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## Original article

# Mucosal T-cell phenotypes in persistent atopic and nonatopic rhinitis show an association with mast cells

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**Background:** Allergic rhinitis is characterized by selective expansion of T cell subsets with a CD4+ phenotype. Recently, we identified a subpopulation of nonallergic rhinitis subjects with increased epithelial mast cell and eosinophil populations, suggestive of local mucosal allergy. Previously, T cell subsets have not been characterized in this subselection of nonallergic subjects and furthermore, their relationship to mast cell and basophil effector cells remain unidentified.
**Objective:** To determine if a subpopulation of nonallergic subjects with idiopathic rhinitis (IR) have localized allergy confined to their nasal mucosa by comparing the T cell subsets and major histocompatibility complex (MHC) II expressing cells to persistent allergic rhinitis (PAR). Furthermore, the relationship between T cell subsets and mast cells/basophils was investigated.
**Methods:** None of the symptomatic patients in this study were clinically allergen-challenged. Nasal turbinate mucosa was removed from patients with PAR, IR and normal controls. Morphometry was performed on immunostained sections for T cell subset populations including CD3+, CD4+, CD8+, CD25+, CD45RA+, CD45RO+, human leucocyte antigen (HLA)-DR$\alpha$ (MHC class II), mast cell tryptase and for basophils.
**Results:** Subjects with persistent allergic rhinitis differed to normal controls in showing significantly increased numbers of total (CD3+), activated (CD25+) and allergen-naïve (CD45RA+) T lymphocytes in their nasal mucosa ($P < 0.025$). The naïve CD45RA+ memory T cells correlated to mucosal mast cells in PAR ($P = 0.03$). IR patients differ to allergic subjects in showing significantly reduced numbers of epithelial HLA-DR$\alpha$+ cells ($P = 0.007$), but increased numbers of CD8+ lymphocytes ($P = 0.02$). The CD8+ T cells correlated with mucosal mast cell numbers ($P = 0.02$). In both rhinitis groups, basophils were present in very low numbers obviating the need for statistical analysis.
**Conclusion:** PAR is characterized by increased numbers of CD3+, CD25+ and CD45RA+ T lymphocytes compared with normal mucosa. Allergic and nonallergic rhinitis groups can be separated by significant differences in the number of epithelial antigen presenting cells (APCs) (HLA-DR$\alpha$+) and sub-epithelial activated (CD25+) T cells. Moreover, IR patients do not significantly differ to their allergic counterparts with respect to total (CD3+) and naïve (CD45RA+) T cell numbers, or numbers of epithelial activated (CD25+) lymphocytes. IR subjects show significantly increased numbers of CD8+ lymphocytes compared with control mucosa and although our findings suggest that the initiating inflammatory events may differ, both rhinitis groups show a similarity in pathology involving mucosal mast cells with an association to infiltrating T cells.

Key words: allergic rhinitis; allergy; basophils; cytokines; idiopathic rhinitis; immunochemistry; mast cells.

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In accordance with the Th1/Th2 paradigm (1), subjects with atopic diseases including asthma and rhinitis, have a predominance of CD4+ $T_{helper}2$ (Th2) subsets in their peripheral blood. Restoration of the Th1/Th2 balance by down-regulation of Th2 cells and their mediators, while maintaining Th1 cells is therapeutically beneficial (2).

Currently, there is a growing awareness of the localized nature of inflammation in allergic rhinitis, with evidence suggesting that key elements of the Th2 pathway occur within the nasal mucosa (3, 4). Allergy is initiated by allergen presentation involving major histocompatibility complex (MHC) class II antigen

presenting cells (APC) (5) resulting in selective activation and expansion of CD4+ Th2 subsets (6). Moreover, the production of Th2 cytokines up-regulate MHC II expression on APCs (7) and these cells reciprocally stimulate Th2 cells, favouring a Th2 disease pathway because of selective down-regulation of interleukin (IL)-12 (8). After allergen-sensitization, atopic subjects retain the ability to respond rapidly and specifically because of surveillance by $T_{helper}$ lymphocytes bearing the leucocyte common antigen, CD45 (9). Two populations of CD45+ T cells are recognized, those that are antigen-naïve with a CD45RA+ phenotype, and an allergen-exposed (memory) subset with a CD45RO+ phenotype (10). Subsequently, Th2 cells induce immunoglobulin isotype switching in B cells (11, 12) to produce immunoglobulin (Ig)E (13) within the nasal mucosa (4, 14). More recently it has become apparent that effector cells other than T cells, are active in maintaining a state of inflammatory perpetuation. Mast cells (15) and basophils (16) have the necessary membrane receptors and cytokine production appropriate for inducing B cell immunoglobulin switching. Furthermore, there is evidence suggesting that mast cells and T cells mutually benefit from being in close association because of the production of cytokines needed to sustain survival and to develop a Th2 phenotype (17, 18).

