The Relative Contribution of Mast Cell Subsets to Conjunctival T_H2-like Cytokines

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Purpose. To investigate the distribution of the T-helper (T_H)2-like cytokines, interleukin (IL)-4, IL-5, IL-6, and IL-13 between mast cell subsets in conjunctival biopsy specimens from normal subjects and those with seasonal allergic conjunctivitis (SAC) during and outside of the grass pollen season.

Methods. Sequential and double in situ hybridization (ISH) and immunohistochemistry (IHC) were performed on thin sections of human conjunctiva to determine the colocalization of the immunoreactivity of IL-4, IL-5, IL-6, and IL-13 to mast cell subsets in normal subjects and subjects with atopy and to detect IL-4 mRNA in conjunctival mast cells.

Results. More than 90% of IL-4^+–immunoreactive cells were observed to be mast cells in conjunctival biopsy specimens from all patient groups. The majority of IL-5^+, IL-6^+, and IL-13^+ cells were also noted to be mast cells for each group. IL-4 preferentially colocalized to the tryptase$^+$-chymase$^+$ mast cell phenotype (MC_{TC}) with MC_{TC} cells comprising 93.3% of cytokine$^+$ mast cells in symptomatic SAC ($P = 0.0017$), 89.2% in asymptomatic SAC ($P = 0.0008$), and 77.8% in normal subjects ($P = 0.0472$). IL-13 appeared to colocalize preferentially to the MC_{TC} phenotype and IL-5 and IL-6 to the MC_T phenotype. ISH showed that 75.8% of mast cells in normal subjects, 78.7% in subjects with symptomatic SAC, and 18.7% in subjects with asymptomatic SAC expressed mRNA for IL-4.

Conclusions. Conjunctival mast cells are an important source of IL-4, IL-5, IL-6, and IL-13 immunoreactivity, with preferential colocalization of IL-4 and IL-13 on the MC_{TC} subset and IL-5 and IL-6 to the MC_T subset. This evidence suggests that differences in protease phenotype may also reflect functional differences evidenced by the different patterns of cytokine distribution. (*Invest Ophthalmol Vis Sci.* 2001;42:995–1001)

Mast cells are recognized as central effector cells in atopic diseases including allergic conjunctivitis. In particular, mast cells are known to play important roles in the pathologic course of seasonal allergic conjunctivitis (SAC), a mild, prevalent form of ocular allergy.[1,2] During active SAC, conjunctival mast cell numbers have been shown to increase,[3] and elevated levels of the mast cell products histamine, tryptase, and leukotriene C_4 have also been detected in tears.[4–6] T-cell numbers are increased in chronic allergic conjunctivitis,[7,8] and they are a well characterized source of cytokines in allergic conditions. It is now recognized, however, that mast cells, in addition to T cells, are a source of pleiotropic cytokines, which are known to play key roles in the regulation of allergic diseases.[9] Mast cells represent a heterogeneous population of cells, however (see review in Reference 10), which varies according to their tissue distribution, their response to immune and nonimmune stimuli, and their biochemical content. In humans, two subsets are described, on the basis of on their content of neutral proteases: those containing tryptase, chymase, carboxypeptidase A, and cathepsin G, which are termed MC_{TC}, and those containing tryptase only, termed MC_T.[11] Interleukin (IL)-4, IL-5, IL-6, and IL-13 have been localized to mast cells at both the protein and mRNA levels,[12–17] and human conjunctival mast cells have also been shown to be a source of cytokines.[18]

In the present study, we sought to investigate the normal pattern of cytokine expression between MC_T and MC_{TC} subsets. To explore changes during natural allergen exposure, we studied patients with SAC who were asymptomatic or symptomatic, with regard to itch, and compared them with normal control subjects. In addition, we sought evidence that conjunctival mast cells are capable of sustained cytokine production through the synthesis of IL-4 mRNA.

Materials and Methods

This research followed the tenets of the Declaration of Helsinki. Informed, written consent was obtained from the subjects after explanation of the nature and possible consequences of the study. The study was approved by the Southampton University Joint Ethics Committee. The diagnosis of SAC was made on the basis of history, slit lamp examination, and positive skin prick test to a panel of mixed grass pollen: *Aspidistra elatior, Dactylis glomerata, Festuca pratensis, Lolium perenne, Phleum pratense,* and *Poa pratensis*, (Soluprick; ALK-Abelló, Frederiksburg, Denmark). The scoring system devised by Abelson et al.[19] was used to score the symptoms and signs of SAC. No patients had concomitant eye disease, wore contact lenses, or had been treated with immunotherapy or systemic steroids. All patients were asked to refrain from any systemic or topical medication for at least 2 weeks before entry into the study. Conjunctival biopsy specimens were obtained from 8 patients with symptomatic (3 women; 5 men; mean age, 50.1 years; range, 23–76) SAC in season (SAC IS), 8 asymptomatic patients with (three women, five men; mean age, 35.9 years; range, 19–70) SAC out of season (SAC OS), and 22 control subjects (14 women, 8 men, mean age, 58.2 years; range 20–92) with no atopy (NOR). There was no statistical difference in age between the groups.

