Archivum Immunologiae et Therapiae Experimentalis, 2003, **51**, 259–265
PL ISSN 0004-069X

Time-Dependent Observations of Secretion Marker Levels in Nasal Secretion after Histamine and Methacholine Provocations

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Abstract. Nasal provocation tests with histamine and methacholine were carried out on 25 healthy men in an effort to assess the dynamic changes of albumin, total IgA, secretory IgA and lactoferrin concentrations in the nasal secretion. The trials were performed with 0.5, 1, and 4 mg of histamine and 8, 16, and 32 mg of methacholine. Each dose of histamine or methacholine was sprayed into the nose every 2nd day, with two days' interval between the two provoking agents. Nasal secretions were collected after saline spraying only, forming the baseline group, after 3, 10 and 15 min of administration of the challenge agent. The baseline levels presented the following values: for albumin 257 ± 230 μg/ml, secretory IgA 608 ± 379 μg/ml, total IgA 1025 ± 423 μg/ml, and lactoferrin 213 ± 156 μg/ml. The increase in albumin level after nasal provocation, particularly significant after histamine administration (to 3713 ± 2311 μg/ml), indicates incessant protein plasma leakage from the blood circulation to the nasal secretion. After administration of both provocating agents, there was a significant gradual decrease in secretory IgA level, even below the baseline value. After the 2nd and 3rd doses of methacholine and histamine spray, the concentration of secretory IgA decreased by 2–3 times and was found to be 200–300 μg/ml, respectively. Also, lactoferrin concentration values decreased gradually after the 2nd and 3rd doses of methacholine and histamine to levels close the baseline value. These observations suggest a time- and dose-dependent, non-specific dysfunction of local immunity response after nasal provocations.

Key words: nasal challange; nasal secretion markers; lactoferrin; IgA; secretory IgA.

Introduction

External specific or non-specific environmental factors are frequently repeated stimuli which lead to an increased production of nasal secretions and rhinitis as well as occasional transient nasal blockade. The diagnosis of rhinitis is mainly based on case history, but an escalation of the symptoms could be determined by a variety of physical, chemical and inflammatory mediators[12, 13, 21]. The pathological effect of the released mediators on changes in vascular permeability or on glandular secretions can be assessed by measurement of some plasma proteins[5, 14, 16]. Plasma protein exudation is a hallmark of mucosa inflammation and reflects

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the action of released mediators on post capillary venular integrity[21].

Macromolecules present in nasal secretions can be derived from multiple sources. They may originate in the transudate from the vascular system, but mucous glycoproteins derive from the goblet cells and/or from serous or seromucous glands. Plasma proteins mainly diffuse into secretions through increased vascular permeability. However, the transudation of serum proteins across fenestrated blood vessels into glandular secretions has also been reported[14–16, 18]. Some other proteins, such as granule proteins, eosinophil cationic protein, basic proteins, interleukins and other proinflammatory mediators, are released to nasal secretions from mononuclear cells, mast cells, granulocyte or epithelial cells[1, 7, 21, 22].

Locally present plasma cells are known to produce mainly IgA and IgE[3]. Albumin, a serum protein synthesized mainly by the liver, circulates as a vascular protein. The presence of albumin in the upper-airway secretion is universal and usually accounts for from 5 to 25% of all the proteins in the nasal secretion. An increased level of albumin mainly reflects increased vascular permeability, although it can also be increased after cholinergic stimulation. RAPHAEL et al.[14, 16] have claimed that with increased vascular permeability, the albumin fraction of total protein of nasal secretions also increases, but remains unchanged if glandular secretions occur. Albumin and immunoglobulins collectively account for about 45% of total protein measured in nasal secretions[14]. The major immunoglobulin in the nose, IgA, is locally synthesized accounts for 20–50% of the total protein, and is represented by approximately 80% as secretory IgA. As is commonly known, lactoferrin is produced and stored in serous cells. It has been assumed that direct cholinergic stimulation or indirect stimulation through nasal response causes an increase of its secretion. Secretory IgA and lactoferrin have been proposed as markers of glandular stimulation[4, 16].

