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T lymphocytes and mast cells express messenger RNA for interleukin-4 in the nasal mucosa in allergen-induced rhinitis

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SUMMARY

We have investigated the phenotype of interleukin-4 (IL-4) mRNA$^+$ cells in the nasal mucosa of six subjects with allergic rhinitis before and 24 hr after local allergen provocation with grass pollen extract. Serial cryostat sections were cut from paraformaldehyde-fixed snap-frozen nasal biopsies, and immunocytochemistry (APAAP) followed by *in situ* hybridization performed on the same sections. For immunocytochemistry, antibodies against CD3, tryptase, major basic protein (MBP) and CD68 were used to identify T cells, mast cells, eosinophils and macrophages, respectively. Hybridization studies were performed using a digoxigenin-labelled IL-4 riboprobe. Nitroblue tetrazolium (NBT) and X-phosphate-5-bromo-4-chloro-3-indoly phosphate (BCIP) served as chromogens to detect hybridization IL-4 mRNA signals. Significant increases in T lymphocytes and eosinophils and in the number of IL-4 mRNA$^+$ cells were observed after allergen challenge. Double immunocytochemistry/*in situ* hybridization demonstrated that the majority of IL-4 mRNA$^+$ cells after allergen challenge were CD3$^+$ (73·7% ± 1·6). Lower numbers of IL-4 mRNA hybridization signals were co-localized to tryptase$^+$ cells (26·0% ± 1·6). In contrast, no IL-4 mRNA hybridization signals were co-localized to either eosinophils or macrophages. These results indicate that after allergen challenge T cells are the principal cellular source of IL-4 mRNA transcripts during human late nasal responses, with a lesser contribution from mast cells.

INTRODUCTION

Allergic rhinitis is characterized by the IgE-dependent release of mast cell mediators[1–3] and cellular infiltration of the nasal mucosa with activated T lymphocytes and eosinophils.[4] For example, we[5] and others[6,7] have shown that seasonal pollen exposure results in epithelial migration of mast cells. Furthermore, allergen-induced late nasal responses are accompanied by increases in CD4$^+$ T lymphocytes, eosinophils and CD25$^+$ cells, presumed activated T lymphocytes.[4] These events appear to be regulated, at least in part, by the elaboration of so-called T-helper 2 (Th2)-type cytokines, particularly interleukin-4 (IL-4) and IL-5.[8–10] Interleukin-4 is of particular interest since this is the major cytokine responsible for B-cell isotype switching in favour of IgE production.[11] Recent studies have shown that IL-4 enhances expression of VCAM-1 on the surface of endothelial cells.[12,13] VCAM-1 selectively enhances eosinophil adhesion via a very-late antigen (VLA-4)-dependent mechanism. Thus IL-4 is pivotal in terms of local IgE regulation and, at least in part, may account for selective eosinophil recruitment at allergic tissue sites. We recently reported that topical corticosteroids inhibited allergen-induced late nasal responses and the associated tissue eosinophilia.[14] Of particular interest, corticosteroids selectively inhibited local IL-4 mRNA expression after allergen whereas allergen-induced increases in IL-5 mRNA in the nasal mucosa remained unchanged.[15]

IL-4 is known to be a product of activated T lymphocytes *in vitro*. However, recent studies have suggested that other cell types, including mast cells (at least in the mouse), are capable of synthesizing IL-4.[16–18] Furthermore, by use of immunohistochemical methods, IL-4 and IL-5 have been localized to mast cells in the human nasal mucosa in patients with perennial allergic rhinitis.[18,19] In the present study, nasal biopsies from allergen-induced late responses have been used in order to determine whether T lymphocytes, mast cells, eosinophils or macrophages are the principal source of IL-4 mRNA transcripts. In order to achieve this we have used a non-isotopic method of *in situ* hybridization and simultaneous *in situ* hybridization/immunocytochemistry in order to identify the phenotype of cells expressing IL-4 mRNA during late nasal responses.

