

Eosinophil degranulation patterns in nasal polyposis: An ultrastructural study

Miguel Armengot, M.D., Ph.D.,¹ Luis Garín, M.D., Ph.D.,¹ and Carmen Carda, M.D., Ph.D.²

ABSTRACT

Background: Eosinophils are possibly the most important inflammatory cells in the pathogenesis of rhinosinusitis with nasal polyposis. Eosinophil degranulation is the mechanism by which these cells exert their inflammatory action. Knowledge of eosinophil state and degranulation mode therefore may help us to better understand this disease. A study is made of eosinophil state and degranulation mode using transmission electron microscopy (TEM), attempting to establish correlations with certain clinical variables considered to be of importance in patients with nasal polyposis.

Methods: A prospective TEM study was made to examine 582 eosinophils under 5000× magnification, classifying them according to their state and degranulation mode. The cells originated from 36 cases of nasal polyposis and were catalogued according to the clinical–radiological presentation of the disorder, the presence of asthmatic disease, or acetilsalicylic acid (ASA) triad syndrome (nasal polyposis, asthma, and nonsteroidal anti-inflammatory drug intolerance), and the degree of nasal tissue eosinophilia of the case of origin.

Results: A total of 30.75% of the eosinophils were inactive, 41.75% exhibited piecemeal degranulation (PMD), 27.5% exhibited cytolysis, and 0.34% were in apoptosis. The degranulation mode was significantly correlated to the clinical and histological parameters studied. Thus, cytolysis tended to be less pronounced and PMD greater, in the cells from cases with intense eosinophilia or severe polyposis (high clinical stage and ASA triad).

Conclusion: Cytolysis and PMD are the principal degranulation modes of eosinophils in nasal polyposis—apoptosis being very infrequent. Nasal polyposis shows a correlation between eosinophil degranulation mode and the clinical and radiological stage and the degree of tissue eosinophilia of the case of origin. (Am J Rhinol Allergy 23, 466–470, 2009; doi: 10.2500/ajra.2009.23.3357)

Key words: ASA triad, asthma, cytolysis, electron microscopy, inflammation, nasal eosinophilia, piecemeal degranulation, rhinosinusitis

Nasal polyposis can be defined as a chronic inflammatory process of the nasal and paranasal sinus mucosal membranes. Although the etiology remains uncertain, eosinophils appear to play an important role.¹ Chronic bacterial infection and allergy are not a major component of nasal polyp etiology.^{2,3} Nasal polyposis is quite often associated with intrinsic asthma and nonsteroidal anti-inflammatory drug (NSAID) intolerance. Patients with all three conditions are said to present acetilsalicylic acid (ASA) triad syndrome. The disease is characterized not only by the development of polyps within the nasal cavity but also by eosinophilic infiltration, which differentiates the condition from other forms of chronic rhinosinusitis.^{4,5}

Eosinophil degranulation is the mechanism by which these cells exert their inflammatory effects. Transmission electron microscopy (TEM) studies indicate that the main eosinophil degranulation modes are slow piecemeal degranulation (PMD)⁶ and cytolysis—the latter being characterized by cell membrane rupture and the appearance of clusters of free extracellular eosinophil granules (Cfges). It has been suggested that these degranulation mechanisms may be caused by different stimuli and can mediate different effects. Thus, PMD would appear to have more immune modulating and repair actions, while cytolysis would have important inflammatory effects.^{6,7}

In vitro studies have shown eosinophilic infiltration in different diseases to be regulated mainly by apoptosis-mediated eosinophil elimination and subsequent phagocytosis by macrophages.^{8,9} *In vivo* studies involving TEM stressed the importance of other eosinophil elimination routes such as transit toward the epithelial surface^{9,10} and cytolysis, which would trigger disintegration and subsequent disappearance of the cells.

The starting hypothesis of the present study is that eosinophils are the most important inflammatory cells in the pathogenesis of nasal polyposis. Given the relevance of the eosinophil intracytoplasmic

granules, the degranulation mode of these cells may be of interest in understanding the disease. A study is made of eosinophil state and degranulation mode using TEM, attempting to establish correlations with certain clinical variables considered important in patients with nasal polyposis.