Histamine and methacholine nasal provocations are frequently used to obtain information on the pathophysiological background of nasal hyperactivity because of the different sites in the nasal mucosa on which histamine[2, 4, 6, 16] and methacholine[2, 6, 14, 19] have an effect. Histamine involves direct stimulation of histamine receptors and indirect stimulation via a nasonasal reflex. It leads to vasodilation and increased vascular permeability, resulting in nasal congestion, and can induce the symptoms of rhinitis[6, 11]. In contrast, methacholine provocation increases nasal airway resistance transiently and has a direct effect on glands[6, 14].

Although the composition of nasal mucosa has been investigated by several workers[2, 6, 10, 14, 16], a clear pattern of protein constituent concentrations after repeated challenge and increased doses of histamine and then methacholine have not been yet demonstrated. In this study we have monitored albumin as well as protein levels in nasal secretions which participate in specific (secretory IgA) and non-specific (lactoferrin) immune defense reactions. These proteins are accepted nasal secretion markers of plasma leakage (albumin) and glandular secretion (secretory IgA, lactoferrin). The nasal secretions were collected before and 3, 10 and 15 min after provocation with three increasing doses of two challenge agents, namely histamine and methacholine.

Materials and Methods

Subjects. Twenty-five healthy soldiers (19–25 year old) volunteered to take part in the trials. They had no history of atopic disease and chronic rhinitis, no medications were being taken, and their nasal mucosa were normal. The trials were performed during their stay at the Laryngology Department at the Regional Military Hospital, Wrocław (Poland). The Ethics Committee of the Wrocław Medical University approved the studies. All participants consented prior to taking part in the study.

Nasal provocation tests and mucosa secretion collection. The nasal provocation tests were performed with three doses of two agents three times a week over a period of two weeks. The challenge was carried out by spraying a volume of 0.3 ml of either histamine dihydrochloride or methacholine dihydrochloride solution into the left side of the nose only, with the patient's head maintained in the upright position. During the first week, increasing doses of 0.5, 1 and 4 mg of histamine were administrated every second day. During the second week, increasing doses of 8, 16, and 32 mg of methacholine were sprayed according to the same schedule. Nasal secretions were collected 3, 10 and 15 min after spraying histamine or methacholine. A solution of phosphate-buffered saline (0.3 ml) was sprayed before the trials with challenge agents and the collected mucus served as the baseline.

The procedure of mucus secretion collection was carried out according to LORIN et al.[9] with small modifications. A strip of Whatman's filter paper no. 50 (size 5×55 mm) was applied to the fundus of the nasal cavity for 10 s. Then the strip, soaked the secretion, as removed from the nose and placed into a tube contain-

ing 100 µl of 0.2 mol/l phosphate buffer, pH 6.5. The biological material was stored at –70˚C for further analysis.

Sample preparation. The samples containing the secretion-soaked strips immersed in 100 µl of 0.2 mol phosphate buffer, pH 6.5 were thawed, and the strips were cut into 4 parts, mixed together in the tube, and rocked slowly for 15 min using a roller-mixer. The liquid content of each tube was moved by pipette to separate Eppendorf's tubes. Next, to each tube still containing the pieces of filter paper, 100 µl of 0.2 mol/l phosphate buffer, pH 6.5, was added, mixed, and rotated again for 15 min. Then the content was put together with the previous portion and the elution procedure was repeated again. The content of the Eppendorf's tubes was carefully mixed and the material was divided into 50 and 100 µl aliquots and stored at –70˚C for subsequent analysis.

Analytical procedures. Protein concentration was measured by bicinchoninic assay using bovine serum albumin (Sigma, St. Louis, USA) as a protein standard[17]. Human albumin was measured by rocket immunoelectrophoresis[20] performed in 1% agarose gel, 10 mmol/l Tris-veronal buffer, pH 8.6, containing 0.55 µl/ml of monospecific goat anti-albumin antibody (Sigma, St. Louis, USA) using human albumin ranged 15–75 µg/ml as a standard.

