

Original article

Involvement of inferior turbinate mucosa in chronic sinusitis – localization of T-cell subset

Background: In chronic sinusitis (CS), different subsets of leukocytes are involved in development of persistent inflammation of the nasal mucosa. The localization and differentiation of these infiltrating lymphocytes may help us to understand the inflammatory interactions in the epithelium, lamina propria, and seromucous glands of the nasal mucosa in CS.

Methods: We examined frozen sections of inferior turbinates from 14 patients with nonallergic CS and 10 normal nonallergic controls. We used the avidin-biotin-peroxidase (ABC) technique with monoclonal antibodies against CD3 (total T cells), CD4 (T-helper/inducer cells), CD8 (T-suppressor/cytotoxic cells), CD22 (B cells), CD56 (natural killer cells), elastase (neutrophil granulocytes), eosinophil cationic protein (eosinophil granulocytes), and CD68 (macrophages).

Results: We found significant increases ($P < 0.05$) of CD3, CD4, and CD8 T cells and B cells in the nasal mucosa of patients with CS. The number of CD68 cells and eosinophils showed no significant rise.

Conclusions: The different types of leukocytes play a key role in the defense of the respiratory tract. The analysis of the distribution of cells in the epithelium, mucosa, and glands of the inferior turbinate confirmed that nonallergic CS is, in fact, chronic, bacterial rhinosinusitis involving the inferior turbinates, and that the pathomechanism is therefore different from that of nasal polyposis.

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Chronic sinusitis (CS) is one of the most common diseases, affecting a great number of patients each year. Moreover, this disorder frequently leads to substantial loss of productivity as well as significant medical expenses. It is estimated that more than \$2 billion/year is spent for over-the-counter medication in the treatment of CS in the USA alone (1–3).

Although CS remains a common clinical entity and studies have shown that patients with CS are significantly more limited in their social activities than patients with angina or back pain/sciatica (3), the pathophysiology of CS is still not yet completely understood. In addition, there is little information on the extent and nature of the infiltration of the nasal mucosa by leukocyte subpopulations and how these infiltrates might vary in different areas of the nasal mucosa. CS is characterized by a continuous inflammation of the nasal mucosa, including symptoms such as postnasal drip and frontal cephalgia; radiologic assessment usually shows abnormality of one or more paranasal sinuses for 3 months or longer (4). The infiltration of inflammatory cells, such as T cells, B cells, and macrophages, is a histopathologic hallmark of inflamed tissue in the intestinal mucosa (5) as well as in the respiratory tract (6, 7). Studies have

demonstrated that leukocytes migrate transendothelially from blood vessels into sites of inflammation, attracted by chemotactic cytokines and mediated by adhesion molecules such as selectins, integrins, and members of the immunoglobulin-superfamily in a three-step mechanism (8–10). Moreover there is local apoptosis of lymphocytes, a feature which may also influence the number of immunocompetent cells localized in the inflamed mucosa (11, 12). Apoptosis may also influence cellular infiltrates in the lung, another pathologic feature of chronic obstructive pulmonary disease (COPD), because the survival of inflammatory cells such as neutrophils, lymphocytes, and macrophages is mainly determined by the mechanism of apoptosis (13). Although the influence of leukocytes and cytokine expression in allergic and chronic inflammation has been clearly demonstrated in recent years (6, 7, 14, 15), there are still questions about the immunologic interactions between different subsets of leukocytes in the nasal mucosa and their role in inflammation.

In this study, we used immunohistochemical techniques to describe the lymphocyte subsets and to investigate the quantity and preferred localization of these leukocyte subpopulations in the nasal mucosa of

patients with nonallergic CS ($n=14$), and we compared our findings with those in healthy nonallergic controls ($n=10$). This comparison indicated the involvement of the inferior turbinates in CS as a sign of inflammation of the nose and paranasal sinuses. The localization and differentiation of infiltrating leukocytes may help us to understand the complex pattern of the local immunologic mechanisms in the epithelium, lamina propria, and paraglandular parts of the nasal mucosa of patients with CS. Moreover, our results may give additional information on local cellular activities and provide a reference basis for the cytokine and adhesion molecule studies we now have underway.

Material and methods

Patients

Fourteen patients ($n=14$), six women and eight men aged 16–54 years (mean 38.1 ± 11.39 years standard deviation [SD]) with nonallergic CS, were included in our study. All were undergoing endonasal sinus surgery and mucotomy of the inferior turbinate. CS was diagnosed by patient history, clinical symptoms, and CT scans. Prominent symptoms included nasal purulent rhinorrhea, nasal congestion, headache, chronic post-nasal drip, and facial pain persisting for more than 3 months. The diagnosis was confirmed in all patients by anterior endoscopy and standard CT of the paranasal sinuses showing abnormalities such as mucosal thickening with a width of over 6 mm. Allergy testing was performed by RAST (radioallergosorbent test) and prick tests. Ten control patients ($n=10$), three women and seven men aged 19–68 years (mean age 36.9 ± 17.06 years SD), underwent septoplasty and mucotomy. None had previous allergic history, infection of the upper respiratory tract, symptoms of nonallergic CS, or positive radiographic findings. In addition, the control patients had negative results in prick test or RAST.

