Rhinology, 37, 179–181, 1999

Technical problems with protein extraction of chemokines featuring RANTES*

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SUMMARY

Chemokines are known to be one of the sources for eosinophilic tissue infiltration in eosinophilic inflammation. Detection of beta-chemokines such as RANTES was possible in nasal tissue with or without eosinophilic infiltration. The concentration of chemokines which has been measured in the same tissue differs often in the literature. Aim of this study was to compare the different techniques of protein extraction and help to understand and interpret the investigation on RANTES secretion. Tissue of nasal polyps, inferior and middle turbinate was cut into halves and every half on its own pulverized using liquid nitrogen. The protein extraction was performed either with citric acid solution (pH 2.5) or phosphate buffered saline (PBS). The samples were then lyophilized. The concentration of RANTES was measured by a specific double sandwich ELISA. Using the citric acid technique the average concentration of RANTES in middle turbinates was 1.3 ng/mg, in inferior turbinates 1.6 ng/mg and in polyps 2.6 ng/mg tissue, using the PBS technique respectively 0.6 ng/mg, 0.5 ng/mg and 0.8 ng/mg tissue. Our data revealed a mismatch of 3.3: 1 for polyps (citric acid: PBS), 3.2: 1 for inferior and 2.2: 1 for middle turbinates, respectively. Consequent comparison between the results of different techniques was not possible. Of special interest was also the fact that different techniques had different efficiencies of protein extraction in different tissues. Present statements on RANTES concentrations as a prognostic factor in nasal tissues need a technically careful standardization as far as this study shows.

Key words: RANTES, chemokines, eosinophils, protein extraction

INTRODUCTION

A number of inflammatory disorders are histologically characterized by the presence of different leukocyte types and subpopulations. The selective appearance of leukocytes in inflamed tissues led to the hypothesis that apart from panleukotactic factors cell-specific leukocyte chemoattractants are secreted.

At that time a new group of chemoattractants, the *chemokines* (*chemotactic cytokines*), have been found. The chemokines represent a family of rather cell-selective or specific leukocyte chemoattractants, which consist of mostly basic and heparin-binding proteins. Structurally, chemokines are divided into a C-C-, C- and C-X-C-branch (Baggiolini and Dahinden, 1994; Schröder, 1997; Teran and Davies, 1996). A number of C-C-chemokines are attractive candidates to explain the disease-specific histological pattern of leukocyte tissue infiltration. Whereas C-X-C-chemokines are mainly neutrophil attractants and do not show any eosinophil chemotactic activity, C-C-chemokines express activity in monocytes, lymphocytes and eosinophils. In contrast to well-known leukoattractants such as plasma-derived C5a, the majority of chemokines seems to be generated in inflamed tissues and to a lesser extent in blood (Kameyoshi et al., 1994). To gain insight in mechanisms for tissue leukocyte infiltration by a particular leukocyte type, biological material should be analyzed for the presence or absence of chemokines.

We have compared two major biochemical techniques used for extraction of chemokines, the PBS (Bachert et al., 1997) and citric acid technique (Maune et al., 1996; Meyer et al., 1998) Therefore, we investigated exemplarily the *RANTES* (*R*egulated on *A*ctivation and *N*ormal *T*-cell *E*xpressed and *S*ecreted) secretion in chronic polypous sinusitis, allergic rhinitis and hyperplasia of the middle turbinate using both techniques.

MATERIALS AND METHODS

Patients

The patients had been treated with endonasal sinus surgery for nasal polyposis and hyperplasia of the inferior and middle turbi-

* Received for publication January 26, 1999; accepted June 14, 1999

nates. We have extracted specimens of 6 patients in each of the three groups. Specimens were removed without the need of any additional resection and frozen immediately in liquid nitrogen. Only specimens with more than 100 eosinophils per high power field were included in this investigation.

Solutions

Citric acid buffer was made by mixing 200 ml 0.1 M citric acid and 100 ml 96% ethanol and the pH adjusted to 2.5 with formamid acid. The Phosphate Buffered Saline (*PBS*), was bought from Gibco Life technology, consists of sodiumcarbonate with a pH of 7.2 +/–0.2.

