

IL-4 and IL-5 mRNA and Protein in Bronchial Biopsies from Patients with Atopic and Nonatopic Asthma: Evidence Against "Intrinsic" Asthma Being a Distinct Immunopathologic Entity

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Intrinsic (nonatopic) asthma is considered to be a distinct pathogenetic variant of asthma since, unlike extrinsic (atopic) asthma, patients with the disease are skin test-negative to common aeroallergens, and have total serum IgE concentrations within the normal range. Nevertheless, the recent demonstration of increased numbers of cells expressing the high-affinity IgE receptor in bronchial biopsies from atopic and nonatopic asthmatic subjects, together with epidemiologic evidence indicating that serum IgE concentrations relate closely to asthma prevalence regardless of atopic status, suggests that IgE-mediated mechanisms may participate in the pathogenesis of both atopic and nonatopic asthma. Furthermore both variants of the disease are associated with bronchial mucosal eosinophilic inflammation. Interleukin-4 (IL-4) is an essential cofactor for IgE synthesis, and there is strong evidence that IL-5 plays a major role in eosinophil accumulation in asthmatic inflammation. For these reasons we compared the expression of IL-4 and IL-5 mRNA and protein product using a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) amplification, *in situ* hybridization, and immunohistochemistry in bronchial biopsies from symptomatic atopic and nonatopic asthmatic subjects and atopic and nonatopic controls. The results showed that as compared with controls, biopsies from both groups of asthmatic subjects had increased numbers of IL-4 and IL-5 mRNA copies relative to β -actin mRNA as detected by RT-PCR. Similarly, *in situ* hybridization and immunohistochemistry demonstrated increased numbers of cells expressing IL-4 and IL-5 mRNA and protein in asthmatic subjects, irrespective of their atopic status. We conclude that individuals with either atopic or nonatopic asthma show infiltration of the bronchial mucosa with cells expressing Th2-type cytokines, providing further evidence for similarities in the immunopathogenesis of these clinically distinct forms of asthma. **Humbert M, Durham SR, Ying S, Kimmitt P, Barkans J, Assoufi B, Pfister R, Menz G, Robinson DS, Kay AB, Corrigan CJ. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity.**

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Asthma is often associated with atopy, a disorder characterized by sustained, inappropriate IgE responses to common environmental antigens (allergens) encountered at mucosal surfaces (1). Interaction of environmental allergens with cells sensitized by the binding of surface Fc receptors to allergen-specific IgE is assumed to play a role in the pathogenesis of atopic asthma (2).

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A minority of asthmatic individuals are not, however, demonstrably atopic by conventional criteria, which has led to the suggestion that asthma may be divided clinically, and by implication mechanistically, into atopic (extrinsic) and nonatopic (intrinsic) variants (3).

In addition to IgE-mediated mechanisms, increased local elaboration of the Th2-type cytokines interleukin-5 (IL-5) and IL-4 has clearly been implicated in the pathogenesis of atopic asthma (4-9). IL-5 has selective biologic effects on eosinophils and their precursors (10), and may regulate selective accumulation of these cells in the asthmatic bronchial mucosa (4, 5). IL-4 is an essential cofactor for IgE switching (11) in B-lymphocytes, and is therefore likely to be involved in situations in which there is inappropriate IgE synthesis. We previously showed that local IL-4 and IL-5 mRNA expression correlated with disease severity and response to treatment in atopic asthma (12, 13). Although individuals with intrinsic asthma are skin test-negative to common aeroallergens (i.e., nonatopic) and have normal total serum

IgE concentrations, there is nevertheless some evidence that atopic and nonatopic asthma have similar pathologic mechanisms. For example, increased numbers of activated T cells, eosinophils, and high-affinity IgE receptor-bearing cells were features of bronchial mucosal biopsies in both atopic and nonatopic asthma as compared with controls (14, 15). These findings raise the possibility of locally increased synthesis of the Th2-type cytokines IL-4 and IL-5 in intrinsic as well as extrinsic asthma. Epidemiologic evidence supports this view, since it was shown that serum IgE concentrations related closely to the prevalence of self-reported asthma regardless of atopic status (16). Accordingly, we have tested the hypothesis that as compared with controls, both atopic and nonatopic asthma are characterized by increased bronchial mucosal expression of IL-4 and IL-5 mRNA and protein product.