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MATERIALS AND METHODS

Patients

Subjects were recruited from the allergy clinic of the Sahlgrens Hospital, Gothenburg, Sweden. Informed written consent was obtained, and the study was approved by the Ethics Committees of the Royal Brompton National Heart and Lung Hospital, London, U.K. and the Sahlgrens Hospital. Biopsies were obtained from six patients (four male, two female, age 22–45 years) with grass pollen seasonal rhinitis in whom previous studies had demonstrated a strongly positive IL-4 mRNA hybridization signal after allergen challenge using ^{35}S-labelled riboprobes. All subjects had positive skin prick tests to Timothy grass pollen extract (*Phleum pratense*; Soluprick, ALK, Horsholm, Denmark). None had received topical or oral medication in the 6 months before the study nor immunotherapy in the previous 5 years. Baseline nasal biopsies were performed outside the grass pollen season at a time when the patients were asymptomatic. Patients were then provoked by the application of 7 μl solution of 1000 biological units (BU) of grass pollen extract on 4-mm filter paper discs to the inferior nasal turbinate, as previously described.[4] At 24 hr, nasal biopsies (2·5 mm) were taken by use of Gerritsma forceps[20] after local anaesthesia using 3% cocaine and 0·025% adrenaline.

Tissue preparation

Nasal biopsies were fixed immediately in freshly prepared 4% paraformaldehyde for 2 hr then washed twice (first for 1 hr and then overnight) with 15% sucrose in 0·1 M phosphate-buffered saline (PBS), pH 7·4. Biopsies were blocked with OCT and kept at −80° until used. Double immunocytochemistry/*in situ* hybridization was performed as previously described.[21]

Preparation of probes

In order to obtain sense and anti-sense digoxigenin-labelled riboprobes, PGH–IL-4, containing a human IL-4 cDNA insert of 500 bp into pGEM-4 vector (a gift from Glaxo Institute for Molecular Biology S.A., Geneva, Switzerland) was linearized with *Eco*RI (for sense) or *Sph*I (for anti-sense), respectively. These linearized templates were labelled with digoxigenin-11-UTP (Boehringer-Mannheim, Mannheim, Germany) by *in vitro* transcription according to standard protocol from the manufacturers.

The amount of labelled riboprobe was measured by electrophoresis of a small aliquot of transcription reaction mixture in a denaturing 2% agarose formaldehyde gel together with serial dilutions of a reference sample of known concentration. For each transcription reaction, about 8 μg of labelled IL-4 RNA transcripts was obtained. The specificity of the labelled IL-4 RNA probe was confirmed by using a dot-blot detection system (Boehringer Mannheim).

Immunochemical staining

Cryostat sections (6 μm) were freshly cut from nasal biopsies, mounted on slides coated with 0·1% poly-L-lysine (Sigma, Poole, U.K.) and air-dried for 2 hr at room temperature. The alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique[4] was used to phenotype cells by use of monoclonal antibodies directed against human T lymphocytes (CD3; Becton Dickinson, Cowley, U.K.), human eosinophil major basic protein (MBP; BMK-13) (which stains total eosinophils),[22] macrophages (CD68; Dako, High Wycombe, U.K.) and human mast cells (tryptase; Chemicon International Inc., Temecula, CA).

The APAAP technique was as previously described[4] with some modifications. Briefly, slides were immersed in TBS (150 mM NaCl, 50 mM Tris–HCl, pH 7·4) for 5 min, then incubated with primary monoclonal antibodies for 30 min. Slides were then incubated with a rabbit–anti-mouse Ig (Dako) followed by APAAP conjugates (Dako) each for 30 min. Rabbit–anti-mouse Ig and APAAP conjugates were diluted in TBS with 20% human serum. In order to avoid loss of the immunochemical signals during the *in situ* hybridization procedure, signals were enhanced by repeating steps 2 and 3 (10 min each). Slides were washed between all steps (3 × 2 min) with TBS. All incubations were carried out at room temperature. In order to avoid RNase contamination, all solutions used were made up in 0·1% DEPC-treated distilled water. Additionally, the 20% human serum used also contained 100 U/ml of RNase inhibitor (RNasin; Promega). To develop the reaction, Fast Red TR (Sigma) was used as the chromogen and the intensity of the immunostaining reaction was adjusted by light microscopy. For negative control preparations, the primary antibody was replaced with either non-specific mouse Ig or TBS.

