Rhinology, 34, 147-150, 1996

Electrophoretic pattern of physiological human nasal secretions*

C. Maremmani[1], B. Fattori[2], M. De Ciccio[2], R. Ceravolo[1], P.L. Ghilardi[2], A. Muratorio[1]

[1] Institute of Clinical Neurology, University of Pisa, Italy
[2] Institute of Otorhinolaryngology, University of Pisa, Italy

SUMMARY

Various proteins have been detected in human nasal mucus, but their electrophoretic pattern has not been extensively investigated. Therefore, we have studied this pattern in nasal mucus samples from a group of 40 healthy subjects (20 males and 20 females). The electrophoretic separations have been performed under both native and denaturing conditions. The electrophoretic pattern of nasal mucus obtained under denaturing conditions and stained with Coomassie brilliant blue R-250, reveals 17 distinguishable areas into which protein bands are divided, with no significant variations from one individual to another. Eight of these areas contain high concentrations of nasal mucus proteins, whereas lower concentrations are seen in the other nine areas. The electrophoretic patterns of nasal mucus differs from that of eye mucus. The method used for sampling is rapid, simple, requires no local anaesthetic, and supplies an adequate quantity of proteins for laboratory testing. Electrophoresis under denaturing conditions proves to be the better of the two techniques, since it permits good separation of proteins. Among the protein bands revealed in our study there are some already known proteins (i.a. pre-albumin, albumin, lysozyme), but the presence of other proteins which have yet to be identified cannot be excluded. Hence, a study is underway for the purification and identification, by amino-acid sequencing, of the various proteins which make up each band. A thorough definition of the protein pattern of nasal mucus might prove useful for detecting anomalies in its composition, for example, in cases of olfactory disorders.

Key words: human nasal mucus, electrophoresis

INTRODUCTION

In the past, nasal secretions were considered to be a biochemically-void entity and nasal mucus was therefore simply thought to be made up of water, electrolytes, lysozyme, immunoglobulins and some glycoproteins difficult to define. However, recent studies carried out on the nasal mucus of non-human vertebrates showed that it contains not only molecules which protect against potentially harmful external agents, but also proteins capable of transporting odours (Pelosi et al., 1981; Pevsner et al., 1986, 1988; Pelosi and Maida, 1990).

In human nasal mucus, various proteins have been identified (Table 1), but their electrophoretic pattern has not been extensively investigated. For this reason, we have studied the electrophoretic pattern of nasal secretions in healthy subjects; the study also aimed at finding out whether the pattern is constant or if it displays significant interindividual, sex- or age-related variations.

MATERIAL AND METHODS

The group studied consisted of 20 males (mean age: 29.35±10.16 years) and 20 females (mean age: 30.90±9.98 years). Subjects with the following criteria were excluded from the study: (1) clinical history of smoking; (2) exposure to volatile irritants; (3) acute or chronic disease of the upper respiratory tract; and (4) pharmacological treatment. The sample of mucus was taken from seated subjects with their heads bent backwards at an angle of 30°. The nasal fossae were made visible under anterior rhinoscopy with a long-valve Killian speculum. The sample was obtained using a square of sterile gauze, 1×1 cm^2 and approximately 2 mm thick, held between the tips of a Hartman forceps. The gauze was gently passed between the inferior turbinate and the nasal septum. From each subject, two specimens were taken from each nasal fossa.

* Received for publication May 16, 1995; accepted January 8, 1996

Table 1. proteins described in the human nasal mucus.

proteins	secretory sites	ref.
immunoglobulins (Ig A,G,M,E)	secretory immune system	1
albumin	serum	2
secretory component	Bowman's glands and other nasal glands	3
lysozyme	serous cells of nasal glands and goblet cells	4-5
antiprotease	submucous gland or serum	6-10
substance P	nerve endings of the nasal mucosa (?)	11
VIP	nerve endings of the nasal mucosa (?)	11
GOT, GPT, LDH, CPK, amlylase	cells of the nasal mucosa	12,13
J chain	?	3
lactoferrin	?	3
transferrin	?	14
fibrinogen	?	14
kallikreins	?	15
prevalbumin	?	16
odorant-binding protein	?	17
apocrine secretion odour-binding proteins	?	18

