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Interleukin 4 Is Localized to and Released by Human Mast Cells

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Summary

Recent attention has focused on the T helper type 2 (Th2) lymphocyte as a source of interleukin 4 (IL-4) in allergic disease. However, Th2 cells themselves require a pulse of IL-4 to initiate this synthesis. Here we provide immunohistochemical evidence of IL-4 localization to human mast cells of the skin and respiratory tract, and demonstrate that immunoglobulin E–dependent stimulation of purified human lung mast cells leads to the rapid release of IL-4 into the extracellular environment. We propose that mast cell activation in an allergic response provides a rapid and local pulse of IL-4 into the local environment essential for the triggering of T lymphocytes into sustained IL-4 production and to initiate inflammatory cell accumulation and activation.

IL-4 is a multifunctional cytokine that appears to play an important role in the pathogenesis of allergic disease. In particular, the isotype switching of B cells to IgE synthesis is under the control of IL-4 (1), and in vivo IgE responses in mice are inhibited after prior administration of neutralizing IL-4 antibodies (2). IL-4-deficient mice, which cannot produce IL-4, do not develop IgE responses (3), whereas transgenic mice with enhanced IL-4 production develop high circulating IgE levels, and a severe chronic conjunctivitis, characterized by mononuclear, mast cell, and eosinophil infiltration (4). These cellular changes are similar to those observed in human allergic asthma, rhinitis, dermatitis, and keratoconjunctivitis. In atopic human subjects increased numbers of cells positive for IL-4 mRNA have been found to be present during the allergen-induced late-phase cutaneous response, suggesting IL-4 is upregulated in human allergic disease (5). Both human and rodent T cells of the Th2 phenotype have been shown to produce this cytokine, but they first require the presence of IL-4 for their own development (6, 7). It is our hypothesis that in an allergic response this signal may come from the mast cell. In support of this, IL-4 generation has also been shown by rodent mast cells (8) and by rodent (9) and human (10) non-B non-T cells, which have mast cell basophil characteristics. We have used immunohistochemistry to study the presence and cellular localization of IL-4 in situ in the human respiratory tract, validated the method in IL-4 gene–transfected hamster CHO cells, supplemented our findings by immunostaining of purified human skin and lung mast cells for IL-4, and demonstrated its release along with histamine from mast cells with IgE-dependent activation.

Materials and Methods

Human Subjects and Biopsy Specimens. Nasal biopsies were taken from the inferior or inferomedial edge of the inferior turbinate of four patients with allergic perennial rhinitis using 2-mm cupped Hartman's forceps (Medicom, Tuttlingen, Germany) under tetracaine local anesthesia. Bronchial biopsies were taken from the right middle lobe carina in four perennial allergic asthmatic patients via a fiber-optic bronchoscope (BFIT20; Olympus Company, Tokyo, Japan) using alligator forceps (Olympus FB15E) (11). All patients gave written informed consent and the biopsy procedures were approved by the Southampton University and Hospitals Ethical Committee. In each instance the biopsy specimens were fixed in ice-cooled acetone containing the protease inhibitors iodoacetamide and PMSF, then stored for 24 h at −20°C before processing into glycol methacrylate (GMA). Biopsies were placed in acetone, then methylbenzoate for 15 min each at room temperature, transferred to GMA JB4 solution (Polysciences, Northampton, UK) at 4°C, followed by embedding in GMA resin.

Immunohistochemistry of Biopsy Specimens. For immunohistochemistry, 2-μm sections were cut by ultramicrotomy, floated on 0.2% ammonia solution in water for 1 min, and dried at room temperature for 1–4 h. Sections were pretreated with 10 ml 0.1% sodium azide solution containing 100 μl 30% hydrogen peroxide for 30 min to inhibit endogenous peroxidase. Mouse IgG1 mAbs 3H4 and 4D9 to human IL-4 (12), AA1 to mast cell tryptase, anti-CD3, and anti-CD4 were applied to sequential sections. A biotinylated rabbit anti–mouse (Dako Ltd., High Wycombe, UK) second stage was applied and subsequently demonstrated using the streptavidin-biotin peroxidase complex detection system. Aminoethylcarbazole (AEC) was used as chromogen giving a red reaction product, and sections were counterstained in Mayers hematoxylin. Control slides were similarly treated, either with the primary antibody omitted, or in the presence of an unrelated mouse IgG1 mAb at the same concentration as the anti-IL-4 antibodies.