METHODS

Patient Population

Thirty-seven volunteers were included in this study (Table 1). Atopic asthmatic, nonatopic asthmatic, and atopic nonasthmatic subjects and normal controls were meticulously defined as previously described (15). Briefly, asthma was defined by: (1) a clear clinical history with current symptoms; and (2) evidence of more than 20% reversibility of FEV₁, either spontaneously or after inhaled β_2 agonists and/or a histamine provocation test result with a provocative concentration causing a 20% decrease in FEV₁ (PC₂₀) of ≤ 6 mg/ml in the previous 2 wk. Asthma severity was assessed according to the scoring system of Aas (14, 15). Atopy was defined by a positive skin prick test with extracts of one or more aeroallergens (including house dust mite, mixed grass pollen, mixed tree pollen, mixed molds, cat fur, and dog dander). With the CAP system (Pharmacia Diagnostics, Uppsala, Sweden), all atopic subjects demonstrated a positive radioallergosorbent test (RAST) > 0.70 IU/ml to one or more of these allergens. Nonatopic subjects were defined by negative skin prick tests with a wide range of local aeroallergens in the presence of a positive histamine control. The nonatopic controls also exhibited a serum IgE concentration within the normal range for our laboratory (0 to 150 IU/ml), and negative RAST tests to 25 common aeroallergens. Atopic controls were asymptomatic or had mild to moderate allergic rhinitis with no history of wheezing or chest tightness, normal baseline lung function with less than 20% reversibility of FEV₁, either spontaneously or after inhaled β_2 agonists, and a histamine provocation test result of PC₂₀ > 16 mg/ml. All subjects were nonsmokers and had not taken oral corticosteroids for the previous 2 mo or inhaled corticosteroids for the previous 2 wk. Exclusion criteria included age < 18 yr or > 65 yr, FEV₁ $< 60\%$ of the predicted value on the proposed bronchoscopy day, and evidence of acute or chronic infection, pregnancy, breast feeding, or any chronic medical illness other than asthma. The study was approved by the Ethics Committees of the Royal

Brompton Hospital, London, and the Hochgebirgsklinik Davos, and all patients and volunteers gave written informed consent.

Fiberoptic Bronchoscopy

All subjects (asthmatic and control) underwent bronchoscopy with bronchial biopsies as previously described (15). Two bronchial biopsies were snap-frozen and stored at -80°C to be used later in the reverse transcriptase-polymerase chain reaction (RT-PCR), two were used for *in situ* hybridization experiments (i.e., fixed in freshly prepared 4% paraformaldehyde in PBS [2 h], washed twice [1 h] with 15% sucrose in phosphate-buffered saline [PBS; pH 7.4], embedded in ornithine carbonyl transferase (OCT) compound [Bayer, Basingstoke, UK], snap-frozen, and stored at -80°C until used), and two were embedded in OCT compound, snap-frozen, and stored at -80°C to be used later for immunohistochemistry.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA extraction and cDNA synthesis. All reagents were from Sigma Chemicals (Poole, UK), unless otherwise indicated. For isolation of total RNA from bronchial biopsies, a commercial reagent (TRI Reagent; Molecular Research Center, Inc., Oxford, UK) based on the acid-guanidinium thiocyanate RNA extraction technique (17) was employed. Biopsies were thawed directly with agitation in 1 ml of TRI Reagent with 4 μl of Microcarrier Gel (Molecular Research Center Inc., Oxford, UK), briefly homogenized using an Omni 1000 homogenizer (Camlab, Cambridge, UK), and mixed with 200 μl chloroform for 5 min. After centrifugation (12,000 rpm, 10 min), the upper aqueous phase containing RNA was carefully transferred to a fresh tube and mixed with an equal volume of isopropanol. The RNA was left to precipitate overnight at -70°C . After centrifugation (12,000 rpm, 10 min), the supernatant was discarded and the pellet washed by adding 1 ml of ice-cold 70% ethanol, which was then gently aspirated.