Lactoferrin. The ELISA procedure was as follows: wells of polystyrene microtiter plates (MaxiSorp, Nunc, Rosklide, Denmark) filled with 100 µl of mouse monoclonal anti-human lactoferrin (Dako, Copenhagen, Denmark), diluted 1:10 000 in phosphate buffered saline (PBS) at pH 7.3, were incubated for 2 h at 37˚C, and then the plate was washed and blocked with 200 µl per well of 0.5% human immunoglobulin in PBS for 1 h at 37˚C and overnight at 4˚C. Samples or standard solutions of lactoferrin, i.e. 1–40 ng/100 µl in PBS-T-BSA (PBS with 0.1% Tween 20 and 0.02% bovine serum albumin (BSA)) were added to the wells and incubated for 1 h at 37˚C. The bound lactoferrin was detected by 200 µl of primary rabbit anti-human lactoferrin antibody diluted 1:10 000 in PBS-T-BSA and then with the secondary goat anti-rabbit IgG (diluted 1:10 000) conjugated with horseradish peroxidase (Sigma, St. Louis, USA). For developing the peroxidase activity, 100 µl of the staining mixture (0.6 ml of solution containing 4 mg TMB dissolved in 1 ml of DMSO, 10 ml citric buffer, pH 6.0, and 1.2 µl of 30% H_2O_2) was added to the wells of the plate and incubated at room temperature for 30 min in darkness. The reaction was stopped by 100 µl of 2 M H_2SO_4. The absorbances were measured by ELISA Stat Fax 2100 Reader at 450 nm and 630 nm as reference filter.

Total IgA and secretory IgA. 100 µl of mouse monoclonal anti-human IgA (Sigma, St. Louis, USA), diluted 1:5000 or secretory IgA (Sigma, St. Louis, USA), diluted 1:10 000, were coated with polystyrene microtiter plates (MaxiSorp, Nunc, Rosklide, Denmark) in PBS, pH 7.3, for 2 h at 37˚C. Next, the plate was washed and the wells were blocked for 1 h at 37˚C and overnight at 4˚C with 0.1% BSA in PBS containing 0.1% Tween 20. Samples or standard solutions of secretory IgA (the binding site) ranged from 1 to 100 ng/100 µl for total IgA estimation or ranged from 0.5 to 50 ng/100 µl for secretory IgA estimation, both in PBS-T-BSA (PBS with 0.05% Tween 20), were added to the wells, respectively. The plate was incubated for 1 h at 37˚C. The specific binding IgA or secretory IgA was detected by primary rabbit anti-human IgA antibody diluted 1:100 000 and then diluted 1:10 000 with the secondary goat anti-rabbit IgG-HRP (Sigma, St. Louis, USA). The peroxidase activity was developed as described for lactoferrin determination.

Statistical analysis. The statistical analysis was carried out by the Statistica 5.0 computer program. For the statistical significance, the U Mann-Whitney test was used. The results are given as means and standard deviations (mean ± SD), and p-values lower than 0.05 were considered as significant ($p<0.05$).

Results

The time-dependent observations of albumin, secretory IgA, total IgA, and lactoferrin levels after three increasing doses of histamine and methacholine are illustrated in Fig. 1–4. The albumin level after provocation with histamine was significantly higher ($p<0.0001$) than after methacholine provocation, and those values were also higher than the albumin level found for the group before provocation i.e. which formed baseline (Fig. 1). The highest level of albumin was observed 10 min after the 2nd dose of histamine spray and the 1st dose of methacholine.

After provocations with either agent, total IgA concentration rapidly increased, particularly after histamine, which exceeded several times the baseline level and reached the maximum value 10 min after the 1st dose (Fig. 2). However, the level of total IgA was significantly lower ($p<0.0001$) after methacholine provocation than after histamine. After the 2nd and the 3rd doses of histamine the level of total IgA was also high, whereas it decreased or even fell beneath the baseline with methacholine.