Conjunctival Biopsy and Tissue Fixation and Processing

We chose to obtain biopsy specimens from bulbar conjunctiva, because the procedure is less traumatic than tarsal biopsy and is consistent with our previous reports on conjunctival pathophysiology. Our previous work[20] and that of others[21] has also demonstrated similar numbers of inflammatory cells at both sites, typically with slightly higher numbers of mast cells in bulbar tissue, and similar correlations between adhesion molecule expression and inflammatory cells at both sites. Lastly, sampling error was thought likely to be similar for a small sample of tissue taken from one site at one time compared with examining the entire conjunctiva.

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After instillation of topical oxybuprocaine (Chauvin Pharmaceuticals, Romford, UK), a snip biopsy was performed in the upper bulbar conjunctiva. Specimens were divided into two pieces, one sample processed for IHC and the other for ISH. Tissue samples for IHC were immersed immediately into chilled acetone containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 20 mM iodoacetamide) and stored overnight at −20°C. They were then processed into a water-soluble resin, glycol methacrylate (GMA), as described previously.[22] The blocks were polymerized overnight at 4°C and stored at −20°C in airtight containers containing silica resin. Samples for ISH were immersed in 10% neutral buffered formalin for overnight fixation and processed into paraffin wax using an automatic processor (Hypercentre 2; Shandon, Cheshire, UK).

Antibody and Riboprobe Sources

The following monoclonal antibodies (mAbs) were obtained from the following sources: AA1, Andrew F. Walls (Clinical Pharmacology, Southampton University, UK); MAB1254, Chemicon (Harrow, UK); 3H4 and 4D9, Christophe Heusser (Ciba–Geigy, Basel, Switzerland); MAB7, Laurie McNamee (Glaxo SmithKline, Oxbridge, UK); 104B11, Paul Hissey (Glaxo SmithKline); MAB 213, R&D Systems (Oxford, UK); UCHT1, Dako (High Wycombe, UK); M9269, Sigma (Poole, UK); streptavidin-biotin-peroxidase complexes (SBP), Dako; fluorescein isothiocyanate (FITC) anti-mouse, Dako; and anti-digoxigenin antibody, Boehringer–Mannheim (East Sussex, UK).

In Situ Hybridization

IHC was performed as previously described.[3] Two-micrometer-thick sections were cut, floated onto 0.2% ammonia water, and picked up on poly-L-lysine–coated slides. Endogenous peroxidase activity was blocked by treatment with sodium azide 0.1% wt/vol and hydrogen peroxide 0.003% vol/vol. Nonspecific binding sites were blocked by incubation in tissue culture medium (Dulbecco's modified Eagle's medium and 20% fetal calf serum). Primary antibodies were applied overnight at room temperature. The following mouse IgG_1 mAbs were used at previously titrated optimal dilutions: AA1 to mast cell tryptase, MAB1254 to mast cell chymase, MAB7 to IL-5, 104B11 to IL-6, MAB 213 to IL-13, and UCHT1 to CD3. Two different mAbs to IL-4 were used, 3H4 and 4D9. 3H4 typically gives a ring staining pattern, suggesting detection of IL-4 epitopes expressed when the cytokine is at the cell surface, 4D9 gives a principally cytoplasmic staining pattern suggestive of stored IL-4.[12] We used both mAbs, because our previous studies on the upper airway mucosa and conjunctiva had shown differences between IL-4 expression with regard to surface compared with stored IL-4.[13,18] Control slides included omission of the primary antibody, and because all other mAbs were IgG, a nonspecific IgG_1 mAb at the highest concentration for a primary antibody was also used (M9269). The absence of IgG_1 cross-reactivity was confirmed by preliminary experiments using an additional biotin-blocking stage after ISH, which showed no difference in staining quality between sections processed with or without this stage. After incubation with biotinylated second-stage antibodies (Dako), detection was achieved with streptavidin-biotin-peroxidase complexes (SBP), using aminoethylcarbazole to obtain a red reaction product.

Double IHC

Four-micrometer-thick sections were cut and treated as for standard IHC. Primary antibodies to mast cell tryptase (AA1) and IL-4 (3H4) were applied as before and labeled with either SBP with fast red as a chromogen or FITC anti-mouse mAb. Propidium iodide was used as a counterstain and the cells detected using a confocal laser microscope (Leica, Cambridge, UK). Stringent controls were performed to ensure the specificity of the detection, with the omission of each primary antibody and, separately, the omission of each secondary antibody, followed by application of both detection systems.

IL-4 Riboprobe Synthesis

A 0.3-kb human IL-4 cDNA insert from recombinant pUC18 (R&D Systems) was excised and subcloned into pSP70 (Promega, Southampton, UK) in its multiple cloning site based on the human IL-4 gene nucleic acid sequence published by Yokata.[23] The insert was oriented so that T7 promoter conduction would yield an antisense RNA transcript. *Hin*dIII or *Eco*RI digestion was then used to linearize the recombinant pSP70 IL-4. Digoxigenin-labeled antisense cDNA probe for IL-4 was synthesized by in vitro transcription (Boehringer–Mannheim) using 1 μg of *Hin*dIII-linearized pSP70 IL-4, 20 nanomoles of digoxigenin-uridine triphosphate, and 20 U of T7 RNA polymerase at 37°C. A sense IL-4 RNA probe was generated using the *Eco*RI digest of pSP70 IL-4 by SP6 RNA polymerase–conducted transcription. The synthesized RNA transcripts were checked by agarose gel electrophoresis to confirm a 300-bp fragment. The digoxigenin labeling of

