Rhinology, 44, 140-144, 2006

Nasal RANTES and Eotaxin production pattern in response to rhinovirus infection*

Jens E. Meyer[1], Stefanie Stangenberg[2], Jan B. Weise[3], Christian Beck[2], Christian Schmidt[4], Kristina Kurz[1], Ulf H. Beier[5], Steffen Maune[3]

[1] Department of Otorhinolaryngology, Head and Neck Surgery, University of Schleswig-Holstein, Campus Lübeck, Lübeck, Germany
[2] Department of Internal Medicine II, University of Schleswig-Holstein, Campus Kiel, Kiel, Germany
[3] Department of Otorhinolaryngology, Head and Neck Surgery, University of Schleswig-Holstein, Campus Kiel, Kiel, Germany
[4] Department of General and Thoracic Surgery, University of Schleswig-Holstein, Campus Kiel, Kiel, Germany
[5] Department of Pediatrics, University of Illinois at Chicago, Chicago, IL, USA

SUMMARY

Tissue eosinophilia is a hallmark of nasal polyposis and its pathogenesis is an area of high interest. RANTES and eotaxin are both known to recruit eosinophils, however, the mechanisms triggering their induction are still uncertain, and viral infections have been suggested to be involved in this process. Therefore, we investigated whether rhinovirus infection is a stimulus for RANTES and eotaxin expression and production. Fibroblasts were cultured from healthy nasal mucosa obtained during endonasal surgery. Cultured cells were infected with human rhinovirus-16 for one to 72 hours. Following total RNA isolation and reverse transcription, RANTES- and eotaxin-mRNA levels were analyzed. In addition, RANTES and eotaxin secretion was measured in culture supernatants by means of an ELISA. Rhinovirus infection induces RANTES-mRNA expression as early as one hour after infection, persisting for up to 72 hours. Eotaxin-mRNA profiles did not alter significantly from control. Protein production was confirmatory for both chemokines, indicating distinct translational latency. Our data suggest that RANTES functions as a host defence mechanism responding to rhinovirus infection, thus supporting a linkage between rhinovirus infections and the pathogenesis of nasal polyposis.

Key words: eosinophilia, eotaxin, nasal polyposis, RANTES, rhinovirus

INTRODUCTION

In 1995, Tos developed a model describing different stages of nasal polyposis development: Initially, inflammation induces a rupture of the epithelium, which is followed by a granulation tissue prolapse due to rising oedematous, submucosal pressure. Subsequently, vascularization and epithelialization complete nasal polyp formation [1]. The mechanisms leading to initial protrusion are still under investigation, and several factors have been discussed to be responsible for initiating this process, including inflammatory changes associated with allergy [2] and aspirin-intolerance [3], fungal infections [4], bacteria [5] and respiratory viruses [6]. Human rhinoviruses (HRV) account for more than 50% of these viral upper respiratory infections in adults [7]. Tissue damage caused by HRV infections is mediated primarily by host immune responses, including the recruitment of eosinophils [8]. Tissue eosinophilia is considered a histological hallmark of nasal polyposis and has been shown to correlate with disease severity as well as recurrence rate after sinus surgery [9]. Eosinophils cause injury to respiratory tissue by releasing cytotoxic substances like major basic protein, eosinophil cationic protein (ECP), eosinophil peroxidase and autocrine production of cytokines and chemokines, which results in a self-sustained inflammatory process [10,11]. The selective recruitment of eosinophils from the microvasculature to the site of inflammation is mediated by CC-chemokines, most prominently RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) and eotaxin [11-13]. Eotaxin is more selective in attracting eosinophils than RANTES due to its exclusive use of CC-chemokine receptor-3 (CCR-3) and the predominant expression of CCR-3 on eosinophils [14]. Although chemokines can be secreted by numerous cell types, production by residential cells is particularly relevant to the eosinophil accumulation in nasal polyps, since fibroblasts are the major cellular source of RANTES and eotaxin production in nasal mucosa [15]. In order to further define a possible role of rhinovirus in the pathogenesis of nasal polyposis, we inoculated primary nasal fibroblast cell cultures with HRV-16 and measured gene expression and protein production of RANTES and eotaxin in a time and dose dependent manner.

