Rhinoviruses stimulate chemotaxis of peripheral lymphocytes from healthy and asthmatic subjects

TERESA ŻAK-NEJMARK, MARIA KRAUS-FILARSKA, JÓZEF MAŁOLEPSZY, MONIKA CEMBRZYŃSKA-NOWAK, GRAŻYNA NADOBNA, MAŁGORZATA BIEŃKOWSKA-HABA

1Wroclaw Medical University, Department of Internal Medicine and Allergology, Wroclaw, Poland; 2Institute of Immunology and Experimental Therapy, Department of Clinical Immunology, Laboratory of Virology, Wroclaw, Poland

Abstract

Respiratory viral infections caused mostly by rhinoviruses (RV) contribute to exacerbation of asthma. It was demonstrated that these viruses reach and penetrate also lower airways. At early stage of infection, infiltration of bronchial submucosa accompanied by peripheral lymphopenia and neutrophilia was found. The crucial importance for the course of RV-induced disease is the presence of RV antigens-specific T lymphocytes which are the first to undergo activation.

The study was performed on cells from 7 healthy subjects and 6 patients with stable, chronic asthma. The influence of live Human Rhinovirus Serotype 16 (HRV 16) (range of the titer 4x10^2 –10^3 TCID_{50}/ml) on chemotaxis of lymphocytes and neutrophils was evaluated. Chemotaxis in response to nonspecific chemoattractant fMLP was determined in parallel experiments.

It was found that HRV-16, at the titer 6x10^3 TCID_{50}/ml, significantly increased chemotaxis of lymphocytes of healthy subjects (p<0.02) and asthmatics (p<0.001) whereas fMLP increased chemotaxis of lymphocytes of the patients only (p<0.001). Migration of neutrophils in fMLP gradient was significantly increased in both groups. RV had no influence on chemotaxis of neutrophils.

These results indicate the presence in circulation antigen-specific lymphocytes recognizing HRV-16 antigens. These lymphocytes may infiltrate airways most early after infection leading, in consequence, to peripheral lymphopenia.

Key words: asthma, rhinovirus, viral infection, chemotaxis, lymphocytes


Introduction

Respiratory viral infections, usually manifested as common cold, are the most frequent and world-wide spread human diseases. It is currently estimated that among viruses infecting airways, the cause of 2/3 cases are rhinoviruses (RV) [1]. Considering documented exacerbation of the course of already existing respiratory diseases, large-scale incidence and lack of etiological therapy, RV are a serious health problem.

Application of molecular diagnostics (reverse transcription polymerase chain reaction, RT-PCR) confirmed the role of RV in exacerbation of ongoing inflammatory processes in the diseases such as asthma, chronic obstructive pulmonary disease (COPD), sinusitis, cystic fibrosis, otitis [2].

Infection with RV is not limited to upper airways, the presence of these viruses was also detected in broncho-alveolar lavage (BAL), sputum and specimens of bronchial mucosa from infected patients [2–4]. Infections with RV are the major cause of exacerbation of asthma in children [5] and adults [6, 7]. It was also found that asthmatic patients are more sensitive to infections with RV than healthy subjects [8, 9]. During infection with RV, peripheral lymphopenia parallel to infiltration of bronchial mucosa with lymphocytes was observed in asthmatics and
Rhinoviruses stimulate chemotaxis of peripheral lymphocytes from healthy and asthmatic subjects

Healthy subjects infected experimentally [3, 10–13]. It was also found that the more severe aggravation of cold symptoms, the more pronounced lymphopenia [10]. Significant increase in bronchial hyperresponsiveness (BHR) positively correlated with an increased number of lymphocytes in bronchial mucosa [14].

Rhinoviruses are nonenveloped RNA viruses (picornaviridae family) of 18–30 nm in diameter. Their antigenic differentiation is very high and there are over 150 currently known strains. They replicate exclusively in epithelial cells of upper and lower airway of man. It was found in recent experiments in healthy and asthmatic subjects infected experimentally that RV may penetrate and replicate in lower airways [3, 4]. Replication of RV in bronchial epithelial cells was confirmed in vitro, in human cell cultures, by determining virus titer and production of viral RNA and proteins [4].

Migration of lymphocytes to the lungs is a key process in development, maintenance and exacerbation of inflammatory state. The aim of our study was the investigation of the influence of live human rhinoviruses type 16 (HRV16) on chemotaxis of lymphocytes and neutrophils of asthmatic patients and healthy subjects.

Methods

The study was performed in 6 non-smoking atopic asthmatics (2 females and 4 males) with a mean age of 39 years (range 25–53). The duration of disease ranged from 2 to 30 years (mean 17.5 years). All subjects showed positive skin prick tests with house dust mite, inhaled corticosteroids or oral corticosteroids for ≥1 month prior to the study, and no subject had experienced an upper respiratory tract infection in the preceding month or during the study. Their asthma was stable and controlled by inhaled β2-agonists as required. The control group comprised 7 non-smoking, healthy subjects (six females and one male), mean age 33 years (range 21–42). The study was approved by the Local Ethics Committee and all subjects gave written informed consent.