MATERIALS AND METHODS

The study samples were obtained from 40 consecutively selected patients with idiopathic nasal polyposis. Patients with nasal allergy, asthma, and NSAID intolerance were included. Patients treated with topical corticoids were accepted. Allergy was diagnosed by *in vitro* test for allergen antibodies. The following exclusion criteria were established: (a) the presence of autoimmune disease, (b) systemic corticoid therapy in the previous 2 months, (c) unilateral polyposis, and (d) pediatric polyposis.

The patients were classified into three clinical groups:

1. Isolated nasal polyposis.
2. Nasal polyposis plus asthma (without NSAID tolerance)
3. ASA triad (nasal polyposis, asthma, and NSAID intolerance).

The clinical stage was determined by the nasal endoscopy findings, based on previously established criteria¹¹:

Stage 1. Polyps not reaching the lower margin of the middle turbinate.

Stage 2. Polyps reaching but not surpassing the lower margin of the middle turbinate.

Stage 3. Polyps surpassing the middle turbinate without reaching the floor of the fossa.

Stage 4. Polyps reaching the floor of the fossa.

The radiological stage in turn was determined by computed axial tomography according to the criteria of Lund and Kennedy.¹² Involvement of the frontal, maxillary, anterior ethmoid, posterior ethmoid, and sphenoid sinuses was scored as follows: 0 = no occupation, 1 = partial occupation, and 2 = total occupation. The osteomeatal complex was scored from 0 (unoccupied) to 2 (occupied). Complete occupation corresponded to 12 points/side.

From the ¹Ear, Nose, and Throat Department, Valencia University General Hospital and Medical School, Valencia, Spain, and ²Pathology Department, Valencia University Medical School, Valencia, Spain

Address correspondence and reprint requests to Miguel Armengot, M.D., Ph.D., c/Mediterrani 33, 46134-Foios, Valencia, Spain

E-mail address: miguel.armengot@uv.es

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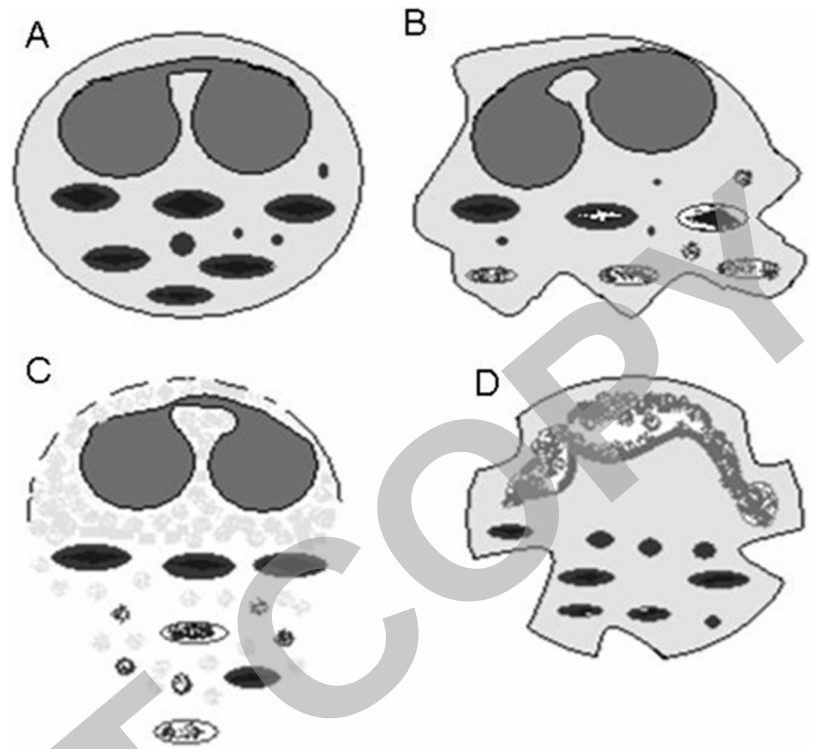


Figure 1. Schematic representation of the types of eosinophils according to their activation state: (A) inactive; (B) piecemeal degranulation; (C) cytolysis; (D) apoptosis.