*Received for publication: June 15, 2005; accepted: December 7, 2005

MATERIAL AND METHODS

Patients

Primary cell cultures used for the stimulation experiments were derived from healthy mucosa samples collected from two female patients (age 72 and 37 at the time of surgery), who have been treated with partial conchotomy for hyperplasia of the inferior turbinates. Aside from nickel hypersensitivity in the 37-year-old woman, there were no allergies as well as no airway hyperreactivities reported in both patients. After patient written consent, specimens have been retrieved without any additional resection (ethical commission approval AZ 22/93). Immediately after resection, the tissue samples were processed for primary cell culture.

Primary cell culture of human nasal fibroblasts

Nasal tissue was cut into small fragments and cultured in fibroblast medium containing DMEM, 10% fetal calf serum (FCS) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (Biochrom, Berlin, Germany) and 2 mM glutamine at 37°C in 5% CO_2 humidified atmosphere. After cells were spread, tissue fragments were removed. The first passage was performed upon a confluent cell layer. The cells were washed in PBS and covered in 0.05% trypsin, 0.02% EDTA for 5 min. Further treatment with trypsin was inhibited by adding FCS-containing DMEM. The cell suspension was centrifuged for 10 min at 1000 rpm, and the cell pellet was resuspended with fibroblast medium and seeded in 75 cm^2 flasks.

Viral stocks

HRV-16 was obtained from the American Type Culture Collection (ATCC, Rockeville, MD). Viral stocks were multiplied by infection of HeLa cells. Cultures were grown in MEM EBS, 2% FCS, and 2 mM glutamine for several days at 37°C, 5% CO_2 under continuous movement until cytopathic effects were obvious. The cultures were then frozen and thawed twice to disrupt cells. The virus-containing fluid was frozen in aliquots at –70°C.

Titration of HRV-16

Rhinovirus was titrated by exposing confluent cell-monolayers of fibroblasts in 96-well plated to serial logarithmic dilutions of virus-containing medium. The plates were incubated at 37°C for 5 days. After medium removal, the cells were washed with 100 μl of PBS and fixed by adding 50 μl of methanol per well for one minute. The methanol was replaced with 100 μl of 0.1% crystal violet for 20 min. Plates were rinsed with PBS and absorbance was measured at 550 nm using a plate reader (Dynatech Laboratories, Chantilly, VA, USA). The amount of specimen required to infect 50% of cells ($TCID_{50}$) was determined using the Reed and Muench formula [16].

Infection of human nasal fibroblast with HRV-16

Nasal fibroblasts were plated in 6-well plates until reaching 80% confluence. FCS-free fibroblast medium containing HRV-16 in the $TCID_{50}$ was added in two different experimental setups. Cells were harvested at 1, 3, 6, 12, 24, 48 and 72 hours. A fibroblast monolayer with non-virus-containing medium served as control. Harvested cells were used for RNA isolation; supernatants were used for RANTES and eotaxin detection with ELISA.

RNA isolation

RNA was isolated using TRIzol® (GIBCO BRL, Eggenstein, Germany) according to the manufacturer's instructions. Briefly, cells were lysed by adding 1 ml TRIzol®, and 0.2 ml chloroform was added and centrifuged at 12,000 rpm for 15 min at 4°C. RNA was precipitated from the aqueous phase by addition of 0.5 ml ice-cold isopropyl alcohol and 1 μl glycogen followed by a 10 min centrifugation at 12,000 rpm and 4°C. The resulting RNA pellets were rinsed with cold 75% ethanol, dried and suspended in 50% formamide. The RNA concentration was quantified by measuring the absorbance of an aliquot in water at 260 nm. The integrity of the RNA was assessed in an ethidium bromide containing 1% agarose gel. RNA was stored at -80°C until further processing.