Total RNA from biopsies was reverse transcribed using oligo-dT12-18 primers (Pharmacia) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco BRL, Paisley, UK) as previously described (18). The total volume of the reverse transcriptase reaction was 40 μl . Reverse-transcribed mRNA was stored at -80°C until used for PCR amplification, and 1:20 dilutions were used for the subsequent PCR analysis.

For PCR amplification of specific complementary deoxyribonucleic acid (cDNA) sequences, 5 μl aliquots of the 1:20-diluted reverse transcription mixture were made up to 40 μl in reverse transcription PCR buffer containing 1.25 mM primers (Clontech, Palo Alto, CA) and 2.5 U of Taq polymerase (BRL), as described previously (18). Samples were overlaid with mineral oil and transferred to a thermal cycler (Hybaid, Teddington, UK). After heating to 94°C for 5 min, samples were subjected to 40 (IL-4, IL-5) or 36 (β -actin) repeated cycles of denaturation (94°C , 1 min), annealing (60°C , 2 min), and extension (72°C , 3 min), followed by a final extension step (72°C , 5 min). The primers used were as follows: PCR 5' primers: AAG GCC AAC CGC GAG AAG ATG

TABLE 1
CLINICAL DETAILS OF PATIENTS AND CONTROLS

Patient group	Age (yr)	Gender (F/M)	Total Serum IgE (IU/ml)	Blood Eosinophils ($10^9/\text{L}$)	FEV ₁ (% pred)	Histamine PC ₂₀ (mg/ml)	Aas Score
Atopic asthma (n = 10)	33 (23–55)	1/9	252 [†] (57–3,749)	0.25 (0.10–0.70)	83 [§] (55–104)	0.34 (0.02–3.60)	3.5 (1–4)
Nonatopic asthma (n = 10)	53* (39–62)	6/4	66 [‡] (17–108)	0.55* (0.10–1.10)	76 [§] (58–112)	0.64 (0.12–6.00)	3.5 (1–4)
Atopic controls (n = 9)	27 (18–36)	1/8	152 [†] (38–808)	0.20 (0.10–0.80)	100 (81–125)	> 16	NA
Nonatopic controls (n = 8)	27 (21–42)	4/4	12 (3–29)	0.10 (0.0–0.20)	110 (90–119)	> 16	NA

All data are expressed as median (range) except for PC₂₀ (geometric mean [range]). NA = not applicable.

* $p < 0.01$ versus atopic asthma, atopic controls and normal controls.

† $p < 0.05$ versus normal controls and nonatopic asthmatic subjects.

‡ $p < 0.05$ versus normal controls.

§ $p < 0.01$ versus controls.

(β -actin), CGG CAA CTT TGA CCA CGG ACA CAA GTG CGA TA (IL-4), and GCT TCT GCA TTT GAG TTT GCT AGC T (IL-5); PCR 3' primer: ACA GGA CTC CAT GCC CAG GAA (β -actin), ACG TAC TCT GGT TGG CTT CCT TCA CAG GAC AG (IL-4), and TGG CCG TCA ATG TAT TTC TTT ATT AAG (IL-5). The primers were designed to span at least one genomic intron, precluding amplification of genomic DNA. The expected product sizes were 480 bp (β -actin), 344 bp (IL-4), and 294 bp (IL-5).

In order to confirm that PCR amplification of IL-5, IL-4, and β -actin cDNA was not saturable under the conditions employed, known numbers of copies of cDNA (Clontech) were amplified along with samples from the subjects. Serial 10-fold dilutions of control cDNA, starting from a maximum number of copies of 1.5×10^8 (2.5×10^{-16} moles), were amplified.