Tissue preparation

Immediately after surgery, each inferior turbinate specimen was transported in RPMI medium (Biochrom KG, Berlin, Germany) and embedded in Tissue TEK (Sakura Finetek, Torrance, CA, USA), shock frozen in liquid nitrogen, and stored at -26°C before cryosectioning and processing by immunohistochemical staining.

Hematoxylin-eosin staining

One section of the left and right inferior turbinate was stained with hematoxylin and eosin (16) in order to observe histologic details such as eosinophil infiltration and control tissue quality.

Immunohistochemistry

Immunostaining was performed by the modified avidin-

biotin-complex (ABC) technique, as previously described (16). Specimens were cryosectioned into 5- μm sections on a Reichert-Jung Frigocut E 2800 and mounted on Super Frost/Plus glass slides (Menzel-Gläser, Germany). The cryostat sections were air-dried overnight at room temperature and fixed in acetone for 10 min. The slides were then washed in phosphate-buffered-saline (PBS), pH 7.6, and the activity of endogene peroxidase was removed in 200 ml of PBS containing 60 μl 30% hydrogen peroxide. After blocking for nonspecific bindings with 20% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany), the slides were incubated for 60 min at room temperature with specific monoclonal antibodies (mAbs). The sections were stained with the mAbs listed in Table 1. These mAbs were replaced by nonimmune serum of the first mAbs for the negative control. Human tonsil tissue (obtained during routine tonsillectomy) was used as positive control on each staining run. Sections were washed twice with PBS for 5 min and then incubated for 30 min with a second antibody (biotinylated horse antimouse immunoglobulin, Vector, Burlingame, CA, USA), diluted 1:200. The tissue sections were washed again in PBS and treated with ABC complex Vecastain ABC Kit (Vector, Burlingame, CA, USA), diluted 1:200, for 30 min. After an additional wash, the slides were stained with a chromogen solution containing 0.01% 3-amino-9-ethyl-carbazole (AEC) (Sigma, Deisenhofen, Germany), 0.0015% hydrogen peroxide, 200 ml natrium acetate-buffer (0.1 M, pH 5.5), and 6% dimethyl-sulfoxid (Merck, Darmstadt, Germany) for 5 min. Sections were counterstained in Mayer's hemalam (16) for 90 s, blued in tap water, and cover-slipped with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Microscopic assessment of staining

All sections were examined under a Zeiss Standard microscope. For counting, we used a Zeiss graticule eyepiece (0.05 mm^2). In each section the numbers of mononuclear (blue-stained) cells and cells that were stained with peroxidase-labeled mAbs were counted. We examined 10 visual fields at $\times 200$ magnification. The ratio of positive cells to mononuclear cells was cal-

Table 1. Monoclonal antibodies used and their specificities

Monoclonal antibodies	Specificity	Source	Clone	Dilution
CD3	Total T cells	Dako	T3-4B5	1:1000
CD4	T cells (helper/inducer)	Dako	MT 310	1:2000
CD8	T cells (suppressor/cytotoxic)	Dako	M 707	1:1000
CD22	B cells	Dako	4 KB 128	1:100
CD56	Natural killer cells	Dako	T 199	1:600
CD68	Macrophages	Dako	EBM 11	1:1750
ECP	Eosinophil granulocytes	Pharm.	EG2	1:1500
Elastase	Neutrophil granulocytes	Dako	NP 57	1:800

Dako, Glostrup, Denmark; Pharm. (Pharmacia), Erlangen, Germany.

