

**Messenger RNA Expression of the Cytokine Gene Cluster, Interleukin 3 (IL-3), IL-4, IL-5, and Granulocyte/Macrophage Colony-stimulating Factor, in Allergen-induced Late-phase Cutaneous Reactions in Atopic Subjects**

By A. B. Kay, Sun Ying, V. Varney, M. Gaga, S. R. Durham, R. Moqbel, A. J. Wardlaw, and Q. Hamid

*From the Department of Allergy and Clinical Immunology, National Heart & Lung Institute, London, SW3 6LY, UK*

**Summary**

Cryostat sections from skin biopsies from 24-h allergen-induced late-phase cutaneous reactions (LPR) in 14 human atopic subjects were hybridized with <sup>35</sup>S-labeled RNA probes for a number of cytokines. mRNA was detected for interleukin 3 (IL-3) (8/14), IL-4 (10/14), IL-5 (11/14), and granulocyte/macrophage colony-stimulating factor (GM-CSF) (13/14). Only 5 of 14 gave hybridization signals for IL-2, and 0 of 14 for interferon  $\gamma$ . Biopsies from diluent controls gave only occasional weak signals. These results suggest that cells infiltrating the site of the 24-h LPR transcribe mRNA for the IL-3, IL-4, IL-5, and GM-CSF gene cluster and support the hypothesis that atopy is associated with preferential activation of cells having a similar cytokine profile to the murine T helper type 2 subset.

We previously showed that infiltrating CD4<sup>+</sup> T lymphocytes together with activated (EG2<sup>+</sup>) eosinophils were characteristic of the immunohistology of the allergen-induced late-phase reaction (LPR) (1). Cytokines produced by subsets of T cells may play a role in regulating some of the features of atopic allergic inflammation. In man, IL-4 serves as an essential co-factor in the induction of IgE synthesis (2). IL-5 promotes the terminal differentiation of eosinophils (3) as well as activating, or priming, the mature eosinophil for more efficient effector functions (4). Furthermore, IL-5, together with IL-3 and granulocyte/macrophage CSF (GM-CSF), increase eosinophil survival in vitro (5-7).

The genes for IL-3, IL-4, IL-5, and GM-CSF have been cloned and are clustered in the region of the long arm of human chromosome 5 (5q 23-32) (8). This very close linkage suggests a common evolutionary origin. Two types of murine T cell clones have been defined on the basis of the cytokines they produce (9). Th1 cells express mRNA and secrete IL-2 and IFN- $\gamma$ , but not IL-4 or IL-5. Th2 cells produce IL-4 and IL-5 but not IFN- $\gamma$  or IL-2. Both cell types produce IL-3 and GM-CSF. Wierenga et al. (10) have shown that in atopic individuals, allergen (house dust mite [HDM])-specific T cell clones produced a Th2-like pattern of cytokine production (IL-4 high, IFN- $\gamma$  low), whereas nonallergen antigen-specific clones from the same patient produced high IFN- $\gamma$  and low IL-4.

In the present study, we test the hypothesis that the infiltrating cells associated with allergen-induced LPR have the IL-3, IL-4, IL-5, and GM-CSF gene cluster cytokine profile and as such have similarity with murine Th2-type cells. For this purpose, we have used the technique of in situ hybridization on sections from skin biopsies taken from the sites of allergen-induced late-phase responses.

**Materials and Methods**

*Human Subjects.* 14 atopic subjects (aged 18-51 yr) were selected using the criteria previously described (1). They all had a positive skin prick test to Timothy grass pollen extract (*Phleum pratense*; Soluprick ALK, Horsholm, Denmark).

The study was performed with the approval of the Royal Brompton and National Heart Hospital Ethics Committee and the patient's written informed consent. Allergen (0.02 ml of an aqueous extract of *P. pratense* [Aquagen SQ; ALK] containing 30 BU) was injected intradermally into the extensor aspect of the forearm. The diluent (0.02 ml of 0.9% sodium chloride with 0.03% human serum albumin and 0.5% phenol) was injected at the same site on the opposite arm. The challenge tests were performed outside the grass pollen season and at the same time of day. The mean diameter of induration of the LPR at 24 h was 70 mm (range, 23-88 mm). The diluent gave zero induration.

At 24 h, two 3-mm punch biopsies were taken from the center of both the allergen and diluent sites in each subject under local

anaesthesia (1% lignocaine), i.e., a total of four biopsies per subject. The biopsies were spaced 1 cm apart across the point at which the needle was introduced. Two biopsies (one allergen and one diluent) were processed by snap freezing for immunohistochemistry and two were placed in 4% paraformaldehyde for in situ hybridization. One batch of allergen was used throughout.