Analysis of PCR amplified product. To verify the size and purity of PCR-amplified products, 5 μ l aliquots of the PCR reaction mixtures were electrophoresed on 2% agarose gels containing 0.5 μ g/ml ethidium bromide in Tris-borate EDTA (TBE) buffer. The gels were photographed under ultraviolet (UV) illumination.

To semiquantify PCR-amplified cDNA, 10 μ l of PCR amplification mixture from all subjects (two samples were analyzed separately with identical cDNA standards) were denatured by adjusting the volume to 200 μ l with TE buffer (10 mM Tris HCl, pH 8.0; 0.1 mM ethylene diamine tetraacetic acid [EDTA]), adding 6 μ l 0.5 M EDTA and 8 μ l 6 M sodium hydroxide, and boiling for 5 min. After quenching on ice and neutralization with 115 μ l 2 M ammonium acetate, the samples were applied with suction to a single nitrocellulose membrane (Hybond-N; Amersham, Aylesbury, UK) using a dot-blotting apparatus (Bio-Rad, Hemel Hempstead, UK), and the cDNA was cross-linked to the membrane by exposure to UV irradiation. The membrane was prehybridized and probe with internal oligonucleotides as previously described (18). The probes were end-labeled with [^{32} P]adenosine triphosphate [^{32} P]ATP (Amersham), using T4 polynucleotide kinase, and separated from free nucleotide on Sepharose G20 (Pharmacia) spin columns. The specific activities of the probes ranged from 9.6 to 15.5×10^6 cpm/ng. The probe sequences were as follows: GGC TGG GGT GTT GAA GGT CTC AAA CAT GAT (β -actin), GTC CTT CTC ATG GTG GCT GTA GAA CTG CCG (IL-4), and ATT TTT ATG TAC AGG AAC AGG AAT CCT CAG (IL-5). In addition to the amplified positive control cDNA sequences, amplified negative control cDNA sequences (β -actin and IL-5 cDNAs in the case of IL-4, and IL-4 cDNA in the case of IL-5) were included on each blot. After a high-stringency wash (18), the membranes were exposed to X-ray film (X-omat; Eastman Kodak, Rochester, NY) for a period sufficient to allow visualization of the blots but not to saturate the autoradiographs.

For each cytokine and β -actin, exposed films were scanned using a flatbed scanner and associated software (Quantity One Software; PDI Inc., New York, NY). The areas under the densitometry peaks from the blot autoradiographs were measured using a uniform trough-to-trough scanning width. To semiquantify the amounts of cDNA encoding IL-4, IL-5, and β -actin in the amplified cDNA samples from the patients and controls, standard curves were first constructed, relating the areas of the blots produced by amplification of the serial 10-fold dilutions of the cDNA standards to the starting concentrations of the standards prior to amplification. Scanning of the areas of the blots produced by coamplification of the serially diluted cDNA standards revealed that, in the case of IL-4, IL-5, and β -actin, the logarithms of the blot areas were approximately linearly related to the starting concentrations of the cDNA standards within the ranges of concentrations measured in the samples from the subjects (Figure 1), as predicted by the exponential nature of PCR amplification under nonsaturating conditions. No signal was obtained from the irrelevant cDNA controls. The areas of the IL-4, IL-5, and β -actin blots from each amplified cDNA sample from the subjects were then expressed in terms of the numbers of starting copies of standard cDNA required to produce a blot of the same area. Finally, for each subject sample, the numbers of starting copies of IL-4 and IL-5 cDNA were expressed as percentages of the numbers of starting copies of the β -actin cDNA.