RANTES protein extraction from nasal specimens

Tissue of nasal polyps, inferior and middle turbinates was cut into two 200 mg pieces and each piece on its own pulverized using liquid nitrogen. The pulver was suspended in 2 ml ethanolic citric acid (1:1 mixture of 96% ethanol and 0.1 M citric acid) (Schröder, 1997) and 2 ml phosphate-buffered saline (PBS) (Bachert et al., 1997), respectively. All further steps have been the same for both preparations. The suspension was further homogenized using an ultrasound homogenizer (Ultraschall-Prozessor 200 H, Dr. Hielscher GmbH) with an amplitude of 70 and a cycle of 0.5 for 1 min chilled on ice. The homogenized specimens were centrifuged at 13.000 rpm for 20 min at room temperature, opalescent supernatant was transferred in a fresh 2 ml tube and frozen in the -70°C freezer. The supernatants were lyophilized and resuspended in 2 ml sodium phosphate buffer, which resulted in a pH of 7.0 and was required for the following ELISA. The RANTES concentration was determined by RANTES-specific double sandwich enzyme linked immunoabsorbent assay (ELISA).

RANTES-ELISA

Polystyrene plates were coated overnight at 4°C with polyclonal anti-RANTES IgG (10 μg/ml) in sodium carbonate buffer, pH 8.9, and blocked with 1% bovine serum albumin (BSA, w/v) in phosphate-buffered saline (PBS) for 1 hour at room temperature. After washing, biotinylated MoAbs were added at appropriate dilutions tested in advance (Sticherling et al., 1995). The enzymatic colour reaction was performed using the avidin-biotin-peroxidase method (ABC-kit, Vector Laboratories) and o-phenylendiamin as substrate. Optical density was measured at 492 nm in a Behring ELISA processor II. A complete calibration curve was used to calculate the concentration of RANTES in ng RANTES per ml.

RESULTS

RANTES protein could be detected by a specific double sandwich ELISA in all of the 36 single preparations.

For the citric acid technique the average RANTES concentration was as high as 1.3 ng/mg for the middle turbinates, 1.6 ng/ml for the inferior turbinate and 2.6 ng/ml for polyps. Using the PBS technique average RANTES concentration for the middle turbinates was 0.6 ng/ml, for inferior turbinates 0.5 ng/ml and for nasal polyps 0.8 ng/ml. The calculated ratio of the citric acid technique to the PBS technique resulted in 3.3 :1 mismatch

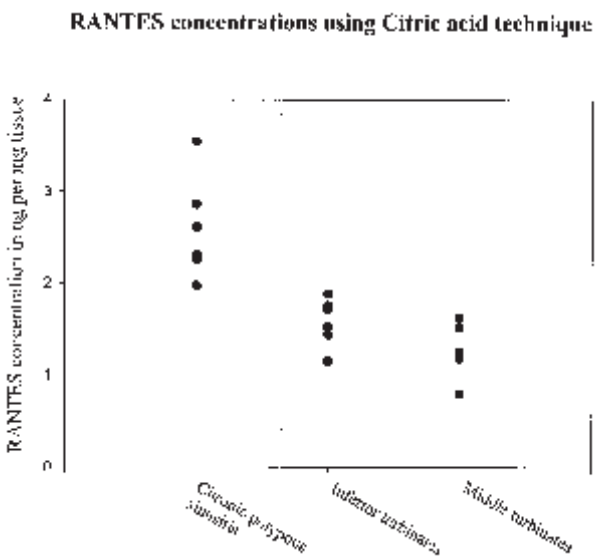

Figure 1. Different tissues were taken to isolate proteins using citric acid technique. RANTES concentrations were quantified with RANTES specific double sandwich ELISA.

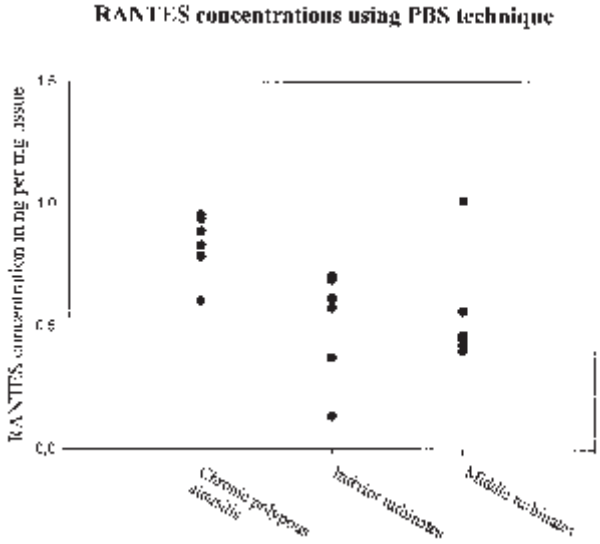

Figure 2. Different tissues were taken to isolate proteins using PBS technique. RANTES concentrations were quantified with RANTES specific double sandwich ELISA.

for the nasal polyps, 3.2 :1 for the inferior turbinates and 2.2 :1 for the middle turbinates (data shown in Figure 3).

