumen of at least 25,000 µm^2, was automatically determined and presented on the screen. We did not exclude damaged epithelium from the analysis, as it is yet unknown as to whether a rhinovirus

infection affects asthmatic bronchial epithelial intactness *in vivo*, thereby potentially introducing a bias.

The automated counting of the number of positively staining cells consisted of the following steps: level off background staining, normalize staining intensity (gray values from 25th percentile to the modus), delete noise, fuse stained fragments, delineate stained clusters, determine cell count by an algorithm. This method has been shown to be fully reproducible (R_i = 1), and to have good agreement with interactive cell counting (R_i = 0.97) (361).

Table 8.2: antibodies for immunohistochemical staining.

Antigen	mAb	dilution	antigen retrieval	sold by
EG2	yes	1:200	trypsine	Kabi Pharmacia[A]
Elastase	yes	1:50	-	Dako[B]
AA1 (tryptase)	yes	1:750	-	Dako[B]
CD3	no	1:400	citrate	Dako[B]
CD4	yes	1:50	EDTA	Thamer Diagnostics[C]
CD8	yes	1:400	EDTA	Novocastra[D]

A: Kabi Pharmacia, Woerden, The Netherlands
B: Dako, Glostrup, Denmark
C: Thamer Diagnostics, Uithoorn, The Netherlands
D: Novocastra, Newcastle upon Tyne, United Kingdom

8.3.9 Statistical analysis

Peripheral blood leukocyte differentials were expressed as percentage of total white blood cell count or cells•10^9/L. FEV_1 was expressed as % predicted (288). The response to histamine was expressed as % fall in FEV_1 from baseline. PC_{20}, being the provocative concentration causing a 20% fall in FEV_1, was calculated by log-linear interpolation of the last 2 adjacent data points. PC_{20} was log-transformed before statistical analysis. The results for PC_{20} are presented as geometric mean ± SEM in doubling dose (DD), and changes in PC_{20} are expressed in doubling dose.

Repeated measures analysis of variance (ANOVA) was applied, analyzing the effect of budesonide pretreatment or RV16 infection within the separate groups, while the interaction of the 2 factors was analyzed in the whole sample. The analysis was either limited to days -17 to -2, being the pretreatment period before RV16 inoculation for analysis of the effect of budesonide alone, or the period from day -4 onward for analysis of the effect of RV16 infection. Significant ANOVA effects were explored using paired and unpaired Student's t-tests.

The number of positively staining cells in lamina propria or epithelium was expressed as cells/0.1 mm^2. Statistical analysis of all cell counts was performed on log-transformed cell count data, after addition of 1 to allow for transformation of zero values (362). Paired and unpaired Student's t-tests were applied to examine the data. The results were expressed as geometric mean ± SEM in doubling cell number (DC). Changes in cell numbers were expressed as doubling cell numbers. Relationships between outcome parameters were investigated using Pearson's correlation test. P values < 0.05 were considered to be significant.

8.4 Results

All 25 patients completed the study. One patient (# 3) did not undergo the bronchoscopies due to strong subjective discomfort. All the other subjects underwent bronchoscopy twice. In few cases the quality and size of the biopsies was inadequate for staining or analysis. RV16 infection was confirmed in all RV16 inoculated subjects, except subjects #2, 8, 17 and 23, who were excluded from the statistical analysis of the effect of RV16. All RV16-treated subjects had an anti-RV16 titer serum ≤ 1:1 before entering the study. However, reassessment just before inoculation of RV16 revealed slightly elevated titers in subjects # 7, 8 and 23, coinciding with symptoms of a common cold in subjects # 8 and # 23, which was confirmed in subject # 8 by rhinovirus-positive (RV16-negative) culture of the nasal lavage. Since low levels of neutralizing antibodies *per se* have not been shown to preclude a symptomatic common cold (148), subject # 7 was not excluded from the analysis.

Table 8.3A: Descriptive and statistical analysis of cell counts in lamina propria

Marker	group	Pre-inoculation: day –2			Post-inoculation: day 6			paired t-test[B]	unpaired t-test Δ[C]
		mean[D]	±	SEM (DC)	mean[D]	±	SEM (DC)	p	p
CD3	All	70.14	±	0.18	94.59	±	0.20	0.03	
	BUD	55.18	±	0.22	76.47	±	0.36	0.14	
	PLAC	85.35	±	0.24	114.55	±	0.18	0.14	
p[A]		0.08			0.18				0.83
CD4	All	94.93	±	0.12	100.99	±	0.13	0.66	
	BUD	84.10	±	0.13	88.14	±	0.21	0.80	
	PLAC	104.82	±	0.17	114.15	±	0.14	0.74	
p[A]		0.18			0.15				0.98
CD8	All	53.97	±	0.18	63.85	±	0.30	0.31	
	BUD	47.89	±	0.30	45.31	±	0.51	0.85	
	PLAC	59.52	±	0.21	86.94	±	0.31	0.04	
p[A]		0.40			0.12				0.19
EG2	All	8.69	±	0.36	6.22	±	0.34	0.34	
	BUD	5.31	±	0.45	6.69	±	0.58	0.71	
	PLAC	13.00	±	0.50	5.87	±	0.42	0.06	
p[A]		0.08			0.79				0.15
Elastase	All	6.59	±	0.30	8.47	±	0.34	0.36	
	BUD	10.18	±	0.22	10.27	±	0.56	0.91	
	PLAC	4.62	±	0.48	7.26	±	0.44	0.30	
p[A]		0.05			0.48				0.51
AA1	All	65.54		0.28	82.42	±	0.27	0.47	
	BUD	87.65	±	0.29	103.76	±	0.23	0.63	
	PLAC	53.10	±	0.43	68.56	±	0.45	0.59	
p[A]		0.21			0.26				0.86

BUD: budesonide group. PLAC: placebo group. All: all subjects. **A**: P-value unpaired t-test for between-group analysis (budesonide *vs.* placebo group) at each time point. **B**: Paired t-test for comparison of day -2 and day 6 within each group. **C**: Unpaired t-test for between-group analysis (budesonide *vs.* placebo group) of changes in cell numbers between days -2 and 6 (Δ).
D: geometric mean.

Table 8.3B: Descriptive and statistical analysis of cell counts in epithelium.

marker	group	pre-inoculation: day -2		post-inoculation: day 6		paired t-test[B]	unpaired t-test Δ[C]
		mean[D]	± SEM (DC)	mean[D]	± SEM (DC)	p	p
CD3	All	19.88	± 0.39	26.23	± 0.29	0.71	
	BUD	13.42	± 0.39	23.07	± 0.47	0.04	
	PLAC	26.45	± 0.59	29.06	± 0.37	0.74	
p[A]		0.22		0.58			0.41
CD4	All	2.89	± 0.40	2.99	± 0.32	0.71	
	BUD	3.39	± 0.47	2.73	± 0.40	0.64	
	PLAC	2.53	± 0.63	3.24	± 0.49	0.42	
p[A]		0.61		0.71			0.35
CD8	All	22.64	± 0.33	26.06	± 0.35	0.52	
	BUD	24.96	± 0.30	20.86	± 0.64	0.74	
	PLAC	20.90	± 0.57	31.83	± 0.33	0.19	
p[A]		0.71		0.40			0.26
EG2	All	1.76	± 0.27	1.27	± 0.19	0.06	
	BUD	1.17	± 0.15	1.08	± 0.11	0.51	
	PLAC	2.44	± 0.42	1.46	± 0.32	0.08	
p[A]		0.04		0.22			0.18
Elastase	All	1.81	± 0.28	2.47	± 0.30	0.33	
	BUD	1.80	± 0.52	3.18	± 0.43	0.35	
	PLAC	1.83	± 0.30	1.98	± 0.42	0.74	
p[A]		0.97		0.27			0.59
AA1	All	2.82	± 0.33	4.75	± 0.45	0.11	
	BUD	4.28	± 0.51	5.74	± 0.75	0.58	
	PLAC	1.98	± 0.43	4.01	± 0.56	0.07	
p[A]		0.11		0.58			0.56

A, B, C: same as in legend of table 3A.

8.4.1 Peripheral blood leukocyte counts

The effects of pretreatment and RV16 on leukocyte counts are depicted in figure 1A. In the placebo group the cold score (table 1) correlated significantly with the increase in the numbers of neutrophils between days 3 and 6 ($r = 0.82$, $p = 0.003$).

8.4.2 Lung function and airways hyperresponsiveness

Before commencement of treatment FEV_1 (% predicted) was not significantly different between the groups (mean ± SEM BUD: 84.6 ± 2.4, PLAC: 89.2 ± 2.5, $p = 0.21$). During the pretreatment period FEV_1 tended to increase in the budesonide group ($p = 0.07$), however, this increase was not significantly different between the groups ($p = 0.63$). RV16 infection had no significant effect on FEV_1 ($p = 0.41$), and changes were not significantly different between the groups ($p = 0.09$).

In the budesonide group PC_{20} showed an increase during the pretreatment period ($p = 0.005$) which was different from placebo ($p = 0.02$). Thus, at day –4, PC_{20} was higher in the budesonide group as compared to placebo ($p = 0.05$). There was no significant effect of RV16 infection on PC_{20} to histamine within either group (PLAC: $p = 0.18$, BUD: $p = 0.65$) and the effect was not significantly different between the groups ($p = 0.20$). However, at day 4, but not

at day 13, PC_{20} was still higher in the budesonide group as compared to placebo ($p = 0.02$ and $p = 0.18$, respectively) (figure 1B).

8.4.3 Lamina propria

The average area of lamina propria per patient that was examined for all stainings and all visits was 266,000 ± 11,000 μm^2 (SEM). The descriptive and statistical analysis are presented in table 3A. The data show an overall increase in the numbers of CD3+ cells ($p = 0.03$), which is not significantly different between the groups ($p = 0.83$). The increase can be attributed in part to a significant increase in CD8+ cells in the placebo group ($p = 0.04$).

In the placebo group cold scores tended to correlate significantly with the increase in the number of CD3+ cells ($r = 0.59$, $p = 0.07$) (figure 2A). In addition, both cold scores and the rise in the numbers of circulating neutrophils between days 3 and 6 were significantly (or borderline -) and inversely associated with the change in AA_1+ cells in either group (cold scores: PLAC: $r = -0.61$, 0.06, BUD: $r = -0.83$, $p = 0.01$, neutrophils: PLAC: $r = -0.62$, 0.07, BUD: $r = -0.71$, $p = 0.05$).

In either group, the number of EG2+ cells at baseline (day –2) correlated significantly with the subsequent RV16-associated decrease in EG2+ cell numbers (PLAC: $r = -0.71$, $p = 0.02$, BUD: $r = -0.80$, $p = 0.02$) (figure 3A). In the placebo group, the largest decrease in the number of EG_2+ cells was associated with less worsening of PC_{20} to histamine ($r = -0.70$, $p = 0.02$) (figure 4A) and with the largest increase in the number of eosinophils in peripheral blood during the acute phase of the cold ($r = -0.74$, $p = 0.04$).

8.4.4 Epithelium

The average area of epithelium per patient that was examined for all stainings and all visits was 90,000 ± 5,000 μm^2 (SEM). The descriptive and statistical analysis are presented in table 3B. The data show a significantly lowered number of EG2+ cells before RV16 inoculation ($p = 0.04$), and thereafter, a significant increase in the number of CD3+ cells in the budesonide group ($p = 0.04$).

Similar to the relationship in lamina propria, the higher the cold score, the larger the epithelial accumulation of CD3+ cells in the placebo group ($r = 0.77$, $p = 0.02$)(figure 2B). This may not be surprising, as the accumulation of CD3+ cells in the epithelium and lamina propria were significantly correlated ($r = 0.66$, $p = 0.04$). Moreover, accumulation of CD8+ cells (in the placebo group), or CD4+ cells (in the budesonide group) was associated with less worsening of PC_{20} during the RV16 cold (PLAC: $r = 0.71$, $p = 0.02$, BUD: $r = 0.67$, $p = 0.047$) (figures 5A, 5B). Finally, the rise in numbers of circulating monocytes (a CD4+ cell) in the acute phase of the cold was significantly but inversely related to infiltration of CD4+ cells ($r = -0.74$, $p = 0.03$) in the placebo group.