Two samples were obtained from each subject: one for light microscopy and the other for TEM. The biopsy specimens were obtained from the same polyp in either the outpatient clinic *via* nasal endoscopy under topical anesthesia or *via* polypectomy in the course of endoscopic surgery under general anesthesia. The pathologist was blinded to the source of the polyp.

The samples for light microscopy were fixed with 10% formaldehyde and processed for paraffin embedding. Sequential 5- μ m sections were cut from each specimen and stained with hematoxylin-eosin and trichromic stain. In each patient, the light microscope (Nikon Eclipse E 200, Nikon, Inc., Melville, NY) was used to select and photograph (Nikon Coolpix 4500 digital camera, Nikon, Tokyo, Japan) four representative fields under 100 $\times$ and 400 $\times$ magnification, with counting of the cells aided by a microgrid.

The samples for the ultrastructural study were immediately immersed in 2.5% glutaraldehyde in Sorensen buffer (pH 7.4, 0.03 M) for 2 hours. The material was then postfixed for 2 hours in buffered 1% osmium tetroxide solution, and after dehydration in graded concentrations of acetone, the tissue fragments were embedded in Epon 812. The blocks of embedded material were cut using a Reichert-Ultracut (Leica, Vienna, Austria) ultramicrotome, and the semithin sections (2 μ m) were stained with toluidine blue for light microscopy and control. Light micrographs were obtained using a Megaview III digital camera (Soft Imaging Systems, Leica, Heerbrugg, Switzerland). The ultrathin sections of the material selected for TEM were contrasted with uranyl acetate and lead citrate and examined with a JEOL JEM 1010 (JEOL, Tokyo, Japan) electron microscope operated at 60 kV.

The observations were realized by two investigators who used homogeneous criteria and that previously were validated by a κ -test (>85%). Tissue eosinophils were identified and classified according to their ultrastructural morphological characteristics into four types⁶ (Fig. 1):

1. Inactive or resting eosinophils. These are characterized by the presence of intact cytoplasmic granules, without signs of degranulation.

2. PMD eosinophils. These are cells with an intact membrane, showing changes in the specific intracytoplasmic granules (at least two partially or totally vacant intracellular granules).
3. Cytolytic eosinophils. These are cells with a ruptured membrane and a partially dissolved cytoplasm. Clusters of free extracellular eosinophils granules (Cfegs) are seen.
4. Apoptotic eosinophils. These are characterized by chromatin condensation, a preserved cell membrane and unaltered cellular organelles.

Following the descriptive study, a statistical analysis was made of the total eosinophils evaluated under 5000 $\times$ magnification. Each cell was classified according to its degranulation mode and according to

Table 1 Patient classification according to clinical and radiological stage and clinical group

	No. Patients	Patients (%)
Clinical stage		
2	10	25
3	25	65
4	5	10
Radiological stage		
10–13	11	27.5
14–17	16	40
18–20	13	32.5
Clinical groups		
NP	19	47.5
NP + asthma (no NSAID intolerance)	13	32.5
ASA triad syndrome	8	20

ASA = acetylsalicylic acid; NP = nasal polyposis; NSAID = nonsteroidal antiinflammatory drug.

Table 2 Degree of tissue eosinophilia (TE) (%) according to the number of eosinophils per 400× magnification field

No. Eosinophils/400× Field	No. Cases	TE (%)
<5	4	10
5–19	13	32.5
20–50	14	35
>50	9	22.5

the case of origin (based on the corresponding clinical group, clinical–radiological stage, and degree of tissue eosinophilia). At last, correlations were made of the state and degranulation mode to the different clinical variables, based on the chi-square test.

RESULTS

The mean patient age was 48.93 years (range, 27–72 years; SD = 11.69). There were 14 women (35%) and 26 men (65%). Ten patients (25%) were diagnosed with allergy, and the remaining 30 (75%) were nonallergic. Table 1 shows the distribution by clinical and radiological stages and the clinical groups.