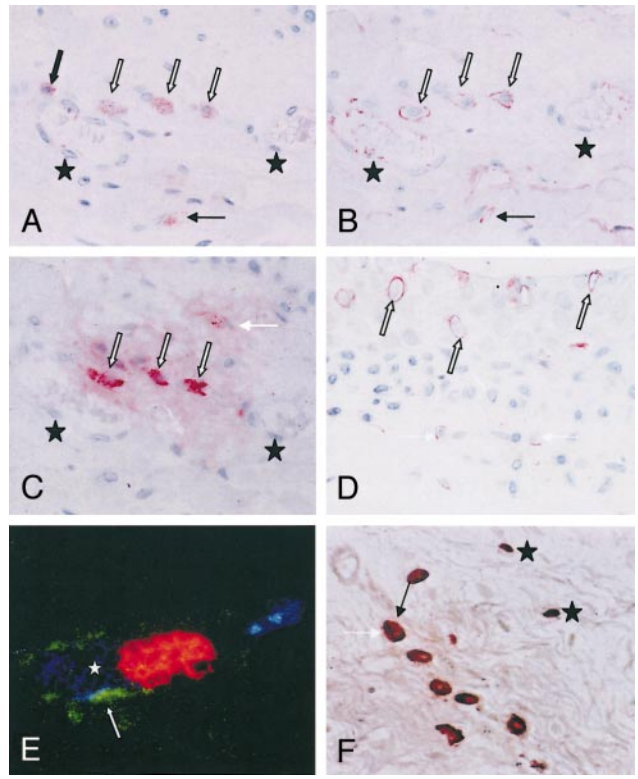

FIGURE 1. Composite images showing immunostaining (**A** through **D**), confocal microscopy (**E**), and double ISH-IHC (**F**) of mast cell cytokines, T cells, and IL-4 mRNA in human conjunctival biopsy specimens. (**A**, **B**, and **C**) Sequential 2-μm sections through the same specimen oriented for examination by using blood vessels (★) as morphologic landmarks. Mast cell tryptase was seen as granular cytoplasmic staining (*thick open arrows*; **A**). The *arrows* highlight the same cells in the adjacent sections, but the labeling was for IL-4 (**B**), which appeared as a ring staining pattern using the mAb 3H4, and for mast cell chymase, which was seen as granular cytoplasmic staining (**C**). (**A**, *thick solid arrow*) $Tryptase^+$ cell that did not colocalize with IL-4 or chymase. (**A**, **B**, *thin solid arrow*) $Tryptase^+$ mast cell that colocalized with IL-4 but not chymase. (**C**, *thin open arrow*) When granular staining alone was present but no nucleus was visualized, the cell was deemed not to show colocalization and was not counted. (**D**) T cells were clearly visible within the epithelium (*thick open arrows*) and the substantia propria (*thin open arrows*) using an mAb to CD3. A confocal micrograph illustrates simultaneous visualization of mast cell tryptase (☆) and surface IL-4 (**E**, *thin open arrow*). (**F**) Double ISH-IHC detected the simultaneous expression of IL-4 mRNA as *dark blue-black* staining (*white arrow*) and mast cell tryptase as a *dark red* chromogen (*thin solid arrow*) within the same cell. Also seen are cells positive for IL-4 mRNA (★), which did not show immunoreactivity for tryptase.

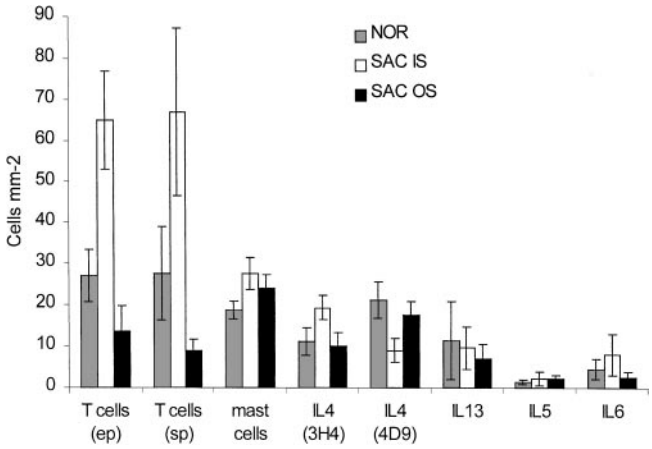

FIGURE 2. Total leukocyte and cytokine counts for each of the three study groups. T cells (EP), epithelial $CD3^+$ T cell count; T cells (SP), substantia propria $CD3^+$ T cell count. Error bars show SEM for each count.

each RNA probe produced was confirmed by dot-blot immunostaining with anti-digoxigenin antibody (Boehringer–Mannheim).