In situ *hybridization*

After immunochemical staining the same slides were immediately immersed in 4% paraformaldehyde/PBS for 5 min, then treated with 0·3% Triton X-100 in PBS for 10 min. After a brief wash in PBS supplemented with 0·1% DEPC, sections were exposed to proteinase K solution (1 μg/ml in 20 mM Tris–HCl and 1 mM EDTA, pH 7·2) for 15 min at 37°. After a brief rinse in PBS, slides were immersed in 4% paraformaldehyde in PBS for 5 min, transferred to PBS supplemented with 0·1% DEPC and then air-dried. Negative controls were slides treated with 100 μg/ml of RNase A (Sigma) at 37° for 30 min to digest all tissue mRNA, including IL-4 mRNA.

Concentrations of digoxigenin (dig)-labelled IL-4 probes, both sense and anti-sense, were adjusted to approximately 100–500 ng/section in hybridization buffer which comprised 50% formamide, 5 × Denhardt's solution, 5 × standard saline citrate buffer (SSC) and 500 μg/ml denatured salmon sperm DNA (Sigma). A volume of 20 μl of the hybridization mixture was added to each section. The sections were covered and incubated overnight at 40° in a humid chamber. Slides were then washed sequentially in 4 × SSC at 42° for 10 min twice, incubated with 20 μg/ml RNase A in 2 × SSC for 30 min at 37° to remove any unhybridized RNA, and washed in 2 × SSC at 42° for 10 min followed by a 0·1 × SSC wash for 10 min at room temperature. After a brief wash in TBS, sections were incubated with 3% bovine serum albumin (BSA; Sigma)/TBS for 10 min to reduce non-specific background, then incubated with 1 : 500–1 : 5000 dilutions of dig–AP conjugate (sheep polyclonal anti-digoxigenin antiserum (Boehringer Mannheim) conjugated with alkaline phosphatase) in TBS containing 0·1% Triton X-100 (in a humid box, overnight at room temperature). The slides were washed three times (15 min each) in TBS followed by incubation with equalization buffer (0·1 M Tris–HCl, 0·1 M NaCl, 50 mM $MgCl_2$, pH 9·5) for 5 min. Colour development was achieved by adding a freshly prepared

substrate solution consisting of 0·175 mg X-phosphate-5-bromo-4-chloro-3-indoly phosphate (BCIP), 0·37 mg nitro-blue tetrazolium (NBT; Boehringer Mannheim) salt/ml of equalization buffer to the slides (for 20–40 min, at room temperature). Slides were transferred to TBS and washed in tap water, counterstained with haematoxylin for 5 seconds, and mounted with glycerol gelatin.

The following control experiments were also performed: (1) running *in situ* hybridization (ISH) experiments without previous immunocytochemistry (ICC); (2) ICC alone (no ISH); (3) omitting the probe in ISH protocol and using a sense dig–IL-4 riboprobe; (4) pretreatment of slides with RNase A before ISH; (5) use of an unrelated anti-sense dig-labelled RNA probe (human atrial natriuretic polypeptide); and (6) omitting the dig–AP conjugate. A separate set of sections was immunostained with monoclonal antibodies (CD3, CD68, BMK-13, tryptase) using the APAAP method, as previously described.[4]

Slides were counted blind in coded random order by two independent observers, using a light microscope with an eyepiece graticule (0·202 mm^2). At least two sections and a minimum of four fields were counted for cells positive for ICC, ISH and double ICC and ISH. Cell counts/high power field were expressed as the percentage of IL-4 mRNA cells that co-expressed immunoreactivity for CD3, CD68, MBP or tryptase. The inter-observer variation was less than 8%.