Cell Cultures

WI-38 human embryonic lung fibroblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Eagle’s culture medium supplemented with 7% fetal calf serum (FCS), 0.09% sodium bicarbonate, 1% L-glutamine (Sigma, St Louis, USA) and the antibiotics 100 IU/ml penicillin and 100 µg/ml streptomycin (Polfa, Tarchomin S.A., PL).

Human rhinovirus 16 procedure

Human rhinovirus serotype 16 (HRV16), strain 11757 was obtained from ATCC and replicated in rhinovirus-sensitive WI-38 cell line. Cells were plated in 75-cm² flasks (Costar, Cambridge) at the density 2x10⁶/ml and cultured for 24 hours at 37°C with 5% CO₂. Confluent monolayers were inoculated with virus suspension at the concentration of 10⁶ of 50% tissue-culture infective dose (TCID₅₀) per ml of culture medium. The virus was allowed to adsorb to the cells for one hour at 33°C with rotation. The cells were than washed tree times and incubation was continued for 48–72 hours until characteristic cytopathic effect (CPE) was achieved. Cell-free culture supernatants from the infected cultures were collected, centrifuged at 10 000 rpm for 15 min. and filtered using 0.22 µm Milipore filter (Milipore Corp., USA). The samples of virus were stored at -80°C for later experiments.

Determination of virus titer

Determination of the titer of RV16 in supernatants was assessed by using endpoint dilution assay on the basis of development of typical cytopathic effect in WI-38 cell cultures in 96-well plates. The confluent monolayers were exposed to tenfold serial dilutions of 100 µl of virus samples. Supernatants from non-infected cell cultures were used as controls. Every dilution was assessed in six monolayers. The plates were incubated at 33°C for 5 days. Next, the medium was removed, the cells were washed with Eagle’s medium, fixed with methanol for 1 min. and stained with 0.1% crystal violet for 20 min. Then, the plates were washed twice and the cells were lysed with 0.5% sodium dodecyl sulphate (SDS) water-solution. The absorbance was measured at 570 nm using the plate reader (StatFax 2100, Awareness Technology Inc., USA). The reciprocal of the highest dilution of virus sample causing infection 50% of cells in monolayers is expressed as virus titer in TCID₅₀ units (Reed&Münch endpoint calculation method) [15].

Separation of leukocytes

Cells were isolated from heparinized (10 UI/ml) whole blood by Gradsisol gradient, using Zeman et al. procedure [16]. This method enables simultaneous separation of two highly purified leukocyte fractions; lymphocytes and neutrophils. The viability of cells determined by trypan blue exclusion, was consistently >97%.

Chemotaxis assay

Chemotaxis tests were performed by modified Boyden method [17] in 48-well chemotaxis chambers (Neuro Probe Inc., USA) with 5 µm pore-size nitrocellulose filters (Sartorius, Gütenberg, Germany) for neutrophils and 8 µm for lymphocytes. The lower chambers were filled with cell-free supernatants from noninfected WI-38 cultures as a control, fMLP (N-formyl methionyl-leucyl-phenylalanine, Sigma, USA) 10⁻⁷M and rhinovirus 16 at
appropriate concentrations (tested dilution HRV 16 in range 4x10^2–10^4 TCID_{50} unit). Cell suspension 5x10^5/ml in medium were placed in the upper chambers. Medium was composed of Hanks’ balanced salt solution containing 0.4% human albumin (ZLB, Switzerland) and gentamycin 40 µg/ml (Polfa, Poland). All tests were performed in duplicates. The chambers were incubated at 37°C, 5% CO_2 in humidified air for 60 min. After incubation the filters were washed in 0.9% NaCl to remove settled cells, fixed, stained and dehydrated according to Boyden. The distance of migration was determined using leading front assay in five high power fields in duplicate micropore filters. The results are presented as a mean values and standard deviation of migration distances.

**Statistics**

Statistical analysis was performed with Student’s t-test. Differences associated with probability values of p < 0.05 were considered to be significant.

**Results**

In preliminary experiments, using various gradients of RV suspension (4x10^2–1x10^4 TCID_{50}/ml) the significant changes in cell motility were found at the virus titer 6x10^3 TCID_{50}/ml. Comparison of changes in motility of lymphocytes from asthmatic and healthy subjects in fMLP and virus suspension gradient is presented on Fig. 1.