The secretory IgA level was significantly higher ($p<0.005$–0.0001) after provocation with methacholine

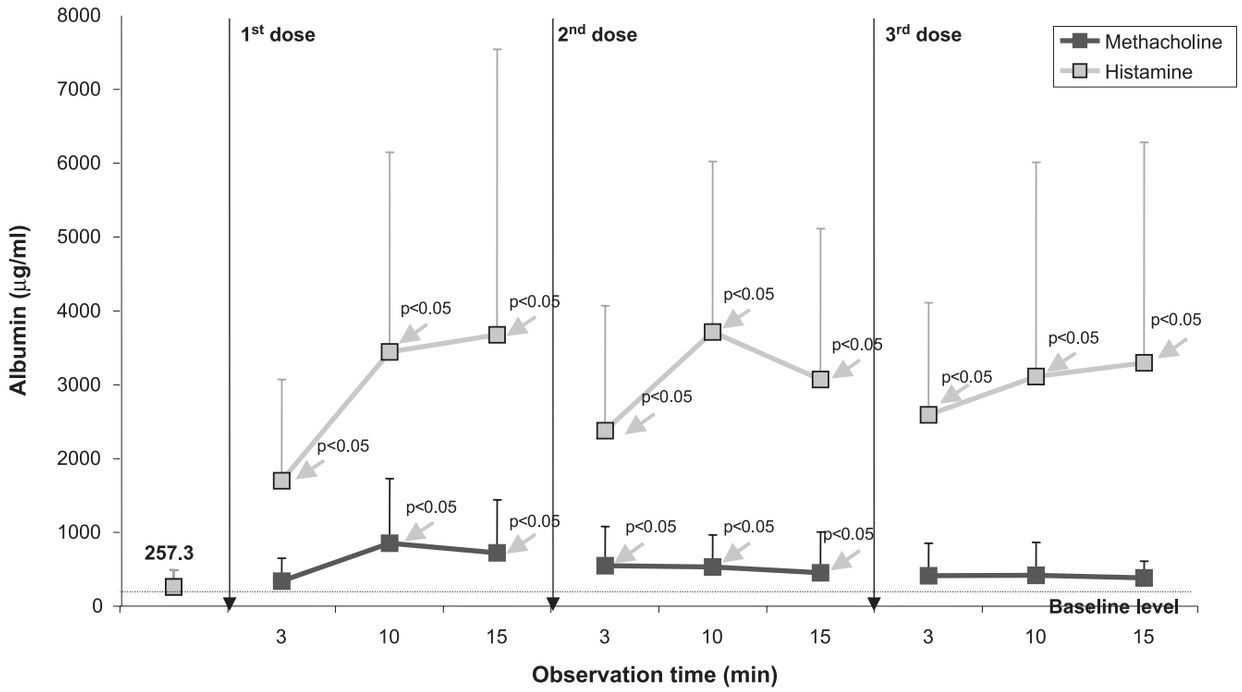

Fig. 1. Time-dependent observations of albumin content in nasal secretions after histamine or methacholine provocation. The nasal secretions were collected before spraying the provocating agent (baseline level) and 3, 10 and 15 min after provocation tests with 3 doses of histamine (0.5, 1, 4 mg) or methacholine (8, 16, 32 mg). The results are given as means and standard deviations. The long black arrows indicate the one-day intervals before the next dose of provocation agent spraying. The grey arrows indicate the statistically significant results: the p values ranged from <0.01 to <0.00001. For other details see Materials and Methods