Immunohistochemistry

ISH was performed as previously described.[24] Conjunctival biopsy specimens were fixed by overnight immersion in 10% neutral buffered formalin and processed as before. Four-micrometer-thick sections were cut, floated onto a water bath at 37°C, and picked up on aminopropyltriethoxysilane coated slides. Sections were dewaxed, rehydrated, and washed in diethylpyrocarbonate (DEPC)–treated water followed by immersion in 0.2 N HCl at room temperature. The sections were then transferred to preheated (70°C) SSC and washed before buffering in 50 mM Tris/HCl. Permeabilization was performed by proteinase K (Sigma) treatment at 5 μg/ml at 37°C. Posttreatment fixation was achieved by immersion in 0.4% paraformaldehyde at 4°C. Before the hybridization of probe to target mRNA, the sections were incubated in the hybridization buffer. Fresh hybridization buffer was then added with digoxigenin-labeled antisense riboprobe at a final concentration of 5 ng/μl at 42°C overnight. Nonspecifically bound probe was removed by stringent washing using progressively decreasing salt concentrations of SSC at 42°C with the addition of formamide (30%) to the last wash. Nonspecific antibody-binding slides were blocked by washing in 3% bovine serum albumin (BSA)/0.1% Triton X-100 in Tris-buffered saline (TBS). Probe detection was achieved using antidigoxigenin alkaline phosphatase–conjugated antibody at 1:600 (Sigma) in TBS. Bound antibody was detected by characteristic blue-black staining with the chromogen nitrobluetetrazolium-5 bromo-4 chloro-3 indolyl phosphate (NBT-BCIP; Sigma). Negative control slides were produced using the sense IL-4 riboprobe and omission of riboprobe. Positive controls were performed using a 300-bp riboprobe for the constitutive cytoskeletal protein β-actin (Ambion, Oxford, UK).

Double ISH-IHC

The ISH protocol was followed by IHC, using the mAb AA1 to mast cell tryptase as described earlier. Control slides were treated identically and comprised a combination of sense probe and irrelevant antibody controls performed as part of the individual protocols for IHC and ISH.

Quantification and Statistical Analysis

Cells were counted without knowledge of their original source. Only nucleated cells were counted, and all cells within each specimen were counted at a magnification of ×400. To measure counting consistency, 20% of the slides were re-counted by the same observer, who was masked to the specimen number and group from which the slides were

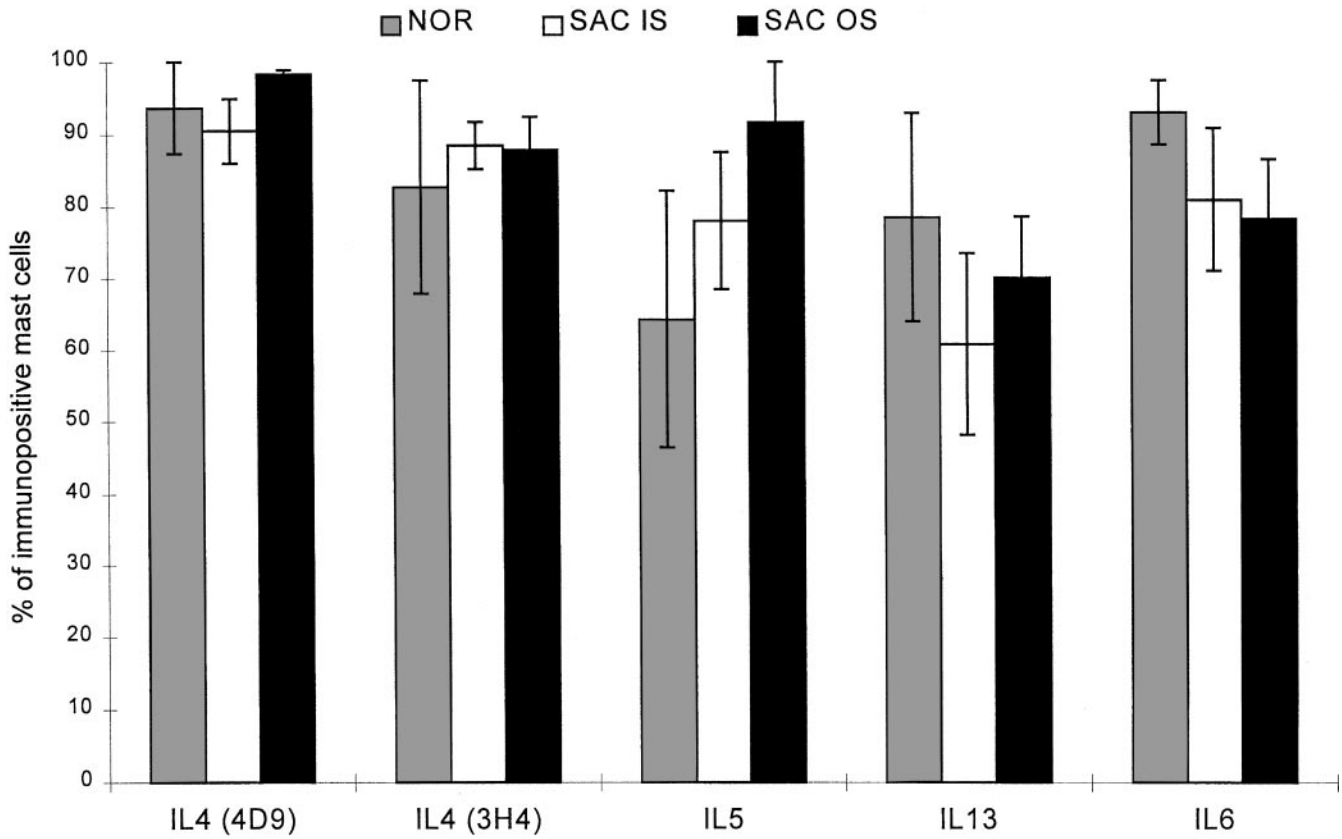

FIGURE 3. Percentages of cells immunopositive for cytokines that were also identified as mast cells. Error bars show SEM for each count.

