Nasal cytology: the "infectious spot", an expression of a morphological-chromatic biofilm

M. Gelardi, G. Passalacqua, M. L. Fiorella, A. Mosca, N. Quaranta

Nasal cytology: the " infectious spot ", an expression of a morphological-chromatic biofilm

Matteo Gelardi MD[1], Giovanni Passalacqua MD[2], Maria Luisa Fiorella MD[1], Adriana Mosca BSC[3], Nicola Quaranta MD[1]

[1] Department of Otolaryngology, University of Bari, Italy

[2]Allergy and Respiratory Diseases, University of Genoa, Italy

[3] Microbiology, Department MIDIM, University of Bari, Italy

Correspondence
Matteo Gelardi, M.D.
Department of Otolaryngology, University of Bari, Piazza G. Cesare, 11 - 70124 Bari, Italy
Tel: + 39 080 5592388
Fax: +39 080 5593315
E-mail: gelardim@inwind.it

Keywords: nasal cytology, Biofilms, Infectious Spot, infectious rhinitis, nasal mucosa.

Abstract

The purpose of study was to describe some "morphological-chromatic" patterns (i.e.spots of cyan colour) identified during the study of nasal cytology in patients with both bacterial and fungal infectious rhinological disorders. These peculiar aspects strongly suggest the presence of a microscopic biofilm. We retrospectively examined 1,410 nasal cytology specimens from subjects who underwent clinical-instrumental investigations (history, ENT visit, nasal endoscopy and nasal cytology) from January to August 2010. The control samples were represented by 30 subjects not suffering from infectious rhinological diseases. The presence of particular spots of "Cyan", was found in colour in 107/1,410 rhinocytograms (7.6 %) within which bacterial colonies and/or fungal spores were found. We called these colored spot formations "Infectious Spots" (IS).

The positivity to PAS staining confirmed the polysaccharide nature of the colored spots and allowed us to relate them to biofilms. This study demonstrates, for the first time, that nasal cytology performed by optical microscope can play an important role in detecting biofilms.

Introduction

Over the last years nasal cytology is increasingly used in the study of either allergic and vasomotor rhinological disorders, or infectious and inflammatory rhinitis [1,2]. Since nasal cytology can detect cellular changes of epithelium exposed to physical-chemical inflammation [3,4], acute or chronic infections of different nature (viral, bacterial, fungal or parasitic) [5,6], it has been matter of interest, both in basic and clinical research.

Several reasons contributed to the increased interest in this diagnostic method: among them, the simplicity of the cytological technique and the lack of invasiveness, which makes possible to repeat the examination in the follow-ups and monitoring of therapy. The nasal cytology has characteristics suitable for its application in the biomedical and clinical research, outpatient care, and it can be performed on subjects of any age [7].

Over the years, several studies on nasal cytology helped to clarify some of the pathophysiological mechanisms underlying IgE-mediated rhinitis, as well as the identification of new classified entities such as non-allergic rhinitis with eosinophilia (non-allergic rhinitis with eosinophils , NARES), with mast cells (non-allergic rhinitis with mast cell, NARMA), neutrophilic forms (non-allergic rhinitis with neutrophils, NARNE) and, finally, the eosinophil-mast cell (non-allergic rhinitis with eosinophils and mast cell, NARESMA) [8-11].

Over the last decade, rhinological diagnosis has been devoted, to the possibility to relate chronic rhinopathies (rhinosinusitis, nasal polyps, adenoid hypertrophy, etc.) with the presence of biofilms[12-16].