RANTES and eotaxin SQRT-PCR

One μg total RNA was reversely transcribed using an Oligo(dT)-18 primer, SuperScript™ II RNAse H-Reverse Transcriptase and standard reagents according to the recommendations of the manufacturer (GIBCO BRL, Eggenstein, Germany). Briefly, in a total of 8 μl volume 1 μg RNA were assembled with 0.5 μg Oligo(dT) (GIBCO BRL, Eggenstein, Germany) and DEPC-water, heated to 70°C for 10 min, and chilled on ice for 5 min. Subsequently, 4 μl of First Strand Buffer, 2 μl of 0.1 M DTT and 5 μl of 2 mM dNTP mix were added to each sample. After 2 min incubation at 37°C, 1 μl of Superscript II was added and samples were incubated at 44°C for 45 min. The enzyme was inactivated by incubation at 70°C for 15 min. RT products were diluted with nuclease-free water to a final volume of 200 μl and kept at 4°C. Intron spanning sets of primers specific for RANTES sense 5'-GCC TCG CTG TCA TCC TCA TTG-3', antisense 5'- TAA CTG CTG CTC GTC GTG GTC-3', eotaxin sense 5'-CCC AAC CAC CTG CTG CTT TAA CCT G-3', antisense 5'-TGG CTT TGG AGT TGG AGA TTT TTG G-3', glycerin*a*ldehyd-3-*p*hosphat*deh*ydrogenase (G3PDH) sense 5'-CCA GCC GAG CCA CAT CGC-3', antisense 5'-ATG AGC CCC AGC CTT CTC CAT-3' were used to enable comparison between different cDNA contents of the samples. Subsequently, cDNA corresponding to 50 ng RNA served as template in a Duplex-PCR-reaction containing 0.8 μM of primers specific for RANTES, eotaxin and 0.1 μM of a G3PDH specific primer pair as internal control. PCR products were subjected to 2% agarose gel-electrophoresis and visualized by ethidium bromide staining. PCR signals were quantified using the analysis software E.A.S.Y Win 32 (Herolab, Belm, Germany). Relative RANTES and eotaxin gene expression was adjusted to G3PDH signal strength.

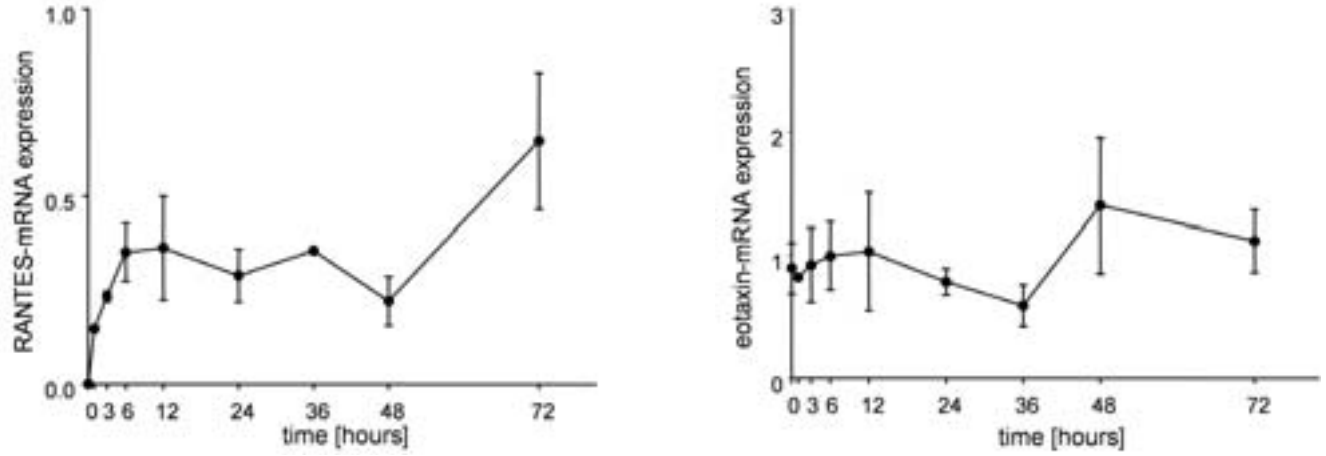

Figure 1. RANTES- (left panel) and eotaxin- (right panel) mRNA expression in nasal fibroblasts at various time points after rhinovirus infection, adjusted to relative G3PDH signal strength. Values are relative to the G3PDH signal. Increased mRNA expression post infection is observed for RANTES, but not for eotaxin. Results are mean ± SEM.