Previous investigations by ourselves and others show that some nonatopic subjects with rhinitis have allergic-type responses when nasally allergen-challenged (19–21). Furthermore, we detected *in situ* nasal allergen-specific IgE in nonatopic idiopathic subjects (22, 23) and proposed the term 'entopy' to define mucosal allergy in the absence of systemic atopy (24). In the knowledge of these recent developments, we hypothesized that a subpopulation of idiopathic patients might show evidence of a Th2 mediated pathway in their nasal mucosa. To determine this, we compared the distribution of MHC class II HLA-DR+ cells and T lymphocyte subsets in the nasal mucosa of patients with nonallergic idiopathic rhinitis to that from persistent (25) allergic rhinitis and normal controls. In addition, we assessed the association between the T cell subsets and mast cells and basophils because (i) these granulocytes have a propensity for B cell activation (15) and (ii) a dependence on T cell-derived cytokines for growth, survival and effector function (17, 18).

## Materials and methods

### Selection of patient groups

Inferior turbinate specimens were surgically removed from consenting patients in the Department of Otorhinolaryngology at Queen's Medical Centre (QMC), Nottingham, with approval from the QMC ethics committee. Three groups of patients were rigorously selected according to strict criteria (Table 1). Briefly, a persistent allergic rhinitis group (PAR) ($n = 21$; range:14–57 years) and a persistent nonatopic group with idiopathic rhinitis (IR) ($n = 22$; range:17–61 years) were compared with normal (nonrhinitic) patients ($n = 19$; range:17–54 years). All patients were skin prick tested (SPT) for common allergens including grass, weed and tree pollen mixes, house dust mite, cat, dog, feather and moulds. A positive response was defined as a weal equal to or $>3$ mm in diameter. Total serum and specific IgE tests were performed on the same patients using the Pharmacia UniCap System (Pharmacia Diagnostics, Sweden). By definition, PAR patients showed a positive skin prick test and the presence of corresponding serum allergen-specific IgE. In contrast, IR patients had persistent rhinitis but did not show a positive response to SPT and had an absence of allergen-specific IgE in the serum. Normal patients had negative tests for SPT and specific IgE. All patients underwent surgery for nasal obstruction. Normal control nasal mucosa was obtained from patients operated on for mechanical obstruction secondary to a septal deviation.

The rhinitis patients had perennial symptoms but none were clinically allergen-challenged prior to surgery. Patients with a history of asthma, eczema, seasonal rhinitis or autonomic rhinitis were excluded to improve on selection homogeneity.

Table 1. Idiopathic rhinitic patient selection criteria

| |
|---|
| Inclusion criteria |
| Age between 16 and 64 years |
| Negative skin prick test and serum IgE enzyme-linked immunosorbent assay (DPC AlaStat, Los Angeles, CA, USA) to allergen panels including house dust mite, dog, cat and moulds mix |
| Symptoms for more than 2 years |
| A minimum of two of three of the following symptoms |
| Periods of nasal discharge, sneezing and nasal obstruction for an average of at least 1 h/day for more than 14 consecutive days |
| Endoscopy shows no purulent disease or evidence of middle meatal disease e.g. polyposis |
| No history suggestive of a viral or bacterial upper respiratory tract infection at the time of their surgery or in the previous month |
| Exclusion criteria |
| Clear rhinorrhoea and response to ipratropium bromide (excludes vasomotor/autonomic rhinitis) |
| The use of systemic or nasal corticosteroids within the past 6 weeks |
| Use of nasal sodium cromoglycate or nedocromil sodium within the last 6 weeks |
| Use of systemic or nasal antihistamines within the previous month |
| Abnormal sinus CT scan |
| Purulent discharge at nasal endoscopy or evidence of middle meatal pathology |
| History of asthma or eczema |
| Pregnancy or lactation |
| Abnormal laboratory test results for |
| Blood: antinuclear cytoplasmic antibody, angiotensin converting enzyme, sodium, potassium, calcium, total protein, albumin, urea, creatinine, bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gammaglutamyl transpeptidase, haemoglobin, red blood cell count, plasma cell volume, mean corpuscular volume, platelets, total white blood cell count, neutrophils, lymphocytes, monocytes, eosinophils, basophils |
| Urine: blood, protein, glucose |