The investigation of biofilms has increased its possibilities with the evolution of the methods of investigation, whereas the early work was based on scanning electron microscopy (SEM) [17], and transmission electron microscopy (TEM) [18], which clarify the ultramicroscopic fine structure. Indeed electron microscopy and specific staining of polysaccharides with ruthenium identify the nature of the extracellular fibers as biofilms and their association with cells [19,20]. However, the solvents necessary for the gradual dehydration of the sample, is responsible for generating images of biofilms with artifacts. In the ‘90 the development of confocal laser scanning microscopy (CLSM) [21] gave the possibility to examine biofilms *in situ* without limitations, although at a lower resolution, in comparison with SEM. Most of studies on biofilms currently available are aimed to understanding either the mechanisms of drug resistance systems [22-24] in order to prevent contamination of medical devices (cochlear implants, cardiac valves, venous catheters, urinary catheters, contact lens, intrauterine devices, etc.) [25-27] or the pathophysiological mechanisms of major diseases (endocarditis, cystic fibrosis, chronic rhinosinusitis, otitis media, etc) [28-31]. This study describes ,for the first time, the significance of "morphological-chromatic" entities found during nasal cytology in patients with rhinological disorders.

Materials and Methods

From January to August 2010 we examined the nasal cytology of 1,410 subjects, 826 (58.5%) males, 584 (41.4%) females, aged between 2 and 74 years (average 32). All enrolled subjects underwent clinical-instrumental investigations (history, ENT visit, nasal endoscopy and nasal cytology).

Control sample

The control sample consisted of 30 subjects (12 males and 18 females) aged between 5 and 68 years (average 44), and admitted to hospital because of non-infectious diseases: voice disorders from chronic vocal abuse (4), tinnitus (6), neurosensorial hearing loss (9), trigeminal neuralgia (5); vertiginous syndromes secondary to cupololithiasis (6).

Nasal cytology

The nasal cytology was performed by anterior rhinoscopy, using a nasal speculum and good lighting. The collection technique consisted of scrapings from the middle portion of the inferior turbinate, using a Rhino-Probe ® [7]. Anesthesia was not necessary. In young and/or uncooperative patients cell sample was obtained by a nasal swab. Briefly, the cellular material was placed on a glass slide, fixed by air drying and then stained by the May-Grunwald Giemsa (MGG) method (Carlo Erba ® , Milan, Italy). MGG staining is the most widely used method in diagnostic nasal cytology, because all the cellular components of the nasal mucosa, from inflammatory cells (neutrophils, eosinophils, mast cells, lymphocytes) up to bacteria, spores, fungal hyphae, and mucous secretions are easly stained. Furthermore, to confirm the nature of the polysaccharide secretions present in the cytological preparations, a special staining method (PAS-Periodic Acid-Schiff) was used [32]. The slide was observed by a Nikon E600 light microscope (Nikon, Canada) equipped with a digital camera (Nikon "Coolpix 3:34") for the acquisition of microscopic images. For the rhinocytogram analysis, 50 microscopic fields were read at a magnification of 1000 x, to assess the presence of normal and abnormal cellular elements, along with any microscopic features (spots, special inclusions, etc.) important for the diagnosis. Cell counts, bacterial and fungal analysis, were carried out by a semi-quantitative grading, as proposed by Meltzer [33]. In particular, bacteria and fungal spore assessment was determined as follows:

Grade 0 (not visible);

Grade 1 + (occasional groups);

Grade 2 + (moderate number);

Grade 3 + (easily visible);

Grade 4 + (many of which cover the entire field of view).

Results

Control group

Control group showed a normal nasal cytology, characterized by the presence of many hair cells and muciparous cells, at the typical ratio 4-5:1. Neither immune-inflammatory cells (eosinophils, mast cells etc.) with the exception of some neutrophils (Grade 1 +), nor items such as bacteria, spores and/or fungal hyphae (Grade 0) were observed. Specific structures and/or colour variations of doubtful significance were not found..

Study Group

193/1,410 (13.6%) rhinocytograms had cytological signs of infectious rhinitis, characterized, at a different degree from grade 1 + to 4 +, by inflammatory infiltration (i.e. neutrophils, intra and extracellular bacteria, and/or fungal spores). In 107/1,410 (7.6%) rhinocytograms specific "Cyan" colour patches corresponding to a wavelength of 480 nanometers, were present (Fig. 1 a, b, c, d).