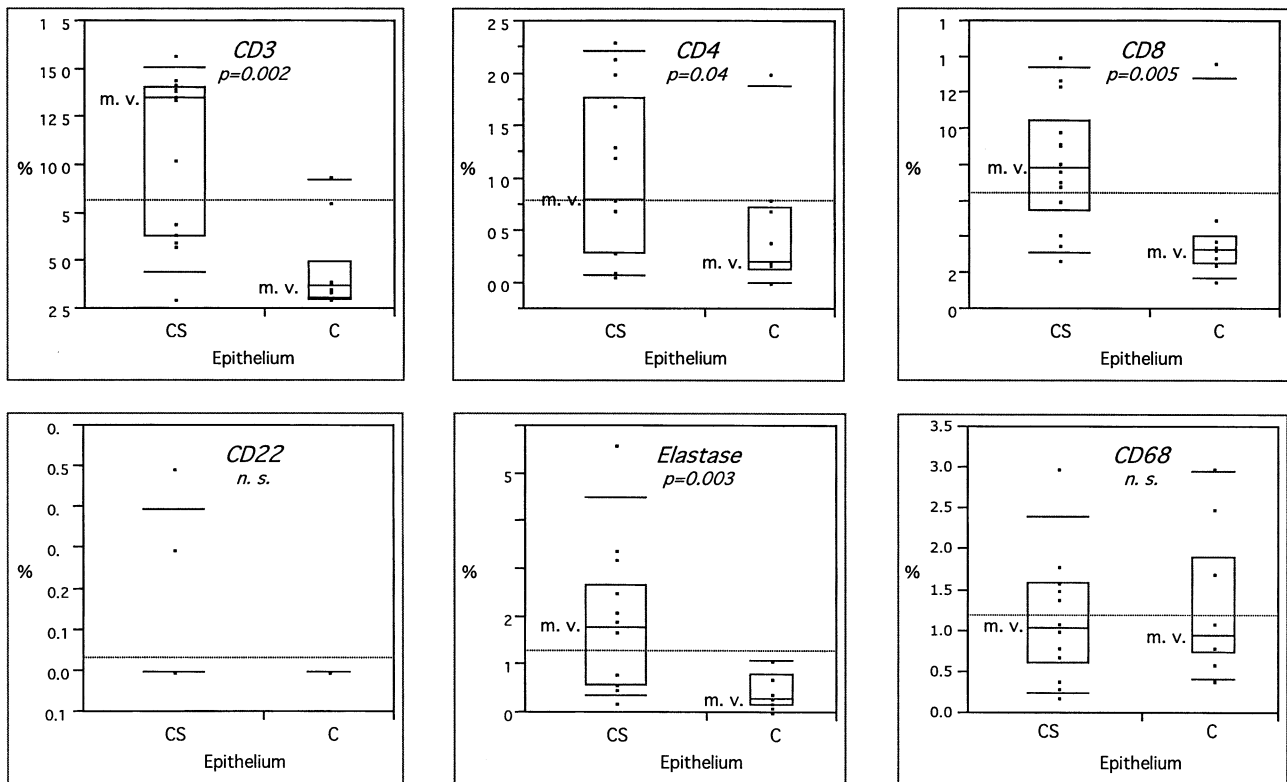


Figure 1. CD3-, CD4-, CD8-, CD22-, CD68-, and elastase-positive immunostained cells in epithelium of nasal turbinate mucosa. Rectangles give range from 25th to 75th percentiles, and horizontal lines indicate range from 10th to 90th percentiles. m.v.: median value; n.s.: not significant.

culated for each mAb. We counted in three histomorphologically different parts of the nasal mucosa: epithelium, lamina propria, and seromucous glands. The specimens were double-blind counted by two persons. The mean intraobserver coefficient of variation for repeat counts was less than 5%.

Statistical analysis

Distributions of cell numbers were compared by Mann-Whitney U tests. Cell numbers in different anatomical structures (epithelium, lamina propria, and seromucous glands) were correlated by Spearman's rank correlation coefficient. All analyses were performed with SAS JMP Version 3.1.6. (SAS, Inc., Cary, NC, USA). *P* values less than 0.05 were considered statistically significant.

Results

The overall leukocyte infiltrate was most prominent in the lamina propria immediately underneath the epithelial basement membrane. Staining with mAb against CD3 (total T cells) demonstrated increased presence of T cells in each defined histomorphologic area of the nasal mucosa of patients with CS. For example, in the seromucous glands (CD3+ mean [m]=11.3%) of CS patients, we found twice as many as in the seromucous

glands of healthy control patients (CD3+ m=5.45%). T cells per unit area in the lamina propria were more numerous in the epithelium and around the seromucous glands. The highest concentration of T cells was found underneath the epithelial layer. The concentration decreased in the deeper areas of the lamina propria. However, the total amount of leukocyte subsets in the epithelium was less than in the lamina propria or around the glandular structures. All results with *P* values are shown in Figs. 1–3.

Epithelial layer

In the epithelial layer, we found hardly any CD22+ or ECP+ marked cells and only a few CD4+ stained cells 0.8% (m). CD8+ cells were the predominant immunocompetent cells residing in the epithelium marked up to 7.9% (m). CD4+ lymphocytes could be observed in the epithelium as well, but in comparison to CD8+ cells in a very small number, with a CD4/CD8 ratio of (1/5) in the epithelial layer. Neutrophil granulocytes were detected not only in intraepithelial groups, but also above the epithelium, the only subpopulation of leukocytes located there (Fig. 4a). Macrophages were noticed in the epithelium of control patients and CS patients, but there was no significant increase (Fig. 1).