Similar to the lamina propria, the larger the number of EG2+ cells present at baseline, the larger the subsequent RV16-associated decrease in eosinophil numbers in either group (PLAC: $r = -0.74$, $p = 0.01$, BUD: $r = -0.88$, $p = 0.004$) (figure 3B). In addition, in the placebo group, a rise in the number of AA_1+ cells and CD3+ cells in the epithelium correlated significantly with a fall in EG_2+ cell numbers in the lamina propria ($r = -0.70$, $p = 0.03$). Finally, accumulation of elastase+ cells was associated with lowering of FEV_1 (%pred.) in the acute phase of the cold ($r = -0.71$, $p = 0.03$, $Rs = -0.76$, $p = 0.02$) (figure 4B). This relationship still tended to be significant after exclusion of the obvious outlier ($r = -0.62$, $p = 0.10$, $Rs = -0.66$, $p = 0.08$).

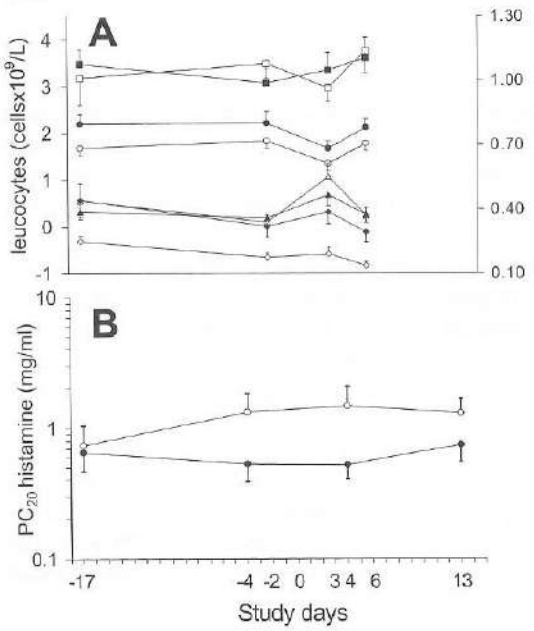

Figure 8.1
A: Peripheral blood leukocytes numbers (cells•10^9/L, mean ± SEM) in the budesonide group (open symbols) and the placebo group (closed symbols). Primary ordinate: □: neutrophils; ○: lymphocytes. Secondary ordinate: Δ: monocytes; ◇: eosinophils.
Effect of pretreatment alone:
At baseline, leukocyte counts were not different between the groups ($p = 0.17$). Pretreatment did not affect leukocyte counts (within-group: $p \geq 0.75$, between-groups: $p = 0.27$). Eosinophil numbers decreased in both groups ($p \leq 0.04$), but this effect was not different between the groups ($p = 0.39$).
Effect of RV16
Placebo: leukocyte and lymphocyte counts decreased at day 3 after RV16 infection, returning to baseline at day 6 ($p \leq 0.03$). neutrophil -, eosinophil -, monocyte - and basophil counts increased ($p \leq 0.03$). Budesonide: lymphocyte counts decreased, whereas monocytes increased significantly ($p \leq 0.001$). Changes in basophil and monocyte numbers were significantly different between the groups ($p \leq 0.02$).
B: PC_{20} to histamine (mg/ml, geometric mean ± SEM -doubling dose-) in the budesonide group (open symbols) and the placebo group (closed symbols).
Effect of pretreatment alone:
Baseline PC_{20} was not different between the groups ($p = 0.79$). The increase in PC_{20} in the budesonide group in the pretreatment period ($p = 0.005$) was different from placebo ($p = 0.02$).
Effect of RV16:
At day –4, PC_{20} was higher in the budesonide group as compared to placebo ($p = 0.05$).
There was no significant change in PC_{20} in either group (PLAC: $p = 0.18$, BUD: $p = 0.65$), and changes were not different between the groups ($p = 0.20$). At day 4, but not at day 13, PC_{20} was still higher in the budesonide group as compared to placebo (day 4: $p = 0.02$, day 13: $p = 0.18$).

8.4.5 ICAM-1 expression

The results of ICAM-1 staining have been reported previously (173). In short, the ICAM-1 score increased significantly in the placebo group (Wilcoxon test: $p = 0.03$), with a similar trend in the budesonide group ($p = 0.07$). These changes were not significantly different between the groups (MWU test: $p = 0.74$). The increase in ICAM-1 expression in the placebo group appeared to be positively associated with the change in epithelial AA_1+ cells numbers ($r = 0.86$, $p = 0.003$), but inversely correlated with the change in numbers of CD3+ ($r = -0.75$, $p = 0.02$) and CD4+ cells ($r = -0.87$, $p = 0.003$) in the lamina propria.

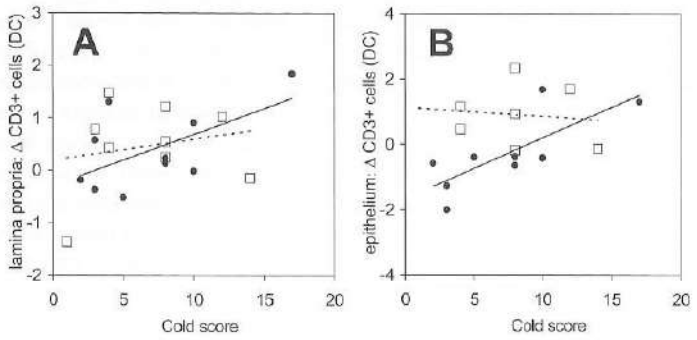

Figure 8.2: Correlation of the cold score *versus* the change in the numbers of CD3+ cells (in doubling cell numbers -DC-) in the lamina propria (**A**) and epithelium (**B**).

legend	8.2A	8.2B
All subjects	$r = 0.40$, $p = 0.09$	$r = 0.50$, $p = 0.03$
Plac (•, —):	$r = 0.59$, $p = 0.07$	$r = 0.77$, $p = 0.02$
Bud (•, -----):	$r = 0.20$, $p = 0.60$	$r = -0.11$, $p = 0.81$

8.5 DISCUSSION

In this study we demonstrated that an experimental RV16 infection in asthmatic subjects is associated with accumulation of T cells, particularly CD8+ cells, and with a trend towards lowering of eosinophil numbers in lamina propria and epithelium. In the placebo group the increase in T cell numbers was positively associated with the cold symptoms. Large epithelial accumulation of T cells was correlated with less worsening of airway hyperresponsiveness. In the placebo group this relationship was significant for CD8+ cells, whereas in the budesonide group the relationship was significant for CD4+ cells. In either group, the decrease in eosinophil numbers was significantly related to the degree of pre-existing eosinophilic inflammation: the more eosinophils present at baseline, the more efflux after infection. Reduced eosinophilia in the lamina propria was associated with less worsening of airways hyperresponsiveness, whilst epithelial accumulation of neutrophils was found to be related to worsening of airways obstruction. Although 2 weeks pretreatment with inhaled budesonide improved airway hyperresponsiveness and lowered the numbers of eosinophils in biopsies, we did not observe any significant effects on the RV16-associated accumulation of any of the inflammatory cell types. Our data suggest that antiinflammatory treatment with inhaled glucocorticosteroids in patients with asthma does not affect rhinovirus-induced airways inflammation, as reflected by cellular infiltration. Yet, patients with asthma may profit from a corticosteroid-induced improvement of their baseline condition.

This is the first study to describe the effects of placebo-controlled pretreatment with inhaled glucocorticoids on lower airways inflammation, as induced by experimental RV16 colds in asthmatic subjects. The observed RV16-associated infiltration of T cells into the lamina propria confirms the results of a previous study on RV16-induced airway inflammation by Fraenkel *et al.* (164). The absence of an increase in eosinophil numbers is in keeping with findings in

normal and atopic subjects after natural rhinovirus infection (363), but is in apparent contrast to the observed increase in EG2+ cells in the epithelium in the before mentioned study (164). Methodological differences such as patient selection (atopic asthmatics only *versus* a mixed sample of normal and asthmatic subjects) and study design (biopsies taken 6 days *versus* 4 days after inoculation) may have contributed in part to the different outcomes in these two studies. The lack of any significant effect of budesonide on rhinovirus-associated changes in lung function, airway hyperresponsiveness, or cellular infiltration into the bronchial mucosa seems to be in keeping with clinical studies, showing a lack of protection against virus-induced exacerbations of asthma (355,356). Fortunately, neither did we observe any detrimental effects of inhaled steroids on any of the parameters, in terms of severity of response and recovery time, which *a priori* could not be excluded, based on potentially impaired viral clearence (119,359).

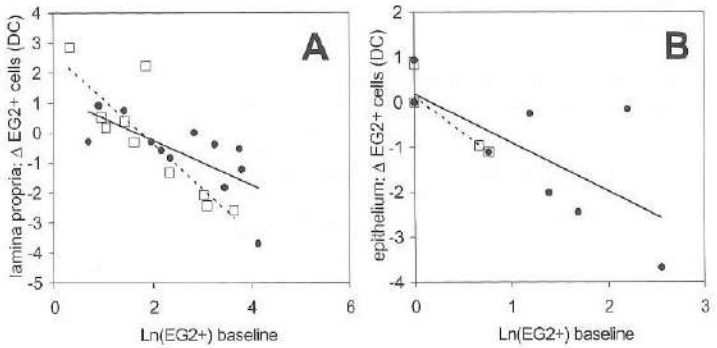

Figure 8.3: Correlation of the number of EG2+ cells present at day -2 (log-transformed) *versus* the subsequent change in the numbers of EG2+ cells (DC) in the lamina propria (A) and the epithelium (B).

legend	8.3A	8.3B
All subjects	r = -0.77, p < 0.001	r = -0.78, p < 0.001
Plac (•, —):	r = -0.71, p = 0.02	r = -0.74, p = 0.01
Bud (•, -----):	r = -0.80, p = 0.02	r = -0.88, p = 0.004

The results of this study were obtained after carefully considering study design and subjects selection, while using validated methods for administering RV16 (162,163) and recording lung function and airway responsiveness (289). By using an automated system to perform biopsy cell counting we were able to analyze relatively large amounts of tissue for each staining (362), in a standardized and highly reproducible way (361). We included only steroid-naï ve patients who had mild persistent asthma, who were eligible for regular treatment with inhaled steroids, according to treatment guidelines (1). However, the dosage used (800 μg b.i.d.) was higher than the recommended dose (1), in order to ensure an optimal effect during a relatively short pretreatment period (364). The decrease in airway hyperresponsiveness and the reduced eosinophil counts in the bronchial mucosa of the budesonide-treated subjects after the 2 weeks pretreatment period fit in with the results of previous studies on the effects of inhaled corticosteroids (336,365), and indicate that the pretreatment with inhaled corticosteroids was adequate to produce anti-inflammatory effects (364).

The present data do not seem to be affected by lack of statistical power, as the power was adequate to detect a two-fold difference in eosinophil numbers between the groups after 2 weeks budesonide treatment, and also to detect rhinovirus-associated within-group changes in cell numbers of similar or smaller magnitude (362). The various correlations between biopsy cell counts and clinical/physiological outcome parameters indicate that even variation around the zero-value, resulting in statistically non-significant changes, may not just be random noise, but can indeed have biological relevance. As the present sample size has previously been shown to allow detection of the effect of placebo-controlled treatment with inhaled steroids on bronchial inflammatory cell counts (336), we speculate that any effects of budesonide treatment on rhinovirus-induced airways inflammation are likely to be smaller than those observed during treatment with inhaled steroids alone.