**Immunohistology.** The APAAP technique was used to enumerate cells staining with the mAbs Leu-4 (CD3), Leu-3a (CD4), and EG2 (cleaved and secreted form of eosinophil cationic protein). The technique was as previously described in detail (1) with the exception that, instead of normal rabbit Ig, 20% human serum in tris-buffered saline was used to prevent nonspecific binding to the second- and third-layer antibody.

**In Situ Hybridization.** Riboprobes, both antisense (complementary RNA) and sense (having an identical sequence to mRNA), were prepared using methodology previously described in detail (11). cDNA for IL-2, IL-4, IL-5, GM-CSF, and IFN- $\gamma$  (or for IL-3, a synthetic DNA of >95% homology) were inserted into different pGEM vectors and linearized with appropriate enzymes before transcription. Transcription was performed in the presence of  $^{35}\text{S}$ -UTP and the appropriate T7 or SP6 RNA polymerases. For in situ hybridization, cryostat sections were permeabilized with Triton X-100 and proteinase K (1  $\mu\text{g}/\text{ml}$ ). To inhibit nonspecific binding of  $^{35}\text{S}$ , the preparations were treated with 10 mM iodoacetamide and 10 mM *N*-ethylmaleimide for 30 min at 37°C and then in 0.5% acetic anhydride (0.1 M) triethanolamine for 10 min at 37°C (12). Prehybridization was carried out with 50% formamide and 2 $\times$  SSC. For hybridization, antisense or sense probes (10<sup>6</sup> cpm/section) diluted in hybridization buffer were used. Dithiothreitol (100 mM final concentration) was added to the hybridization buffer (13) to ensure blocking of any further nonspecific binding of the  $^{35}\text{S}$ -labeled probes. Post-hybridization washing was performed in decreasing concentrations of SSC (4–0.05  $\times$  SSC) at 45°C. Unhybridized single-stranded RNAs were removed by RNase A (20  $\mu\text{g}/\text{ml}$ ). After dehydration, the sections were immersed in K-5 emulsion and exposed for 7–10 d. The autoradiographs were developed in Kodak D-19 and counterstained with hematoxylin.

As positive controls for IL-2, IL-3, IL-4, IL-5, and GM-CSF, cytopins from a peripheral blood T lymphocyte clone obtained from a patient with the hyper-IgE syndrome were prepared. This clone was treated with Con A and, after stimulation, expressed mRNA for IL-2, IL-3, IL-4, IL-5, and GM-CSF (D. J. Quint, E. D. Zanders, and B. Champion, personal communications). PHA-stimulated blood mononuclear cells were used for the IFN- $\gamma$ -positive controls. Cytopins were processed as described for the biopsies. For the negative controls, skin biopsies and cytopins were hybridized using sense probes for the relevant cytokines. In addition, sections were treated with RNase-A solution before the pre-hybridization step with antisense probes. Specific hybridization was recognized as clear dense deposits of silver grains in the photographic emulsion overlaying the tissue sections or cytopin preparations. Cells were identified as dense, discrete, well-circumscribed areas of silver grains. When hybridizing cells were in close proximity, their numbers were determined by visualizing the individual nuclei using dark field illumination. Positive cells were only observed with an antisense probe; preparations treated with sense probes or pretreated with RNase were negative with only baseline background signals.

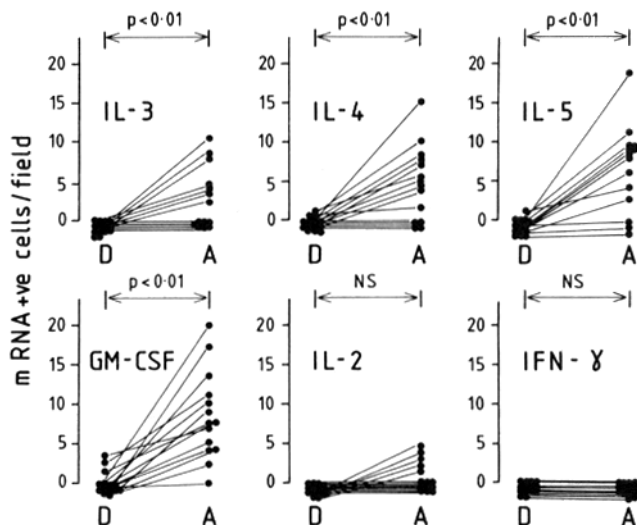
**Quantitation.** For both immunohistology and in situ hybridization slides were counted "blind" using an eyepiece graticule. For each biopsy at least two sections were immunostained or hybridized from which two to six fields were counted. Results were ex-

pressed as positive cells per field (0.202 mm<sup>2</sup>). The within observer coefficient of variation was <5%.