There were no CD56+ (natural killer [NK]) cells detected in the epithelial layer. In some specimens, we

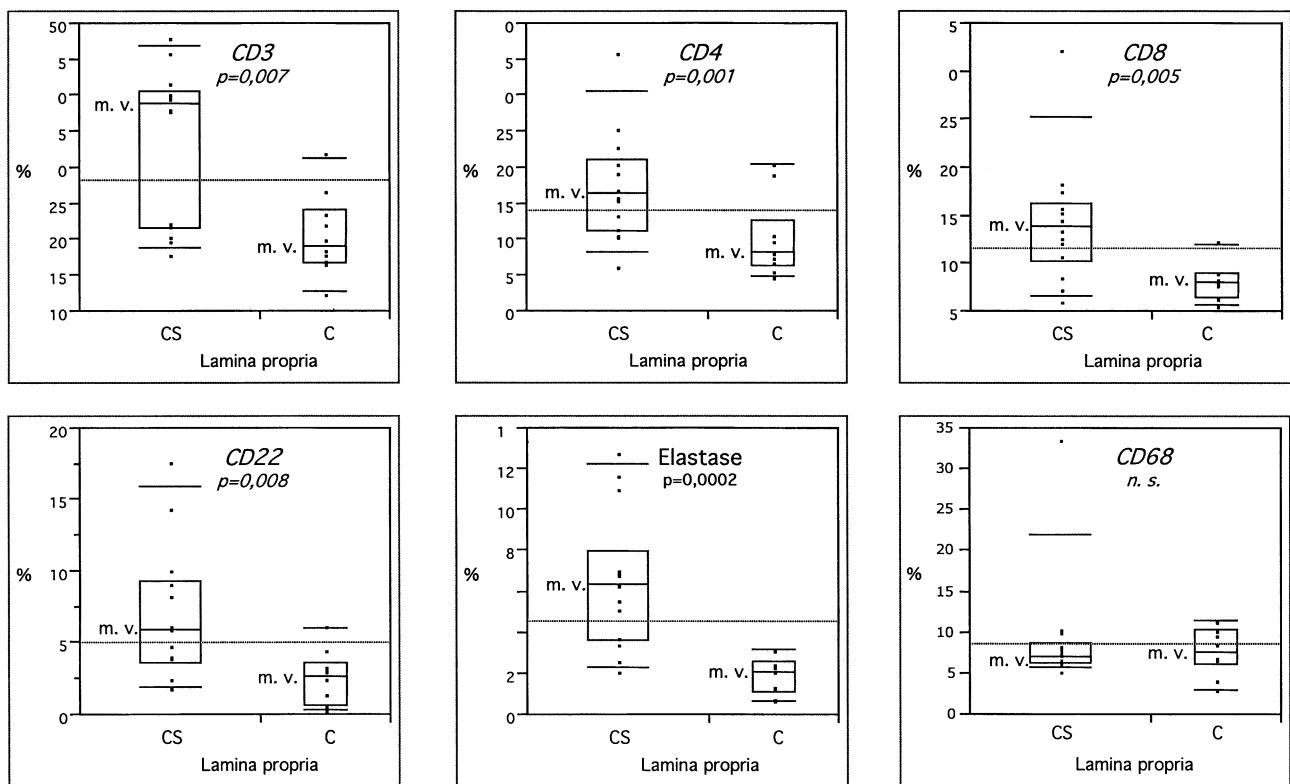


Figure 2. CD3-, CD4-, CD8-, CD22-, CD68-, and elastase-positive immunostained cells in lamina propria of nasal turbinate mucosa. Rectangles give range from 25th to 75th percentiles, and horizontal lines indicate range from 10th to 90th percentiles. m.v.: median value; n.s.: not significant.

could not examine all leukocyte subsets in the epithelium, due either to their limited number or to the epithelial detachment sometimes found in the specimens of patients with CS.

Lamina propria

The lamina propria of patients with CS showed a mixed pattern of inflammatory cells (Figs. 4 and 5); CD3+ cells (T cells) comprised the major population of lymphocytes in the lamina propria ($m=38.85\%$). In CS patients, CD8+ and CD4+ T cells were more frequently observed than in control specimens. In contrast to CD4+ T cells, which were clustered, CD8+ cells were scattered in the lamina propria (Fig. 5). Macrophages were predominantly noticed in the upper parts of the lamina propria. CS and control patients showed no significant difference (Fig. 2).

In CS specimens, CD4+ (T-helper/inducer) cells were arranged in clusters in the lamina propria mixed with CD22+ B cells. The subepithelial parts of the lamina propria were infiltrated by neutrophil granulocytes, which were primarily located adjacent to the basal lamina (Fig. 4b), with CD22+ and CD4+ cells lying close to them. NK cells (CD56+) were observed in the lamina propria in 50% of the patients with CS. NK cells and eosinophil granulocytes (ECP) were very

rare in all specimens, constituting less than 1% of the lymphocytes, and therefore not being statistically evident.

Seromucous glands

The seromucous glands were also infiltrated by CD3+ T cells. As in the other defined areas, total T cells were predominant (Figs. 1–3). Nearly every stained T cell belonged to the subset of CD8+ lymphocytes. Cytotoxic/suppressor T cells were located between the glandular structures and between the glandular cells (Fig. 5c). There were fewer CD4+ lymphocytes ($m=2.25\%$) and CD22+ B cells ($m=1.3\%$) in contact with the seromucous glands, in control subjects as well as in patients with CS (Fig. 3). Neutrophil granulocytes as well as macrophages were seen in only a few CS specimens within the glandular tissue area, whereas CD56+ and ECP+ stained cells were completely absent.