### Immunohistochemistry

Full-thickness mucosa was fixed in 10% formal saline and four micron thick paraffin wax sections were produced. For immunohistochemistry (IHC), a streptavidin immunoperoxidase technique was used with a selection of mouse monoclonal antibodies

specific to HLA-DRα (MHC class II; Dako, Cambridge, UK), CD3 (Dako), CD4 ($T_{helper}$; NovaCastra, Newcastle, UK), CD8 ($T_{Cytotoxic}$; NovaCastra), CD25 (activated T cells-clone 4C9; Novacastra), CD45RA/RO (Dako), and mast cell tryptase (clone AA1; Dako). Biotinylated secondary antibodies and a peroxidase conjugated avidin biotin complex was used with the chromogen DAB for visualization. Positive staining was indicated by brown cytoplasmic colouration. For the demonstration of basophils, we used a monoclonal antibody (BB-1; 26) on frozen sections with an alkaline phosphatase anti-alkaline phosphatase (APAAP) technique and the chromogen NBT (Sigma, Dorset, UK). Positive staining was indicated by blue cytoplasmic colouration. Memory T cells that have had allergen exposure have a CD4+ CD45RO+ phenotype whilst naïve T cells are CD4+ CD45RA+. Negative control sections had the primary antibody omitted.

### Morphometry

Morphometry was performed on full-length, full-thickness immunostained sections of mucosa according to the method previously reported (27). Briefly, three mucosal strata were defined comprising the epithelium, superficial and deep submucosa. Total counts were manually made using an eyepiece graticule and positively stained cells converted to mean counts/mm (2). The antigen presenting cells (HLA-DR+) stained cells were enumerated in only two strata (epithelium and adjacent submucosa) because previously, APCs were shown to differ between allergic and normal nasal mucosa in the epithelium (5). To facilitate this, semi-automated interactive morphometry was performed using an AnalySIS program (Soft Imaging System, Münster, Germany). The percentage area stained per selected field was calculated and used for comparison between the patient groups. Sections were counted without prior knowledge of their pathologic origin.

### Statistics

The three patient cohorts were compared for their T cell subsets and mast cell counts using the Mann–Whitney nonparametric technique with a Bonferroni correction applied. A $P$-value $\leq 0.025$ was considered significant.

Tests for association between the different mucosal T lymphocyte subsets and mast cells were investigated using linear regression (GraphPad Prism, San Diego, CA, USA).

## Results

### HLA-DRα MHC II+ cells

Brown cytoplasmic HLA-DRα staining was seen in the epithelial (stratum 1) and adjacent superficial submucosa (stratum 2) (Fig. 1). Comparison of HLA-DRα stained sections showed a significantly increased epithelial median cell count for the allergic group compared with the idiopathic group ($P = 0.007$) but not to the normal group ($P = 0.06$) possibly because of three normal control patients with increased APC counts (Fig. 2).

### CD3+ cells

CD3 antibody is a pan-marker of T lymphocytes and after detection resulted in brown staining (Fig. 1). The highest CD3+ cell counts were seen in the epithelium of all cases. All subject groups showed considerable overlap of cell counts (Fig. 2) but the PAR group had significantly more T lymphocytes in the superficial submucosa compared with normal subjects ($P = 0.01$). Five of 22 (22.7%) IR patients had CD3+ cell counts in excess of the median counts obtained in the superficial submucosal region of the allergic group. No correlation was found between the number of CD3+ T cells and mast cells in the PAR and IR groups.

### CD4+ cells

Positively stained CD4+ cells were stained brown (Fig. 1) and all patient groups showed considerable overlap in CD4+ T cell counts with no significant difference seen between them (Fig. 2). Generally, increased CD4+ cell counts were seen compared with CD8+ cells in all patient groups. No correlation was found between CD4+ T cells and either mast cells or eosinophils in the PAR and IR groups.

### CD8+ cells

Fewer CD8+ T lymphocyte counts indicated by brown staining, were seen in the epithelium and superficial submucosa compared with CD4+ cells (Figs 1 and 2). In contrast, increased CD8+ cells were seen in the deeper submucosa of all groups compared with CD4+ counts. CD8+ lymphocytes were significantly increased in the superficial submucosa of IR compared with normal controls ($P = 0.02$) but did not show a significant difference to allergic subjects ($P = 0.17$).

A correlation was found between CD8+ T cells and mast cells in the superficial submucosa of IR patients ($r^2 = 0.26$, $P = 0.02$; Fig. 3).