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Four double immunostaining sections were initially treated with the first antibody as described using the streptavidin-biotin peroxidase detection system. This process was then repeated for the second antibody but by utilizing the streptavidin-biotin alkaline phosphatase detection system (Dako Ltd.) using fast blue B.B as the chromogen. No counterstain was applied. Controls were performed with no antibody, either the anti-tryptase, anti-CD3, or anti-IL-4 antibody absent, and with each antibody applied first, to show that the detection system for the second antibody did not crossreact with the first antibody.

Mast Cell Purification. Foreskin mast cells were dispersed from circumcision specimens using collagenase (1.5 mg/ml) and hyaluronidase (0.75 mg/ml) as previously described (13). The isolated cells were layered onto a discontinuous gradient of 60–80% Percoll (density, 1.076–1.1 g/ml) and centrifuged at 500 *g* for 20 min at 4°C. Mast cells at the bottom of the gradient and between the 70/80% interface were 90% pure. T lymphocytes were removed by incubation of resuspended cells for 30 min at 4°C with magnetic beads coated with a mAb specific for the CD2 T cell antigen (Dynal, Oslo, Norway). Two 15-min exposures to a magnet removed 99% of CD2-positive T cells. Lung mast cells were dispersed from macroscopically normal human lung, obtained within 1 h of resection as described for the skin above. Due to the different nature of contaminating cells in the dispersate, it was not possible to use density sedimentation alone to purify lung mast cells. Erythrocytes were removed by centrifugation through a 65% continuous Percoll gradient (1.084 g/ml), and the nucleated cells were incubated for 30 min with 5 μg/ml of the mAb YB5.B8 (kindly donated by L. Ashman, Melbourne, Australia), which recognizes the mast cell c-*kit* receptor. Cells were then washed and incubated for 60 min with a 500-μl suspension of magnetic beads coated with goat anti–mouse IgG antibody (Dynal) at 4°C on a roller. Purified mast cells attached to magnetic beads were removed from the suspension with a magnet.

Immunohistochemistry of Purified Mast Cells. For immunocytochemical staining, purified mast cells were fixed in buffered 4% paraformaldehyde (20 min), and cytospin preparations were made. The mast cells were then stained for IL-4 with three mAbs, 3H4, 4D9, and 8F12 (12), using the alkaline phosphatase anti–alkaline phosphatase (APAAP) detection system with fast red as the chromogen for skin cells and fast blue as the chromogen for the lung cells to help distinguish cells from dynabeads. Negative controls were performed with primary antibody absent and an unrelated mouse IgG1 mAb. Specificity of immunostaining was demonstrated in Chinese hamster ovary (CHO) cells transfected with cDNA for human IL-4. IL-4 immunoreactivity was shown with the mAb 3H4, which could be effectively inhibited when the 3H4 mAb was preincubated with rhIL-4 (Fig. 1). Positive immunostaining of IL-4-transfected CHO cells was also observed with the anti-IL-4 mAbs 4D9 and 8F12.

IL-4 Release by Purified Mast Cells. After purification mast cells were resuspended in cold $HBSS^+$ and allowed to warm slowly to room temperature. Aliquots of the cell suspension were made (225 μl containing ~5–10 × 10^4 mast cells/tube) and preincubated for 15 min before addition of 25 μl of 10× concentrated anti-IgE or $HBSS^+$ (control). Cells were then incubated for 1, 2, 4, or 6 h, and the release reaction was stopped by centrifugation (5 min, 500 *g*, 4°C). Supernatants were decanted and frozen at −80°C. For histamine analysis, TCA was added to a final concentration of 5% before freezing. Histamine was measured spectrophotofluorimetrically and IL-4 assayed by ELISA, which has a lower detection limit of 40 pg/ml of IL-4 and a coefficient of variation of 4% in the range of 60–2,000 pg/ml.