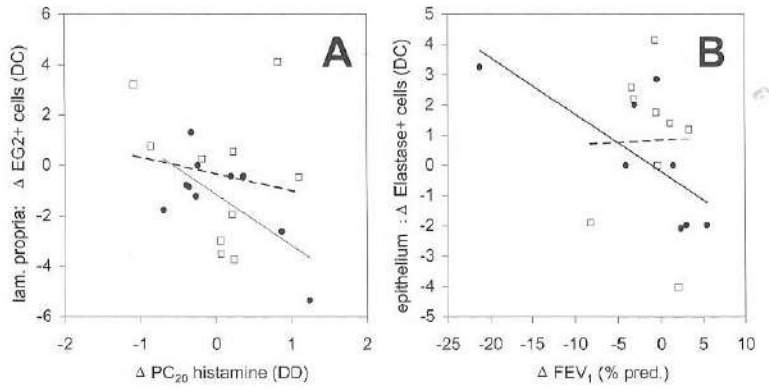

Figure 8.4: A: Correlation of the change in PC_{20} between days –4 and 4 (doubling dose -DD-) *versus* the change in the number of EG2+ cells in the lamina propria (DC).
B: Correlation of the change in FEV_1 (% predicted) between days –4 and 4 *versus* the change in the number of elastase+ cells in the epithelium (DC).

legend	8.4A	8.4B
All subjects	r = -0.38, p = 0.12	r = -0.05, p = 0.86
Plac (•, —):	r = -0.70, p = 0.02	r = -0.71, p = 0.03
Bud (o, -----):	r = -0.20, p = 0.64	r = 0.02, p = 0.95

In the present study, RV16 inoculation resulted in successful infection in 21 out of 25 patients. Yet, in contrast to previous studies by others (162) and ourselves (148), the RV16 colds in the present study were not associated with a significant increase in airway hyperresponsiveness to histamine. The severity of infection, as reflected by cold scores and rise in numbers of circulating neutrophils, has been shown to be linked to the rhinovirus-associated enhancement of airway hyperresponsiveness (148), such that only the severest colds lead to a significant decrease in PC_{20}. As the cold scores were generally lower than those described in a previous study (148), we speculate that this might explain the observed lack of increase in airway hyperresponsiveness. The present data extend previous findings by showing that the severity of cold symptoms and rise in numbers of circulating neutrophils correlate positively and significantly with the accumulation of T cells and inversely with mast cells in the bronchial

mucosa. This indicates that mild rhinovirus infections exacerbate airway inflammation, even in the absence of marked clinical worsening of asthma.

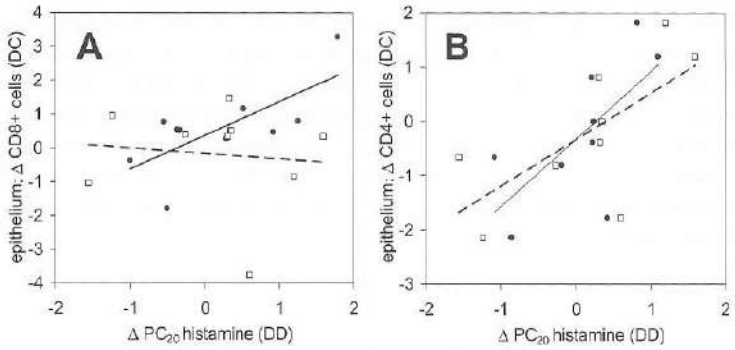

Figure 8.5: Correlation of the change in PC_{20} between days –4 and 4 (DD) *versus* the change (DC) in CD8+ cell numbers (A) or CD4+ cell numbers (B) in the epithelium.

legend	8.5A	8.5B
All subjects	r = 0.25, p = 0.30	r = 0.49, p = 0.03
Plac (•, —):	r = 0.71, p = 0.02	r = 0.39, p = 0.27
Bud (o, -----):	r = -0.11, p = 0.78	r = 0.67, p = 0.047

We have previously shown that rhinovirus infection in asthmatics induced an increase of ECP in sputum 2 days after RV16 inoculation, associated with worsening of airway hyper-responsiveness (35). Furthermore, a small increase in the eosinophil numbers in the bronchial epithelium was demonstrated 4 days after RV16 inoculation in a sample of both normal and asthmatic subjects (366). At 6 days after infection we now find a trend towards a decrease in eosinophil numbers in both the epithelium and lamina propria, the latter being associated with an increase in numbers of circulating eosinophils, and with the absence of enhancement of airways hyperresponsiveness. Taken together, one could postulate that a rhinovirus cold causes eosinophils to migrate towards the airway lumen, thereby temporarily depleting the tissue from activated eosinophils just after the acute phase of infection, which might be beneficial during the recovery from an initial enhancement of airways hyperresponsiveness. Yet, the decrease in EG2+ cells in the lamina propria was associated with an increase in numbers epithelial CD3+ and AA1+ cells, the latter also being correlated with the increase in ICAM-1 expression, as assessed in a previous study (173). These associations suggest that such eosinophil migration indicate in fact an active and ongoing inflammatory process. Based on the consistent association between eosinophil numbers present at baseline and the subsequent infection-related decrease in eosinophil numbers within the same tissue compartment, one might consider the possibility that eosinophils migrate or degranulate as result of a bystander effect rather than a specific eosinophil-mediated immune response.

There is ample evidence that rhinovirus infections induce neutrophil migration towards the nasal mucosal surface (248,256), along with the release of neutrophil chemoattractants such as IL-8 into the airway secretions (35,148,251). The observed association between worsening of lung function and RV16-associated changes in epithelial neutrophil numbers, in the absence of significant neutrophil accumulation within the epithelium, is in keeping with the above.

Moreover, it raises the suggestion that neutrophil products not only provide a defense mechanism, but may also be involved in virus-induced lung injury (367,368), thereby potentially causing airway wall swelling with excessive airways obstruction (177,285).

We also observed the RV16-associated accumulation of CD3+ cells, and particularly CD8+ cells in the lamina propria. These findings are in keeping with a MHC class I restricted cytotoxic T cell response. Such an immune response is considered to be most efficient for viral clearence and recovery (215,369,370), and adds to previous suggestions of rhinoviral infection of the bronchial tissues themselves (169,276). Indeed, the increase in epithelial CD8+ cell infiltration was associated with improvement of airway hyperresponsiveness in the placebo group. In the budesonide-treated subjects, however, it was the migration of CD4+ cells that appeared to be associated with changes in airway hyperresponsiveness. Based on the observations that the average numbers of CD4+ cells exceed CD3+ cell numbers in the lamina propria, and that accumulation of CD4+ cells correlates significantly with the shift in numbers of circulating monocytes, it seems likely that CD4+ cell infiltrate represent both T helper cells and monocytes. Therefore, additional studies are required in order to assess the relative contribution of monocytes and T helper cells in a MHC class II restricted response to rhinovirus-induced airway pathology, particularly in relation to ICAM-1 expression and to corticosteroid therapy.

We did not observe an effect of budesonide pretreatment on rhinovirus-induced cellular infiltration of the bronchial mucosa. The interaction of glucocorticosteroids, rhinovirus infection and underlying allergic airway inflammation is likely to be complex. For example, glucocorticosteroids down-regulate cytokine expression (371). However, some cytokines (IL-4, IFN-γ) counteract the glucocorticoids-induced inhibition of the effects of IL-1β (357,358), which is a pivotal cytokine in the rhinovirus-induced immune response (182,248). Moreover, all three factors may affect monocyte responsiveness in various ways (190,191,357). Based on the present results one could argue that glucocorticoids reduce allergic airway inflammation, possibly even during a rhinovirus infection, whereas they do not seem to have a detrimental effect on the anti-viral immune response.

Regular treatment with inhaled corticosteroids may not prevent colds (355) or reverse the asthma exacerbations associated with them (356). However, our data implicate that, when the improved baseline condition with less eosinophilic airway inflammation is taken into account, asthmatics do benefit from prophylaxis with inhaled steroids during rhinovirus-induced exacerbations of their disease. Thus, we postulate that asthma therapy that aims to achieve improvement of allergic airways inflammation (360) provides a better baseline condition to sustain the consequences of a rhinovirus cold.

In summary, our data demonstrate that rhinovirus colds induce accumulation of T cells, particularly cytotoxic T cells, in the bronchial mucosa. Physiological worsening of asthma after RV16 infection is inversely associated with this accumulation of T cells and migration of eosinophils, while being positively associated with accumulation of neutrophils. Pretreatment with inhaled steroids improves eosinophilic airway inflammation, but does not appear to prevent RV16-induced inflammatory cell migration. Our data suggest that the merits of prophylaxis with inhaled steroids lie in improvement of the baseline condition of patients with asthma.

ACKNOWLEDGMENT:
The authors wish to thank AstraZeneca for providing the study medication.

9 CONCLUSIONS, SUMMARY AND GENERAL DISCUSSION

9.1 INTRODUCTION

Rhinovirus infections are the most common cause of colds, for which no specific prevention or therapy is available to date. In patients with asthma rhinovirus infections appear to have a more severe impact than in otherwise healthy humans, in that it induces temporary worsening of their disease. Rhinovirus infections appear to be a major risk factor for exacerbations of asthma, even during regular asthma treatment with inhaled corticosteroids. Treatment of exacerbations of asthma according to the current guidelines does not take the cause of the acute worsening into account. This is understandable, as evidence on the effectiveness of various existing treatment modalities for (rhino)virus-associated exacerbations of asthma is scarce. Yet, severe, and potentially fatal exacerbations still occur, indicating that there is room for improvement of therapy or prevention.

The pathogenic mechanisms for rhinovirus-associated exacerbations of asthma were largely unclear. The studies in this thesis describe the effects of experimental rhinovirus infections in asthmatic patients with respect to the pathophysiologic and inflammatory effects of rhinovirus infections in asthmatic patients. In addition, we investigated the effect of antiinflammatory pretreatment in the experimental setup in patients with asthma. The general aim of the studies in this thesis was to gain insight in the pathogenic mechanisms of rhinovirus-associated exacerbations of asthma. Such insight might provide clues as to how to tailor existing or new treatment modalities to the cause of the exacerbation for more effective asthma control.

9.2 CONCLUSIONS OF THIS THESIS

Given the objectives of this thesis, the present data lead to the following conclusions:

1. Experimentally induced RV16 colds in atopic asthmatic humans increase asthma symptoms in conjunction with enhancement of variable airways obstruction *(chapter 3)*.
2. Experimentally induced RV16 colds in atopic asthmatic humans induce an increase in airway hyperresponsiveness to histamine during the acute phase of the cold *(chapters 4, 5)*.
3. The degree of worsening of variable airways obstruction and airway hyperresponsiveness after RV16 infection is positively associated with the severity of the cold symptoms and the inflammatory response, as reflected by an increase in IL-8 in nasal lavage, drop in numbers of circulating lymphocytes, and rise in numbers of circulating neutrophils *(chapter 3, 4)*.
4. Unmyelinated airway sensory nerves are likely to be involved in rhinovirus infections in asthma, as indicated by rhinovirus-associated abrogation of the tolerance to repeated bradykinin challenges *(chapter 5)*. However, their exact role in the development of rhinovirus-induced exacerbations of asthma remains uncertain.
5. Rhinovirus 16 infections in atopic asthmatic subjects induce the release of pro-inflammatory and chemotactic mediators (IL-8, IL-6, ECP), both in the upper and lower airway secretions *(chapters 4, 6)*.

6. The early rise in sputum ECP levels after rhinovirus infection *(chapter 6)* is associated with enhancement of airway hyperresponsiveness, while depletion of bronchial tissue eosinophils later on is associated with lack of enhancement of airway hyperresponsiveness *(chapter 8)*. This suggests that eosinophils and their products mediate the initial deterioration, while tissue depletion of eosinophils is beneficial for the recovery of airway hyperresponsiveness.

7. The number of sputum neutrophils that contain IL-8 increases after RV16 infection in atopic asthmatic subjects *(chapter 6)*. This is indicative of a role of neutrophils in the response to a rhinovirus infection. Moreover, bronchial epithelial neutrophil accumulation was found to correlate significantly with worsening of airway obstruction *(chapter 8)*.

8. Rhinovirus 16 colds increase the expression of ICAM-1 in the bronchial epithelium of atopic asthmatic subjects *(chapter 7)*, which is positively correlated with a rise in epithelial mast cell numbers and a rise in the number of CD3+ and CD4+ cells in the lamina propria *(chapter 8)*, indicating its association with worsening of airway inflammation after RV16 infection.

9. RV16 infection induces a rise in the numbers of T cells in the bronchial mucosa in steroid-naive patients and corticosteroid-pretreated patients alike. Epithelial accumulation of T cells correlates with less worsening of airway hyperresponsiveness *(chapter 8)*. This observation suggests that the antiviral immune response interferes with the allergic inflammation, such that the effect outweighs the potential detrimental consequences of a cold.