Discussion

In this study, we used the immunoperoxidase technique (ABC) and several mAbs against antigens (Table 1) expressed on B cells, T-cell subsets, neutrophil granulocytes, macrophages, eosinophil granulocytes, and NK

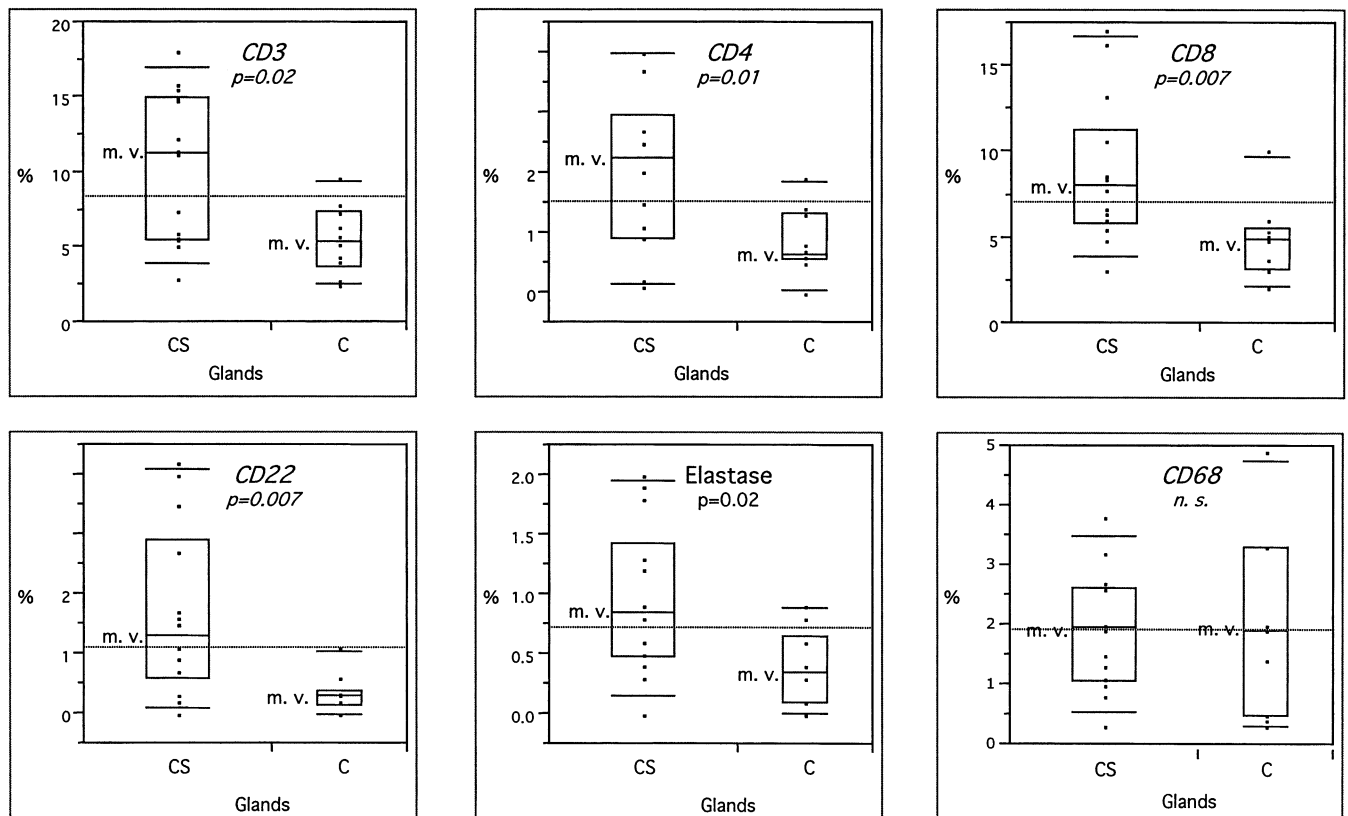


Figure 3. CD3-, CD4-, CD8-, CD22-, CD68-, and elastase-positive immunostained cells in glandular area of nasal turbinate mucosa. Rectangles give range from 25th to 75th percentiles, and horizontal lines indicate range from 10th to 90th percentiles. m.v.: median value; n.s.: not significant.