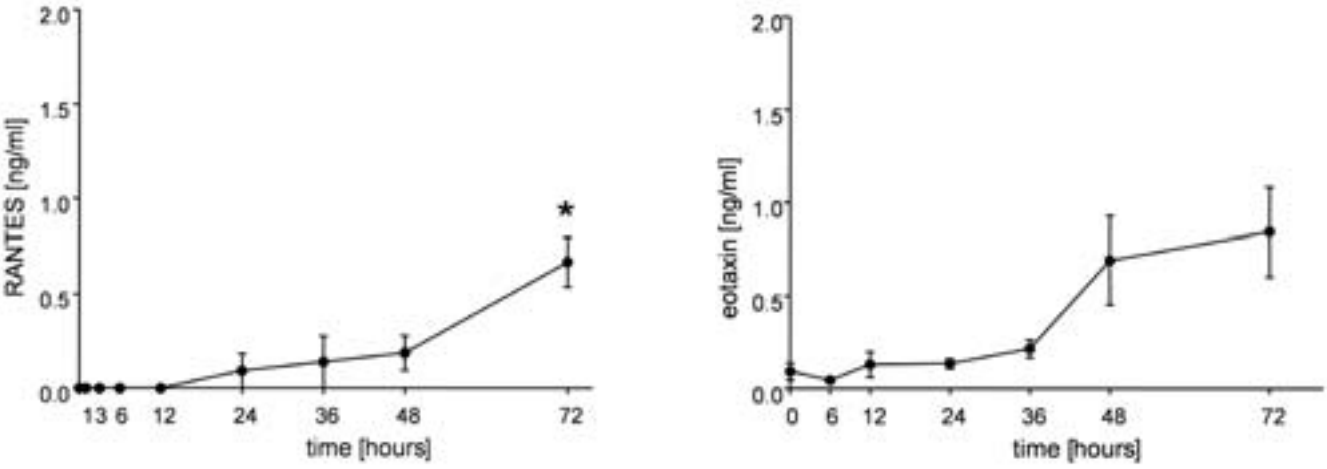

Figure 2. Time-courses of RANTES (left panel) and eotaxin (right panel) production after exposure of nasal fibroblasts to HRV-16. Both chemokines tend to increase with time. RANTES production becomes significantly elevated at the 72 hour-time point. Results are mean ± SEM of nasal cells from two patients. *$p < 0.05$.

RANTES and eotaxin ELISA

Polystyrene plates were coated overnight at 4°C with 2 μg/μl polyclonal anti-RANTES IgG (R&D Systems, Minneapolis, MN, USA) in sodium carbonate buffer (pH 8.9) and blocked with 1% (w/v) BSA in PBS for 1 hour at room temperature. After washing the plate with Tween (0.05% in PBS), the protein supernatants were added and allowed to bind to the polyclonal anti-RANTES antibody for one hour. Subsequently, biotinylated monoclonal antibodies were added at appropriate dilutions tested in advance [17]. The enzymatic color reaction was performed using the avidin-biotin-peroxidase method (ABC-kit, Vector Laboratories, Burlingame, CA, USA) with o-phenylendiamin as substrate. Optical density was measured at 492 nm in a Behring ELISA processor II (Behring, Liederbach, Germany). A complete calibration curve was used to calculate the concentration of RANTES in ng/ml. For the eotaxin ELISA, the same protocol was used with monoclonal eotaxin capture antibody and biotinylated eotaxin detection antibody purchased from R&D (Minneapolis, MN, USA).

Statistical evaluation

Statistical analysis was performed using SPSS 9.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA). Both G3PDH adjusted chemokine expression as well as protein production in the virus-stimulated cells were compared to the control at various time intervals, and analyzed using the paired t-test. A $p < 0.05$ was considered significant.

RESULTS

Agarose gel electrophoresis assessment of the RNA samples revealed sufficient quality and quantity.

Effects of HRV infection on chemokine mRNA expression
RANTES-mRNA could not be detected before stimulation. RANTES-mRNA levels were elevated as soon as one hour after infection (15% of the relative G3PDH value) and reaching a plateau at six hours (35%). Highest RANTES-mRNA levels were measured at the 72 h time point with 65%. In contrast, eotaxin-mRNA was constitutively expressed (Figure 1).