10. As anticipated, two weeks pretreatment with inhaled corticosteroids results in reduction of the numbers of activated eosinophils in the bronchial mucosal biopsies, and improvement of airway hyperresponsiveness. However, such anti-inflammatory treatment does not alter the rhinovirus-associated effects on ICAM-1 expression *(chapter 7)* or T cell accumulation *(chapter 8)*. This indicates that corticosteroids have limited value in preventing the airway inflammation associated with a rhinovirus infection.

Briefly, we have demonstrated that rhinovirus infections in patients with asthma induce a mild exacerbation of asthma. The pathofysiological characteristics of such an exacerbation are a temporary increase in variable airways obstruction and airways hyperresponsiveness. The pathogenic mechanisms associated with such worsening involve airway sensory nerves and airway inflammation. The immune response to a rhinovirus infection appears to be mediated by T cells, which accumulate in the bronchial mucosa during the cold. In addition, the evidence suggests that eosinophils, neutrophils, and their products contribute to deterioration of airway hyperresponsiveness. The expression of ICAM-1 (the rhinovirus receptor) in the bronchial epithelium increases in conjunction with the enhanced airway inflammation. Anti-inflammatory pretreatment with inhaled corticosteroids does not seem to prevent either the accumulation of T cells or increased ICAM-1 expression, suggesting that its value during rhinovirus-induced worsening of asthma is limited.

9.3 Summary and General discussion

9.3.1 Host susceptibility to infection and asthma exacerbation

The susceptibility to "catch the cold" varies among and within hosts (80), which may be related to personality, stress, and recent life events (102-104), or factors associated with atopy or asthma (147,161). Experimental rhinovirus infections apparently (partly) overcome low susceptibility to infection, as we were able to obtain an infection rate as high as 89 % (46/52 subjects), and even succeeded in inducing several secondary infections in subjects with low titers against RV16 *(chapter 3)*. Yet, experimental RV16 infections did not consistently cause exacerbations of asthma, and the effects were generally mild. This might be ascribed to the mildness of the colds that were induced *(chapters 4, 8)*. Alternatively, it could be postulated that in addition to a shared pathogenetic background of both colds and rhinovirus-associated exacerbations of asthma, additional factors might increase the susceptibility to exacerbations of asthma. Such factors might include host characteristics associated with age (73,84,86,87,89), co-morbidity (85,88,101,372), smoking habits, medication usage, and the severity of asthma and allergic rhinitis (147,161), which is possibly associated (with the timing of) recent or chronic allergen exposure (162,169,179) or ICAM-1 expression (280). For obvious safety concerns, patients with characteristics that were assumed to increase the risk of a severe exacerbation were initially excluded from the studies. On the other hand, in view of the mildness of the observed clinical effects of experimental RV16 infections thus far, is seems feasible to study the contribution of various host factors. For example, the interaction of viral infection and prolonged low dose allergen exposure, using experimental RV16 infections in the mild, stable asthmatic patients. Such studies and others will hopefully provide more insight in host- and virus-related factors that influence the response to infection, and the severity of asthma exacerbations in particular. In view of the above, it is likely that exacerbations of asthma associated with natural rhinovirus infections, as opposed to experimentally induced infections in relatively stable, mild asthmatics, show larger variability in their time course and severity. This may not only be a consequence of variation in host characteristics and environmental exposure, but also of variation in rhinovirus serotype, virulence and the infectious dose (80).

9.3.2 Pathophysiological changes during rhinovirus infections in asthma

As previously stated, both empirical evidence and population-based studies point towards a role for virus infections in exacerbations of asthma (77,78,142-144). There is no doubt that exacerbations of asthma associated with (rhino)viral infections can be severe enough to require hospitalization (73-76). However, the pathophysiological changes that underlie these exacerbations were largely unclear. Therefore, we studied the effects of experimental rhinovirus 16 infection on asthma symptoms, usage of β_2-agonist rescue medication, variable airways obstruction and airways hyperresponsiveness to histamine in a placebo-controlled study *(chapters 3, 4, and chapters 5 and 8, in part)*.

Our data show an increase in asthma symptoms, without significantly increased usage of rescue medication after experimental rhinovirus 16 infections in asthmatic patients, fitting in with previous findings (163). In addition, we were able to demonstrate that experimental RV16 infection increases variable airways obstruction during the first week after infection *(chapter 3)*, which was found to be associated with asthma symptoms in every day life. Such an observation has not been made by using lab recordings of FEV_1 *(chapter 4)*(160,163), suggesting that home recordings may be more sensitive in picking up changes in airways obstruction. Alternatively, one could speculate that airways obstruction may improve spontaneously during the day, or secondary to frequently repeated deep-breath maneuvers as are being performed in the lung function laboratory. Moreover, it could be postulated that perception of dyspnea associated with airways obstruction reminds participants to record their FEV_1, thereby shifting the timing of measurements towards moments of more severe obstruction. The increase in variable airways obstruction points

towards either increased sensitivity to bronchoconstrictive stimuli and/or a reduced bronchodilating effect of a deep breath (57).

RV16 infection was shown to temporarily increase airway hyperresponsiveness to histamine *(chapters 4, 5)*. This observation fits in with the previously described increase in the maximal bronchoconstrictive response to methacholine after RV16 infection in asthmatics (163). The increased airway hyperresponsiveness in asthma suggests an interactive effect of virus-induced airways inflammation with features of the underlying disease, such as altered airway geometry (54,180), airway smooth muscle phenotype (208,244) or airways inflammation (24,181).

The degree of physiological worsening, as reflected by the degree of spontaneous airways obstruction and the change in airway hyperresponsiveness after infection, appeared to be positively associated with the severity of the cold symptoms and the inflammatory response to the cold, as reflected by an increase in IL-8 in nasal lavage, drop in numbers of circulating lymphocytes and rise in numbers of circulating neutrophils *(chapters 3, 4)*. This indicates that the severity of the cold and the subsequent inflammatory response are major determinants for the degree of worsening of asthma subsequent to infection.

Taken together, the data presented in these chapters confirm and extend empirical and epidemiological evidence that symptomatic rhinovirus colds in asthmatic patients may induce exacerbations of asthma (142-144), by describing the pathophysiological changes underlying such an exacerbation (77,78).

9.3.3 Role of neurogenic mechanisms in rhinovirus-associated exacerbations of asthma

Experimental infections in animals have provided ample evidence of the involvement of small, unmyelinated airway sensory nerves (C-fibers) in the development of virus-induced airway hyperresponsiveness (242,311). In order to investigate whether a rhinovirus cold would increase the sensitivity of airway sensory nerves, we examined the effect of rhinovirus colds on airway responsiveness to bradykinin, a sensory nerve stimulus, in patients with asthma *(chapter 5)*. We observed marked tolerance to a repeated bradykinin challenge, which was abrogated by experimental RV16 infection. This indicates that neurogenic mechanisms are somehow involved in the response to rhinovirus infections in patients with asthma. However, this marked tolerance to inhaled bradykinin over such a long period of time (10 days) has not been described before, and we are uncertain as to its mechanisms. Therefore, any neurogenic explanation for the pathogenesis of rhinovirus-associated exacerbations of asthma remains speculative. Specific receptor antagonists for sensory nerve stimuli such as bradykinin and capsaicin or for the various neuropeptides will be required in order to further investigate the role of neurogenic mechanisms in airway narrowing and airway hyperresponsiveness in humans *in vivo*, and during virus-induced exacerbations of asthma in particular.

9.3.4 Rhinovirus-induced airways inflammation

The observed pathophysiological changes are suggestive of an RV16-induced increase in airway inflammation. How can the rhinovirus-associated airway inflammation be characterized? The studies in this thesis address this question by investigating the effects of rhinovirus colds on soluble inflammatory mediators in sputum and nasal lavages *(chapters 4, 6)*, as well as the inflammatory cells in/of sputum and the bronchial mucosa *(chapters 6, 8)*. In addition, we addressed the bronchial epithelial expression of the intercellular adhesion molecule ICAM-1, which is also the major rhinovirus receptor *(chapter 7)*. By relating the observed inflammatory changes to physiological changes, we aimed to pinpoint inflammatory mechanisms that are particularly relevant for clinical worsening of disease.

9.3.4.1 Eosinophils

With respect to eosinophilic inflammation we observed an increase in the levels of eosinophil cationic protein (ECP) in the lower airway secretions at day 2 of a rhinovirus 16 infection, that was not paralleled by a significant increase in ECP levels in the nasal lavage in atopic asthmatic subjects *(chapter 6)*. Previously, a small increase in the numbers of eosinophils (EG2+ cells) has been observed in the bronchial epithelium at 4 days after RV16 inoculation in a sample of normal and asthmatic subjects (164). However, at day 6 after RV16 inoculation, we observed a trend towards a decrease in the numbers of eosinophils in the bronchial mucosa *(chapter 8)*, suggesting either migration, degranulation or degradation of eosinophils in response to infection. The initial rise in sputum ECP was associated with enhancement of airway hyperresponsiveness *(chapter 6)*, while tissue depletion of EG2-positive-staining cells at day 6 after rhinovirus infection was associated with improvement of airway hyperresponsiveness *(chapter 8)*.

The observations are not easy to interpret. They suggest that eosinophils and their products mediate the deterioration of airway hyperresponsiveness in the early phase of a rhinovirus infection (between days 0 and 2), while subsequent bronchial mucosal depletion of eosinophils may be associated with recovery of airway hyperresponsiveness after rhinovirus infection.

Our findings in asthmatics differ from previous observations in normal non-atopic subjects, in whom few eosinophils were present at baseline (24,177), and in whom rhinovirus infection did not cause changes in eosinophil numbers in the bronchial tissue (164,177,262). Therefore, our data suggest that rhinovirus colds interact with underlying pre-existing airway inflammation in asthma. The nature of such interaction is largely unclear, and deserves attention in further studies (179).

9.3.4.2 Neutrophils

Neutrophils may contribute to airway inflammation in asthma by means of the release of cytotoxic components (373). Accumulation of neutrophils is not commonly observed in asthma, but has been described in the airways of patients who suddenly died from asthma (285,374), suggesting a role for neutrophils in exacerbations of asthma, possibly in those of infectious origin (255,375,376). In our studies we observed that rhinovirus 16 infections in atopic asthmatic subjects induces the release of the chemokine IL-8 both in the upper and lower airway secretions *(chapter 4, 6)*. IL-8 may be produced by various inflammatory cells and tissues, including neutrophils and epithelium. Indeed, increased numbers of sputum neutrophils were shown to contain IL-8 after RV16 infection, suggesting that these cells contribute to the increased amount of IL-8 in airway secretions. Such IL-8 production is indicative of a role for neutrophils in the response to a rhinovirus infection *(chapter 6)*. The correlation between neutrophil accumulation in the airway epithelium and worsening of airways obstruction suggests that cellular damage, smooth muscle contraction, and/or airway wall swelling may be consequences of such a response *(chapter 8)*.

Apparently, both subtypes of granulocytes, neutrophils and eosinophils are involved in rhinovirus induced airways inflammation. This may not be surprising, as they share many functional and cytotoxic characteristics. Yet, obvious differences exist, and there is evidence to suggest that effects of both types of granulocytes might add up or even potentiate each other's effects (377). Therefore, the mutual interaction of the two cell types during rhinovirus infection in asthma deserves attention in future studies.

9.3.4.3 T cells

Experimental rhinovirus 16 infection induced a fall in the numbers of circulating lymphocytes *(chapters 4, 8)* and a concomitant rise in the numbers of T cells in the bronchial mucosa in our

patients with asthma *(chapter 8)*. The more epithelial accumulation of T cells (notably, CD8+ cells in the corticosteroid-naï ve subjects and CD4+ cells in the corticosteroid pre-treated subjects), the less worsening of airway hyperresponsiveness was observed. These findings are in keeping with the observed rise in T cell numbers (CD3+ cells) in the bronchial mucosa after RV16 infection in normal non-atopic subjects (164), and suggest that rhinovirus colds induce an immune response that is likely to be orchestrated by T cells. In asthmatic patients, this response appears to involve both CD8+ cells and CD4+ cells *(chapter 8)*.