### CD25+ cells

Significantly increased numbers of activated CD25+ T lymphocytes were seen in the epithelium of PAR compared with normal subjects ($P = 0.001$) but not to IR (Fig. 2). CD25+ cells were significantly increased in the superficial submucosal layer of PAR compared with IR ($P = 0.02$), but 4/22 (18%) subjects had T cell counts above the PAR median count for this region. No significant difference was seen between the IR and normal subjects but the IR group showed a trend towards significance ($P = 0.05$). No correlation was found between (CD25+) T cells and mast cells in the PAR and IR groups.

### CD45RA+ T cells

The PAR group had significantly increased numbers of brown-stained allergen-naïve T cells in the superficial submucosa compared with normal subjects ($P = 0.015$) (Figs 1

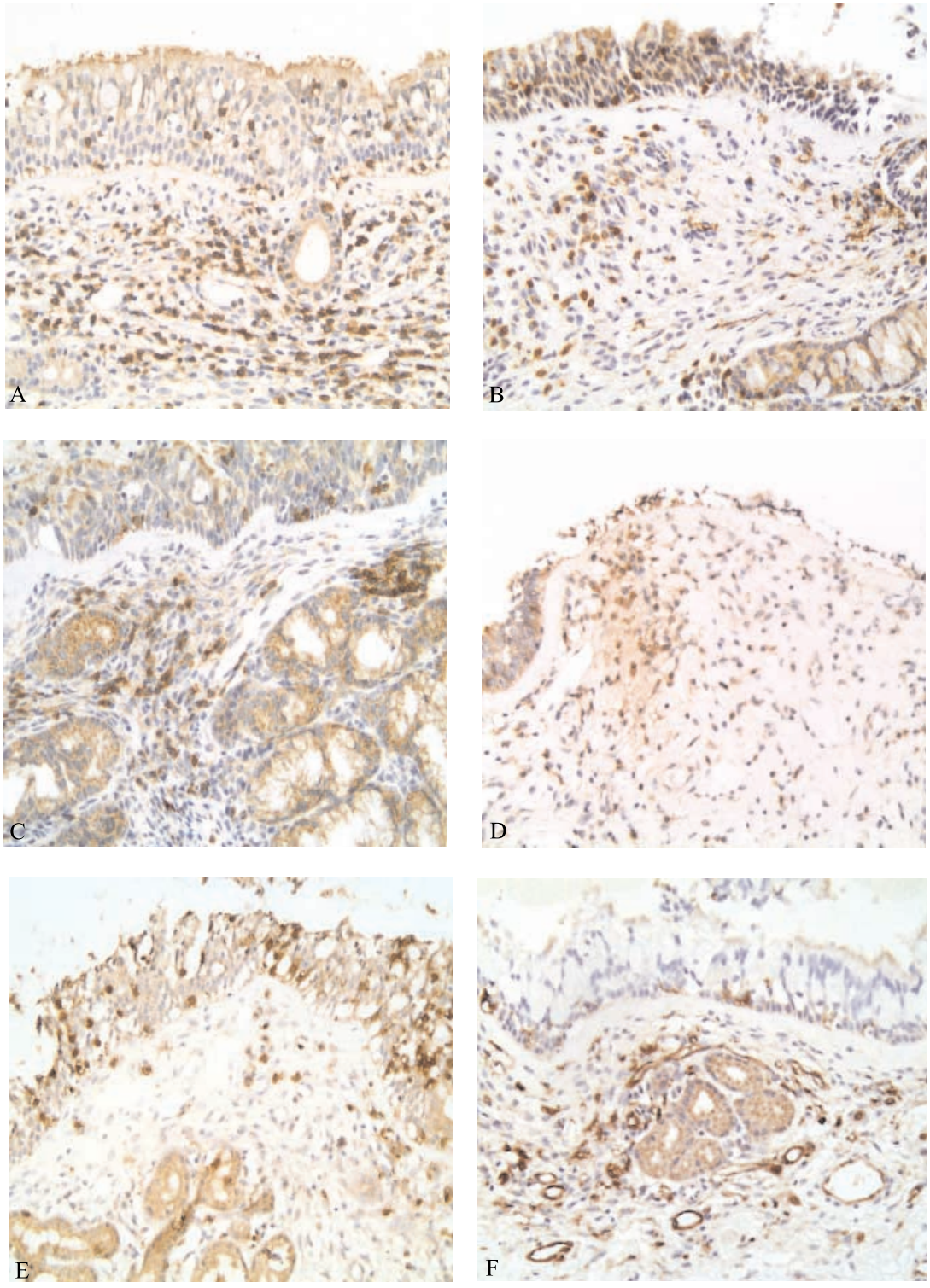


*Figure 1*. Lymphocyte T subsets were detected immunohistochemically in sections of nasal mucosa. CD3+ in (A) persistent allergic rhinitis (PAR) and (B) nonatopic idiopathic rhinitis (IR) tissue; CD4+ in (C) IR and (D) PAR; CD8+ in (E) PAR; HLA-DR in (F) PAR.