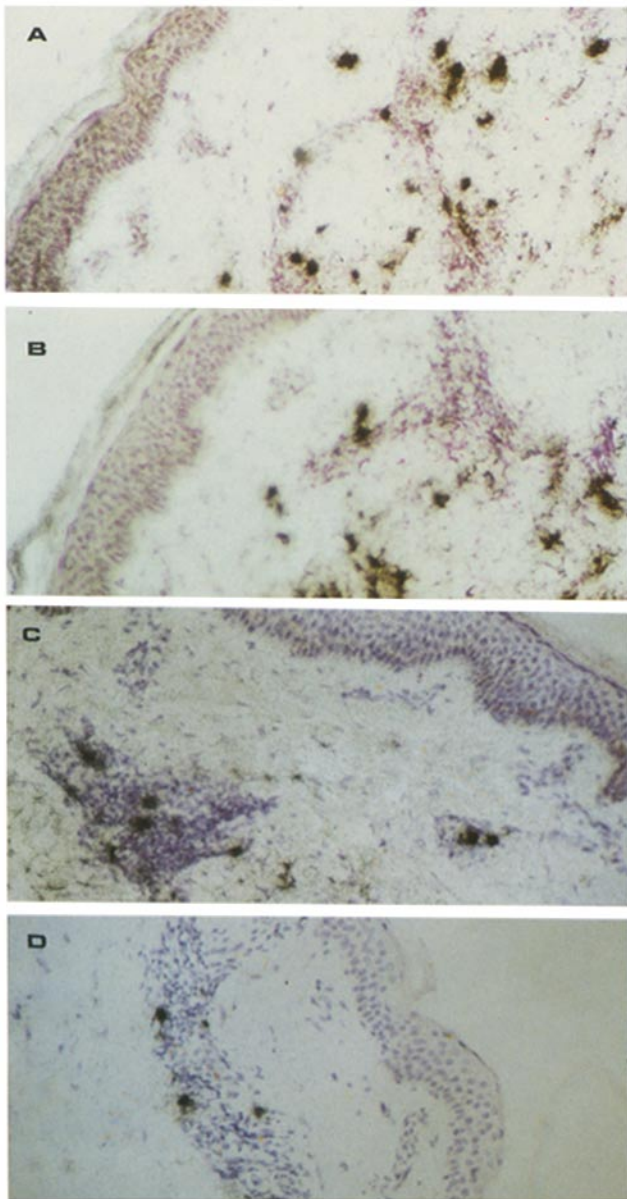
## Results and Discussion

At allergen sites, there were significant increases (compared with diluent) in the numbers of infiltrating CD3<sup>+</sup> ( $p < 0.001$ ), CD4<sup>+</sup> ( $p < 0.01$ ), and EG2<sup>+</sup> ( $p < 0.001$ ) cells. This confirmed our previous findings (1). At the allergen-injected sites, most biopsies had positive hybridization signals for IL-3 (8/14), IL-4 (10/14), IL-5 (11/14), and GM-CSF (13/14), whereas only 5 of 14 were positive for IL-2 mRNA (Fig. 1). At the diluent sites, there were a few cells that expressed weak hybridization signals for IL-4 (2/14), IL-5 (1/14), and GM-CSF (3/14). No hybridization was detected for IFN- $\gamma$  mRNA in either the allergen- or diluent-challenged biopsies. When the allergen-injected site was compared with the diluent control, there were significant increases in the numbers of cells expressing mRNA for IL-3, IL-4, IL-5, and GM-CSF ( $p < 0.01$ ); the differences for IL-2 and IFN- $\gamma$  were not significant.

In general, the cytokine mRNA-positive cells were located among the inflammatory infiltrate at the upper part of the dermis. There was no hybridization in the epithelial or connective tissue cells. Some examples of the strong signals obtained in allergen-injected sites using the antisense probe are shown in Fig. 2. The specificity of the hybridization signals obtained with the probes was confirmed by the presence of similar intense labeling of cells expressing mRNA for IL-2, IL-3, IL-4, IL-5, and the GM-CSF in a Con A-stimulated T cell clone from the hyper-IgE syndrome, and IFN- $\gamma$  mRNA in cytopins obtained from lymphoblast cultures 24 h after PHA stimulation (Fig. 3). The absence of hybridization signals with the appropriate sense probes and/or RNase pre-treatment