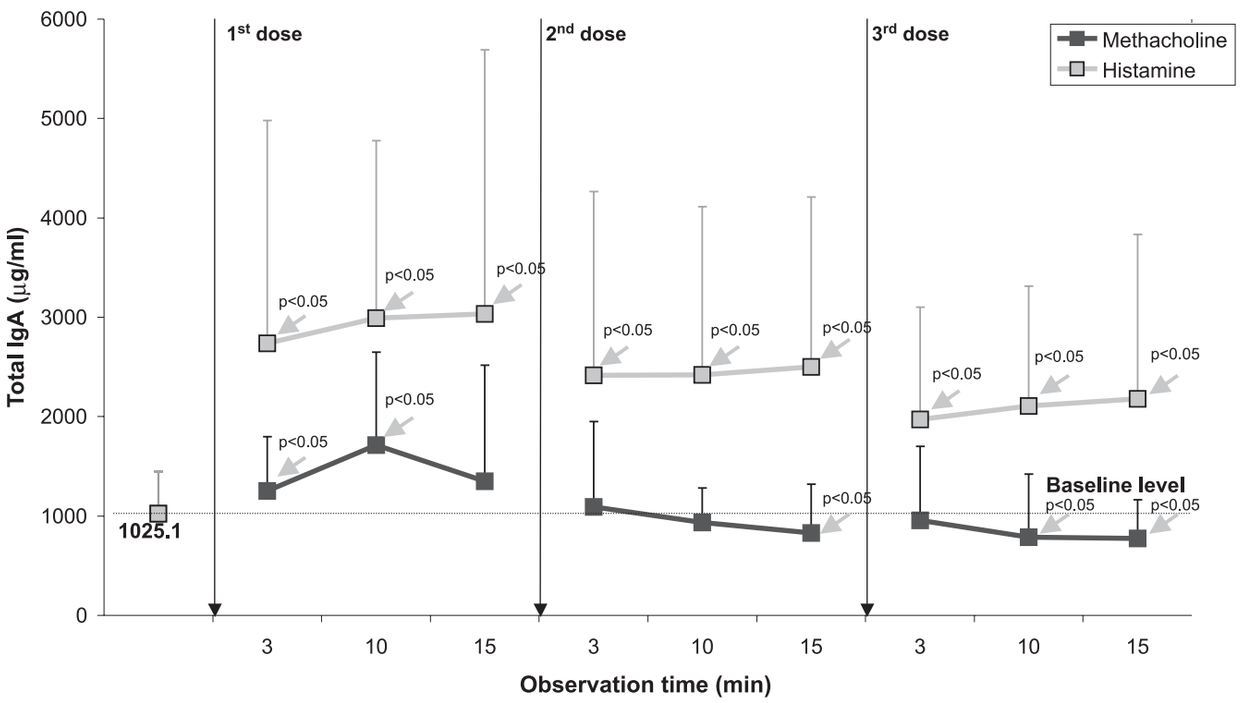

Fig. 2. Time-dependent observations of total IgA content in nasal secretion after histamine or methacholine provocation. For explanations see the Fig. 1