Effects of HRV-16 stimulation on chemokine protein production in nasal fibroblasts
No basal RANTES protein production was found in supernatants of uninfected fibroblasts. RANTES was furthermore not detectable the first 12 hours after incubation of fibroblasts with rhinovirus. RANTES production started at 24 hours although increased levels of RANTES in all cultures were not measured before 48 hours (0.19 ng/ml). Maximal elevated RANTES levels were detected at 72 hours (0.67 ng/ml). Eotaxin protein production trended, like RANTES, to increase with latency. During the first 24 hours of HRV exposure, eotaxin levels were within the basal production (0.09 ng/ml). After 36 hours of HRV-stimulation, eotaxin was detected at a concentration of 0.22 ng/ml. The level of eotaxin production continued to rise up to 0.69 ng/ml, reaching a maximum level of 0.84 ng/ml, at 48 and 72 hours, respectively. In summary, a trend of late rhinovirus-stimulated eotaxin production in nasal fibroblasts can be seen, although none of the elevated eotaxin levels became statistically significant (Figure 2).

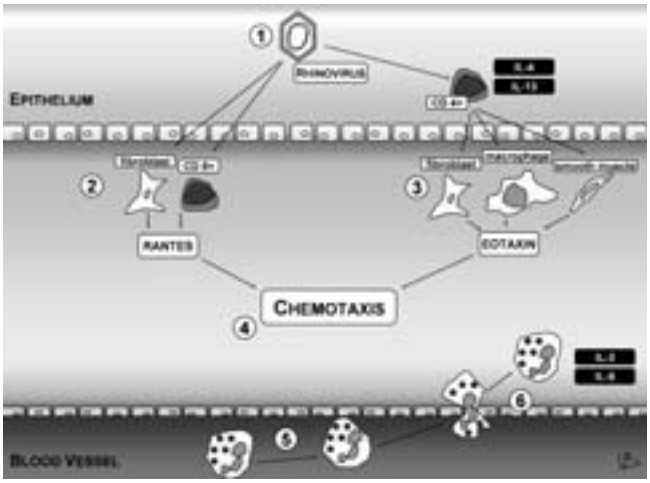

Figure 3. Schematic illustration of the potential effects of human rhinovirus induced release of RANTES and eotaxin in the mucosa: (1) Human Rhinovirus is infecting nasal mucosa, which triggers (2) RANTES and (3) eotaxin production. Both mediators lead to eochemotaxis (4), which promote proinflammatory cytokines (PIC) and CC receptor expression in the endothelium. CCR-3 positive eosinophils in the bloodstream start the process of leukodiapedesis first by rolling and adhesion (5), than by migration through the endothelium into the submucosa (6), from where on they follow their chemotactic gradient.

DISCUSSION
The exact localization of CC-chemokine production within the nasal tissue is of importance for understanding the sequential accumulation of different inflammatory cells. Nasal RANTES production is mainly localized to fibroblasts and endothelial cells, while eotaxin production is connected to endothelial cells, fibroblasts and also epithelial cells [18]. This is suggestive for a role for RANTES as first general immune response mediator in the vicinity of blood vessels involving various inflammatory cells, including eosinophils, T lymphocytes and monocytes. On the other hand, eotaxin is a more selective eosinophil chemoattractant. It is therefore not surprising that its concentration increases with proximity to the epithelial luminal side of the nose, where nasal pathogens, e.g. viruses get in first contact with the nasal mucosa. Eotaxin seems to be involved in a precise selection of eosinophils to migrate to the airway lumen to defend pathogens at this site.