The mean number of eosinophils per 400× magnification field was 42.98 (range, 1–311; SD = 57.48; Table 2). Tissue eosinophilia in clinical stage 2 was significantly less than in clinical stages 3 and 4 ($p = 0.0342$). No differences were found between stages 3 and 4. No statistically significant differences were observed in tissue eosinophilia level between the allergic and nonallergic subjects or among clinical groups. To the effects of statistical comparison, the cases studied were reduced to 36, since 4 patients (with eosinophilia grades of <5 eosinophils per 400× magnification field) showed no eosinophils in the biopsy processed for ultrastructural study and these 4 patients might comprise a separate disease.

A total of 582 eosinophils were studied under 5000× magnification (Fig. 2). The mean number of eosinophils studied per patient was 16.11 (range, 2–62; SD = 12.97). The number of eosinophils used in some clinical or tissue eosinophilia comparisons were reduced from

582 to 580, after eliminating the two cases of apoptosis, which may be regarded as exceptional.

Eosinophil classification according to degranulation mode is shown in Table 3. The percentage of inactive eosinophils compared with the degranulated cells (PMD and cytolysis) was quite constant and independent—few differences being observed on analyzing according to other epidemiologic, clinical, or histological variables. The percentage of cytolytic eosinophils tended to decrease, whereas those showing PMD tended to increase in patients with ASA triad syndrome, a high clinical–radiologic stage, and in those cases of polyposis with a mean tissue eosinophilia of >50 eosinophils per 400× magnification field (Tables 3; Fig. 3). No statistically significant differences were observed between those polyps obtained by local or general anesthesia or between allergic and not allergic patients ($p > 0.05$). Cytolysis was more frequent in the patients treated with topical steroids ($p < 0.007$).

At ultrastructural level, the statistical analysis of the global eosinophils showed a significant correlation ($p < 0.05$) between degranulation mode (excluding the two cases of apoptosis) and the clinical and histological parameters studied. Thus, cytolysis tended to decrease and PMD tended to increase in the cells derived from patients with intense eosinophilia or severe polyposis (high clinical stage and ASA triad).

DISCUSSION

No statistical differences were observed between degranulation mode and clinical group, clinical staging, radiological score, or tissue eosinophilia, with exception of patients treated with topical steroids ($p < 0.007$). Only statistical tendencies can be obtained. The study is fundamentally of a cellular nature. In effect, we have photographed and quantified almost 600 eosinophils under 5000× magnification—thus, ensuring that the global data are sufficiently reliable. Inevitably, at patient level, the number of eosinophils studied depends on their tissue concentration; as a result, in those cases characterized by scant eosinophilia, fewer eosinophils were available for study.

We selected an exclusively morphological study design, based on TEM, to observe the specific granules and thus identify and quantify the structural changes associated with their release.⁸ With this tech-