It is presently unclear whether such an immune response in the lower airways is antigen-dependent, or whether it occurs independently, as a bystander effect (183,189). This issue deserves further study in view of the potentially protective effects of prolonged corticosteroids treatment against antigen-independent T cell-mediated inflammation (371). Whatever the mechanisms, the T cell-mediated response is likely to promote abnormal airway function, as was demonstrated by others in normals (164,261,262). Yet, the presently observed inverse relationship between T cell accumulation and worsening of airway hyperresponsiveness after RV16 infection in asthmatic patients *(chapter 8)* suggests that such an immune response might interfere with pre-existing allergic airways inflammation in asthma, potentially outweighing the pathophysiological consequences that were seen in normals (261). It would be interesting to study these phenomena in more detail, addressing the RV-16-induced T cell cytokine profiles, their antigen specificity, the role of antigen presenting cells, and possible differences herein between normals (non-atopic, non-asthmatic) and asthmatics.

9.3.4.4 ICAM-1

The adhesion molecule ICAM-1 promotes infiltration and migration of inflammatory cells and provides a costimulatory signal for T cell activation. It is also the major receptor for 90% of the rhinovirus serotypes (including RV16). *In vitro* studies show that its expression may be enhanced by various inflammatory conditions, including rhinovirus infection itself (197,209,215,216,218).

Our *in vivo* studies have demonstrated that ICAM-1 is expressed in the basal and parabasal bronchial epithelial cells of atopic asthmatic subjects, and that subsequent rhinovirus 16 colds further increase the epithelial expression of ICAM-1 *(chapter 7)*. These findings are in keeping with the observed enhanced epithelial expression of ICAM-1 in asthma (213,214), and the effect of rhinovirus infections on ICAM-1 expression in normal, non-asthmatic subjects (262,378). The enhanced ICAM-1 expression was positively correlated with a rise in epithelial mast cell numbers and with a rise in the number of CD3+ and CD4+ cells in the lamina propria *(chapter 8)*, indicating its association with worsening of airways inflammation during a rhinovirus infection. Whether upregulation of ICAM-1 expression occurs as a consequence of direct exposure to rhinovirus particles or as a consequence of secondary inflammatory changes or both, is unclear. However, there is evidence that upregulated ICAM-1 expression may promote both spreading of and repeated infection with airways inflammation, thereby functioning as an amplifying mechanism of disease. Therefore, the role of ICAM-1 in the lower airways should be taken into account when candidate therapies for the common cold, for example soluble ICAM-1 (125,137) are investigated.

9.3.5 Corticosteroids as prophylaxis against rhinovirus-induced exacerbations of asthma

As stated previously, evidence on the effectiveness of regular asthma treatment in preventing or treating virus-induced exacerbations is rather scarce. The final study of this thesis addresses the issue of corticosteroid prophylaxis of virus-induced exacerbations in asthma. We investigated the effect of placebo-controlled pretreatment with the inhaled corticosteroid budesonide on

rhinovirus-associated effects on lung function, airway hyperresponsiveness, bronchial epithelial ICAM-1 expression *(chapter 7)*, and infiltration and accumulation of inflammatory cells in the bronchial mucosa *(chapter 8)*.

As anticipated, our data confirm the anti-inflammatory potential of inhaled corticosteroids, as subjects who had received two weeks pretreatment with inhaled budesonide had lower numbers of activated eosinophils in their bronchial mucosal biopsies, whilst airway hyperresponsiveness had improved as compared with the placebo group. However, we did not observe significant effects of such anti-inflammatory treatment on rhinovirus-associated airways obstruction or airway responsiveness, although it allowed asthmatic patients to maintain their initial improvement of airway hyperresponsiveness *(chapter 8)*. The lack of effectiveness of inhaled corticosteroids against RV16-induced worsening is difficult to interpret. This is due to the fact that in this particular study RV16 infection failed to cause significant deterioration of airways obstruction and airway responsiveness. Yet, we did observe an increase in epithelial ICAM-1 expression *(chapter 7)* and a small increase in CD8+ cell numbers in the bronchial mucosa *(chapter 8)* after RV16 infection. Again, corticosteroid pretreatment did not alter the rhinovirus-associated effects on these parameters *(chapters 7, 8)*.

Recent evidence from *in vitro* studies using bronchial epithelial cell lines suggests that corticosteroids reduce rhinoviral replication, in conjunction with inhibition of enhancement of ICAM-1 expression and cytokine production (379,380), which strongly supports the rationale for testing the effectiveness of corticosteroid prophylaxis *in vivo*. The apparent discrepancy between data from cell culture models and the data in this thesis underlines the complexity of the *in vivo* situation, which precludes simple extrapolation of data obtained in *in vitro* models using epithelial cell cultures. Indeed, the *in vivo* usage of corticosteroids (either nasal or systemically) have not been shown to be effective against rhinovirus infection and its symptoms in non-asthmatic subjects (119,134). The lower airways response to a rhinovirus infection in humans may involve both a systemic antiviral cell-mediated immune response generated in response to the upper airways infection *(chapter 4)*, as well as a direct effect of rhinovirus particles in the lower airways *(reviewed in chapter 2)*. For these reasons, (topical) treatment of the lower airways with corticosteroids might not parallel the observations in *in vitro* studies.

It can be speculated that more aggressive and prolonged corticosteroid pretreatment, by minimizing airway inflammation (including reduction of T cell numbers/activation) and consequently, optimizing the airway mechanical characteristics, would have been more effective (381). However, even in non-asthmatics RV16 infections increase in the maximal bronchoconstrictive response (382), associated with the infiltration of T cells (164,261,262). It should be considered that such a response might be the side effect of an appropriate anti-viral immune response. Aggressive corticosteroid pretreatment (either inhaled or oral) might even be detrimental to such an antiviral immune response, thereby potentially worsening or prolonging the infection (119,359).

Taken together, pretreatment with inhaled corticosteroids reduces eosinophilic inflammation, possibly even during infection, but does not seem to interfere with the inflammatory response to a rhinovirus infection, as reflected by increased ICAM-1 expression or accumulation of T cells. Thus, inhaled corticosteroids may have limited value in preventing the airway inflammation associated with a rhinovirus infection *in vivo*. Therefore, in the ongoing debate on how to determine the appropriate level of corticosteroid treatment for optimal control of asthma (1,25) our conclusions favor optimization of anti-inflammatory maintenance treatment in order to allow the patient to sustain subsequent worsening during an exacerbation. It should be considered that corticosteroids might well have beneficial effects on

other aspects of virus-associated airway inflammation that were beyond the scope of the study in this thesis (383). Nevertheless, the addition of existing treatment modalities or newly developed drugs (see below) during virus-induced exacerbations deserves consideration and study, as they might have additional value for asthma control.

9.3.6 Methodological considerations

Thanks to the close collaboration with Dr. Elliot Dick and Rebecca Dick from Madison, Wisconsin, USA our lab has been able to perform experimental rhinovirus infections in humans. The investigational model of experimental rhinovirus in asthmatic patients has enabled us to make numerous observations that would have been very difficult to obtain using natural rhinovirus infections in asthmatics. However, any experimental model has its limitations in extrapolating the findings to every day life. These limitations are inherent to the advantage of simplifying the complexity of real life conditions. In addition, studies in humans come with limitations for ethical reasons that preclude too extensive, too invasive or too frequent testing in human volunteers. Some issues regarding experimental rhinovirus infections in patients with asthma are the following:

9.3.6.1 Is the inoculation method for experimental infection realistic?

Natural rhinovirus infections are probably mainly transmitted by aerosol (generated by coughing and sneezing), although transmission by infectious fresh mucous may also take place (80). Dr. David Cheung first started out in Leiden by applying the method for rhinovirus inoculation that had been shown to effectively cause symptomatic colds in the Madison experiments (162,169). This method resembled the "fresh mucous method" and involved nasal spraying and instilling droplets into the nose (80). As the first experimental infections in asthma in our lab did not result in any worsening of asthma, the method was modified. Nasal inhalation of nebulized small particles was added in order to mimic aerosol transmission and to facilitate the deposition of virus particles in the lower airways. This resulted in a significant increase in the degree of maximal airway narrowing in response to inhaled methacholine (163), and from then on this "Leiden variant" was used in patients with asthma and healthy subjects.

What makes the Leiden variant effective? One possible explanation may be that much of the nasal spray and droplets often simply end up being swallowed, which surely inactivates the virus (81). The additional aerosol inhalation takes several minutes of tidal breathing, and may therefore be more effective for mucosal deposition of the viral particles, thus, in effect, resulting in administration of a higher viral dose on the nasal mucosa. In addition, the deposition of rhinovirus particles in the lower airways might be crucial for induction of a lower airway immune response. However, this remains speculative. As inhalation of infectious aerosol is an effective way of transmission (80), it is well possible that lower airway deposition of rhinovirus particles also occurs during natural rhinovirus infections, and the "Leiden variant" therefore seems to be realistic.

Assuming that a direct effect of virus particles on lower airway tissues is indeed required to cause lower airways inflammation, and considering the unlikelihood of a florid infection of the lower airways *(chapter 2)*, it can be speculated that just the 2 experimental exposures to rhinovirus particles might be insufficient to cause lower airways inflammation that lasts for up to 2 weeks. As an alternative idea I postulate that lower airways inflammation might be boosted continuously by ongoing rhinovirus deposition in the lower airways, possibly by frequent inhalations of an infectious aerosol, generated in the nose by intermittent sniffing. However, to my knowledge no evidence is available to date to support or reject this postulate.

9.3.6.2 Safety aspects, risk of severe exacerbations

Obviously, severe and potentially dangerous virus-associated exacerbations are a great concern to patients and doctors. As stated before, experimental RV16 infection in mildly asthmatic patients generally causes a mild exacerbation of asthma, without signs of severe and/or prolonged worsening or need for additional medication. However, one of our asthmatic volunteers developed a moderately severe exacerbation of asthma that required prednisone treatment *(chapter 4)*. This patient did not differ from other participants, as she fulfilled the inclusion criteria for the study. However, the patient had severe airways hyperresponsiveness (PC_{20} to histamine: 0.12 mg/ml) and FEV_1 (% predicted) at the lower end of the normal range (70 %predicted), and had already at baseline a higher consumption of a short acting β_2-agonist relative to the other volunteers. This suggests that in this patient the asthma was less stable relative to the other participants' asthma. The observed fall in PC_{20} and FEV_1 after infection was of the same magnitude as that observed in the other participants. Even though this single observation cannot be generalized, it underlines that even small changes in airways obstruction and responsiveness can be enough to cause intolerable clinical deterioration in those who have moderate or severe asthma, and are therefore clinically relevant. An additional complicating factor may be found in the high consumption of a β_2-agonist, that might have rendered this patient less responsive to extra β_2-agonists when needed during further worsening (384). The safety of the experimental model would benefit from insight in the host factors and virus factors that contribute to the susceptibility to infection and rhinovirus-induced asthma exacerbations.

9.4 OPEN QUESTIONS, FUTURE RESEARCH

Some unanswered questions and ideas for future research have been mentioned above. Some additional questions are summarized below:

Is the response to a rhinovirus infection disease-specific?

The issue of disease-specificity of the consequences of a rhinovirus infection in asthma is essential for the interpretation of the observed effects in asthma, and needs to be addressed in further detail, with respect to both immunological mechanisms (does the antiviral immune response in asthmatics differ from normal subjects?), neurogenic and inflammatory mechanisms (what is the contribution of pre-existing airways inflammation to the inflammatory response to a rhinovirus infection?) and pathophysiological mechanisms (to what extent do pre-existing structural changes contribute to the pathophysiological effect of a rhinovirus infection?). Such insight may require adjusting the scope of research on either asthma (either with or without allergic rhinitis), other airway diseases like COPD, cystic fibrosis, broncho-pulmonary dysplasia, or healthy normals. It could also help to identify patients at risk for developing severe virus-associated exacerbations of their lung disease.

Rhinovirus infections coinciding with other respiratory virus infections: mere coincidence?

Rhinovirus infection in combination with and one or more other respiratory viruses is not an uncommon finding. Even repeated infections with the same rhinovirus serotype have been described *(chapter 4)*. It is presently unclear whether this is coincidence, or whether specific characteristics of either (rhino)viruses or hosts predispose to contracting multiple, repeated or more frequent infections. What is the impact of such multiple infections on pre-existing respiratory diseases? In this respect, our observation that secondary rhinovirus infection leads to worsening of airway hyperresponsiveness is interesting, but requires confirmation. If interaction between rhinoviruses and other respiratory infections exists, the benefit of a

(pharmacological) therapy for rhinovirus infections may stretch even further than curing half of all the common colds.