Analysis of results

Cell counts for *in situ* hybridization and for immunohistology in biopsies obtained after allergen were compared with baseline biopsies using Student's paired *t*-test. P values $< 0{\cdot}05$ were considered statistically significant.

RESULTS

Immunohistology of the nasal mucosa was performed separately on baseline nasal biopsies and biopsies obtained 24 hr after local nasal allergen provocation (Table 1). At baseline the majority of cells were T cells ($CD3^+$) or macrophages ($CD68^+$), with fewer mast cells and only occasional eosinophils scattered throughout the submucosa. Twenty-four hours after allergen there was a marked increase in MBP^+ cells (eosinophils) and a modest but significant increase in $CD3^+$ T lymphocytes (Table 1, Fig. 1b).

Table 1. Phenotype of cells in the nasal mucosa before and after allergen

	T cells (CD3)	Macrophages (CD68)	Eosinophils (MBP)	Mast cells (tryptase)
Before challenge	85·8 ± 2·1	57·4 ± 4·2	2·1 ± 0·9	12·1 ± 1·9
After challenge	96·9 ± 2·6*	63·8 ± 5·1	38·0 ± 4·9*	10·3 ± 1·8

*$P < 0{\cdot}05$.

Cell counts (mean ± standard error) in the nasal mucosa in biopsies obtained from six subjects before and after local nasal allergen provocation. Immunostaining was performed using monoclonal antibodies directed against the markers shown.