1) Rossen and Waldmann, 1967; 2) Mikulewicz, 1968; 3) Getchell and Mellert, 1991; 4) Ogawa et al., 1979; 5) Van den Oord et al., 1982; 6) Tachibana et al., 1986a; 7) Ohlsson and Tegner, 1976; 8) Hochstraber, 1983; 9) Thompson and Ohlsson, 1986; 10) Frysmark et al., 1989; 11) Chaen et al., 1993; 12) Schorn and Hochstrasser, 1976; 13) Tachibana et al., 1986b; 14) Widdicombe and Wells, 1982; 15) Proud et al., 1989; 16) Redl et al., 1992; 17) Pelosi et al., 1990; 18) Spielman et al., 1995

The pieces of gauze imbibed with nasal mucus were then placed in a plastic tip (into which a piece of sterile gauze had been pushed previously to act as a filter for the larger particles). The tip was then placed in a test tube and 150 µl of chilled (5°C) buffer (20 mM Tris/HCl buffer, pH 7.4) were added. The mucus was eluted by centrifugation at 3,000 rpm for 15 min at a constant temperature of 5°C. The mucus sample was then aspirated from the bottom of the test tube and filtered through an 0.2-µm filter. Total protein concentrations were immediately determined according to Bradford (1976). The calibration curves were achieved using samples of known concentrations of bovine serum albumin (BSA). At this point the sample was divided into aliquots and stored at -80°C. To facilitate pattern comparison, varying quantities of chilled (5°C) buffer (20 mM Tris/HCl buffer, pH 7.4) were added to each sample, prior to electrophoresis, to obtain the same concentration of total proteins (100 mg%).

The day after nasal mucus sampling, a sample of eye mucus was taken from each subject by means of a rectangular strip of sterile gauze (0.5×1 cm^2; approximately 2 mm thick) which was placed for 1 min in the lower eyelid fornix. The piece of gauze imbibed with eye mucus was placed in a small plastic tip which was then put into a test tube. Fifty microlitres of chilled (5°C) buffer (20 mM Tris/HCl buffer, pH 7.4) were poured over the mucus-soaked gauze. The eye mucus was eluted by centrifugation at 3,000 rpm for 15 min at a constant temperature of 5°C. The mucus sample was then aspirated from the bottom of the test tube, filtered through an 0.2-µm filter and stored at -80°C.

The electrophoretic separations were performed under denaturing (Laemli, 1970) and native conditions. Native separations were performed in the same way as under denaturing conditions, except for the use of sodium dodecyl sulfate and ß-mercaptoethanol. Electrophoresis was carried out with a Bio-Rad Mini-Protean II System, with runs of 45 min each at 200 V. The concentration of acrylamide in the running gel was 12%, with 3% of bis-methyleneacrylamide. The following standard proteins were used as references: bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.2 kDa). The proteins on the gels were then stained with Coomassie brilliant blue R-250.

RESULTS

The average volume of the nasal mucus samples was 316.57±52.62 µl, with an average total protein concentration of 335.40±72.07 mg%. The total volume of each sample also included the 150 µl of buffer used to elute the mucus-soaked gauze, with the consequent constant elution of the nasal mucus total proteins. The electrophoresis carried out under native conditions did not yield adequate separation of the nasal mucus proteins (Figure 1), whereas that performed under denaturing conditions achieved a better separation of the protein bands, with molecular weights ranging from 14 to 70 kDa. The

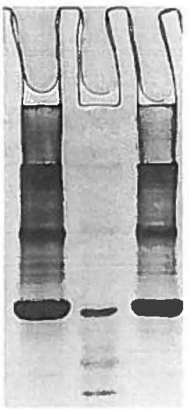

Figure 1. Native electrophoresis. Nasal mucus in lanes 1 and 3 (diluted in sample buffer 1:2 and 1:4, respectively). Standard proteins (bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin) were loaded in lane 2.