Rhinoviruses infection of the lower airways?

Only recently, direct evidence of rhinovirus infections of the lower airways has been published (277,278). This finding suggests that viral infection, or at least the presence of viral particles in the lungs accounts for the observed pro-inflammatory response to a common cold. The next question is about the involvement of other tissues beside the epithelium (antigen presenting cells, smooth muscle, others). Do these tissues become infected or do they participate in the immune response through indirect mechanisms? Finally, it would be of great interest to know which factors promote susceptibility and spreading of the infection (ICAM-1, other factors)? Such factors may potentially be accessible to therapy.

Examining bronchial tissue after rhinovirus infection: what to focus on?

So far, when examining bronchial biopsies and sputum others and we have focused mainly on inflammatory cells. However, it is clear that also resident tissues and - cells are involved in allergic airway inflammation and are very likely to be involved in rhinovirus-associated pathology (*see review chapter 2*). Therefore, as a next step, lung tissues such as the epithelium (which may be the primary target for rhinovirus infection) antigen presenting cells, (myo)fibroblasts and airway smooth muscle (in view of the involvement in airway hyperresponsiveness) deserve further study with respect to integrity/permeability (epithelium), production of inflammatory mediator and growth factors, expression of adhesion – and co-stimulatory molecules, proliferation and contractility (myofibroblast, smooth muscle), and the interaction with inflammatory cells.

What to do in case of an exacerbation of asthma associated with a rhinovirus infection?

The wish to improve the sometimes failing treatment regimens is obviously what has driven the investigations on the pathogenetic mechanisms of rhinovirus-induced exacerbations of asthma. At this point, one still has to base the choice of therapy on general guidelines, as few pharmacological studies have been conducted in asthmatics during rhinovirus-associated exacerbations. The development of compounds against the common cold is promising (138,208), and might solve the problem of rhinovirus-induced exacerbations of asthma. In the meantime, the accumulating insight in the effects of rhinoviruses on human airways provides a rationale for re-investigating the usefulness of some existing drugs against asthma, such as anticholinergics (43,208), cromoglycates (385-387) and antioxidants (80,235,388) or NK-antagonists (389,390) particularly during virus-associated exacerbations of the disease.

10 REFERENCES

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11 ABBREVIATIONS

AA_1+ cell	:	Tryptase-positive cell, mastcell
AHR	:	Airway hyperresponsiveness
ALP	:	Antileukoproteinase
BAL	:	Bronchoalveolar lavage
BALT	:	Bronchus-associated lymphoid tissue
Bk	:	Bradykinin
BSA	:	Bovine serum albumin
CD	:	Clusters of differentiation
CI	:	Confidence interval
COPD	:	Chronic obstructive pulmonary disease
cv	:	Coefficient of variation
DC	:	Doubling cell number
DD	:	Doublin dose
ECP	:	Eosinophil cationic protein
EG_2+ cell	:	ECP-positive cell, eosinophil
ELISA	:	Enzyme-linked immunosorbent assay
FEV_1	:	Forced Expiratory Volume in 1 second
FEV_1%pred.	:	FEV_1 as percentage of predicted value
GM-CSF	:	Granulocyte-macrophage colony stimulating factor
HBSS	:	Hanks' balanced salt solution
HEL	:	Human embryonic lung fibroblast
HRV	:	Human rhinovirus
ICAM-1	:	Intercellular adhesion molecule-1 (CD54)
IFN	:	Interferon
Ig	:	Immuneglobulin
IL-.. (6, 8, etc.)	:	Interleukin-.. (6, 8, etc.)
LFA-1	:	Lymphocyte functional antigen-1
MANOVA	:	Multivariate analysis of variance
MCP	:	Monocyte chemoattractant protein
MHC	:	Major histocompatiblity complex
MIP-1α	:	Macrophage inflammatory protein-1 α
MMAD	:	median mass aerosol diameter
NEP	:	Neutral endopeptidase
NKA	:	Neurokinin A
NO	:	Nitric oxide
PBS	:	Phosphate buffered saline
$PC_{20}FEV_1$	:	Provocative concentration causing a 20% fall in FEV_1
$PC_{40}V_{40p}$	:	Provocative concentration causing a 40% fall in V_{40p}
PEFR	:	Peak expiratory flow rate
r	:	Correlation coefficient
RANTES	:	regulated on activation, normal T expressed and secreted
Ri	:	Intraclass correlation coefficient
RV	:	Rhinovirus
SD	:	Standard deviation
SEM	:	Standard error of the mean
SLPI	:	Secretory leukocyte proteinase inhibitor
SP	:	Substance P

Tc	:	Cytotoxic T cell
Th	:	T-helper cell
TLC	:	Total lung capacity
TNF-α	:	Tumor necrosis factor-α
V_{40p}	:	Flow at 40% of FVC on the partial expiratory flow-volume curve

12 WEBSITES ON RHINOVIRUSES

http://www.bocklabs.wisc.edu/Rhinovirus.html
http://www.bocklabs.wisc.edu/movies/r16_ico-2.html
http://leahi.kcc.hawaii.edu/~johnb/micro/m130/readings/Rhinovirus.htm
http://mmtsb.scripps.edu/viper/1aym.html
http://www.nbif.org/bioimage/mmedia.html
http://pharminfo.com/gallery/molecules/molecule.html
http://news.uns.purdue.edu/html4ever/9804.Rossmann.receptor.html
http://bilbo.bio.purdue.edu/~baker/projects/picorna/rhino/rhino.html
http://scop.berkeley.edu/data/scop.1.009.005.001.001.001.html
http://www.iah.bbsrc.ac.uk/virus/picornaviridae/SequenceDatabase/3Ddatabase/3D.HTM
http://seqaxp.bio.caltech.edu/www/nt_manuals/local_spdbv/SHORT-MANU.HTML#Build multimer
http://www.ncbi.nlm.nih.gov/htbin-post/Taxonomy/wgetorg?id=31708
http://www.asmusa.org/division/c/virotxt.htm

13 SAMENVATTING

13.1 Inleiding

13.1.1 Wat is astma?

Astma is een aandoening van de longen die gekenmerkt wordt door wisselende klachten van benauwdheid, kortademigheid en drukkend gevoel op de borst. De klachten gaan gepaard met piepende ademhaling en vernauwing van de luchtwegen, als gevolg van overprikkelbaarheid voor diverse prikkels zoals inspanning, hyperventilatie, hypertone of hypotone stimuli, inhalatie van koude lucht of prikkelende stoffen zoals rook en zwaveldioxide, en soms ook aspirine. Deze verschijnselen zijn meetbaar als variabele luchtwegobstructie en bronchiale hyperreactiviteit. Tekenen van ontsteking van de luchtwegen zijn in alle stadia van de ziekte te vinden. Inhalatie van luchtwegverwijdende medicatie, met name β_2-adrenerge stoffen geven meestal onmiddellijke verlichting van de klachten. De ernst van de klachten, de functionele afwijkingen en onderliggende ontsteking van de luchtwegen varieert gedurende de dag (ernstiger in de nacht en vroege ochtend) en kan toenemen door blootstelling aan allergenen, bepaalde chemicaliën, ozon (o.a. in zomersmog) en virale luchtweg infecties. Inhalatie van ontstekingsremmende corticosteroïden is meestal een effectieve manier om het optreden van klachten te voorkómen.

13.1.2 Vóórkomen van astma

Momenteel lijdt 10-20% van de westerse bevolking aan astma. In de laatste tientallen jaren wordt er een duidelijke toename in het vóórkomen van allergische aandoeningen en astma waargenomen. Zowel genetische factoren als omgevingsfactoren dragen bij aan het ontstaan van de aandoening. Het toenemend optreden van astma lijkt echter vooral samen te hangen met factoren die geassocieerd zijn met westerse levenstijl en toegenomen welvaart, mogelijk geassocieerd met voedingsgewoontes en hygienische omstandigheden op de kinderleeftijd. Enkele risicofactoren voor het krijgen van de aandoening zijn: het hebben van een ouder met astma, allergie, blootstelling aan allergenen, aantal broers en zussen en rangnummer in de kinderrij (afnemen risico voor hoger rangnummer), en passief roken (vooral bij kinderen van ouders met astma).

13.1.3 Astma-aanvallen en luchtweginfecties

De toename in ziekenhuisopnames en overlijden door astma houdt gelijke tred of overtreft zelfs de toename in het vóórkomen van astma. Astma behandeling volgens de geldende richtlijnen, o.a. met inhalatiesteroïden geeft bij de meeste astma patiënten een duidelijke vermindering van de klachten, verbetering van de functionele afwijkingen en van de onderliggende ontsteking van de luchtwegen. Echter, ernstige, en potentieel fatale exacerbaties (episodes van verslechtering) komen nog steeds voor, ook bij patiënten met een lichte vorm van astma, en ook ondanks veelal agressieve behandeling van het astma. Het is mogelijk dat dergelijke ongecontroleerde aanvallen verlopen via andere ziektemechanismen, bijvoorbeeld samenhangend met virale respiratoire infecties. Het is gebleken dat er vaak virale luchtweg infecties in het spel zijn als opname wegens astma nodig is. Er zijn aanwijzingen dat behandeling met inhalatie steroïden geen bescherming biedt tegen het optreden van verkoudheden en evenmin tegen de verslechtering van het astma als gevolg van dergelijke virale infecties. Populatiestudies laten

zien dat ongeveer 44% (volwassenen) tot 85% (kinderen) van de exacerbaties van astma geassocieerd is met een virale luchtweg infectie door diverse veel voorkomende verwekkers zoals influenzavirus, para-influenzavirus, respiratory syncytial virus (RSV), coronavirus of rhinovirus. Rhinovirus infecties maken ongeveer de helft uit van alle gedetecteerde virussen bij astma, en leveren dus een belangrijke bijdrage aan de last die het hebben van astma met zich meebrengt.

13.1.4 De rol van rhinovirussen

Rhinovirussen zijn de meest voorkomende verwekkers van verkoudheden. Binnen deze virusfamilie zijn er ongeveer 100 verschillende serotypes bekend. Daarvan circuleren er meestal enkele, wisselende serotypes binnen een populatie, zodat mensen gemiddeld 0,25 tot 2 maal per jaar een rhinovirus infectie doormaken, waarbij de frequentie afneemt met de leeftijd. Een specifiek en effectief geneesmiddel of vaccin is er niet. Een rhinovirus infectie leidt bij anderszins gezonden meestal "slechts" tot een bovenste luchtweg infectie ofwel verkoudheid die binnen enkele dagen geneest. Daarentegen kan een dergelijke verkoudheid bij mensen met astma ernstiger gevolgen hebben, en met name een astma-exacerbatie teweeg brengen. De ziektemechanismen die leiden tot een dergelijke verslechtering zijn nog grotendeels onduidelijk. Beter inzicht in deze ziektemechanismen zou kunnen leiden tot (ontwikkeling van) meer gerichte en specifieke behandeling voor dergelijke exacerbaties.

13.1.5 Experimentele rhinovirus infecties

Experimentele rhinovirus infecties, dat wil zeggen opzettelijke besmetting van vrijwilligers in het kader van wetenschappelijk onderzoek maakt het mogelijk om de effecten van een rhinovirus infectie intensief te bestuderen bij zorgvuldig geselecteerde patiënten, onder gecontroleerde (laboratorium-)omstandigheden, en op welomschreven tijdstippen te bestuderen. Ook is het mogelijk gebleken om de effecten van dubbelblinde placebo-gecontroleerde interventie met medicijnen tegen verkoudheid of astma exacerbaties in een dergelijke opzet te testen. De toepassing van experimentele rhinovirus infecties blijkt veilig te zijn, ook bij allergische patiënten en astma patiënten. Dankzij de samenwerking met Dr. Elliot Dick en Rebecca Dick uit Madison, Wisconsin, in de Verenigde Staten, konden wij beschikken over een rhinovirus dat geschikt en veilig was voor gebruik bij mensen. Het rhinovirus type dat in onze studies gebruikt werd, wordt aangeduid als RV16.