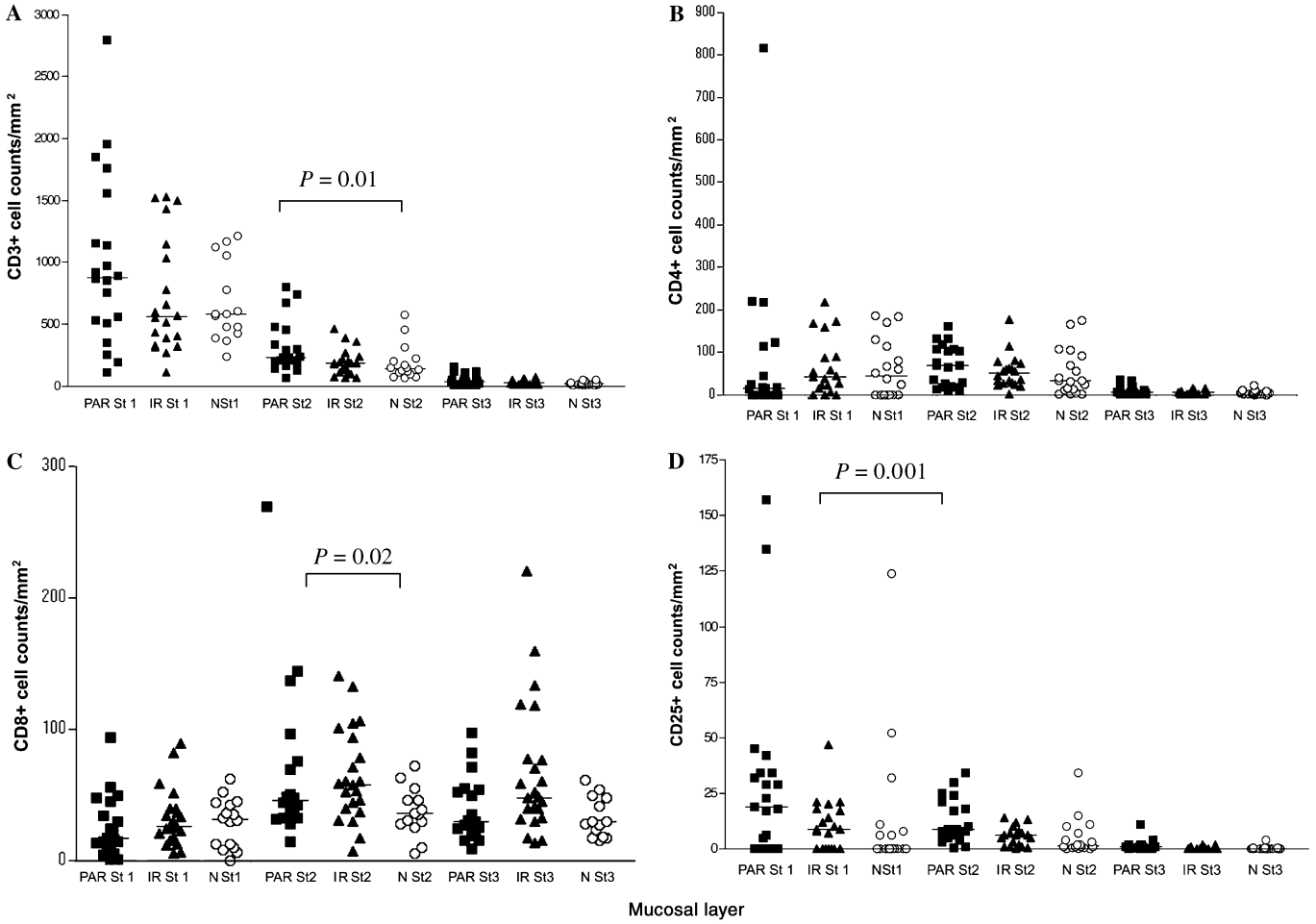


*Figure 2.* Cell counts were measured in three regions across the mucosa comprising the epithelium (St 1), superficial (St 2) and deep (St 3) submucosa. Cell counts for (A) CD3+ (pan) T lymphocytes, (B) activated CD4+ T lymphocytes, (C) CD8+ T lymphocytes, (D) activated CD25+ T lymphocytes, (E) allergen-naïve CD45RA T lymphocytes, (F) allergen-exposed CD45RO T lymphocytes, and (G) HLA-DRα expressing cells. Median values are shown by the horizontal bar in each column.

and 2) but not to IR patients. A correlation was found between CD45RA+ T cells and mast cell numbers in the superficial submucosa of PAR ($r^2 = 0.26$, $P = 0.03$; Fig. 3).