Results and Discussion

In all the biopsies from both the rhinitic and asthmatic subjects, cells staining positively for IL-4 were present (Fig. 2). No significant immunostaining was identified in either the absence of antibody or in the presence of an unrelated antibody control. On identifying the same nucleated, IL-4-immunoreactive cell in adjacent 2-μm sections, positive staining was seen with both the 3H4 and 4D9 mAbs, which recognize different epitopes on human IL-4, thereby confirming the identity of immunoreactivity as IL-4. When immunostaining with AA1, an antibody against mast cell tryptase, was also performed in adjacent sections (Fig. 2), or in the same section (Fig. 3), IL-4 immunoreactivity was found to be localized to mast cells. Comparison of sequential sections stained in the eight biopsies using the camera-lucida system (Leica UK Limited, Milton Keynes, UK) has shown that the percentage of IL-4-containing cells that were also tryptase positive ranged from 78 to 100%, while the number of tryptase-positive cells that were also IL-4 positive ranged from 68 to 85%. No IL-4 immunoreactivity was localized to either CD3- or CD4-positive T cells with either double staining or comparison of sequential sections. No crossreaction was observed in the double-stained controls.

Investigation of IL-4 immunoreactivity using the anti–human IL-4 antibodies 3H4, 4D9, and 8F12 in mast cells, dispersed from both human lung and skin purified to >95%, confirmed the intracellular localization of IL-4 (Fig. 4). Anti-IgE challenge resulted in specific release of IL-4 from purified human lung mast cells (n = 3), with mean levels in cells challenged with anti-IgE and unchallenged controls of 13.2 ng/10^6 cells and <0.5 ng/10^6 cells, respectively (Fig. 5).

The immunocytochemical and ELISA techniques we have used, chosen in preference to estimation of mRNA by in situ hybridization, which does not always reflect protein synthesis and cannot detect storage, have clearly demonstrated that human mast cells contain and release IL-4. The studies with biopsies and isolated cells have allowed us to examine mast cells from several tissue sites, all of which show IL-4 immunoreactivity with three different mAbs. This observation strongly suggests that the presence of IL-4 is not restricted to one mast cell subset, the mast cells of the skin and lung being phenotypically distinct (14). Thus, mast cells at both connective tissue and mucosal sites may participate in allergic inflammation through IL-4 release. In experiments to assess IL-4 release from lung mast cells, we have used an ELISA to quantitate IL-4 after anti-IgE challenge. The observation that anti-IgE released similar amounts of IL-4 at 1, 2, 4, and 6 h suggests that this cytokine seems to be present within the cells in a preformed state similar to that reported for TNF-α (15).

The specificity of our immunostaining for IL-4 is suggested by several factors. First, in the tissue section, the two specific IL-4 antibodies used, 3H4 and 4D9, stain the same cells in spite of reacting with different epitopes on human IL-4, while an isotype control antibody at the same Ig concentration was negative. Second, with the purified cells, the three antibodies used, 3H4, 4D9, and 8F12, all give positive staining, whereas