cells to determine the phenotypic characteristics of these cells in specimens from normal human nasal mucosa, as compared to CS specimens. The nasal mucosa contains well-developed defense mechanisms composed of non-specific and specific elements. The cells presented in the nasal mucosa are known to undergo a permanent change in their composition, function, and number, depending on the kind of mucosal irritation (7) and the functional implications and behavior of the vascular endothelium (17). We observed various kinds of leukocytes already present in the nasal mucosa of healthy volunteers. Confirming the results of Igarashi et al. (18), we found a high number of acutely recruited cells especially CD8+ (T-suppressor/cytotoxic lymphocytes), CD4+ (T-helper/inducer lymphocytes), and CD22+ B cells, and neutrophil granulocytes in patients with CS. We also demonstrated T cells and neutrophil granulocytes to be more frequent in patients with identified CS than in asymptomatic individuals. Inflammation in CS seems to be maintained by CD8+ T cells, as compared to patients with allergic rhinitis, in whom CD4+ and eosinophil granulocytes were found to be the predominant subpopulations (14, 15). Other authors mention a significant tendency for an increase in total T-cell counts (CD3+), as well as a significant rise in the number of CD4+ eosinophils and CD25+

interleukin (IL)-2 receptor-bearing cells, after allergen challenge in the nasal mucosa, without increase in CD8+ cells (18, 19). The accumulation of CD8+ cells in the epithelium and next to the seromucous glands might indicate that selectivity of cell infiltration is one of the immunologic reasons for the persistent and chronic character of inflammation in CS. T suppressor cells tend to suppress immune reactions and provide negative feedback to the immune system (7).

Neutrophils in the epithelium showed an increased density compared to the controls, and seemed to be able to penetrate through the basement membrane, and thereby constitute one of the first and most important factors of mucosal defense. Bachert et al. (21) found that epithelial cells accommodate neutrophil granulocytes producing strong neutrophil chemotactic proteins such as IL-1, IL-8, and GM-CSF (granulocyte-macrophage colony-stimulating factor) (22, 23). The localization of neutrophil granulocytes above the epithelial layer is in accordance with studies that have found neutrophil granulocytes in sinus lavage fluids and a high level of neutrophil-derived mediator (MPO) and neutrophil chemoattractant cytokine (IL-8) in patients with CS (24). In the early stages of inflammation, neutrophil granulocytes could be found in the inflamed tissue within 15 min (25). Infiltration of neutrophil

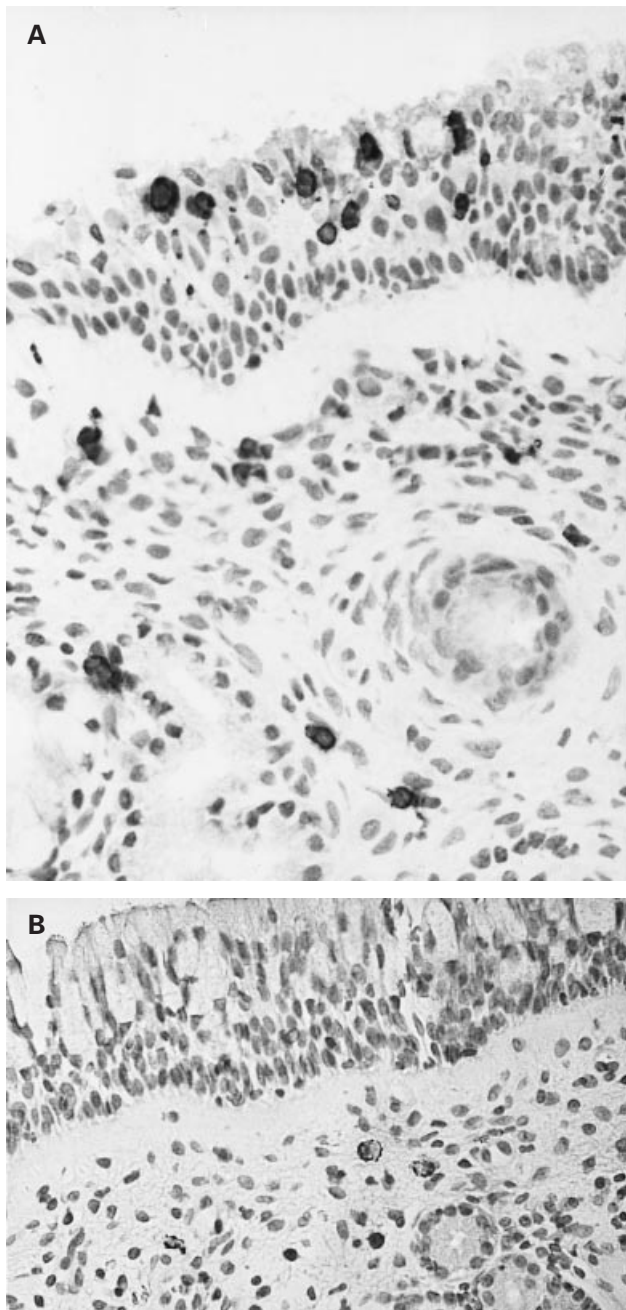


Figure 4. A) Patient with CS. Biopsy of nasal turbinate mucosa, immunostained with elastase mAb with aminoethycarbazole (AEC, brown) chromogen ($\times 400$). B) Patient with CS. Biopsy of nasal turbinate mucosa, immunostained with eosinophil cationic protein (ECP) mAb with aminoethycarbazole (AEC, brown) chromogen ($\times 400$).

granulocytes is orchestrated by a number of receptors belonging to distinct families of adhesion molecules (10). Although there are fewer neutrophil granulocytes than T cells, neutrophil granulocytes are known to be not only terminal effector cells. They affect other cells releasing various cytokines such as IL-1 α , IL-1 β , IL-6, IL-8, interferon (IFN)-alpha, and tumor necrosis factor (TNF) (21), being able to activate B cells and T cells.