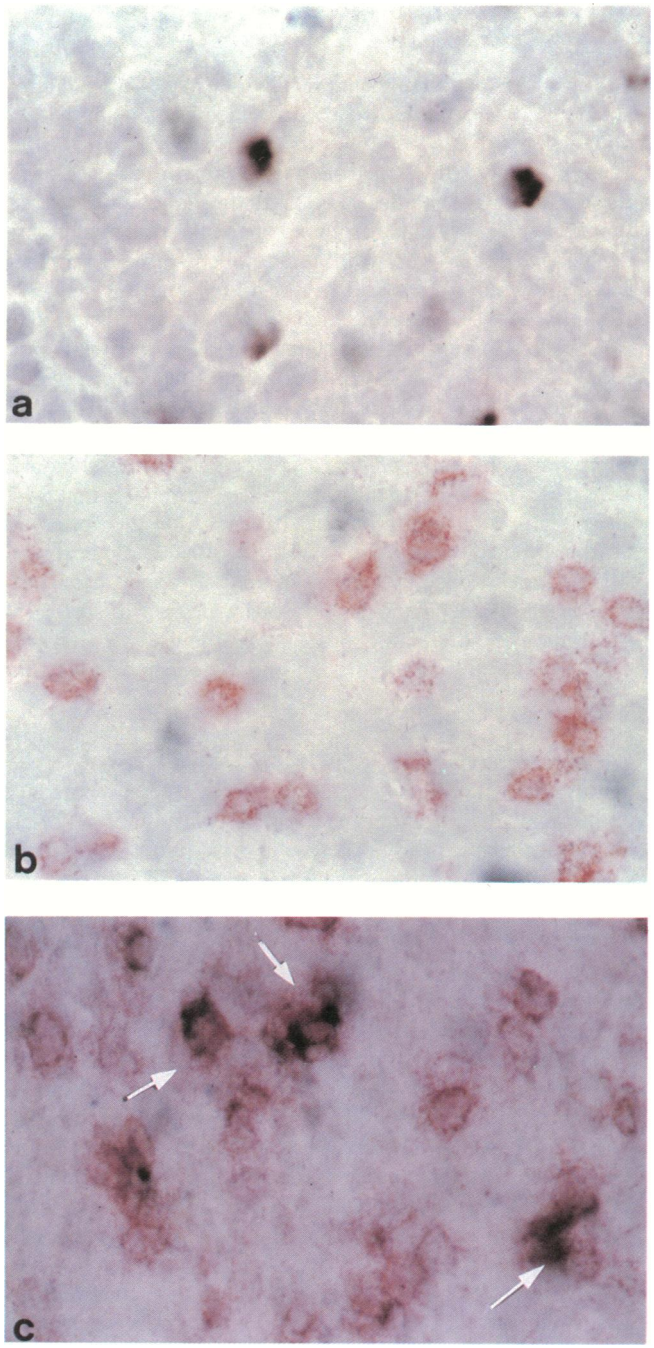

Figure 1. (a) Single *in situ* hybridization of section from the same biopsy using a dig-labelled IL-4 riboprobe and developed with NBT–BCIP. Few cells showed positive hybridization signals (dark blue) (magnification ×1000). (b) Cryostat section of nasal biopsy after allergen challenge immunostained with anti-CD3 using the APAAP method. Positive cells stained red (magnification ×1000). (c) Simultaneous *in situ* hybridization and immunocytochemistry of a section from a nasal biopsy after allergen showing a few cells (arrowed) which were double stained (purple) for CD3 and IL-4 mRNA (magnification ×1000).

In situ hybridization was performed separately using a dig-labelled riboprobe coding for IL-4 mRNA (Fig. 2). Only occasional IL-4 $mRNA^+$ cells were observed at baseline. Highly significant increases in IL-4 $mRNA^+$ cells were

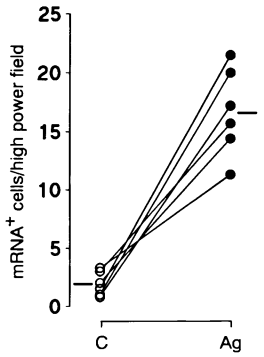

Figure 2. *In situ* hybridization of nasal biopsies using dig-labelled riboprobes directed against mRNA for IL-4. Cell counts before (C) and after (Ag) local nasal allergen provocation are shown. Mean values are represented by the horizontal bars. $P < 0.001$.

observed after allergen challenge (1·9 ± 0·42 increasing to 16·7 ± 1·5) ($P < 0.001$). Cells identified using dig-labelled riboprobes developed with NBT–BCIP appeared blue/black (Fig. 1a).

In order to co-localize cytokine mRNA with cytoplasmic or surface markers on the same cell, simultaneous immunohistology and non-radioactive *in situ* hybridization were employed. Double-stained positive cells exhibited a mixed dark-purple colour (Fig. 1c). Table 2 summarizes the results of simultaneous detection of IL-4 mRNA compared with the various cell markers using biopsies obtained following allergen challenge. After allergen a mean of 73·7% (± 1·6) of cells expressing cytokine mRNA for IL-4 during the late response were also $CD3^+$ (Fig. 1c, Table 2). Lower numbers of IL-4 mRNA hybridization signals were co-localized to $tryptase^+$ mast cells (26·0%) (± 1·6). In contrast, no IL-4 mRNA hybridization signals were co-localized to $CD68^+$ cells (putative macrophages) or MBP^+ eosinophils. Although the number of IL-4 $mRNA^+$ cells at baseline was very small (Fig. 2), the proportion of IL-4^+ cells which co-expressed CD3 (78·6%) or tryptase (21·5%) was similar to those observed after allergen (Table 2).

Conversely, when the data were represented as the percentage of T lymphocytes and mast cells which co-expressed IL-4 mRNA the results were 5·3% (± 1·0) $CD3^+$/IL-4 $mRNA^+$ and 17·6% (± 3·3) $tryptase^+$/IL-4 $mRNA^+$ cells after allergen, and 0·76% (± 0·2) $CD3^+$/IL-4 $mRNA^+$ cells, and 2·4% (± 0·9) $tryptase^+$/IL-4 $mRNA^+$ cells at baseline. Thus the majority of IL-4^+ cells (approximately 75%) were T cells both at baseline and after allergen. A greater proportion of mast cells present expressed IL-4 mRNA, although in view of the fewer mast cell numbers this only amounted to approximately 25% of the total IL-4 $mRNA^+$ cells.