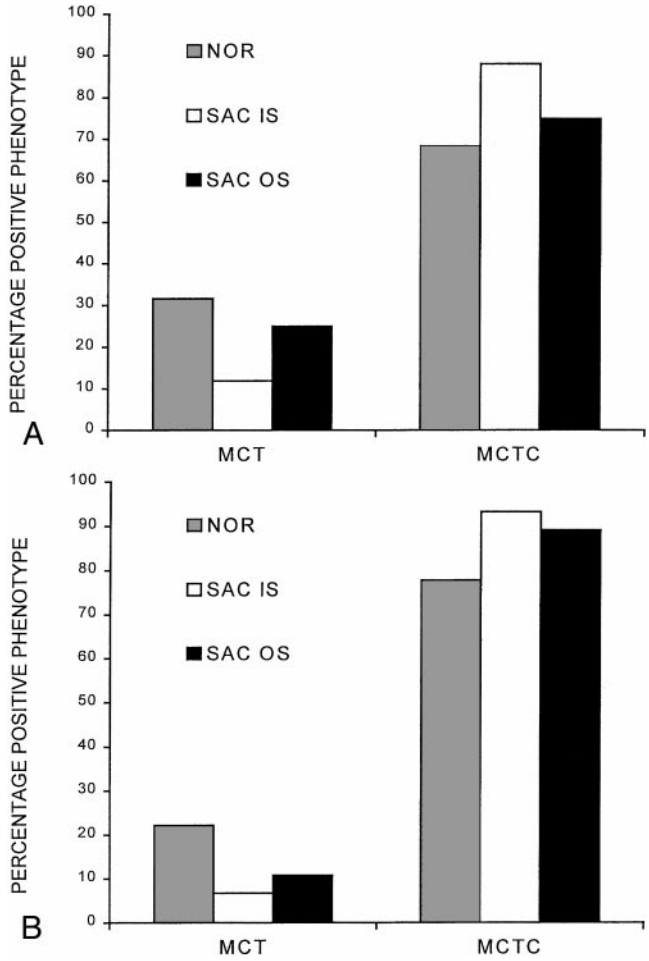

FIGURE 4. Distribution of mast cell phenotype in the total population of mast cells that were immunopositive for IL-4, using two different labels: (**A**) cell surface IL-4 using mAb 3H4 and (**B**) cytoplasmic IL-4 using mAb 4D9. The total proportion of MC_{TC} and MC_T cells in each case was 100%.

chosen. The area of lamina propria (in square millimeters) and length of the epithelium (in millimeters) was measured using a semiautomated image analysis system (Colorvision 1.7.4a; ImproVision, Warwick, UK; and Symantec, Cupertino, CA.). Using the method of Bland and Altman,[25] a coefficient of repeatability (twice the SD of the log-transformed differences in counts) was calculated for the recounted slides.

Tryptase$^+$ and/or chymase$^+$ cells showed colocalized positivity to both the 3H4 or 4D9 mAbs for IL-4 and for IL-5, IL-6, and IL-13 by using a camera lucida to identify the same cells in adjacent sections. In addition, IL-4 was colocalized to CD3$^+$ cells by using the 3H4 and 4D9 IL-4 mAbs and UCHT1 (CD3) mAb. Three random microscope fields with high cell counts were examined at ×250 magnification. In cases in which immunoreactivity of positive granules could not be shown to colocalize on nucleated cells (Fig. 1C), the cells were not counted. Sections processed for double ISH-IHC were counted at a magnification of ×400, and the number of cells positive for IL-4 mRNA, tryptase, and both IL-4 mRNA and tryptase was noted.

A nonparametric test, the Mann–Whitney test, was used to explore the relationship between data sets, with $P < 0.05$ accepted as significant. All values are expressed as mean ± SEM.

RESULTS

All symptomatic patients with SAC experienced itch, redness, and lacrimation, with an average symptom score of 6.75 (range, 5–8). Asymptomatic patients and normal control subjects had symptom scores of 0, with significant differences between scores of symptomatic and asymptomatic patients ($P = 0.0008$) and between symptomatic patients and normal subjects ($P = 0.007$).

Clear immunohistochemical staining for IL-4, IL-5, IL-6, IL-13, mast cell tryptase and chymase, and CD3$^+$ T cells was observed, with no staining appearing on the control slides. Examination of sequential sections using morphologic landmarks to orient the specimen under the microscope enabled the identification of the same cells and the colocalization of cytokine product to mast cell subtypes (Figs. 1A, 1B, 1C).