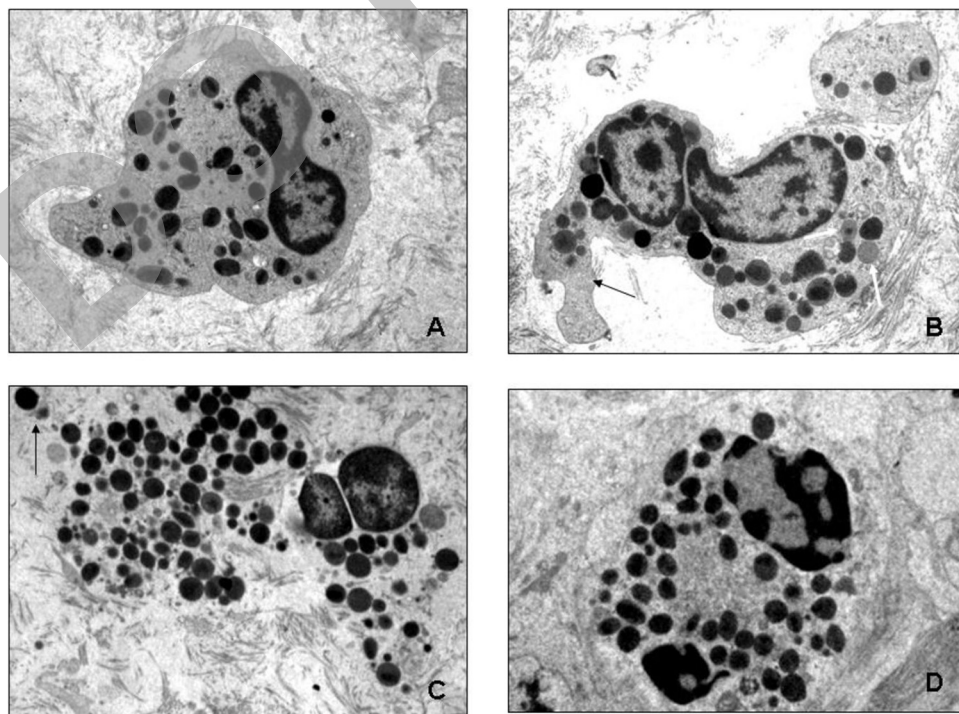


Figure 2. Types of eosinophils according to their activation state, observed under 5000× magnification (transmission electron microscopy [TEM]). (A) Inactive or resting eosinophil in connective tissue: intact cytoplasmic granules. (B) Eosinophils showing piecemeal degranulation, with pseudopods (black arrow) and intracytoplasmic granules partly or completely empty (white arrows). (C) Cytolytic eosinophil: ruptured membrane, partially dissolved cytoplasm, and clusters of free extracellular eosinophils granules (arrow). (D) Eosinophil in the early stages of apoptosis; note the chromatin condensation with an intact cell membrane. (Modified from Ref. 6)

Table 3 Distribution of eosinophil degranulation mode according to clinical stage and group

Transmission Electron Microscopy, 5000×		Clinical Stage			Clinical Group		
		2	3	4	NP	NP + Asthma	ASA Triad
Total eosinophils	582	120	384	78	239	167	176
Inactive	179	42	110	27	76	54	49
	30.75%	35%	28.64%	34.61%	31.79%	32.33%	27.84%
Piecemeal degranulation	243	38	177	28	103	59	81
	41.75%	31.66%	46.09%	35.89%	43.09%	35.32%	46.02%
Cytolysis	158	40	95	23	58	54	46
	27.14%	33.33%	24.73%	29.48%	24.26%	32.33%	26.13%
Apoptosis	2	0	2	0	2	0	0
	0.34%		0.52%		0.83%		

ASA = acetilsalicylic acid; NP = nasal polyposis.

nique it is possible to determine whether the cell is activated and to establish the degranulation mode. The data in our study were obtained using TEM in accordance with the descriptions of Pearson,⁸ Erjefält,¹³ and Ahlstrom-Emanuelson.⁷

Our clinical findings and the results of tissue eosinophil quantification are comparable with the data generally reported for nasal polyposis. The sample therefore can be considered representative. Ninety percent of our patients presented 5 or more eosinophils per 400× magnification field. Most of the cases presented between 20 and 50 eosinophils per 400× magnification field. The mean number of eosinophils per 400× magnification field was similar to that reported by other investigators.^{8,13,14}

At ultrastructural level, the comparison of our findings with those of other authors^{13,15} reveals a larger proportion of inactive eosinophils, a lesser proportion of eosinophils showing PMD, and a larger proportion of cytolytic cells. These differences may be explained at least in part by the larger number of patients involved in our study. The difference in the percentage of inactive eosinophils and PMD cells could be caused by differences in criterion on evaluating such cells—because we classified as inactive all eosinophils that did not clearly show at least two diffuse or vacant granules. Regarding cytolysis, the phenomenon proves highly evident under the electron microscope, and our results coincide with those published by Uller in 2004.¹⁵

Regarding the percentage of activated eosinophils, our data approach those reported by Kanai¹⁶ and Stoop.¹⁷ These studies were based on immunohistochemistry with EG2—the latter being considered a marker of the released form of eosinophil cationic protein—

and found approximately three-quarters of the eosinophils in nasal polyps to be activated.^{16,17}

Cytolysis is a much more potent degranulation mode than PMD. In this sense, although the proportion of degranulated eosinophils in our series was smaller than reported by Erjefält,¹³ percentage cytolysis was greater. Thus, our results agree with the hypothesis that eosinophil activation plays a key role in the pathogenesis of nasal polyposis. These results in relation to cytolysis, together with cell transit toward the epithelial surface, probably reflect the main mechanism underlying eosinophil clearance from the airways, because very few cases of apoptosis were observed.