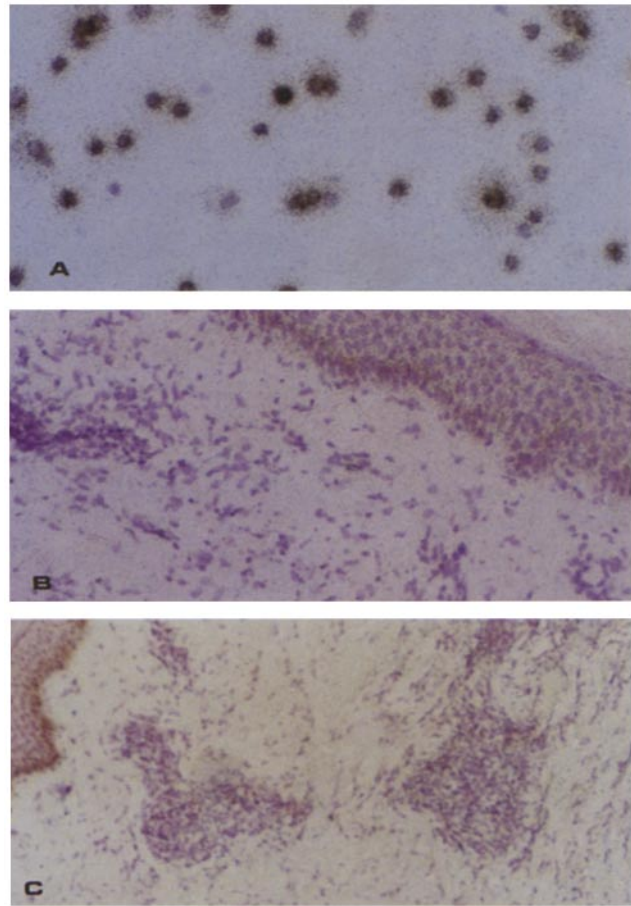
**Figure 1.** In situ hybridization of allergen-induced late-phase cutaneous reactions in atopic subjects using riboprobes for IL-3, IL-4, IL-5, GM-CSF, IL-2, and IFN- $\gamma$ . Comparisons were made between the allergen (A) and the diluent (D) challenged sites using the Wilcoxon Rank test for paired differences.



**Figure 2.** Autoradiographs of cryostat sections of skin biopsies from the antigen site. Sections were hybridized with <sup>35</sup>S-labeled antisense probes. (A–C) Examples of strong hybridization signal for IL-4 (A), IL-5 (B), and IL-3 (C) mRNA. The occasional cell expressing IL-2 mRNA is illustrated in D.

(Fig. 3) provided further support for the specificity of the hybridization reaction.

We found significant correlations between IL-5 mRNA-positive cells and IL-3 ( $r = 0.80$ ,  $p < 0.01$ ), IL-4 ( $r = 0.92$ ,  $p < 0.01$ ), and GM-CSF ( $r = 0.82$ ,  $p < 0.01$ ) mRNA-positive cells at the allergen-injected sites ( $n = 14$ ); the correlation between IL-5 and IL-2 mRNA-positive cells was not significant. This supports the possibility of linked transcription of the IL-3, IL-4, IL-5, and GM-CSF gene cluster. There was also evidence of an association between the degree of



**Figure 3.** Autoradiographs showing examples of controls used throughout the study. (A) A cytospin of PHA-stimulated blast cells hybridized with <sup>35</sup>S-labeled IFN- $\gamma$  antisense probe, showing strong hybridization signal. (B and C) Cryostat sections of skin biopsy from the antigen site hybridized with sense IL-4 probe (B) or pretreated with RNase (C) before the application of IL-4 <sup>35</sup>S-labeled IL-5 antisense probe. Note the absence of significant silver grain deposits among cells of the inflammatory infiltrate.

eosinophilic inflammation and cytokine expression since there were significantly more EG2<sup>+</sup> cells in those biopsies that had the highest number of positive signals for IL-5 and IL-4 mRNA (i.e., counts of  $>4$ /high power field [Mann Whitney U test for unpaired data;  $p < 0.01$ ]). There was a similar trend for IL-3 and GM-CSF, but this was not significant.

The detection of mRNA does not necessarily equate with protein synthesis and it needs to be shown that translation and secretion of these lymphokines also occurs. In addition to T lymphocytes, mast cells also produce lymphokines (14), as do macrophages and fibroblasts. Nevertheless, we believe that the cytokine profile observed in the 24-h LPR provides evidence that, in atopic subjects, allergen induces activation and/or proliferation of cells having the Th2 pattern. That the antigen itself determines the cloning frequency of murine Th1 and Th2 cells was recently stressed by Street et al. (15).

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Address correspondence to A. B. Kay, Department of Allergy and Clinical Immunology, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY, UK.

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