Published November 1, 1992

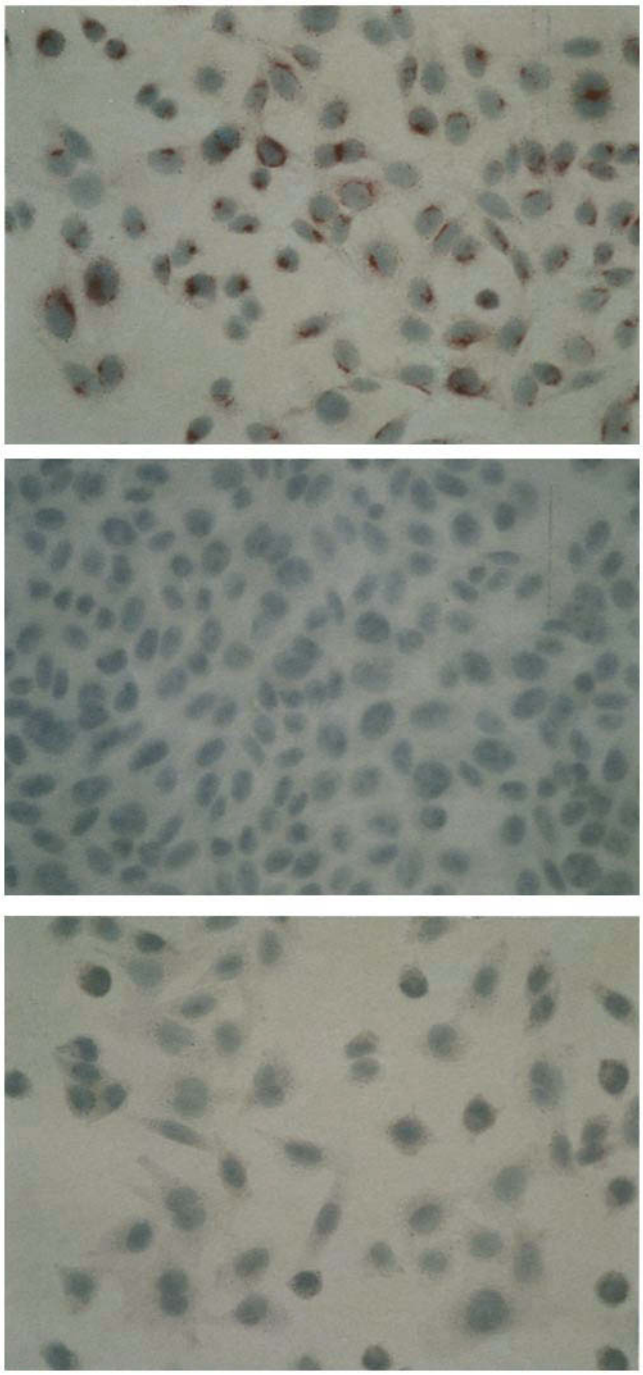


Figure 1. Cytocentrifuged IL-4-transfected (CHO) cells (*top*) and non-transfected CHO cells (*middle*) showing positive and negative staining, respectively, with mAb 3H4. Incubation of mAb 3H4 with an excess of IL-4 before staining of transfected cells greatly reduced IL-4 immunostaining (*bottom*).