DISCUSSION

Eosinophils and memory T-cells are present in affected tissues of patients with chronic polypous sinusitis and allergic rhinitis (Finotto et al., 1994; Klementsson 1992; Linder et al., 1993; Stoop et al., 1989). The amount of activated eosinophils in nasal polyps is significantly increased in comparison with non-affected nasal tissue. This suggests that eosinophils might play an important role in diseases of the nasal mucosa with eosinophilic infiltration although their exact role in pathogenesis and the fashion of mediators influencing eosinophilic tissue infiltration are still unknown.

Since heparinbinding, but not non-heparinbinding proteins from nasal polyps, revealed Eo-chemotactic activity, chemoki-

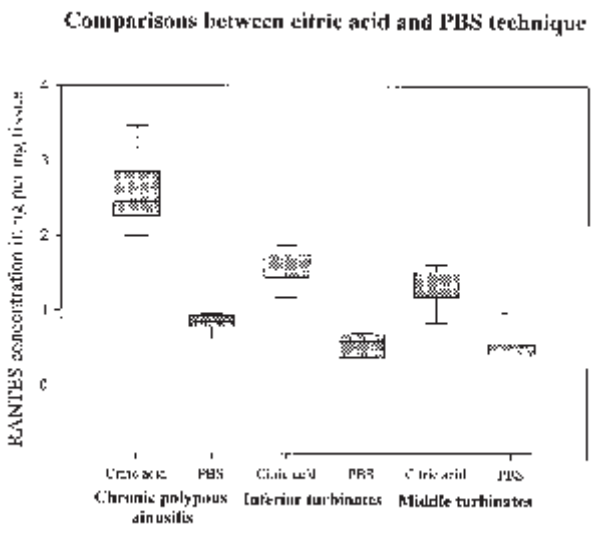

Figure 3. Different tissues were taken to isolate proteins using citric acid or PBS technique. RANTES concentrations were quantified with RANTES specific double sandwich ELISA.

nes could be likely candidates to be main chemoattractants in nasal polyps (Maune et al., 1996) and allergic rhinitis (Teran and Davies, 1996). Structurally, chemokines are divided into a C-X-C-, a C- and a C-C-branch. Different substances are identified in the C-C branch: macrophage inflammatory proteins-1-alpha (MIP-1-alpha) and -beta (MIP-1-beta), I-309, monocyte chemotactic protein-1 (MCP-1), MCP-2 and RANTES (Schröder 1998; Teran and Davies, 1996). Studies on the chemotactic activity of these mediators revealed a high specificity for their target cells. RANTES is very potent, chemotactic substance that activates eosinophils (Kameyoshi et al., 1994; Kuna et al., 1993; Schall 1991). It also provokes degranulation of eosinophilic cationic protein (ECP) in eosinophils. These reports indicated that the local production of RANTES by fibroblasts in a time- and dose-dependent manner might play an important role in chronic inflammatory diseases of the nasal mucosa with eosinophilic cell infiltration such as chronic polypous sinusitis or allergic rhinitis (Kameyoshi et al., 1994; Kuna et al., 1993; Schröder et al., 1994). Our results show that the citric acid technique is up to 3.3-fold more effective compared with the PBS-technique for nasal polyps. Comparison between both techniques shows that addition of ethanol is essential for optimal extraction of chemokines. Omission of this organic solvent leads to strongly increased amounts of fine material in the supernatants, which cannot be filtered and thus impairs subsequent further investigations. Additional extraction of tissue with acidic ethanolic buffers allows direct application of samples to high performance liquid chromatography (HPLC) columns for further investigations. High molecular mass proteins, which are present in huge amounts when extraction is performed at neutral pH in the absence of ethanol, e.g. PBS, are present only in low concentrations. Plus the use of acidic buffers leads to a better extraction of extracellular matrix-bound chemokines, whereas the stickiness of the very hydrophobic chemokines will be reduced by adding organic solvents such as ethanol. Furthermore, drastic losses of activity, possibly by binding of the highly cationic and hydrophobic chemokines to tissue glycosaminoglycans and/or hydrophobic structures, were seen (Schröder 1997).

ACKNOWLEDGEMENT

This work was supported by Deutsche Forschungsgemeinschaft, grant CH38/7-1.

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