Since bacteria and/or fungal spores were observed within colour samples of different, size the possibility that these formations could be the expression of biofilm prompted us to perform a specific staining for polysaccharides. (ì.e. periodic acid staining - Schiff's reagent - PAS), which are the main constituents of the matrix of biofilm [34]. The positivity to PAS staining (Fig. 2) confirmed the polysaccharide nature of chromatic stains thus, confirming their association with biofilms. This morphological-chromatic expression was termed “infectious spot” (IS).

DISCUSSION

Already in 1600 Anton van Leeuwenhoek, scraping the surface of teeth, observed with his primitive microscope, the "animaluculae", which were nothing but microbial aggregates. It took many years of research with advanced diagnostic equipment to identify the chemical and genetic structure of these bacterial colonies, which were later named "biofilm". Thanks to electron microscope studies, we presently know that biofilm consists of bacterial colonies (around 15% of the volume), surrounded by an organic matrix produced by themselves, whose skeleton is composed of exopolysaccharides [34]. The amount of polysaccharides is variable, and tend to increase as the age of biofilms increases. Extracellular proteins and DNA have been also identified in the biofilm matrix. The physico-chemical nature of the biofilm, confer resistance to antimicrobial agents such as antibiotics, disinfectants and detergents. This is not a genetic antimicrobial resistance (i.e. carried by plasmids, or related to mutational events), but is rather due to the ability of individual cells inside the biofilm to differentiate into a phenotypically antibiotic tolerant state [35]. Other mechanisms intervene, including the delayed penetration of the antimicrobials through the matrix of the biofilm or the abnormal growth of organisms within the biofilm [36,37]. On this ground, identification of biofilms has a not negligible clinical relevance also in rhinology [31]. Nonetheless, the electron microscope techniques are expensive, poorly accessible and require special processing of samples, which can also result in artifacts. For this reason we attempted to identify biofilms in nasal mucosa, by means of nasal cytology, evaluating a large number of scrapings.

During the cytological study, in a large proportion of patients with infectious rhinitis, we repeatedly observed particular chromatic spots, which were variable in size, had

irregular and blurred margins, and an intense cyan coloration (wavelength around 480 nm). Such colour attracted our attention, since this chromatic band does not fall in the colour spectrum of nasal cytology, when stained with MGG. In addition, the presence of bacteria or fungal spores, suggested that those spots (infectious spots) could be the optical microscopy expression of biofilm. Thus, when infectious spots were observed, a staining with PAS, which is polysaccharide-specific, was carried out. That staining resulted invariably positive when micro-organisms were present, thus we confirmed the biofilm-related nature of the infectious spots. Of note, the infectious spot, which always remain in the spectrum of the cyan color, may have variable shades. This is probably attributable to the age of biofilms, which when more mature is more rich in polysaccharides, and consequently has a more intense coloration. Nasal cytology in infectious rhinological diseases, has been predominantly focused the presence or absence of bacteria in the planctonic state, which are known to represent only a small percentage (<10%) of bacterial biomasses in nature which, in contrast, are mostly organized in biofilms. The present study demonstrates for the first time that nasal cytology is able to identify biofilms in nasal scrapings, by a relatively simple and economic technique. In the light of these results, we are confident that the nasal cytology will find applications not only in experimental fields, but also in diagnosis and therapy of allergic and nonallergic rhinitis. Further comparative studies are, nonetheless, needed to validate our observations.

Figure Legends

Fig 1 A,B,C,D: Spot infectious. Visible colonies of bacteria (a, c) or fungal spores (b, d) embedded in a brightly colored cyan matrix. Staining MGG - 400 and 1000X magnification