13.2 DIT PROEFSCHRIFT

Zoals eerder beschreven, zijn er duidelijke aanwijzingen dat acute verslechtering van astma (exacerbaties), ook de ernstige exacerbaties, vaak samengaan met virale luchtweginfecties, en met name rhinovirus infecties. Echter, er was (en is) nog veel onduidelijkheid omtrent de ziektemechanismen die leiden tot zulke tijdelijke verslechtering bij astma. De studies die we verricht hebben hadden als algemeen doel het inzicht in de effecten van rhinovirus infecties bij astma te vergroten.

Het proefschrift beschrijft de effecten van experimentele rhinovirus infecties bij mensen met astma, op het gebied van astmasymptomen en medicatiegebruik, verandering van longfunctie, bronchiale hyperreactiviteit voor diverse stimuli, en facetten van de immuunrespons en ontstekingsreactie op de infectie, gemeten in neusspoelsel, bloed, opgehoest slijm uit de longen, en biopten van het longslijmvlies. Verder hebben we onderzocht wat het effect is van voorbehandeling met inhalatiesteroïden op zulke rhinovirus-geïnduceerde veranderingen.

In hoofdstuk 1 wordt een algemene inleiding gegeven en worden de doelstellingen per deelstudie geformuleerd. Hoofdstuk 2 bevat een overzicht van de literatuur over rhinovirus infecties in relatie tot allergie en astma. De hoofdstukken 3 tot en met 8 bevatten artikelen met daarin de resultaten van de studies, waarvan hieronder een korte beschrijving. In hoofdstuk 9 worden de beschreven studies samengevat. Tevens wordt besproken hoe onze bevindingen passen in het denken over het ontstaan van virus-geassocieerde exacerbaties van astma, en hoe die bevindingen kunnen bijdragen aan het inzicht in de ontstaanswijze van zulke exacerbaties. Verder worden er suggesties gedaan voor mogelijk verder onderzoek op dit gebied.

13.2.1 Luchtwegobstructie

Variabele luchtwegobstructie is kenmerkend voor astma. Blootstelling aan bepaalde prikkels, waaronder allergenen, leidt vaak tot toename van luchtwegobstructie en benauwdheid. Luchtwegobstructie is het gevolg van diverse factoren die samenhangen met ontsteking van de luchtwegen, waaronder structurele veranderingen van de luchtwegen, slijmvlieszwelling, slijmophoping, en het samentrekken van spiertjes in de luchtwegwand. Bij gezonden wordt luchtwegobstructie voorkomen door geregeld diep in te ademen, waardoor de longen als het ware weer even opgerekt worden. Bij astmapatiënten lijkt een diepe inademing minder effectief te zijn, het is moeilijker om luchtwegobstructie te voorkómen of om te keren. Toename van luchtwegobstructie is te meten als een afname van de FEV_1, ofwel de hoeveelheid lucht die iemand in 1 seconde maximaal kan uitblazen na een maximale inademing. *Hoofdstuk 3* beschrijft het effect van een RV16 infectie op FEV_1, die met behulp van een draagbare spirometer 3 maal daags gemeten werd door de patiënten zelf. Het blijkt dat RV16 infecties een toename van variabele luchtwegobstructie teweegbrengt, die samen gaat met verergering van de astmasymptomen. Hoe erger de verkoudheid, hoe meer toename van luchtwegobstructie. Deze bevinding wijst enerzijds op een mogelijk toegenomen bronchiale hyperreactiviteit (prikkelbaarheid van de luchtwegen). Anderzijds is het mogelijk dat het luchtwegverwijdend effect van een diepe inademing nog verder is verminderd.

13.2.2 Bronchiale hyperreactiviteit

Luchtwegobstructie (bronchoconstrictie) ontstaat doordat de luchtwegen zich vernauwen als reactie op blootstelling aan bepaalde prikkels, zoals inademen van koude of droge lucht (vergelijkbaar met het effect van inspanning), allergenen, prikkelende stoffen (ozon, zwaveldioxide, diverse farmacologische stoffen). De luchtwegen van mensen met astma zijn overprikkelbaar, ofwel hyperreactief. Inhalatie van bepaalde prikkelende stoffen leidt al bij lage concentraties tot vernauwing van de luchtwegen. Die vernauwing kan bovendien sterker zijn dan bij gezonden. De prikkeldrempel kan bepaald worden in het longfunctielaboratorium door het inhaleren van oplopende concentraties van bijvoorbeeld histamine (een lichaamseigen stof), en wordt vaak uitgedrukt als PC_{20}. De PC_{20} is de provocerende concentratie die leidt tot 20% daling van de FEV_1. In *hoofdstuk 4* wordt beschreven dat RV16 infectie leidt tot een toename van de bronchiale hyperreactiviteit, ofwel een daling van de PC_{20}. Deze daling van de PC_{20} is het meest uitgesproken bij de patiënten met ernstige verkoudheidsymptomen. Deze toegenomen bronchiale hyperreactiviteit vormt een verklaring voor de variabele luchtwegobstructie die na infectie gezien wordt. Verder wijst deze bevinding in de richting van de betrokkenheid van bijvoorbeeld fijne zenuwvezels, en/of toegenomen ontsteking van de luchtwegen.

13.2.3 Zenuwvezels en bronchiale hyperreactiviteit

Het onderzoek beschreven in *hoofdstuk 5* is gebaseerd op het idee dat de fijne gevoelszenuwen (C-vezels) in de long betrokken zijn bij de toename van bronchiale hyperreactiviteit als gevolg van virale luchtweginfecties, zoals dit ook bij diermodellen is aangetoond. Het is mogelijk dat de stoffen die door de C-vezels worden uitgescheiden (neurokininen) een versterkt samentrekkend effect hebben op het spierweefsel van de luchtwegen doordat de prikkeldrempel voor uitstoot van deze stoffen lager wordt, of omdat er meer uitstoot c.q. minder afbraak van neurokininen is. Bradykinine is een stof die via prikkeling van de C-vezels tot luchtwegvernauwing leidt, althans bij mensen met astma. Bradykinine wordt evenals de neurokininen afgebroken door het enzym NEP. We hebben onderzocht of de bronchiale hyperreactiviteit voor bradykinine verandert onder invloed van een RV16 infectie. Dat blijkt wel het geval, maar anders dan we verwacht hadden. Herhaalde provocaties met bradykinine bleken te leiden tot verminderde gevoeligheid voor die stof (tolerantie). Na RV16 infectie trad echter nauwelijks tolerantie op. Er was dus geen absolute maar wel een relatieve toename van de bronchiale hyperreactiviteit na RV16 infectie. Hoewel moeilijk te verklaren is dit toch een aanwijzing dat C-vezels een rol spelen bij de toegenomen bronchiale hyperreactiviteit na een RV16 infectie. Het Lijkt er echter op dat er ook andere mechanismen in het spel zijn.

13.2.4 Tekenen van ontsteking: sputumonderzoek

Onderzoek van sputum (slijm uit de longen) wordt al van oudsher toegepast in de geneeskunde. De hoeveelheid, kleur, geur, consistentie van sputum, en de aanwezigheid van bepaalde ontstekingscellen of microorganismen in sputum kunnen soms veelzeggend zijn over de ziekte waaraan een patiënt lijdt. De groene kleur van het sputum van sommige astmapatiënten wordt bijvoorbeeld verklaard door het grote aantal "witte" bloedcellen in het sputum. De toepassing van moderne immunologische technieken heeft aangetoond dat het sputum van astmapatiënten tevens verhoogde concentraties van onstekingsmediatoren (o.a. signaalstoffen) bevat. De samenstelling het sputum met betrekking tot deze mediatoren kan een indicatie geven over de aard van de ontsteking. De eerder beschreven veranderingen van de functie en prikkelbaarheid van de longen na RV16 infectie wezen al in de richting van een toename van ontsteking. In *hoofdstuk 6* wordt beschreven dat een RV16 infectie inderdaad gepaard gaat met een toename van concentraties van ontstekingsmediatoren in het sputum, hoewel er geen duidelijke verschuivingen waren in de percentages van de verschillende typen witte bloedcellen. Deze stoffen zijn mogelijk deels afkomstig van eosinofiele granulocyten (zoals ECP), deels van andere witte bloedcellen waaronder de neutrofiele granulocyten (IL-8), en deels uit de slijmvliesbekleding van de long (IL-8). Het is opvallend dat de sterkste verhoging van ECP gevonden werd bij de patiënten met de grootste toename van bronchiale hyperreactiviteit.

13.2.5 Tekenen van ontsteking: onderzoek van het longslijmvlies

Gezien de toegenomen luchtwegobstructie en hyperreactiviteit die samengaat met tekenen van verergerde ontsteking in de luchtwegen, lijkt het aannemelijk dat de afweer en ontsteking zich ook in het longweefsel zelf manifesteren. Witte bloedcellen bereiken de luchtwegen door uit de bloedvaten te treden en door het weefsel te migreren. Een afweerreactie van witte bloedcellen in het weefsel zou diverse veranderingen tot gevolg kunnen hebben die de bronchiale hyperreactiviteit doen toenemen. Ook het longweefsel zelf zou een actieve rol kunnen spelen in de ontsteking, onder andere door de migratie van witte bloedcellen te bevorderen. Een volgende stap was daarom het onderzoeken van het longweefsel zelf *(hoofdstukken 7 en 8)*. Dit weefsel werd verkregen door tijdens een bronchoscopie kleine hapjes van het longslijmvlies te nemen.

Deze weefselstukjes werden zodanig bewerkt dat microscopisch onderzoek mogelijk was. Daarna werden de microscoopbeelden gedigitaliseerd. Met behulp van de computer hebben we vervolgens de verschillende types witte bloedcellen geteld. Dit werd gedaan in welomschreven gebieden, namelijk de cellaag die de luchtwegen bekleed (epitheel) en de laag die daar direct onder ligt (lamina propria). Het bleek dat er na infectie een toegenomen aantal was van het type witte bloedcel dat lymfocyt genoemd wordt. Lymfocyten zijn cellen die onder meer gespecialiseerd zijn in het regiseren van de afweer tegen virale infecties, maar ook de allergische ontsteking zoals die bij astma gezien wordt. Hoe groter de toename van lymfocytenaantallen in het epitheel, hoe geringer de toename van bronchiale hyperreactiviteit. Dit zou er op kunnen wijzen dat de functie van lymfocyten mede bepalend is voor het al of niet verslechteren van astma tijdens infectie.

Nadere bestudering van het epitheel toonde een toename van een molecuul aan dat de migratie van witte bloedcellen bevordert, namelijk ICAM-1 *(hoofdstuk 7)*. Het is niet toevallig dat we dit molecuul onderzochten, aangezien het de specifieke receptor (hechtingstructuur) is voor de meeste rhinovirussen, inclusief RV16. Hechting aan ICAM-1 katalyseert de infectie van de cel. Er zijn aanwijzingen dat een infectie de expressie van ICAM-1 doet toenemen, maar het is ook mogelijk dat ICAM-1 expressie toeneemt als gevolg van de afweerreactie. Hoe dan ook, de toegenomen expressie zou in potentie de verspreiding van de infectie kunnen bevorderen. Of infectie van de longen daadwerkelijk op uitgebreide schaal plaatsvindt, moet nog worden vastgesteld.

13.2.6 Behandeling van virusgeassocieerde exacerbaties van astma

Wat te doen tegen een astma-aanval door een rhinovirus infectie? Het tekort schieten van de therapeutische mogelijkheden waren (en zijn) de drijfveren voor onderzoek naar de ziektemechanismen van virusgeassocieerde exacerbaties van astma. Op dit moment baseert men de keuze van therapie op de geldende algemene richtlijnen voor behandeling van astma in het algemeen en exacerbaties in het bijzonder. Corticosteroï den, hormoonachtige ontstekingremmende stoffen die per inhalatie (inhalatiesteroï den) of soms per tablet genomen worden, vormen de basis van astmatherapie. In de laatste studie van dit proefschrift hebben we onderzocht of onderhoudsbehandeling met inhalatiesteroï den al of niet een gunstig effect heeft op het astma gedurende een rhinovirus infectie *(hoofdstuk 8)*. We vonden dat onderhoudsbehandeling op zich een resulteerde in een verbetering van de bronchiale hyperreactiviteit en verlaging van de aantallen eosinofiele granulocyten in longweefsel. Dit zijn bekende effecten van inhalatiesteroï den. Nieuw was dat deze verbetering behouden bleef gedurende en na de infectie. Echter, er waren geen significante verschillen met de patiënten die geen voorbehandeling met inhalatiesteroï den gehad hadden, óók niet met betrekking tot virus-geï nduceerde de toename in ICAM-1 expressie en de aantallen lymfocyten in longweefsel. Hoewel een duidelijk gunstige (of ongunstige) werking van inhalatiesteroï den op de rhinovirus-geassocieerde effecten in deze studie niet kon worden aangetoond, zouden astmapatiënten wel kunnen profiteren van een betere uitgangspositie door gebruik van deze medicatie.