### CD45RO+ T cells

Increased numbers of brown-stained antigen-exposed CD45RO+ compared with CD45RA+ T cells were seen in all groups (Fig. 2). No significant difference was seen between the three subject groups for CD45RO+ lymphocytes.

No correlation was found between CD45RO+ T cells and mast cells in PAR or IR.

### Mast cell counts

Mast cells were detected in all three patient groups but were significantly increased in the epithelium of the PAR and IR groups ($P > 0.05$) compared with normal mucosa (data not shown).

### Basophil counts

The BB-1 antibody detected cells with a granular cytoplasm and a smaller size than that of mast cells, compatible with the morphology of basophils. These were predominantly localized to vessels in the superficial submucosa. Less than two cells per square millimetre were found in any of the cases.

## Discussion

The interaction between antigen presenting cells (APCs) and T cells is important in the development of a Th2 pathophysiology as seen in allergic rhinitis (6, 28). Most previous studies have phenotyped T cell populations present in the peripheral blood of allergic subjects. Here, we determined the local population of cells with antigen presenting properties, HLA-DRα+ MHC II, and different T cell subsets in the nasal mucosa of

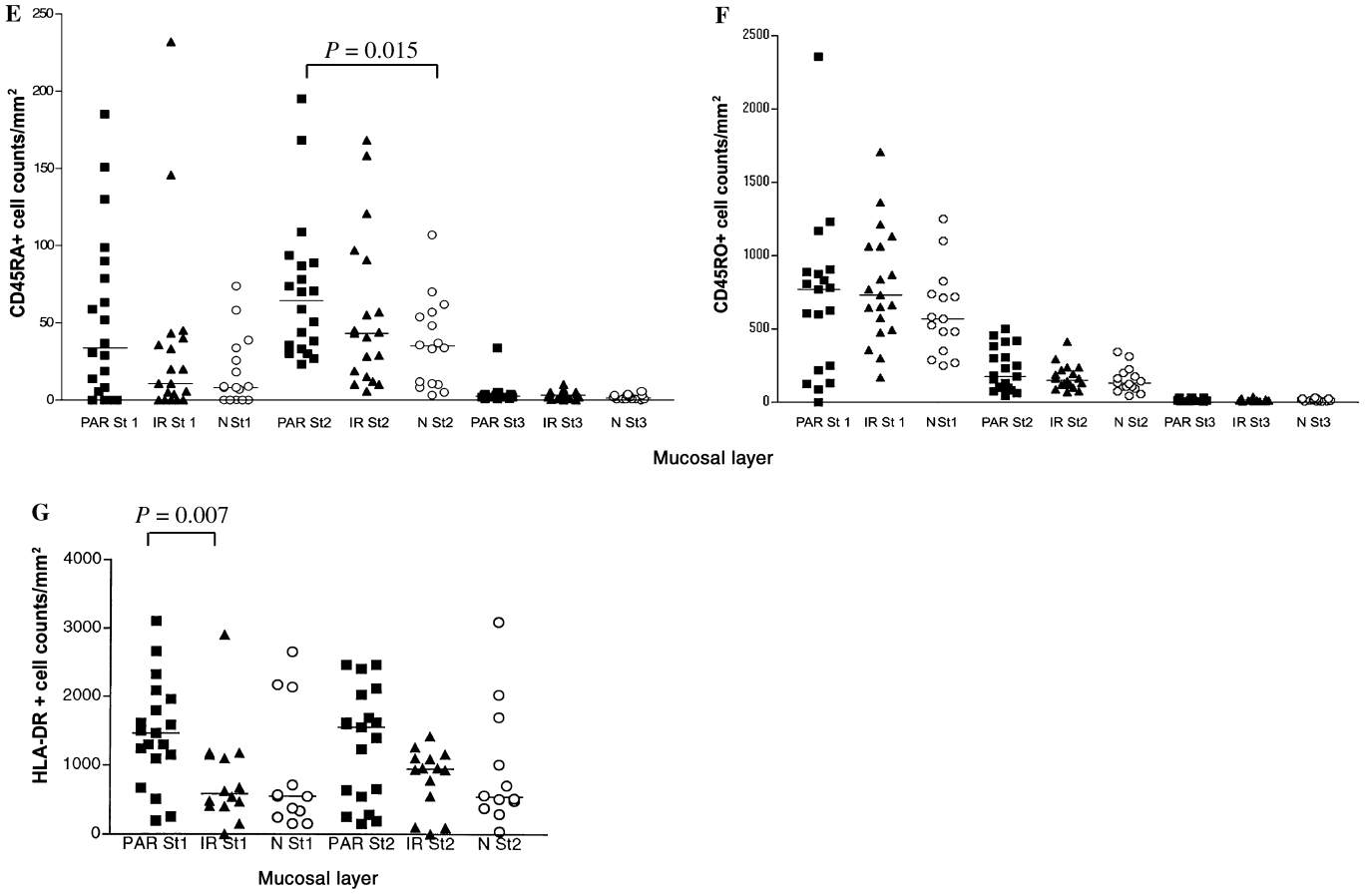


*Figure 2.* Continued.

patients with persistent allergic rhinitis (PAR) and nonatopic idiopathic rhinitis (IR). Comparison with normal mucosa allowed us to characterize these two forms of rhinitis. Moreover, we investigated if a relationship existed between different T cell subsets with mast cells and basophils as it has been proposed that there is amutual association between these inflammatory cells (17, 18).