DISCUSSION

We have confirmed that allergen-induced late nasal responses are accompanied by T lymphocyte and eosinophil recruitment in the nasal mucosa and an increase in the number of IL-4 $mRNA^+$ cells. By use of combined *in situ* hybridization/immunocytochemistry we have demonstrated that the majority of cells expressing IL-4 mRNA are $CD3^+$ T lymphocytes. In biopsies from all six subjects the majority (approximately 75%) of cells were $CD3^+$, the remaining IL-4 $mRNA^+$ cells being mast cells. In contrast, no IL-4 mRNA signal was demonstrated in MBP^+ eosinophils or $CD68^+$ macrophages. This evidence supports our hypothesis that T lymphocytes have a pivotal role in atopic allergic inflammation, at least in part mediated via IL-4.

Simultaneous *in situ* hybridization and immunocytochemistry, performed on the same sections, provided a precise and visually more accurate method of identifying double-positive cells in the same rather than serial sections. In agreement with the present finding, by use of the same

Table 2. Phenotype of cells expressing messenger RNA for IL-4

Subject	%IL-4^+/$CD3^+$	%IL-4^+/$CD68^+$	%IL-4^+/MBP^+	%IL-4^+/$tryptase^+$
1a	74·0	0	0	25·8
1b	75·0	0	0	24·5
2a	78·9	0	0	20·6
2b	71·4	0	0	26·0
3a	72·7	0	0	27·0
3b	75·6	0	0	22·0
4a	67·8	0	0	32·0
4b	81·8	0	0	22·5
5a	76·9	0	0	22·8
5b	83·0	0	0	20·0
6a	71·8	0	0	27·7
6b	85·0	0	0	14·2
Mean ± SE	a: 73·70 ± 1·6%			26·0 ± 1·6%
	b: 78·60 ± 2·2%			21·5 ± 1·7%

Percentage of IL-4 $mRNA^+$ cells which co-express CD3, CD68, MBP or tryptase immunoreactivity in the nasal mucosa after allergen challenge (a) and at baseline (b) in individual subjects.

methodology, we recently confirmed that the principal cell source of IL-5 mRNA was also the T lymphocyte.[21] In the present study, in the absence of immunocytochemistry there was a number of cells in biopsies that exhibited strong dark blue staining, indicating expression of IL-4. The specificity of these hybridization signals was confirmed by the absence of staining with the sense probe after RNAase pretreatment, or with unrelated probes. Furthermore, there was no signal when the dig–Ag conjugate was omitted in the developing stage. Probes applied at concentrations of less than 100 ng/section did not hybridize specifically to mRNA. For simultaneous ISH/ICC it was essential to add initially DEPC and RNAase inhibitor during the ICC processing steps to preserve mRNA for subsequent ISH. At the developing stage of the latter each slide was individually monitored. In the course of determining the sequence of staining and hybridization we observed that when ISH was performed first and followed by ICC, faint ICC signals (stained by anti-CD3) were obtained which disappeared almost completely after mounting. This was probably due to cellular antigen denaturing as a result of either proteinase K digestion or the high temperature required for ISH. We also attempted to amplify our ISH signals by using triple steps of anti-dig–biotin-labelled rabbit anti-mouse IgG–streptavidin-labelled–AP and obtained results similar to the direct detection system used in this study. Digoxigenin is a plant alkaloid which is not constitutively expressed on mammalian cells. As such, it does not generate endogenous background to the same extent as biotin in immunohistochemical detection systems.