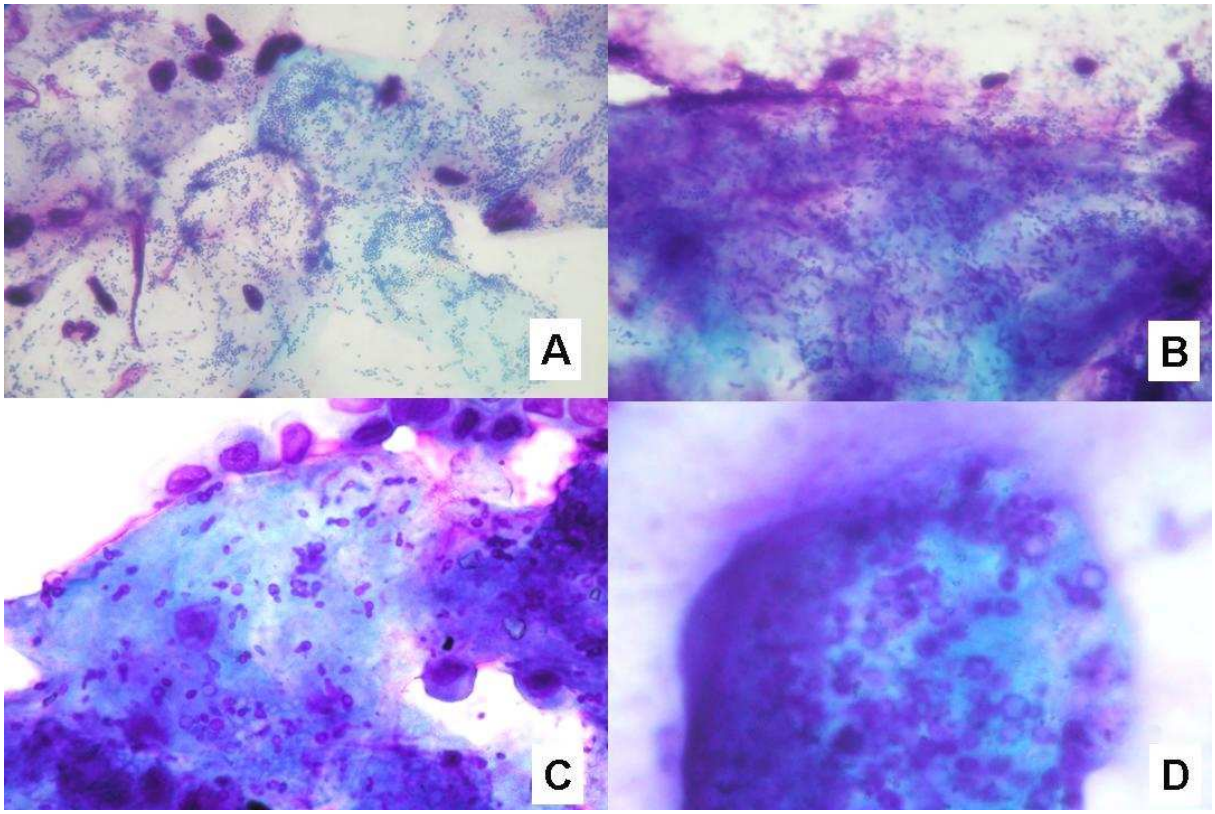
Figure 2 A,B,C,D Spot infectious. The PAS staining confirmed the polysaccharide nature of the matrix. PAS staining - 1000X magnification