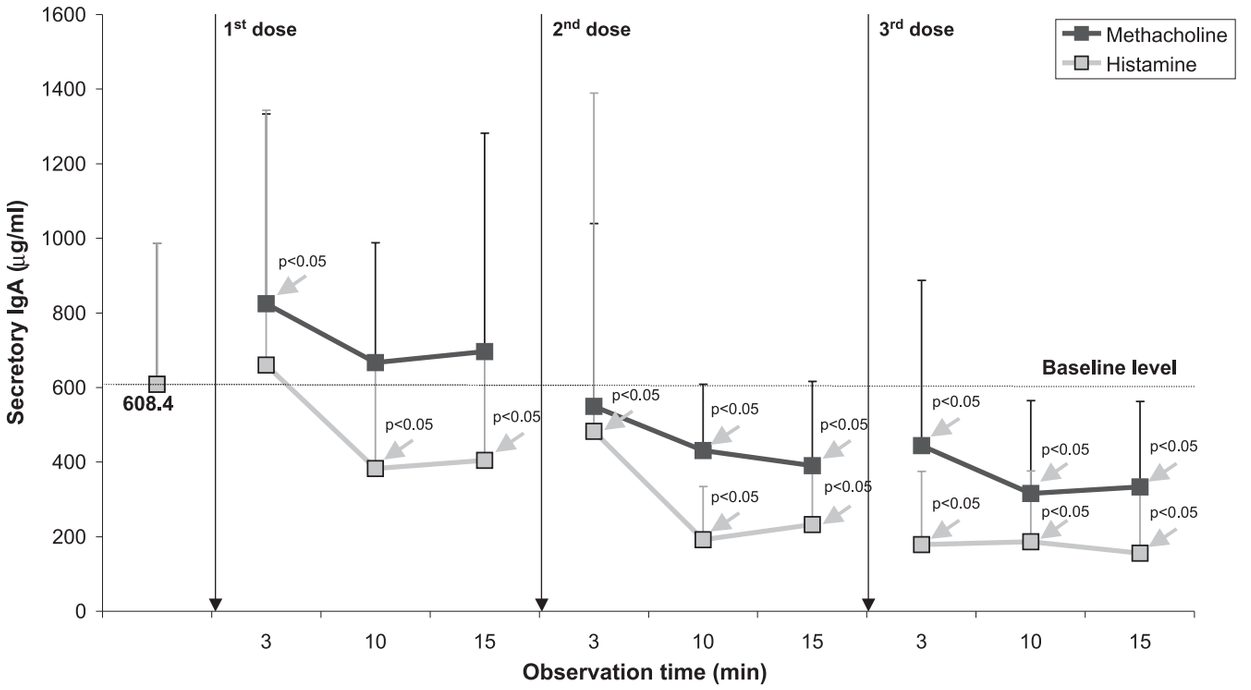

Fig. 3. Time-dependent observations of secretory IgA content in nasal secretion after histamine or methacholine provocation. For explanations see the Fig. 1

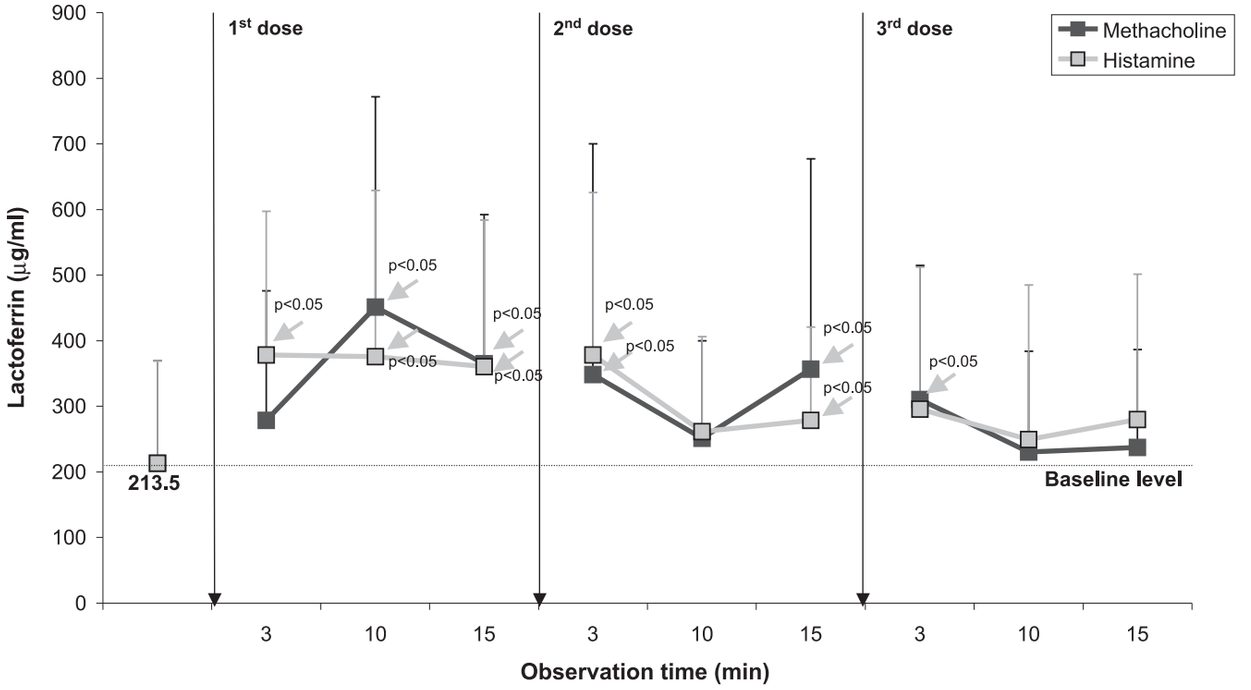

Fig. 4. Time-dependent observations of lactoferrin content in nasal secretion after histamine or methacholine provocation. For explanations see the Fig. 1

than after histamine. However, compared with the values before provocation, these values were higher only after 3 min of the 1st methacholine dose (Fig. 3). After that secretory IgA level gradually decreased, and after the 2nd and the 3rd doses fell even below the level of the baseline.