It is now well established from murine[8] studies and more recent studies in humans[9] that distinct Th1 and Th2 subsets of T lymphocytes may be distinguished on the basis of their profile of cytokines which they produce in response to antigenic stimulation. Our own studies, involving *in situ* hybridization of biopsies obtained from skin,[23] nose[10] and lung[24,25] during late responses after allergen provocation, confirm preferential mRNA expression towards IL-4 and IL-5. This is in contrast to enhanced expression of cytokine mRNA for IL-2 and interferon-γ during classical tuberculin-induced delayed-type hypersensitivity in the skin.[26]

IL-4 has a number of important biological properties relevant to human allergic inflammation. It is the dominant cytokine responsible for B-cell switching in favour of IgE production.[11,27] It promotes selective, VLA-4-dependent eosinophil transendothelial migration.[12,28] Moreover, IL-4 plays a dominant role in the differential development of Th2-type cells.[29,30] The precise genetic control of IL-4 transcription and translation is unknown, although regulatory elements have been identified in the 5′ flanking region of the IL-4 gene.[31] Corticosteroids are the most effective agents currently available for treating human allergic disease. It is therefore of interest that in a recent study it was shown that hydrocortisone inhibited mitogen-induced production of human IL-4 by T lymphocytes at the secreted protein as well as at the mRNA level.[32] We recently observed that topical corticosteroids selectively inhibited IL-4 mRNA expression in the nasal mucosa concomitant with inhibition of the observed clinical late response.[15] The importance of IL-4 in human nasal mucosal responses is further supported by the observation that IL-4 given as cancer chemotherapy provokes dose-limiting nasal congestion and detectable increases in mediators in nasal lavage fluid.[33] Local tissue inflammation in mice provoked by Th2 cells injected into the footpads or ears of naive syngeneic recipient mice was inhibitable by monoclonal antibodies directed against IL-4 or by soluble recombinant IL-4 receptor.[34]

Taken together, these studies provide strong evidence for a pivotal role for IL-4 in allergic inflammation. In the future, therapy might usefully be directed against cells producing IL-4 and IL-4-induced effects in humans. It is therefore of interest that in the present study the majority of cells expressing IL-4 *in vivo* in humans in the target organ in response to specific allergen challenge were indeed T cells. In agreement with recent studies involving *in vitro* polyclonal activation of normal human peripheral blood T cells, IL-4 mRNA expression was restricted to less than 5% of the T-cell population present.[35] With our present methodology employing double *in situ*/immunostaining it has not been possible to establish whether IL-4 is produced predominantly by CD4 or CD8 T cells. However, *in vitro* studies confirm that both can be primed to produce IL-4.[35,36]

In addition to T lymphocytes there is now good evidence that alternative cells may also produce IL-4 at the level of mRNA and secreted product. Thus IL-4 production has been detected in rodent mast cells[16,17] and shown to be localized to and released by mast cells in the human respiratory tract.[18,19] In the present study we confirm that mast cells may also express mRNA for IL-4, although threefold less cells than the number of T lymphocytes. It seems likely that both T lymphocytes and mast cells express messages for IL-4 and secrete IL-4, whereas only mast cells store IL-4. This is the likely explanation of why IL-4 product was specifically co-localized to mast cells but not T lymphocytes by use of immunocytochemical staining.[18,19] We could not co-localize IL-4 mRNA to either macrophages or eosinophils. To our knowledge there are no previous reports which suggest that these cell types may produce IL-4.

In summary, our results provide direct evidence that $CD3^+$ cells are the principal source of IL-4 mRNA in human allergic inflammation associated with the nasal late-phase response. IL-4 mRNA is also produced from mast cells and it has been suggested that initial mast cell activation with IL-4 release may trigger T lymphocytes into sustained IL-4 production.[18,19] The relative importance and interrelationships between IL-4-induced effects on either IgE-dependent or eosinophil-dependent mechanisms in the development of late responses remains to be determined. However, IL-4 synthesis and release from either T lymphocytes or mast cells may represent an important target for future anti-allergic therapy.

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