In accordance with the Th1/Th2 paradigm (1, 29), we found significant differences between the atopic and nonatopic groups. Significantly fewer numbers of antigen presenting cells (APC) with an HLA-DR$\alpha$+ phenotype were present in the epithelium of the nonatopic IR subjects compared with their allergic counterparts. Interestingly, IR patients did not significantly differ to the PAR group with respect to numbers of total (CD3+) lymphocytes or epithelial activated (CD25+) and naïve (CD45RA+) T cells. This is unlikely because of natural biological variation because of significant differences seen between the allergic and normal controls for these subsets. Both rhinitis groups showed increased numbers of sub-epithelial cytolytic CD8+ lymphocytes compared with normal controls but the difference was only significantly different in the IR group.

There is evidence showing that APCs can induce a Th2-biased response by a reduction in the Th1 cytokine, IL-12 (8, 30). In agreement with others (5), we detected a significant increase in APCs in the epithelium of the allergic control group compared with the nonatopic rhinitis group but there was overlap seen between the patient groups. The significance of this finding pertaining to the normal controls is interesting and might be explained by these normal patients having asymptomatic 'covert' allergy (24) or natural biological variation.

CD4+ $T_{helper}$ lymphocytes are associated with allergic status because of Th2 cytokine secretion and account for $<5\%$ of the total CD3+ T cell population in peripheral blood (31). During an allergic response, increased numbers of CD4+ $T_{helper}$ cells occur in the nasal mucosa of persistent allergic (32, 33) and seasonal allergic rhinitis subjects (34). Here, we found no significant difference for CD4+ cells in the nasal mucosa between our three subject groups compared with the exaggerated CD4+ response seen in clinical allergen provocation studies (35). In contrast, it is reported that smaller 'natural' doses of allergen result in negligible alteration of the CD4+ and CD8+ lymphocyte population (36). Our study patients did not receive clinical allergen challenge prior to surgery