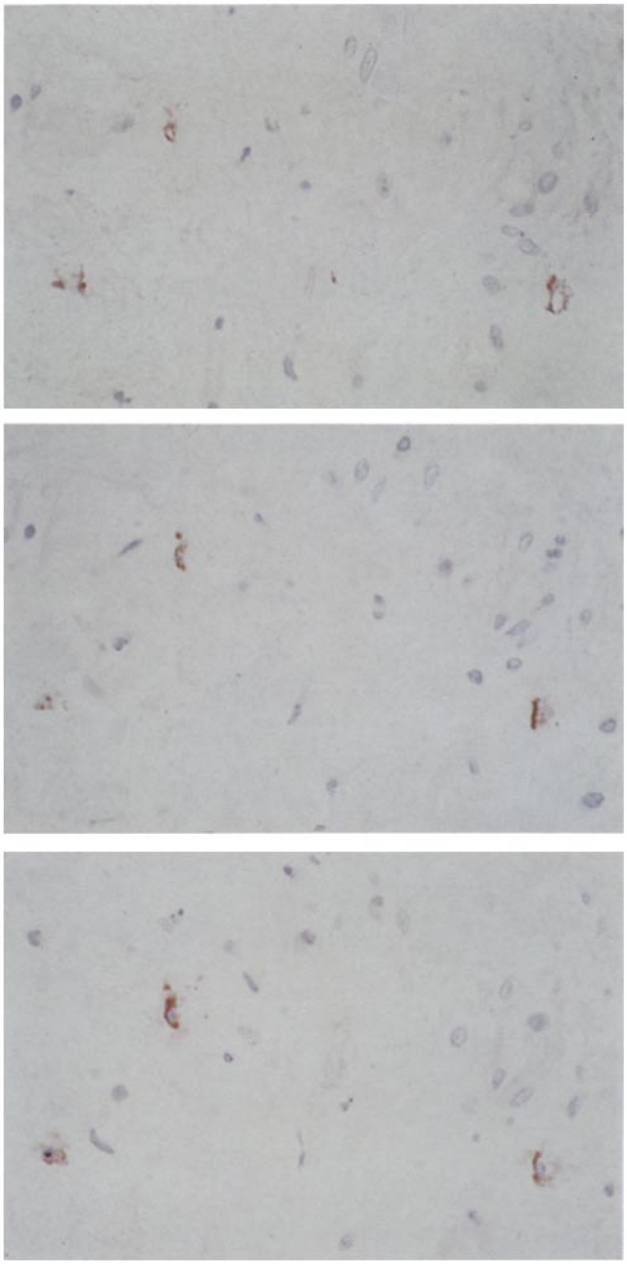


Figure 2. Immunohistological staining with antibodies 3H4 against human IL-4 (*top*), AA1 against mast cell tryptase (*middle*), and 4D9 also against human IL-4 (*bottom*) in sequential 2-μm sections of a mucosal biopsy from a patient with atopic asthma showing cellular staining with both anti-IL-4 antibodies and the anti-mast cell tryptase antibody.

an isotype control was again negative. Third, CHO cells transfected with the human IL-4 gene and known to secrete large quantities of IL-4 stained positively with the IL-4 antibodies, whereas CHO cells without gene transfection were negative. Incubation of antibodies with IL-4 before staining greatly reduced staining of IL-4-positive CHO cells but was unhelpful in tissue sections due to marked subsequent background staining, probably due to nonspecific binding of immune complexes to resin sections, or binding of immune complexes via the IL-4 receptor, as neither antibodies employed in the tissue sections were neutralizing.

These findings have considerable relevance to the pathogenesis of chronic allergic inflammation and in particular for the role of the tissue mast cell in its initiation. IL-4 has many effects that promote allergic inflammation. These include:

Published November 1, 1992

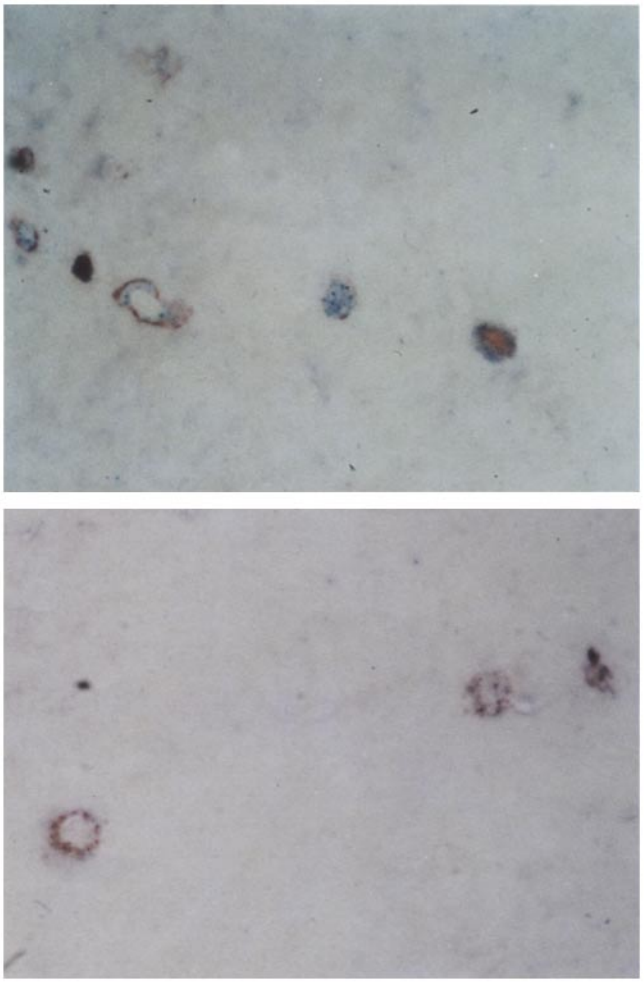


Figure 3. Double immunostaining of 2-μm sections from a nasal biopsy with antibody AA1 against mast cell tryptase and 3H4 against human IL-4 (*top*), and a bronchial biopsy with AA1 and 4D9 also against human IL-4 (*bottom*) identifying the localization of IL-4 immunoreactivity to mast cells.

upregulation of IgE synthesis (1); stimulation of T cell proliferation and Th2 cell development (6, 7, 16); induction of MHC class II molecules on B cells and monocytes (17, 18); induction of F_cER_{II} (CD23) on B cells and macrophages (17, 19); induction of LFA-1 and LFA-3 on B cells (20); activation of macrophages (21); proliferation of fibroblasts (22); induction of VCAM-1 on endothelial cells (23); increased mast cell basal and stimulated histamine release (24); the promotion of eosinophil and mast cell accumulation at the site of allergic reactions (25, 26); and mast cell growth and maturation (27).