electrophoretic pattern of the nasal mucus, obtained under denaturing conditions and by staining the gels with Coomassie brilliant blue R-250, showed no variation from one individual to another, nor was there a relationship with age and sex. The pattern displayed 17 distinct areas, in which the protein bands were grouped together. The most abundant proteins, which were also visible in the gels obtained from the mucus samples diluted with greater quantities of sample buffer, produced eight areas (numbered 1 to 8; cf. Figure 2, lane 6). The first area (approximately 70 kDa) showed two bands; the second one, ranging from 50-65 kDa, showed a triplet; the third (45 kDa), had a group of 2-3 thinner bands; the fourth (approximately 38 kDa) one band only; the fifth (approximately 27 kDa) was an intense but rather blurred line; the sixth (approximately 25 kDa) was one band, as were the seventh (16 kDa) and the eighth (<14 kDa). The pattern also included nine fainter lines (numbered I to IX; cf., Figure 2, lane 2) corresponding to nasal mucus proteins present in lower concentrations. These bands, which

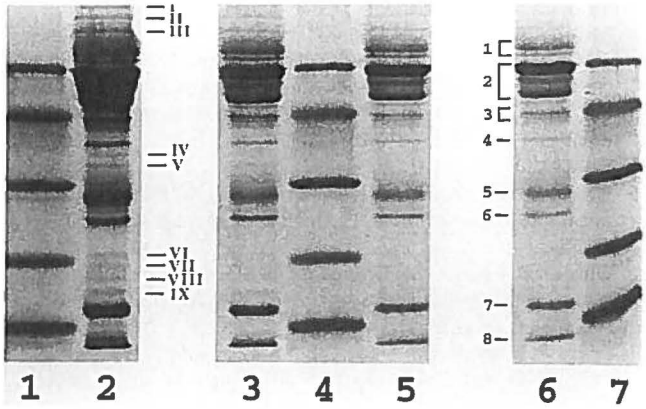

Figure 2. Electrophoretic pattern under denaturing conditions. Nasal mucus in lanes 2, 3, 5, and 6 (diluted in sample buffer 1:2, 1:4, 1:5, and 1:7, respectively). Standard proteins of 66, 45, 29, 20, and 14.2 kDa (from top to bottom) were loaded in lanes 1, 4, and 7.

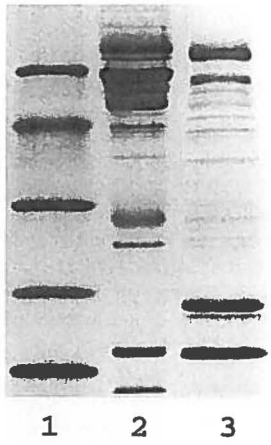

Figure 3. Electrophoretic pattern of nasal (lane 2) and eye mucus (lane 3) under denaturing conditions. Standard proteins of 66, 45, 29, 20, and 14.2 kDa (from top to bottom) were loaded in lane 1.

were visible only in those gels belonging to the samples of mucus which had been diluted less with the sample buffer, were distributed as follows: three bands (I-III) >70 kDa; two bands (IV-V) between 32 and 35 kDa, and four lines (VI-IX) between 17.5 and 21 kDa. The eye mucus pattern was not superimposable, however, to that of the nasal mucus (Figure 3).

DISCUSSION

To date there appears to be no common opinion with regard to the best technique for collecting nasal mucus secreted under physiological conditions. In fact, each method has its advantages and disadvantages. Some authors (Eichner, 1983) use gauze pads placed inside the nasal fossae which are kept there for 20 min before removing them for centrifugation to obtain the mucus. With this method the amount of mucus collected can be calculated; however, the lengthy, albeit necessary, irritative stimulation of the mucus can alter the actual composition of the mucus (particularly with contamination by ocular mucus).

Other authors (Naclerio et al., 1983; Klementsson et al., 1991) prefer flushing each of the nasal fossae with 5 ml of normal saline solution. The advantage of this method is that it reduces irritation to a minimum; nevertheless, the pitfall lies in the fact that it is rather difficult to retrieve all of the flushing liquid and it is, therefore, impossible to establish an exact measurement of the mucus. This makes the method difficult to standardize. Moreover, the protein concentration achieved in this way is rather low, which is a drawback when using the sample for biochemical techniques (such as separation and purification of protein molecules).