In nasal polyps, increased levels of RANTES and eotaxin have been measured, suggesting a role in the pathogenesis of this disease [13,19], however the factors causing their increased production have not been identified yet. In consideration of previous reports on other viral induced airway diseases supporting a triggering effect of antiviral host response, we suspected that these host defense mechanisms could also trigger a nasal polyposis in reaction to a rhinovirus infection [20,21]. Our data show that the stimulation of fibroblasts, which are the major source of CC-chemokines in the nose, with rhinovirus, induces production of RANTES. This present observation identifies a possible link between rhinovirus infection and eosinophilic inflammation of the nose. Evidence for a HRV-induced nasal eosinophil accumulation was also published by Greiff et al., who found increased ECP and eotaxin in the nasal lavage of patients undergoing experimental HRV-16 infection [22,23]. The upregulation of RANTES in nasal cells in response to rhinovirus inoculation is consistent with previous investigations in the lower respiratory tract, where interactions of rhinovirus and eosinophils have been studied more extensively due to the known association of common colds and asthma exacerbations: An increase of RANTES and eotaxin following rhinovirus infection has been shown for bronchial cells [24,25].

The recruitment of eosinophils into the nasal mucosa is a part of an antiviral immune response. The eosinophil secretory products eosinophil-derived neurotoxin (EDN) and ECP have both ribonuclease activity, and have recently been shown to inactivate single-stranded RNA virions: both ECP and EDN promoted a dose-dependent decrease in respiratory syncytial virus infectivity [26]. Antiviral activity of EDN has also been demonstrated against pneumonia virus of mice, parainfluenza virus and the human immunodeficiency virus [27-29]. This suggests also an antiviral effect of these eosinophil products against other single-stranded RNA viruses like HRV. However, the antiviral activity of eosinophil released products is accom-

panied by their detrimental features causing ciliostasis, epithelial destruction and selfsustained inflammatory changes. Tissue eosinophilia once initiated by RANTES and eotaxin is prolonged by IL-3, IL-5, and GM-CSF, which are known to inhibit eosinophil apoptosis and will be produced by eosinophils in an autocrine manner [30-32].

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the German Research Foundation (DFG).