The increased level of lactoferrin in the nasal secretions after provocation with both agents was noticed after the 1st dose either. Then, the lactoferrin level gradually decreased, particularly 10 min after the 2nd and 3rd doses of both agents (Fig. 4).

Discussion

The time-dependent observations of marker levels of blood plasma leakage (albumin), glandular secretion (secretory IgA and lactoferrin) and a marker of both (total IgA) in nasal secretions prove that a series of provocations, performed with histamine, have a negative influence on the cholinergic stimulation of glandular challenged with methacholine. The statistically significant ($p< 0.0001$) increases in the levels of albumin (Fig. 1), total IgA (Fig. 2), non-secretory IgA (not shown) and the relative albumin amounts in total protein after histamine provocation of the nose (not shown) indicate incessant protein plasma leakage from the blood circulation into nasal secretion. These results of blood plasma leakage are in agreement with others[2, 14, 16]. The dramatic, statistically ($p<0.0001$) significant decrease of secretory IgA levels after methacholine challenges, even below the baseline value, as well as the gradual decrease of lactoferrin concentration after challenge with the 2nd and the 3rd doses of provocation agents were observed, contrary to the results published by RAPHAEL et al.[14, 16].

However, the current results should not be compared with the results of other investigators[8, 9, 16] uncritically because of some differences in the material sampling and protein elution. Nevertheless, the relative courses of concentration changes of total protein (not shown), albumin (Fig. 1), and total IgA (Fig. 2) after nasal provocation were consistent with those mentioned above. Moreover, in contrast to RAPHAEL et al.[14, 16], who performed two independent trials with methacholine[14], and histamine[16] provocations, we carried out a quite different schedule of the experiment. Our provocations of the nose were performed as a sequence of challenges to the nose of the same subject: first with histamine and then with methacholine in one day intervals. Instead of an increased secretory IgA level, as was observed by RAPHAEL et al.[14] after cholinergic stimulation provocated by methacholine only, we found a significant gradual decrease of secretory IgA ($p<0.01–0.0001$) and lactoferrin levels after nasal provocations with methacholine followed histamine.

As is commonly known, IgA is produced by plasma cells, especially of the periglandular nasal mucosa area. Released dimeric IgA diffuses through the periglandular intersitial connective tissue, then it binds to a membrane-bound, specific receptor for polymeric immunoglobulin on the basolateral surface of the glandular serous cells or on the ductalepithelial cells. The complex of receptor plus dimeric IgA is endocytosed and processed within the serous cell and the complete secretory IgA molecule is released into the lumen of the gland[3]. Thus, glandular secretions are the most important contributing source of secretory IgA, which plays a very important part in local immunity[3]. The hypothetical explanation of our results may be as follows: during the 15 min after nasal provocation with histamine and then with methacholine, a non-specific secretory IgA transportation blockade on the surface of epithelium cells or on the glands takes place, which may, of course, disappear after a longer period of time. Our research results referring to lactoferrin are consistent with the data of RAPHAEL et al.[15]. However, an increase in provocation agent dose results in a decrease of lactoferrin in total protein after histamine administration (regardless of the value increase in μg/ml), which is caused by a much bigger response from the side of, for example, albumin which results in rarefaction of lactoferrin in the total protein (Fig. 4).

In conclusion our results suggest a non-specific inhibition of the synthesis or/and secretion of secretory IgA and lactoferrin after a mixed type of nose provocation. As a consequence, this can lead to a significant decrease of specific and non-specific defense immunity of the nose membranes and a higher susceptibility of the upper airway mucosa to bacterial or viral infections.

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Received in January 2003
Accepted in April 2003