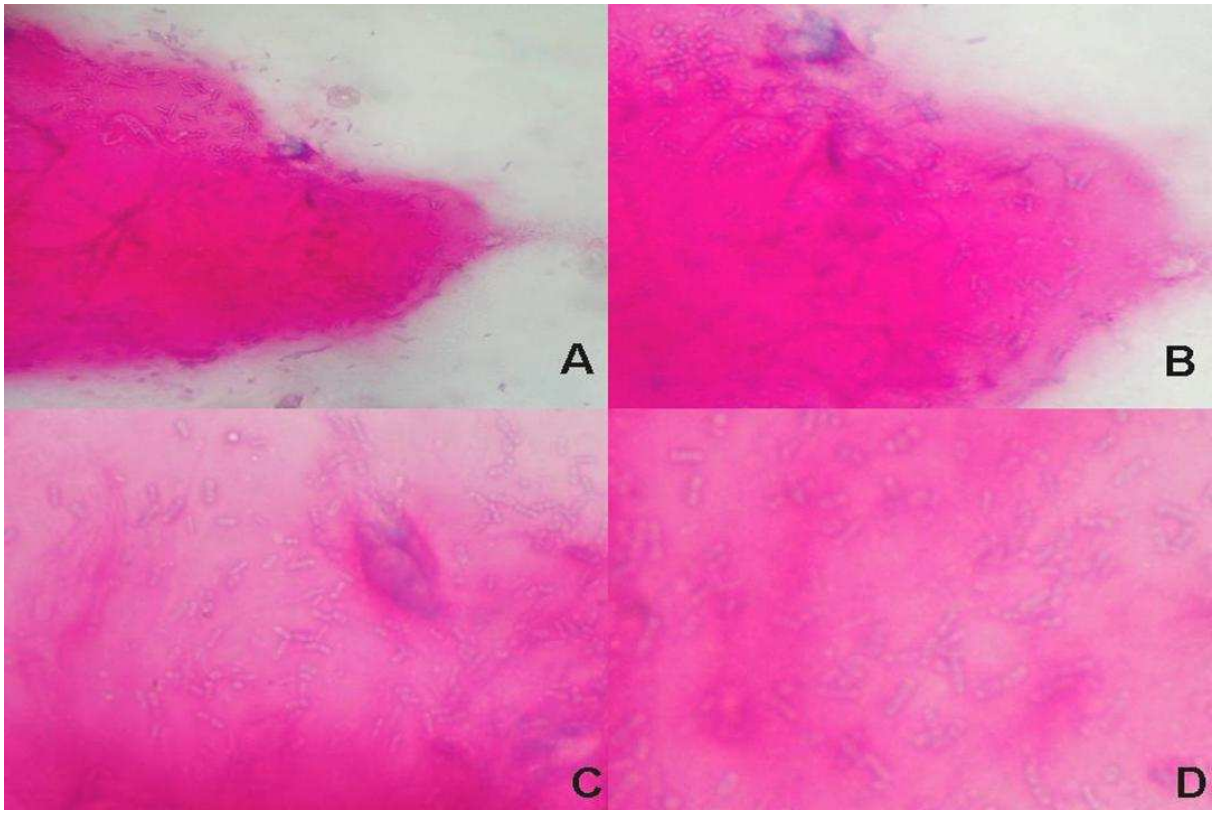
REFERENCES

1) Bogaerts P, Clement P (1981) The diagnostic value of a cytogram in rhinopathology. Rhinology 19:203-208
2) Malmberg H, Holopainen E (1979) Nasal smear as a screening test for immediate-type nasal allergy. Allergy 34:331-337
3) Glück U, Schütz R, Gebbers JO (2003) Cytopathology of the nasal mucosa in chronic exposure to diesel engine emission: a five-year survey of Swiss customs officers. Environ Health Perspect 111:925-929
4) Boysen M, Zadig E, Digerne V (1990) Nasal mucosa in workers exposed to formaldehyde: a pilot study. Br J Ind Med 47:116-121
5) Gelardi M, Tomaiuolo M, Cassano M, Besozzi G, Fiorella ML, Calvario A, Castellano MA, Cassano P (2006) Epstein-barr virus induced cellular changes in nasal mucosa. Virology Journal 3:6-10
6) Gelardi M, Fiorella M.L, Leo G, Incorvaia C (2007) Cytology in the diagnosis of rhinosinusitis. Pediatr Allergy Immunol 18:50-52
7) Gelardi M (2006) Atlas of nasal cytology. Centro Scientifico Editore -Turin (Italy)
8) Bickmore JT, Marshall ML (1976) Cytology of nasal secretions: further diagnostic help. Laryngoscope 86:516-523
9) Jacobs RL, Freedman PM, Boswell RN (1981) Non−allergic rhinitis with eosinophilia (NARES syndrome): clinical and immunologic presentation. J Allergy Clin Immunol 67:253-257
10) Connell JT (1969) Nasal Mastocytosis. J Allergy 43:182-189
11) Gelardi M, Maselli Del Giudice A Fiorella ML, Fiorella R, Russo C, Soleti P, Di Gioacchino M, Ciprandi G (2008) Non-allergic rhinitis with eosinophils and mast cells constitutes a new severe nasal disorder. Int Journal Immunopathol Pharmacolol 23:325-331
12) Ferguson BJ, Stolz DB (2005) Demonstration of biofilm in human bacterial chronic rhinosinusitis. Am J Rhinol 19:452-457