The source of IL-4 necessary to maintain these responses is considered to be the T lymphocyte, particularly its Th2 subset (28). However the Th2 lymphocyte itself requires IL-4 to initiate and support its development and cytokine synthesis (6, 7). It is our hypothesis that the release of IL-4 from the mast cell after IgE-dependent activation provides a localized initiating stimulus for the activation of Th2 cells. Furthermore, we suggest that mast cell cytokine release within minutes of allergen provocation plays a major role in the early accumulation of inflammatory cells observed. Thus, in addition to being the source of histamine and eicosanoids, which are responsible for most of the symptoms of the early phase of an allergic response, mast cells may play an obligatory role in the initiation, and possibly the maintenance, of allergic inflammation through the production and release of IL-4, TNF-α, and possibly other cytokines.

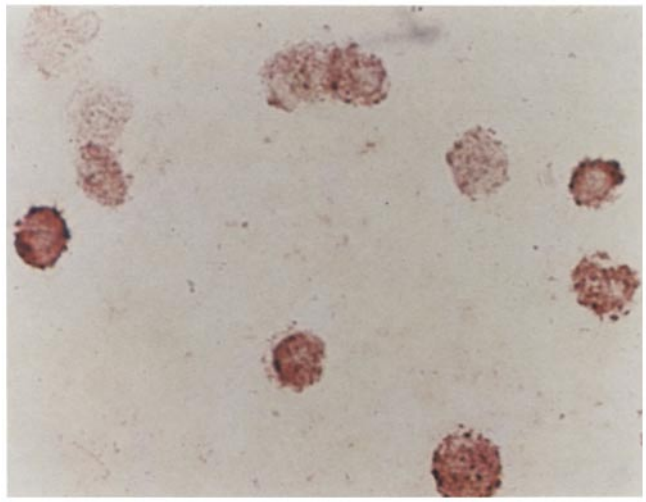


Figure 4. IL-4 immunostaining of human mast cells dispersed and purified from resected foreskin stained with the 3H4 and anti-human IL-4 antibody.

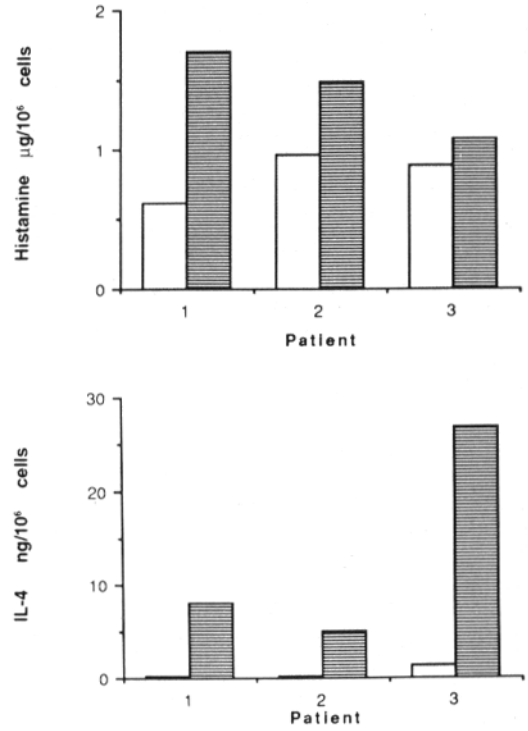


Figure 5. Levels of histamine (expressed as μg/10^6 mast cells; *top*) and IL-4 (ng/10^6 cells; *bottom*) in culture supernatants from purified lung mast cells after either saline control (*open bars*) or anti-IgE challenge (*striped bars*).

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