REFERENCES

1. Tos M (1995) Nasal polyps. Curr Opinion Otolaryngol Head Neck Surg 3: 31-35.
2. Bernstein B, Gorfien J, Noble B (1995) Role of allergy in nasal polyposis: a review. Otolaryngol Head Neck Surg 113: 724-732.
3. Perez-Novo C, Kowalski ML, Kuna P, et al. (2004) Aspirin sensitivity and IgE antibodies to Staphylococcus aureus enterotoxins in nasal polyposis: studies on the relationship. Int Arch Allergy Immunol 133: 255-260.
4. Gosepath J, Brieger J, Vlachtsis K, Mann WJ (2004) Fungal DNA is present in tissue specimens of patients with chronic rhinosinusitis. Am J Rhinol 18: 9-13.
5. Bachert C, van Zele T, Gevaert P, De Schrijver L, Van Cauwenberge P (2003) Superantigens and nasal polyps. Curr Allergy Asthma Rep 3: 523-531.
6. Klima A, Burkle H, May A, Braun W (1993) Studies on viral and allergic genesis of nasal and paranasal polyps. Laryngo Rhino Otol 72: 131-135.
7. Papadopoulos N, Psarras S (2003) Rhinoviruses in the pathogenesis of asthma. Curr Allergy Asthma Rep 3: 137-145.
8. Winther B, Gwaltney JM, Hendley JO (1990) Respiratory virus infection of monolayer cultures of human nasal epithelial cells. Am Rev Respir Dis 141: 839-845.
9. Stoop A, van der Heijden HA, Biewenga J, van der Baan S (1991) Lymphocytes and non-lymphoid cells in nasal polyps. J Allergy Clin Immunol 87: 470-475.
10. Nishioka, K, Saito C, Nagano T, Okano M, Masuda Y, Kuriyama T (1993) Eosinophil cationic protein in the nasal secretions of patients with mite allergic rhinitis. Laryngoscope 103: 189-192.
11. Meyer J, Berner I, Teran LM, et al. (1998) RANTES production by cytokine-stimulated nasal fibroblasts: its inhibition by glucocorticoids. Int Arch Allergy Immunol 117: 60-67.
12. Shin S, Park JY, Jeon CH, Choi JK, Lee SH (2000) Quantitative Analysis of Eotaxin and RANTES messenger RNA in nasal polyps: association of tissue and nasal eosinophils. Laryngoscope 110: 1353-1357.
13. Allen J, Eisma R, LaFreniere D, Leonard G, Kreutzer D (1998) Characterization of the eosinophil chemokine RANTES in nasal polyps. Ann Otol Rhinol Laryngol 107: 416-420.
14. Heath H, Qin S, Rao P, et al. (1997) Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J Clin Invest 19: 178-184.
15. Maune S, Berner I, Sticherling M, Kulke R, Bartels J, Schröder JM (1996) Fibroblasts but not nasal epithelial cells obtained from human nasal mucosa produce the chemokine RANTES. Rhinology 34: 210-214.
16. Reed L, Muench H (1938) A simple method of estimating fifty per cent endpoints. Am J Hygiene 27: 493-497.
17. Sticherling M, Kupper M, Koltrowitz F, et al. (1995) Detection of the chemokine RANTES in cytokine-stimulated human dermal fibroblasts. J Invest Dermatol 105: 585-591.
18. Pods R, Ross D, van Hulst S, Rudack C, Maune S (2003) RANTES, eotaxin and eotaxin-2 expression and production in patients with aspirin triad. Allergy 85: 1165-1170.
19. Bartels J, Maune S, Meyer JE, et al. (1997) Increased eotaxin-mRNA expression in non-atopic and atopic nasal polyps: comparison to RANTES and MCP-3 expression. Rhinology 35: 171-174.
20. Fleming H, Little FF, Schnurr D, et al. (1999) Rhinovirus-16 colds in healthy and in asthmatic subjects: similar changes in upper and lower airways. Am J Respir Crit Care Med 160: 100-108.
21. Furukawa E, Ohrui T, Yamaya M, et al. (2004) Human airway submucosal glands augment eosinophil chemotaxis during rhinovirus infection. Clin Exp Allergy 34: 704-711.
22. Greiff L, Andersson M, Svensson C, Linden M, Myint S, Persson CG (1999) Allergen challenge-induced acute exudation of IL-8, ECP and alpha2-macroglobulin in human rhinovirus-induced common colds. Eur Respir J 13: 41-47.
23. Greiff L, Andersson M, Andersson E, et al. (1999) Experimental common cold increases mucosal output of eotaxin in atopic individuals. Allergy 45: 1204-1208.
24. Schroth M, Grimm E, Frindt P, et al. (1999) Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. Am J Respir Cell Mol Biol 20: 1220-1228.
25. Papadopoulos N, Papi A, Meyer J, et al. (2001) Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells. Clin Exp Allergy 31: 1060-1066.
26. Rosenberg H, Domachowske JB (2001) Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens. J Leukoc Biol 70: 691-698.
27. Domachowske J, Dyer KD, Adams AG, Leto TL, Rosenberg HF (1998) Eosinophil cationic protein/RNase 3 is another RNase A-family ribonuclease with direct antiviral activity. Nucleic Acid Res 26: 3358-3363.
28. Lee-Huang S, Huang PL, Sun Y, et al. (1999) Lysozyme and RNases as anti-HIV components in beta-core preparations of human chorionic gonadotropin. Proc Natl Acad Sci USA 96: 2678-2681.
29. Klebanoff S, Coombs RW (1996) Virucidal effect of stimulated eosinophils on human immunodeficiency virus type 1. AIDS Res Hum Retroviruses 12: 25-29.
30. Shinagawa K, Trifilieff A, Anderson GP (2003) Involvement of CCR3-reactive chemokines in eosinophil survival. Int Arch Allergy Immunol 130: 150-157.
31. Bachert C, Wagenmann M, Hauser U, Rudack C (1997) IL-5 synthesis is upregulated in human nasal polyp tissue. J Allergy Clin Immunol 99: 837-842.
32. Simon H, Yousefi S, Schranz C, Schapowal A, Bachert C, Blaser K (1997) Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. J Immunol 158: 3902-3908.

Dr. med. Jens Eduard Meyer
Department of Otorhinolaryngology
Head and Neck Surgery
University of Schleswig-Holstein
Campus Lübeck
Ratzeburger Allee
23538 Lübeck
Germany

Tel: +49-451-500-2256
Fax: +49-451-500-4196
E-mail: jens.meyer@hno.uni-lubeck.de