The lymphocytes of asthmatic group only showed significantly increased motility (p<0.001) in fMLP (10^{-8}M) gradient. Significantly increased chemotaxis of the lymphocytes of healthy subjects (24.7±2.3 µm) at the virus titer 6x10^3 TCID_{50}/ml was found as compared to spontaneous motility in medium (22.3±2.1 µm), p<0.02. Motility of lymphocytes of asthmatic patients in medium was 21.2±0.7 µm whereas in gradient of virus suspension was increased significantly to 23.5±0.7 µm (p<0.001). Motility of neutrophils of healthy subjects and asthmatics in fMLP gradient (10^{-8}M) and various titers of HRV16 (range 4x10^2–1x10^4 TCID_{50}/ml) is presented on Fig. 2. Chemotaxis of neutrophils in fMLP gradient was significantly increased in both groups (healthy subjects p<0.05, asthmatics p<0.02) while the presence of HRV16 had no influence.

**Discussion**

We found significantly increased chemotaxis of lymphocytes of healthy subjects and asthmatic patients in the presence of HRV16 (6x10^3 TCID_{50}/ml). In response to bacterial chemotaxtractant fMLP, a nonspecific activator of cell metabolism causing an increase in secretion, adhesion of cells to an endothelium and increased expression of membrane receptors [18, 19], the lymphocytes of patients only showed increased chemotaxis. Considering a chronic inflammatory processes being background of asthma [20] the presence of activated subpopulation among peripheral lymphocytes of these patients may be expected. Lymphocytes infiltrate airways at the earliest stages of viral infection. It is recently widely accepted that the recruitment of lymphocytes and infiltration of the bronchial mucosa is caused by numerous cytokines and...
Rhinoviruses stimulate chemotaxis of peripheral lymphocytes from healthy and asthmatic subjects

chemokines produced by epithelial cells of respiratory tract, following activation by the RV [4, 11, 13, 14, 21].

The main chemoattractant of CD4 lymphocytes is IL-16 which, in asthma, regulates the infiltration of bronchi and is also an activator of eosinophils and macrophages. This interleukine is produced by CD8 lymphocytes and epithelial cells after stimulation with histamine [22]. The investigations of Papadopouls et al. [4] have shown that this cytokine is produced also following an experimental infection with RV. The authors suggest that IL-16 is a main factor regulating the intensity of infiltration of bronchi with lymphocytes during RV infection. It was also found, that IL-16 appeared earlier than other cytokines, 6 h after the RV infection. In our in vitro experiments, lymphocytes were exposed to HRV 16 for 1 hr and already their activation, expressed as increased chemotaxis, was observed. This fact prompts to assume that RV may directly influence the recruitment from circulation antigen-specific lymphocytes, recognizing viral antigens. Due to wide incidence of illnesses caused by RV, a major part of population has the history of infection with these viruses and possesses memory lymphocytes, recognizing serotypes among numerous RV strains. Gem et al. [3] showed that RV-specific clones obtained from peripheral T lymphocytes of healthy subjects may proliferate in response to 2−5 serotypes of RV. This observed cross-reactivity does not prevent numerous, repetitive infections with RV, due to a large number of existing serotypes. Viral infections induce pro-inflammatory immunological response of TH1 type [19] and production of IFNγ and GM-CSF, the cytokines enhancing inflammation being a hallmark of asthma [23−25]. IFNγ increases also an expression of adhesion molecule ICAM-1 (CD 54), a main receptor for majority of RV strains, thus enabling spread of infection [3]. It was found that the expression of ICAM-1 on resting lymphocytes was low [26]. Numerous observations indicate that ICAM-1 on lymphocytes participates in signal transmissions and modulates activation, proliferation and production of cytokines. It was also found that its expression was markedly increased in asthma [27]. Vrtis et al. [28] have observed that HRV-16 stimulated peripheral lymphocytes to the production of IFN-γ through ICAM-1, whereas Gem et al. [29] have shown that incubation of peripheral mononuclear cells caused 30−70% increase in expression of CD69, the marker of early activation of T lymphocytes that could be blocked by antibodies to ICAM-1. One may assume that early and direct influence of HRV-16 on motility of lymphocytes, observed by us, may be at least in part responsible for lymphopenia accompanying RV infection in vivo and being the result of selective migration of lymphocytes to the bronchial mucosa.

Neutrophils appear in airways at later stage of RV infection, during which neutrophilia is observed. In experimental RV infection, the number of neutrophils in BAL was unchanged after 48 h, and showed an increase later, after 96 h [12]. We have not observed any significant influence of HRV 16 on migration of neutrophils from healthy and asthmatic subjects. In parallel experiments, fMLP significantly increased chemotaxis in both groups.
Conclusions

1. HRV 16 increased chemotaxis of lymphocytes from healthy and asthmatic subjects. This fact indicates the presence in circulation antigen-specific lymphocytes for serotype 16. Increased chemotaxis of circulating lymphocytes may cause lymphopenia due to recruitment of antigen-specific lymphocytes to the site of infection.

2. No influence of HRV 16 on migration of neutrophils was found.

Acknowledgment The authors would like to thank Mrs. Krystyna Baramiecka for helpful technical assistance.

References