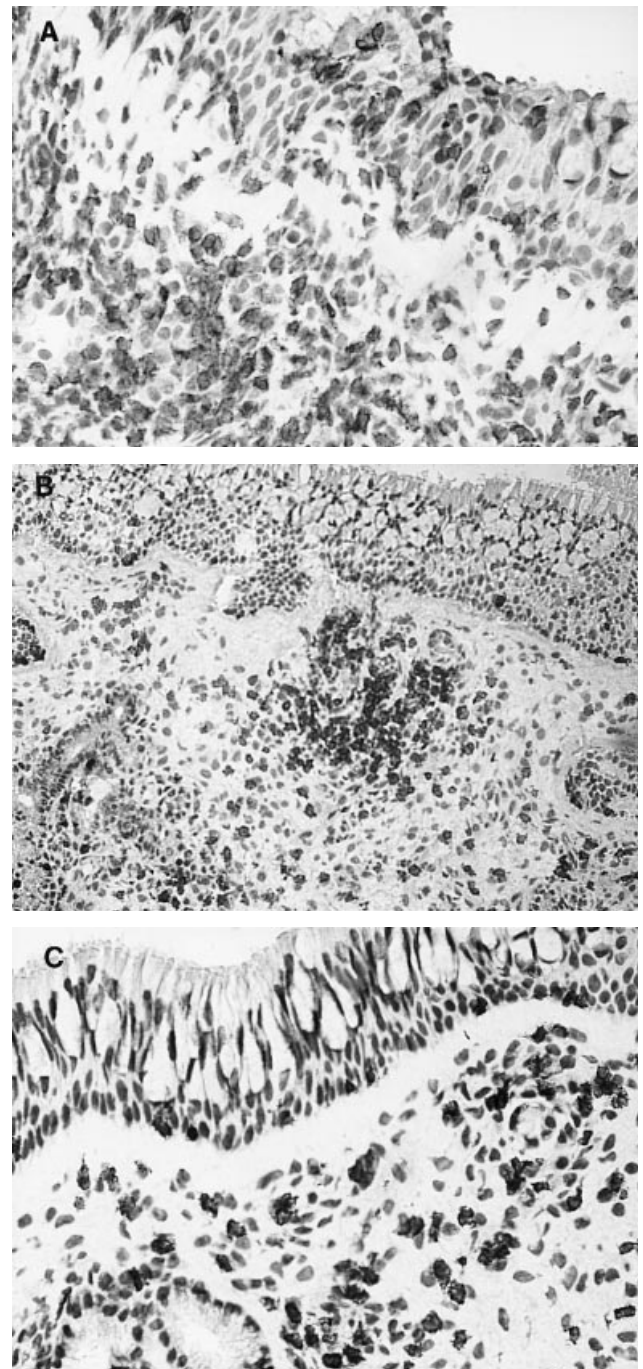


Figure 5. T-cell subsets in nasal turbinate mucosa in CS by ABC-PO staining with mAb. A) CD3+ cells in strong infiltration in CS ($\times 400$). B) CD4+ cells tend to cluster in lamina propria ($\times 200$). C) CD8+ cells scatter in lamina propria and appear in epithelium and next to glands ($\times 400$).

Several studies dealing with the recruitment and migration of neutrophil granulocytes (22, 26) confirm our finding that neutrophil granulocytes are the most important leukocytes in the pathogenesis of CS. Neutrophil granulocytes play an important role in mucosal defense either in the epithelium or forming a barrier directly underneath the epithelium under the thickened basement membrane (27).

We observed more CD8⁺ than CD4⁺ T cells in the epithelial layer, and conclude that CD8⁺ lymphocytes belong mainly to the group of cytotoxic T cells. According to Pawankar et al. (15), there were more cytotoxic T cells (CD8⁺) than CD4⁺ cells in the epithelial layer. Driscoll et al. (28) found significantly more CD4⁺ cells in the epithelium of children with chronic sinusitis than in normal sinus mucosa, whereas the number of CD8⁺ cells was not significantly different. These observations indicate that there might be a difference between the immunologic response in the nasal mucosa of children and that in adults because we found a predominance of CD8⁺ cells.