In contrast, the cytolytic phenomena were not observed within the context of tissue necrosis but in tissues where the rest of cells remained in good condition. In other words, the images of cytolysis were not artifacts or a consequence of sample manipulation—this aspect already having been stressed by other authors.¹³

The comparative study of the clinical groups and stage or degree of eosinophilia versus eosinophil degranulation mode is difficult to establish, and no studies have been found in this sense in the literature. The method used in our study analyzes the eosinophils one by one, classifying them according to their degranulation state and according to the case of origin—with results that we believe are of interest.

Additional studies with a larger number of cases will help to confirm or refute our findings of lesser cytolysis in severe polyposis (ASA triad syndrome and patients with high clinical and radiological stages) with intense tissue eosinophilia. The lesser percentage cytolysis seen in such cases may be caused by still unknown regulatory

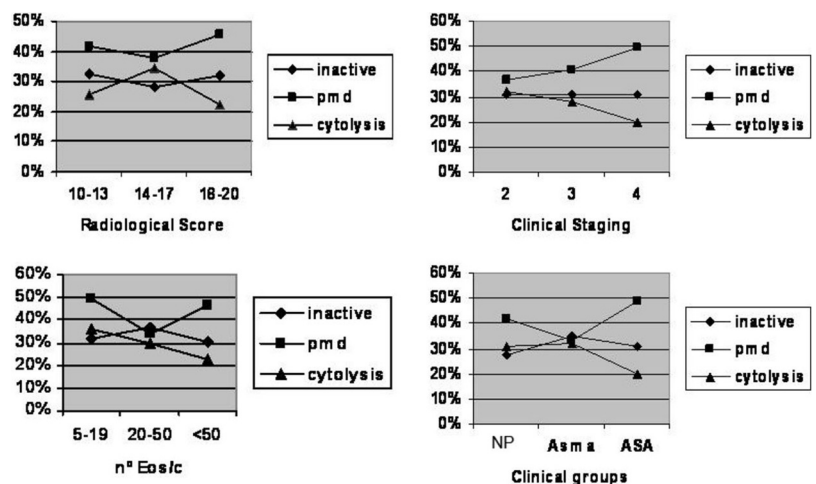


Figure 3. The figures show the relationship between eosinophil degranulation mode (ordinate axis, expressed as a percentage) and the clinical groups, stages, and degrees of tissue eosinophilia. The percentage of cytolytic eosinophils is seen to decrease, whereas those showing piecemeal degranulation increase in cases with a high clinical–radiological stage, ASA triad syndrome, and tissue eosinophilia involving >50 eosinophils per 400× magnification field. NP, isolated nasal polyposis; ASMA, nasal polyposis and asthma; ASA, ASA triad; n° Eos/c, eosinophils per 400× magnification field.

mechanisms capable of arresting cytolysis, thereby limiting the tissue damage that intense eosinophilia may cause.

CONCLUSIONS

Systematic ultrastructural study is able to define the degranulation mode and state on eosinophils in nasal polyposis. The proportion of degranulated eosinophils with respect to the inactive cells appears quite constant and is independent of the different study variables. Two-thirds of the eosinophils observed in nasal polyposis showed signs of degranulation. Cytolysis was seen in over one-quarter (27%) of the eosinophils in nasal polyposis, whereas eosinophil apoptosis was very infrequent.

Nasal polyposis shows a correlation between eosinophil degranulation mode and the clinical and radiological stage and the degree of tissue eosinophilia of the case of origin. In this sense, cytolysis tends to decrease and PMD tends to increase in the cells derived from patients with intense eosinophilia or severe polyposis (high clinical stage and ASA triad syndrome).

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