13.3 CONCLUSIES IN DIT PROEFSCHRIFT

1. Verkoudheden door experimentele rhinovirus 16 infectie leiden tot een toename van astma symptomen en variabele luchtweg obstructie in de eerste week na infectie *(hoofdstuk 3)*.

2. Experimentele rhinovirus 16 infecties leiden tot een toename van bronchiale hyperreactiviteit voor histamine in de acute fase van de verkoudheid *(hoofdstuk 4,5)*.
3. De mate van verslechtering van de variabele luchtweg obstructie en bronchiale hyperreactiviteit is positief gerelateerd aan de verkoudheidsymptomen en de afweerreactie, weerspiegeld door toename van interleukine-8 in het neusspoelsel, en de daling van het aantal lymfocyten en stijging van het aantal neutrofiele granulocyten in het bloed *(hoofdstuk 3 en 4)*.
4. Het is aannemelijk dat ongemyeliniseerde sensibele zenuwen (C vezels) betrokken zijn bij de respons of rhinovirus infecties bij mensen met astma, gezien de verdwijning van tolerantie voor herhaalde bradykinine provocaties *(hoofdstuk 5)*. Echter, hun rol bij de ontwikkeling van rhinovirusgeassocieerde exacerbaties van astma is vooralsnog onduidelijk.
5. Rhinovirus 16 infecties induceren de vrijmaking van pro-inflammatoire en chemotactische mediatoren *(IL-8, IL-6, ECP)*, zowel in het secreet van de neus als in de lagere luchtwegen *(hoofdstuk 4 en 6)*.
6. De vroege toename van de hoeveelheid ECP in sputum is geassocieerd met een toename van de bronchiale hyperreactiviteit *(hoofdstuk 6)*, terwijl de latere depletie van eosinofiele granulocyten in de bronchiale mucosa geassocieerd is met een verminderde toename van bronchiale hyperreactiviteit *(hoofdstuk 8)*.
7. Na rhinovirus 16 infectie bij astmapatiënten bevatten de neutrofiele granulocyten in sputum een toegenomen hoeveelheid IL-8, hetgeen laat zien dat neutrofielen betrokken zijn bij de ontstekingsreactie op een rhinovirus infectie *(hoofdstuk 6)*. Verder bleek de accumulatie van neutrofielen in het bronchiale epitheel te correleren met de verslechtering van luchtwegobstructie *(hoofdstuk 8)*.
8. Verkoudheden door een rhinovirus 16 infectie gaan gepaard met een toename van ICAM-1 expressie in bronchiaal epitheel, hetgeen positief gecorreleerd is met een toename van het aantal mestcellen in het epitheel, en een toename in de aantallen CD3+ en CD4+ cellen in de bronchiale lamina propria *(hoofdstuk 7 en 8)*.
9. Rhinovirus 16 infectie induceert een toename in het aantal CD3+ cellen in de bronchiale mucosa, zowel bij steroid-naïve patiënten, als bij astmapatiënten die voorbehandeld zijn met inhalatiesteroïden. Accumulatie van T cellen in het epitheel, met name CD8+ cellen bij voorbehandelde patiënten, en CD4+ cellen bij steroid-naïve patiënten, is gecorreleerd met een verminderde toename van bronchiale hyperreactiviteit *(hoofdstuk 8)*.
10. Voorbehandeling met inhalatiesteroïden gedurende 2 weken resulteert in reductie van het aantal geactiveerde eosinofiele granulocyten in de bronchiale mucosa, en geringere verbetering van de bronchiale hyperreactiviteit. Deze anti-inflammatoire therapie blijkt evenwel geen effect op de rhinovirus 16-geinduceerde toename van ICAM-1 expressie *(hoofdstuk 7)* of T cel accumulatie *(hoofdstuk 8)* te hebben.

13.4 Tot slot

13.4.1 Perspectief

Het doel van het onderzoeken in dit proefschrift was het verkrijgen van inzicht in de klinische en pathofysiologische effecten van een rhinovirus infectie, en de onderliggende mechanismen van virus-geassocieerde verslechtering van astma. Wat is er bereikt? Ten eerste is het duidelijk geworden dat experimentele rhinovirus infecties een milde exacerbatie van astma kunnen

induceren, die in tijdsverloop, maar niet in de aard van de fysiologische effecten verschilt van verslechtering zoals we die bijvoorbeeld kennen na blootstelling aan allergenen, en meer lijkt op exacerbaties zoals die in de dagelijkse praktijk gezien worden. De aandacht is gevestigd op frequente metingen van luchtwegobstructie door de patiënt zelf als "vinger aan de pols" voor het tijdig detecteren en behandelen van verslechtering van het astma na een virale luchtweginfectie.

Bij het ontstaan van rhinovirus-geinduceerde exacerbaties spelen de antivirale immuunrespons en een ontstekingsreactie in het slijmvlies van zowel de neus als de longen een rol. Naast zenuwvezels zijn er diverse types ontstekingscellen betrokken bij deze processen, en deze reageren met een toegenomen productie van ontstekingsmediatoren in de luchtwegen. Voorbehandeling met ontstekingremmende inhalatiesteroiden had geen duidelijk effect op de gemeten verandering gedurende de rhinovirusinfectie. Deze gegevens bieden aanknopingspunten om het nut van zowel bekende als nieuw te ontwikkelen geneesmiddelen juist in deze specifieke situatie te onderzoeken. Zodoende kan de behandeling van aanvallen van astma wellicht verbeteren door deze (anders dan nu het geval is) aan te passen aan de oorzaak van de verslechtering. Met name de toegenomen expressie van ICAM-1 in de lagere luchtwegen is interessant in het licht van de ontwikkeling van de soluble ICAM-1, bedoeld als antiviraal middel. Juist bij astma zou toepassing van dit middel in de long wellicht van nut zijn.

13.4.2 Verder onderzoek

Het is nu duidelijk dat een rhinovirus infectie bij astmapatiënten tot verslechtering van hun aandoening kan leiden, waarom gebeurt dit juist bij mensen met astma? Mogelijk zijn er verschillen in de antivirale afweerreactie, of in de (funktie van) celtypes en weefsels die bij de ontstekingsreactie betrokken zijn. Vormen de structurele afwijkingen aan de longen een reden waarom een virale luchtweginfectie harder toeslaat bij mensen met astma? Het ontrafelen van deze aspecten zal ons in staat stellen om mensen met een verhoogd risico voor het krijgen van een ernstige exacerbatie te identificeren. Verder zou de kennis die hierover opgedaan wordt bij astma mogelijk ook betrekking kunnen hebben op andere longziekten zoals COPD, cystic fibrosis, en bronchopulmonale dysplasie. Experimentele rhinovirusinfecties bij mensen met astma en gezonden zouden een nuttige methode zijn om in de bovenstaande zaken meer duidelijkheid te brengen.

14 ACKNOWLEDGEMENT

The knowledge, skills, enthusiasm and devotion of many highly appreciated colleagues at the Leiden University Medical Center has been indispensable for the result that lies in front of you. I would like to express my gratitude to all the people I have had the privilege to work with, and thank especially:

- The volunteers who participated in our studies, for their selflessness, enthusiastic cooperation, and patience.
- Elliot and Rebecca Dick for their generosity to allow us to use their RV16 stock, and for their friendship.
- Hermelijn Smits, for her skillful and diligent collaboration, and ongoing support.
- Erik de Klerk and the technicians from the department of Virology, for performing rhinovirus cultures and titrations, even in busy times.
- Mieke Timmers, for countless flow-volume curves and for caring for our volunteers.
- Raffi Sharon, Medical Student, for his enthusiastic participation in chapters 7 and 8.
- Annemarie van Schadewijk, for skillfully processing the priceless bronchial biopsies
- The pulmonary physicians, especially Hans in 't Veen and Joey Brahim, for performing the bronchoscopies and for caring for our volunteers.
- The department of Clinical Hematology, for performing the automated leukocyte counts.
- The department of Clinical Pharmacology and Toxicology, for their participation in the bradykinin study and the budesonide study.
- My roommates Jaap, David, Suzanne, Henk, Heidi, Diana, Christa, and José for their support through the years, both mentally and at times physically.

15 CURRICULUM VITAE

Katrien Grünberg was born on Feb 7th 1966 in Waddinxveen, The Netherlands. After graduating from secondary school (Cals College, Nieuwegein) in 1984 she took courses in Physics and Spanish. She entered Erasmus University Medical School in Rotterdam in 1985, and graduated in November 1992 (Cum Laude). After working for one year as a resident in Pediatrics in the St. Anthonius Hospital in Nieuwegein, she started the research for this thesis with Prof. dr. P.J. Sterk at the department of Pulmonology in the Leiden University Medical Center (current head: Prof. dr. K.F. Rabe). After a break in 1999, during which the family stayed in the United States, she finalized this thesis. In June 2000 she started working as a resident in the department of Clinical Genetics in Rotterdam (head: Prof. dr. H. Galjaard). In September 2000 she commenced her clinical training at the department of Pathology of the University Hospital of the Vrije Universiteit in Amsterdam (head: Prof. dr. C.J.L.M. Meijer).

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Stellingen behorend bij het proefschrift

RHINOVIRUSES IN ASTHMA
AN EXPERIMENTAL APPROACH

1. Experimentele rhinovirus 16 infecties bij astmapatiënten induceren een milde exacerbatie die pathofysiologisch gekenmerkt wordt door toegenomen variabele luchtwegobstructie en toegenomen bronchiale hyperreactiviteit. (dit proefschrift)

2. De relatieve toename van de bronchiale hyperreactiviteit voor bradykinine duidt op de betrokkenheid van ongemyeliniseerde sensibele zenuwen in de luchtwegen bij rhinovirus 16-geïnduceerde exacerbaties van astma. (dit proefschrift)

3. Experimentele rhinovirus 16 infecties bij astmapatienten leiden tot het vrijmaken van ontstekingsmediatoren, zowel in de neus als in de lagere luchtwegen. (dit proefschrift)

4. Rhinovirus 16 infectie bij astmapatienten gaat gepaard met accumulatie van T-cellen en versterkte expressie van ICAM-1, de specifieke rhinovirus receptor, in de bronchiale mucosa van astmapatiënten. Dit gebeurt ongeacht een 2-weekse voorbehandeling met inhalatiesteroïden. (dit proefschrift)

5. De ernst van de verkoudheid zoals die door de patiënt ervaren wordt is een goede afspiegeling van objectief meetbare ernst van de ontsteking en de pathofysiologische consequenties in de luchtwegen. (dit proefschrift)

6. Vooral bij ernstig acuut astma kan het nagenoeg afwezig zijn van systemische effecten van moderne inhalatiesteroïden een nadeel zijn. (S. Schuh *et al.*, N Engl J Med 2000;343:689-94)

7. De volkswijsheid dat men verkouden wordt door het dragen van natte sokken kan door gebrek aan wetenschappelijk verkregen observaties onderbouwd noch verworpen worden.

8. The recognition that most atopic disorders have their origin in childhood, are increasing in incidence in developed and developing countries, and have strong links to the environment suggests that solutions to stop the epidemic are more likely to come from public health than pharmacological interventions. (S.T. Holgate, Nature 1999;402:B2-B4)

9. Hoewel de uitdrukking: "Wer die Wahl hat, hat die Qual" in het licht van de toegenomen technische mogelijkheden voor (presymptomatische) DNA diagnostiek voor ernstige erfelijke aandoeningen ook omgedraaid zou kunnen worden tot: "Wer (möglicherweise) die Qual hat, hat die Wahl", is het goed te bedenken dat de eerste versie onverminderd van toepassing blijft.

10. De glimlach van een kind doet je beseffen dat je leeft. (J. Kluger, J. Brousolle, G. Thibault, L. Post)

Katrien Grünberg
Sassenheim, 31 oktober 2000