The method proposed in our study is not only rapid and easy to perform, but is also minimally irritant since the small square of gauze is gently passed between the inferior turbinate and the nasal septum for only a few seconds (5-10 s). For this reason, the method can be applied to large-scale screening of both normal subjects and patients.

This technique also permits a precise measurement of the mucus collected by calculating the difference between the total volume obtained after centrifugation of the gauze and the known quantity of buffer solution used for washing the gauze itself. Previous studies have shown that total protein concentrations of nasal mucus samples display some variability (Eichner, 1983), and this is in agreement with what has been described for other biological fluids. This fact is confirmed by our study; however, with the method we used samples demonstrate more homogenous levels of total protein. This suggests that the method of sampling, in itself, might influence the relative biochemical result.

Electrophoretic separation performed under native conditions is not suitable for adequate characterization of the protein bands from nasal mucus; in fact, under these conditions the proteins migrate while retaining their quaternary structure but the high-molecular-weight glycoproteins of nasal mucus are unable to penetrate the gel, and many remain trapped at the bottom of the well. The pattern obtained under denaturing conditions in this study was much more stable and the protein bands were decidedly more numerous and better defined than those in previous investigations (Eichner, 1983). The type of gel used in this study produced a good separation of the proteins with molecular weights ranging from 14 to 70 kDa (the majority of nasal mucus proteins are to be found in this range). Consequently, proteins with either higher or lower molecular weights will be defined better with gels that have either a lower or greater concentration of acrylamide, respectively, or even with gradient gel acrylamide.

The pattern showed no difference between the two sexes, nor was there any age-related variation in the group we investigated. We found that when the gels were stained with Coomassie brilliant blue, there were 17 areas in which the protein bands were constantly present. We did not use any of the more sensitive analysing techniques, such as silver staining (Switzer et al., 1979), since we were only interested in defining the electrophoretic pattern of those proteins that were quantitatively greater in nasal mucus. Already known proteins make up some of the protein bands we detected. For example, albumin runs at approximately 66 kDa; apocrine secretion odorant-binding protein-I (Spielman et al., 1995) and pre-albumin, the latter a member of the lipophilic-ligand carrier protein superfamily (Redl et al., 1992), run at 45 and 20 kDa, respectively. Lysozyme

runs at approximately 16 kDa. It must be stressed, nevertheless, that each band visible on electrophoresis may be composed of just one protein or of different molecules with very similar molecular weights. Lastly, a single band may be composed of various isoforms, as is the case in odorant-binding protein-I present in vertebrate species other than human (Felicioli et al., 1993). It is therefore essential to find out whether the electrophoretic bands are made up entirely of already known proteins or if there are molecules that have not yet been investigated. In this respect a study is underway to purify and, subsequently, identify by means of amino-acid sequencing, the single molecules that make up the various bands mentioned above. Nasal mucus contains proteins which are also found in eye mucus, such as lysozyme, lactoferrin, secretory immunoglobulin A (Fleming and Allison, 1922; Broekhuyse, 1974; Allansmith and Hutchison, 1967), and prealbumin (Redl et al., 1992). Nevertheless, the electrophoretic pattern of the two secretions is not superimposable due to the fact that there are more protein bands in nasal mucus (cf., Figure 3). To a certain extent this may be related to the fact that the proteins in common can be present in different concentrations in the two secretions, or even that certain proteins are exclusive to either one of both secretions.

This study has led to a more precise definition of the electrophoretic pattern of nasal mucus of normal subjects, by means of a standardized mucus sampling method. This is undoubtedly preliminary knowledge, but nevertheless indispensable for further investigations aimed at the identification of possible anomalies in the pattern and in the protein composition of nasal mucus of patients affected by nasal disease or by olfactory alterations of various origin.

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Dr. Bruno Fattori
ENT Clinic
University of Pisa
Via Savi 10
I-56100 Pisa
Italy