Another interesting fact is the presence of CD8⁺ cells between the glandular structures and adjacent to glandular cells (Fig. 5c). Cytotoxic/suppressor T cells are able to secrete cytokines (21, 23) that might stimulate the glandular cells to increase their productivity in order to remove antigens. CD8⁺ cells are able to recognize MHC-I presented antigen to lysate and kill their target cells. A similar pattern of leukocyte infiltration is found in COPD. In COPD with airflow limitation, an increased number of CD3⁺ T cells and CD8⁺ lymphocytes, neutrophils, and CD68⁺ macrophages was seen (29). The precise role of these cells in the pathogenesis of similar chronic diseases such as CS and COPD still remains to be determined (30).

CD4⁺ lymphocytes were the smallest intraepithelial population. In the lamina propria, CD4⁺ cells typically tend to cluster and form aggregates consisting of about 100–200 cells in the subepithelial areas (Fig. 5b). CD4⁺ and CD22⁺ cells interact, clustering in the submucosa. It is possible that CD4⁺ and CD22⁺ lymphocytes tend to “compartmentalize” in the lamina propria, whereas neutrophils appear in nasal secretions as well (24). CD4⁺ T cells can induce nearly all the immunologic responses, and their presence indicates an active immunologic process (14). CD4⁺ cells are able to process and present antigen; to recognize antigens presented by the MHC II receptor of macrophages, B cells, and dendritic cells; and to produce various cytokines, such as IL-2, TNF- β , and IFN- α (14, 15, 21). CD4⁺ T-helper cells activate B cells to differentiate into plasma cells and to produce antibodies.

CD22⁺ B cells were weakly stained in the glandular part of the mucosa. We conclude that CD8⁺ T cells are located adjacent to the glands, because these cells are able to defend the glands in the “front line” and allow CD4⁺/CD22⁺ cells to reside in the “backyard”, where they induce the humoral immunologic response and synthesize immunoglobulins. The contact between CD4 and CD8 T cells seems to be less important than the contact between CD4 and CD22 cells.

We tried to evaluate NK cells in patients with CS, because Fort et al. (31) recently demonstrated that NK cells have a critical role in controlling the T-cell response in chronic inflammation. CD56⁺ NK cells are T-cell

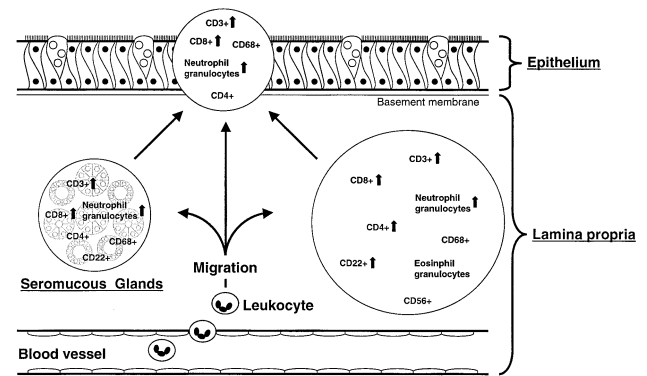


Figure 6. Accumulation of leukocyte subsets in different mucosa compartments in chronic sinusitis (↑ arrows indicate significant elevation of leukocyte subsets).

receptor-negative lymphocytes with large granules that mediate both spontaneous and antibody-dependent lysis of virally infected and neoplastic cells (32). These cells are known to be potent inducers of cytokines, and they might control the differentiation and migration of leukocyte subsets. Their exact pathogenetic role in the chronic inflammatory process has not yet been elucidated. In accordance with other studies of the presence of NK cells in the intestinal mucosa (31), we noticed few NK cells in the nasal mucosa of control patients or in patients with CS. Nevertheless, their reduced appearance cannot be compared with the extensive functional effects these cells can have on the local mucosal immune reactivity. It is suggested that NK cells might have both enhancing and suppressive effects on the B-cell response (32).

However, in addition to the increase of CD4⁺ cells in allergy (13, 14), we could demonstrate a significant increase of CD8⁺ cells. This might be interpreted as an indication of abnormal reactivity in the nasal mucosa of CS patients with a preponderance of CD8⁺ T cells and neutrophils. Although we could show enhanced cell-mediated immune reactions in the nasal mucosa, we also noticed activation of the humoral part of the mucosa immune system.

In conclusion, we could show that leukocyte subpopulations are increased in certain localizations in chronically inflamed nasal mucosa (Fig. 6). CD8⁺ cells and neutrophil granulocytes cells play a very important role in CS in the epithelial layer. CD8⁺ cells are scattered in the lamina propria, whereas CD4⁺/CD22⁺ lymphocytes, which also take part in mucosa inflammation in CS, tend to cluster. NK cells were detected, and although they were sparsely present, they may take part in chronic inflammatory processes (31, 33). Clarification of the leukocyte localization and balance in the inflamed nasal mucosa will help us to develop better therapeutic strategies to prevent these cells from reaching their common sites in chronic inflammation.

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