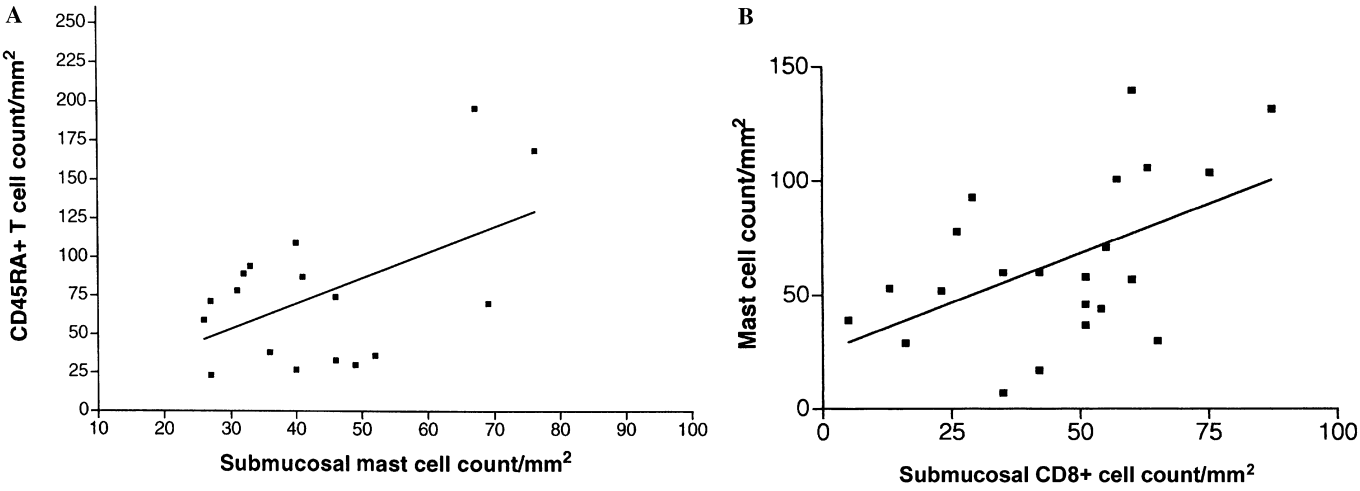


*Figure 3.* A correlation was found for superficial submucosal mast cells and (A) allergen-naïve CD45RA in patients with persistent allergic rhinitis, and (B) CD8+ T lymphocytes in patients with idiopathic rhinitis.

with the intention of maintaining the *in vivo* status of inflammatory cells. We found that the nonatopic idiopathic group had a significant increase in the cytolytic CD8+ T cell subset compared with their normal controls but not to the allergic group. Increased cytolytic cells are more commonly associated with MHC class I associated antigen recognition and in this respect the pathology seen in our nonatopic patient group resemble that found in cases of sudden asthma death (37). In fatal asthma and IR disease, increased numbers of CD8+ T lymphocytes and eosinophils (27) have been reported with relatively normal proportions of antigen presenting cells in the lung, and these differ to stable asthma (37), prompting the suggestion of a possible viral cause. As we found little difference between the allergic and nonallergic rhinitis group for CD8+ counts, the role of CD8+ cells requires further investigation. Although it is accepted that cells with a CD4+ phenotype belong to the $T_{helper}$ population, there are circumstances when $T_{Cytotoxic}$ cells also express CD4 (38).

We investigated the association between T cells and mast cells/basophils because these effector cells can substitute/supplement the function of Th2 cells because of their production of Th2 cytokines (39–41) and possession of CD40 receptors required for B cell immunoglobulin switch induction (15, 42). Previously, it has been suggested that mast cells might prolong the nonresolution of mucosal inflammation by providing alternative or supplementary B cell activation (41, 43, 44) and we propose that this may lead to redundancy of CD4+ cells. This function might account for the insignificant difference seen in numbers of CD4+ T cells between our patient groups. We found that the atopic and nonatopic rhinitis groups showed an association between T cells and mast cells in their nasal mucosa. Allergic patients showed a positive correlation between mucosal mast cells with antigen-naïve CD4+ CD45RA+ T cells. The nonatopic rhinitis group showed a positive correlation between mucosal mast cells and the cytolytic CD8+ lymphocyte population. There are several possible explanations for these findings. The recruitment of T cells and mast cells might involve a common factor, or one cell type might elicit the recruitment of the other. The former would appear more likely as there did not appear to be obvious co-localization between the lymphocytes and mast cells. While both cell types occurred in the same mucosal region (stratum 2), the T cells tended to form aggregates within the nasal airway lymphoid tissue (NALT), while the mast cells were randomly and diffusely distributed. There are reports suggesting the two cell populations may be mutually beneficial to each other because T cell-derived cytokines are needed for the growth, survival and effector function of mast cells and basophils (17, 18) and mast cell-derived cytokines promote the selection of a Th2 pathway. Basophils also have the capacity to induce IgE switching (16) and produce Th2 cytokines but here, we detected only small numbers of basophils and of those seen, they were predominantly localized to blood vessels.

## Conclusion

By not allergen-challenging our patients prior to turbinate sample collection we aimed to retain the fidelity of the T cell infiltrate seen in chronic rhinitis. Although we found overlap between the allergic and nonallergic rhinitis groups for T cell subsets, the two groups show distinct differences with respect to the number of APCs. Allergic patients are characterized by increased numbers of T cells with an activated CD25+ and allergen-naïve CD45RA+ phenotype. In contrast, patients with nonatopic idiopathic rhinitis have a predominance of T cells with a CD8+ phenotype. Interestingly, while our findings suggest that the initiating events of their rhinitis may

involve a different mechanism, both groups show similarities involving a relationship between T cell subsets and mast cells suggesting a common pathway in persistent (24) nasal inflammation.

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