The mAb 3H4 to IL-4 gave predominantly a ring staining pattern (Fig. 1B), although cytoplasmic staining was observed in a significant proportion of cells. This pattern was also imaged by double immunofluorescence confocal microscopy, which clearly demonstrated 3H4$^+$ staining at the mast cell surface (Fig. 1E). Staining with the mAb 4D9 to IL-4 resulted in predominantly cytoplasmic staining, although ring staining was noted in a few cells. Immunostaining for IL-5, IL-6, and IL-13 was cytoplasmic, with very few cells displaying surface staining. Mast cells were observed to stain for both 3H4 and 4D9 mAbs in sequential sections. Although 3H4$^+$ mast cells were often positive for both 3H4 and 4D9 epitopes of IL-4, this was not always the case. 4D9$^+$ mast cells were not always 3H4$^+$, although the numbers in each group were too small to achieve a statistically significant comparison. In sequential sections from two biopsy specimens, one from a normal subject and one from a symptomatic patient, some mast cells were observed to be positive for IL-4, IL-5, and IL-6. No CD3$^+$ cells were noted to show colocalization of either 3H4 or 4D9 immunoreactivity. Because CD3$^+$ cells are recognized as an important source of cytokines in allergic diseases, it is possible that the failure of cytokine product to colocalize on these cells was due to the rapid export of cytokine from the cell with insufficient cytokine epitope available for immunodetection. Mast cells were predominantly located subepithelially and around blood vessels, with no mast cells noted in the epithelium in control specimens or in those from asymptomatic subjects. Two intraepithelial mast cells were observed in two symptomatic patients with SAC. Although the large majority of chymase$^+$ cells were also tryptase$^+$, some chymase$^+$ cytokine$^+$-only cells were observed.

Leukocyte and Cytokine Counts

Mast cell numbers were elevated in symptomatic patients with SAC (27.6 ± 3.9/mm^2) compared with asymptomatic (24.1 ± 3.3/mm^2) and normal subjects (18.6 ± 2.2/mm^2; Fig. 2), although the difference did not reach statistical significance. Epithelial CD3$^+$ cell numbers were noted to be significantly higher in symptomatic patients (64.9 ± 12.0/mm^2) than in asymptomatic patients (13.6 ± 6.3/mm^2, $P = 0.014$) and normal control subjects (26.9 ± 6.6 mm^2, $P = 0.03$). CD3$^+$ cell numbers in the substantia propria were also significantly higher in symptomatic patients (66.9 ± 20.3/mm^2) than in asymptomatic patients (9.0 ± 2.8/mm^2, $P = 0.0019$; Fig. 2) but not normal subjects (27.7 ± 6.8/mm^2, P = nonsignificant [ns]). Although mean epithelial and substantia propria CD3$^+$ cell numbers were almost double in normal control subjects compared with asymptomatic patients, these differences were not statistically significant, probably because of the data variation.

Intraepithelial IL-4$^+$ mast cells were observed in two symptomatic patients, but immunoreactivity was otherwise restricted to substantia propria cells. Of interest was the increased number of 3H4$^+$ cells in tissue specimens from symptomatic patients (19.3 ± 2.9/mm^2) compared with asymptomatic patients (9.9 ± 3.6/mm^2) and normal subjects