13) Głowacki R, Strek P, Zagórska-Swiezy K, Składzień J, Oleś K, Hydzik-Sobocińska K, Miodoński A (2008) Biofilm from patients with chronic rhinosinusitis. Morphological SEM studies. Otolaryngol Pol 62:305-310
14) Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald PJ (2007) Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. Laryngoscope 117:1302-1306
15) Sanclement JA, Webster P, Thomas J, Ramadan HH (2005) Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis. Laryngoscope 115:578-582
16) Vlastarakos PV, Nikolopoulos TP, Maragoudakis P, Tzagaroulakis A, Ferekidis E (2007) Biofilms in ear, nose, and throat infections: how important are they? Laryngoscope 117:668-673
17) Ganderton L, Chawla J, Winters C, Wimpenny J, Stickler D (1992) Scanning electron microscopy of bacterial biofilms on indwelling bladder catheters. Eur J Clin Microbiol Infect Dis 11:789-796
18) Marrie TJ, Costerton JW (1984) Scanning and transmission electron microscopy of in situ bacterial colonization of intravenous and intraarterial catheters. J Clin Microbiol 19:687-693
19) Bryers JD, Characklis WG (1982) Processes governing primary biofilm formation. Biotechnol Bioeng 24:2451-2476
20) Siebel MA, Characklis WG (1991) Observations of binary population biofilms. Biotechnol Bioeng 37:778-89
21) Palmer RJ Jr, Sternberg C. (1999) Modern microscopy in biofilm research: confocal microscopy and other approaches. Curr Opin Biotechnol 10:263-268
22) Hoyle BD, Costerton JW (1991) Bacterial resistance to antibiotics: the role of biofilms. Prog Drug Res 37:91-105
23) Prince AS (2002) Biofilms, antimicrobial resistance, and airway infection. N Eng J Med 347:1110-1111
24) Davies DG (2003) Understanding biofilm resistance to antibacterial agents. Nature 2:114-122
25) Tonnaer EL, Mylanus EA, Mulder JJ, Curfs JH (2009) Detection of bacteria in healthy middle ears during cochlear implantation. Arch Otolaryngol Head Neck Surg 135:232-237
26) Dickinson GM, Bisno AL (1989) Infections associated with indwelling devices: infections related to extravascular devices. Antimicrob Agents Chemother 33:602-607

27) Schumm K, Lam TB (2008) Types of urethral catheters for management of short-term voiding problems in hospitalized adults: a short version Cochrane review. Neurourol Urodyn 27:738-746
28) Liu YC, Post JC (2009) Biofilms in pediatric respiratory and related infections. Curr Allergy Asthma Rep 9:449-455
29) Mladina R, Poje G, Vuković K, Ristić M, Musić S (2008) Biofilm in nasal polyps. Rhinology 46:302-307
30) Ramadan HH (2006) Chronic rhinosinusitis and bacterial biofilms. Curr Opin Otolaryngol Head Neck Surg 14:183-186
31) Post JC, Hiller NL, Nistico L, Stoodley P, Ehrlich GD (2007) The role of biofilms in otolaryngologic infections: update. Curr Opin Otolaryngol Head Neck Surg 15:347-351
32) Nevalainen TJ, Laitio M, Lindgren I (1972) Periodic acid Schiff (PAS) staining of epon-embedded tissues for light microscopy. Acta Histochem 42:230-233
33) Meltzer EO, Jalowayski AA (1988) Nasal cytology in clinical practice. Am J Rhinol 2: 47-54
34) Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010; 8: 623-33
35) Drago L, Ripa S, Zampaloni C, De Vecchi E, Vitali LA, Petrelli D, Prenna M (2005) Activity of ceftibuten, cefaclor, azithromycin, clarithromycin, erythromycin and telithromycin against Streptococcus pyogenes clinical isolates with different genotypes and phenotypes. Chemotherapy 51:268-271
36) Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167-193
37) Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318-1322


A
B
C
D


A
B
C
D