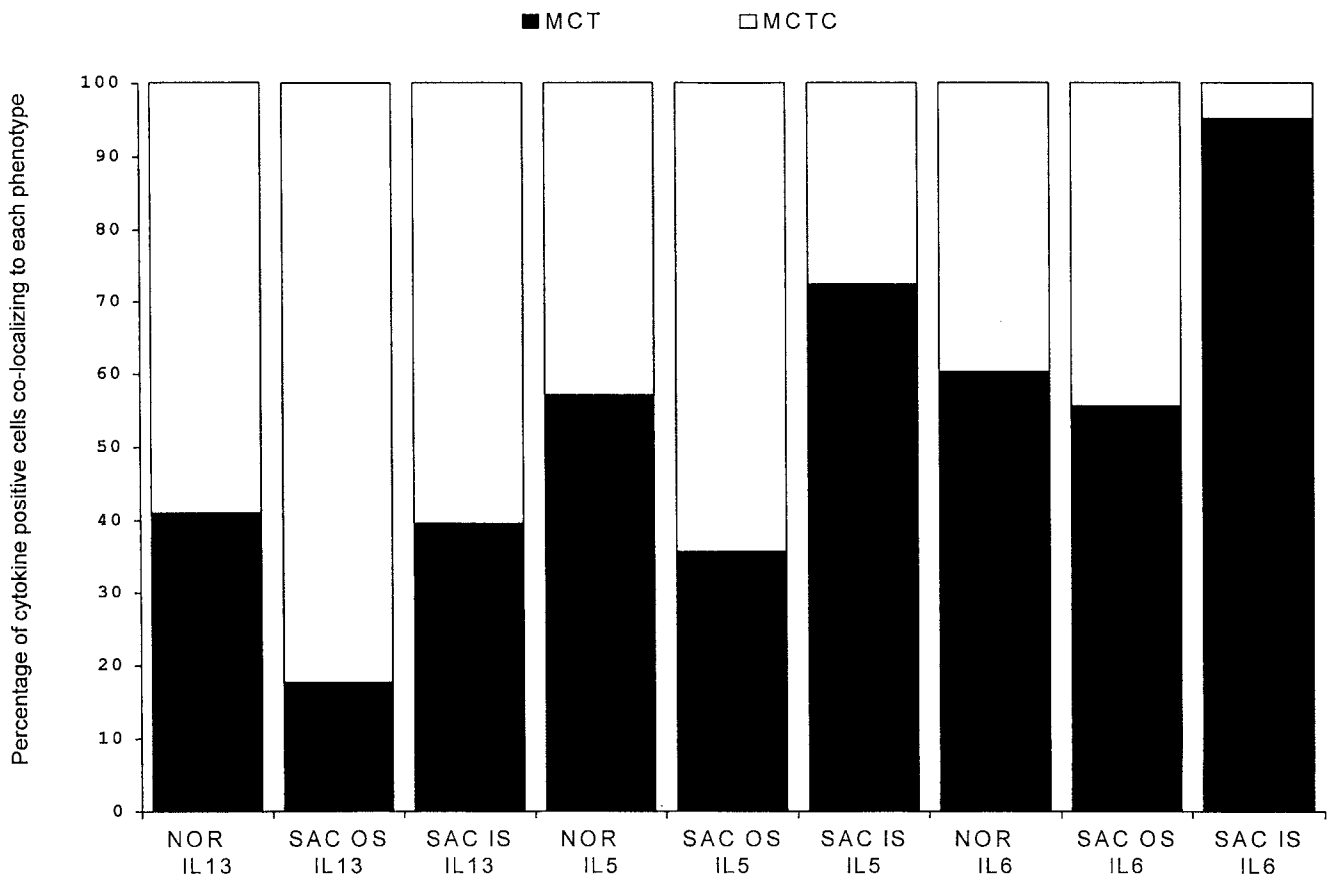

FIGURE 5. Distribution of mast cell phenotype in the total population of mast cells that were immunopositive for IL-5, IL-6, and IL-13. Bars represent the distribution of the total population of cytokine$^+$ cells between MC_T and MC_{TC} subtypes for each patient group.

(11.2 ± 1.2/mm^2; P = ns) and the decreased number of 4D9$^+$ cells in the same specimens (9.0 ± 2.9/mm^2 in SAC IS, 17.6 ± 3.4/mm^2 in SAC OS, 21.1 ± 1.2/mm^2 in normal tissue; P = ns; Fig. 2). The differences in raw counts for IL-5$^+$, IL-6$^+$, and IL-13$^+$ cells showed no significant differences between groups. By recounting the same slides, a coefficient of repeatability of 3.1 cells/mm^2 was calculated, indicating that the counts were reproducible and reliable.

The large majority of cytokine$^+$ cells were mast cells (Fig. 3). This was most marked for IL-4, with which more than 90% of 4D9$^+$ and 80% of 3H4$^+$ immunoreactivity was observed in tryptase$^+$ cells, irrespective of group of origin. For IL-5, IL-6 , and IL-13, the majority of cytokine$^+$ cells were also noted to be tryptase$^+$, irrespective of group of origin (Fig. 3). We did not seek to identify the minority of cytokine$^+$ positive cells that were, by exclusion, neither mast cells nor CD3$^+$ cells. No significant differences in the proportion of cytokine$^+$ mast cells were found between the disease groups and the control group.

Mast Cell Heterogeneity

In all patient groups the majority of mast cells were of MC_{TC} phenotype, with the greatest difference noted in symptomatic subjects with SAC (79.1% MC_{TC} versus 20.9% MC_T, P = 0.0008). This difference was less marked although significant in asymptomatic patients with SAC (65.1% MC_{TC} versus 34.9% MC_T, P = 0.015) and normal subjects (% 71.5 MC_{TC} versus 28.5% MC_T, P = 0.02). No statistical difference was noted between the proportions of MC_T or MC_{TC} cells between the groups.

IL-4$^+$ mast cells displayed clear phenotypic heterogeneity. Surface 3H4$^+$ immunoreactivity colocalized significantly to MC_{TC} cells in symptomatic (P = 0.0008) and asymptomatic (P = 0.006) subjects with SAC (normal subjects, P = ns; Fig. 4A), whereas 4D9$^+$ immunoreactivity colocalized significantly to MC_{TC} cells in all groups (normal tissue, P = 0.0472; symptomatic SAC, P = 0.0017; asymptomatic SAC, P = 0.0008; Fig. 4B). A less consistent pattern was observed for IL-13$^+$ mast cells, with the greatest difference noted in asymptomatic SAC (P = ns; Fig. 5). IL-5$^+$ and IL-6$^+$ immunoreactivities were variable but appeared to colocalize preferentially to MC_T cells, except for IL-5$^+$ mast cells in subjects with asymptomatic SAC (Fig. 5). The differences in distribution did not achieve statistical significance.

ISH demonstrated clear labeling of cells in the conjunctival sections with no staining in the sense probe or no primary antibody controls. Double ISH-IHC (Fig. 1F) demonstrated that 75.8% of mast cells were positive for IL-4 mRNA in normal subjects with 78.7% positive in subjects with symptomatic SAC and 18.7% positive in subjects with asymptomatic SAC. The majority (62%) of IL-4 mRNA$^+$ cells in normal subjects were tryptase$^+$, but the level decreased to 42.5% in subjects with symptomatic SAC (P = ns).

DISCUSSION

In the present study we demonstrate for the first time that the human conjunctival mast cell population is heterogeneous in the pattern of T_H2-like cytokines distributed between the MC_T and MC_{TC} subsets. In particular, IL-4 and IL-13, known to drive

T_H2-like allergic responses, were observed to immunolocalize preferentially to the MC_{TC} phenotype, whereas IL-5 and IL-6 appeared to localize preferentially to MC_T cells. These data suggest that mast cell subsets may serve different roles in atopic reactions.

Our observation of an increase in mast cell numbers during active disease was consistent with our earlier reports[3,18,26] and with their known role in SAC.[1,2] The increases in $CD3^+$ cell number in both the substantia propria and epithelium were also consistent with the observation of increased expression of the adhesion proteins intracellular adhesion molecule-1 (CD54) and vascular cell adhesion molecule-1 (CD106) whose ligands are found on T cells and granulocytes in conjunctival biopsy specimens from patients with allergic conjunctivitis.[20]

Although T cells are known to be an important source of cytokines in allergic diseases, they require activation and, in the case of T_H2-like cells, priming with IL-4 before they can release it.[27] We observed mast cells to be the source of the large majority of IL-4, IL-5, IL-6, and IL-13 immunoreactivity, irrespective of the group from which the tissue was obtained. Because these cytokines appeared to be stored within the cytoplasmic secretory granules, it is likely that they are released during IgE-mediated stimulation, resulting in a rapid pulse of these immunomodulatory proteins into the surrounding microenvironment. By demonstrating that the large majority of $IL\text{-}4^+$ immunoreactivity and IL-4 mRNA in normal human conjunctival tissue was localized to mast cells, these results also suggest that this cell is capable of sustained IL-4 production. The presence of IL-4 was confirmed, not just by the colocalization of 3H4 and 4D9 immunoreactivity to both MC_T and MC_{TC} phenotypes in serial sections, but also by double IHC, which demonstrated simultaneous expression of IL-4 and the unique mast cell protease marker tryptase in the same cells. In contrast, no IL-4 product colocalized to $CD3^+$ cells, perhaps because these cells do not have the necessary structures to store sufficient cytokine for immunodetection.

We noted that IL-4 and IL-13 colocalized preferentially to the MC_{TC} subset in disease and control groups. Because IL-4 is well recognized to upregulate humoral and suppress cell-mediated immunity by stimulating the differentiation of the T_H2 subset of T-helper cell,[27] this evidence suggests that MC_{TC} cells are capable of participating in the local regulation of IgE. It is known that mast cells can induce B-cell isotype switching to IgE synthesis through direct CD40/CD40 ligand binding and through the release of IL-4 and IL-13 independent of T-cell help.[28] IL-4 has also been shown to upregulate the high-affinity IgE receptor component, FcϵRIα chain, on human mast cells.[29] IL-5 and IL-6 appeared to colocalize to the MC_T subset although the difference in distribution was less marked, and IL-5 and IL-6 cell counts were low in all groups including symptomatic patients. Because MC_T cells remained in the minority among mast cells in all groups, the low levels of IL-5 and IL-6 immunoreactivity appeared to be consistent with the mild nature of SAC compared with chronic allergic eye diseases in which IL-5–driven eosinophil activation plays a central role.

The preferential distribution of T_H2-like cytokine immunoreactivity between MC_{TC} and MC_T cells observed in this study supports the hypothesis that these subsets may play different roles during physiological or pathologic conditions. Both 3H4 and 4D9 mAbs to IL-4 displayed a significant preferential colocalization to the MC_{TC} subset. The ring staining pattern observed with mAb 3H4 may have reflected cell surface cytokine possibly in the process of release. This would be in agreement with the observed increase in $3H4^+$ cells in symptomatic patients compared with control subjects. The number of cytoplasmic $4D9^+$ cells declined in symptomatic patients compared with numbers in both asymptomatic and normal control subjects, consistent with cytokine depletion during degranulation. It is possible that the 3H4 staining reflected IL-4 bound to the IL-4 receptor (IL-4R) at the cell surface, but the absence of immunoreactivity on the surface of other cells known to express the IL-4R made this possibility less likely. The high proportion of mast cells observed to express IL-4 mRNA in conjunctiva taken from symptomatic patients compared with the low proportion in asymptomatic patients suggests that induced transcription of IL-4 mRNA may play an important role in the regulation of IL-4 activity. The relatively high proportion of mast cells positive for IL-4 mRNA in conjunctiva from normal control subjects, however, suggests that cytokine storage and release from mast cell granules could also play a role in cytokine regulation. Clearly, future functional studies are needed to clarify this issue.

The findings that mast cells are an important source of the preformed conjunctival T_H2-like cytokines IL-4, IL-5, IL-6, and IL-13 and that the distribution of these cytokines is strongly related to cell phenotype enhances our understanding of the role of the conjunctival mast cell in the local immunoregulation of SAC. As a source of IL-4 and IL-13, these observations provide a potential new mechanism for MC_{TC} cells to bias the conjunctiva toward the development of a T_H2-like immune response before IgE-mediated activation. Although the concept of mast cell subsets based on cytokine secretory pattern has yet to be explored, the functional significance of this heterogeneity suggests different roles for MC_T and MC_{TC} cells in atopic and nonatopic disorders of the conjunctiva.

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