In situ Hybridization

Bronchial biopsies from six normal controls, five atopic controls, nine atopic asthmatic subjects, and 10 nonatopic asthmatic subjects were analyzed by *in situ* hybridization. Sections (6 μ m) were freshly cut from

frozen bronchial biopsies onto slides coated with 0.1% poly-L-lysine (BDH, Leicester, UK) and air-dried for 2 h at room temperature. All reagents were from Sigma Chemicals (St. Louis, MO) unless otherwise indicated. Riboprobes were prepared from cDNAs for IL-4 and IL-5, as previously described (19). Briefly, IL-4 and IL-5 cDNAs were inserted into pGEM 1 and pGEM 4 vectors, respectively. They were then linearized with *Sph* I or *Eco* RI (IL-4) and *Xba* I or *Bam* H I (IL-5) to produce antisense or sense probes, respectively. Labeled riboprobes were synthesized in the presence of ATP, guanosine triphosphate (GTP), cytosine triphosphate (CTP), and [^{35} S]uridine triphosphate [^{35} S]UTP, and SP-6 polymerase or T7 polymerase, to generate antisense or sense probes, respectively. Slides were then defrosted for the experimental procedure. Permeabilization, prehybridization, and hybridization protocols were as described previously (19). Incubation in N-ethyl maleimide, iodoacetamide, and triethanolamine reduced nonspecific binding of the ^{35} S-labeled probes. Negative controls employed included hybridization with the sense probe and pretreatment of slides with ribonuclease A (RNase A) (Promega, Southampton, UK) prior to hybridization with the antisense probe. For autoradiography, slides were dipped into NTB-2 emulsion (Eastman Kodak) and exposed at 4° C for 2 wk in absolute darkness in a desiccated environment. The slides were developed in Kodak Uni-fix, rinsed, counterstained with Harris hematoxylin, and coverslipped. Dense deposits of silver grains on autoradiographs were present over cells expressing cytokine mRNA. Slides were counted in duplicate, under blind conditions to the patients' clinical status, using an eyepiece graticule, as previously described (14). Results were expressed as the total number of positive cells per square millimeter of submucosa. The coefficient of variation (CV) of the duplicate counts obtained from all slides was less than 5%.

Immunohistochemistry

Bronchial biopsies from seven normal controls, nine atopic controls, nine atopic asthmatic subjects, and 10 nonatopic asthmatic subjects were studied with immunohistochemistry. Sections (6 μ m) were freshly cut from

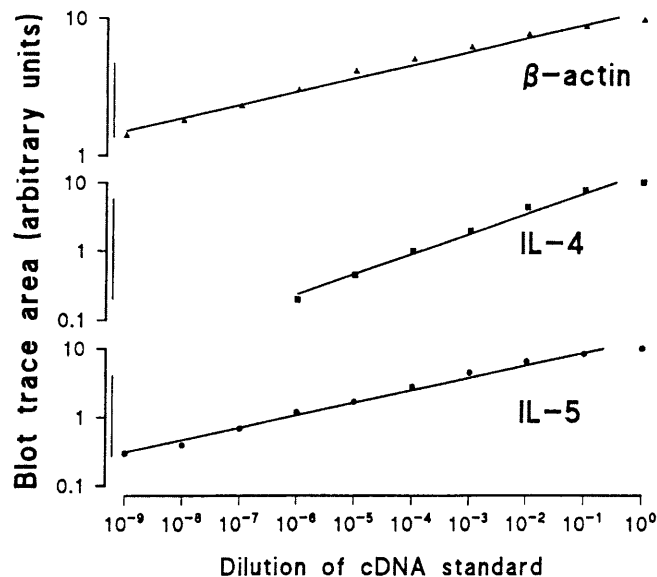


Figure 1. Semiquantitative RT-PCR. The graph shows a standard curve of dilutions of PCR-amplified IL-4, IL-5, and β -actin controls plotted against the respective Southern blot densities. The blot densities from amplification of the biopsy specimens fell within the range of the standard curve (shown by straight lines on y axes). In the lower part of the figure, the dot-blot analysis of PCR-amplified IL-5 cDNA standards from which the IL-5 standard curve was constructed is shown. Amplified IL-4 and β -actin standards were similar.

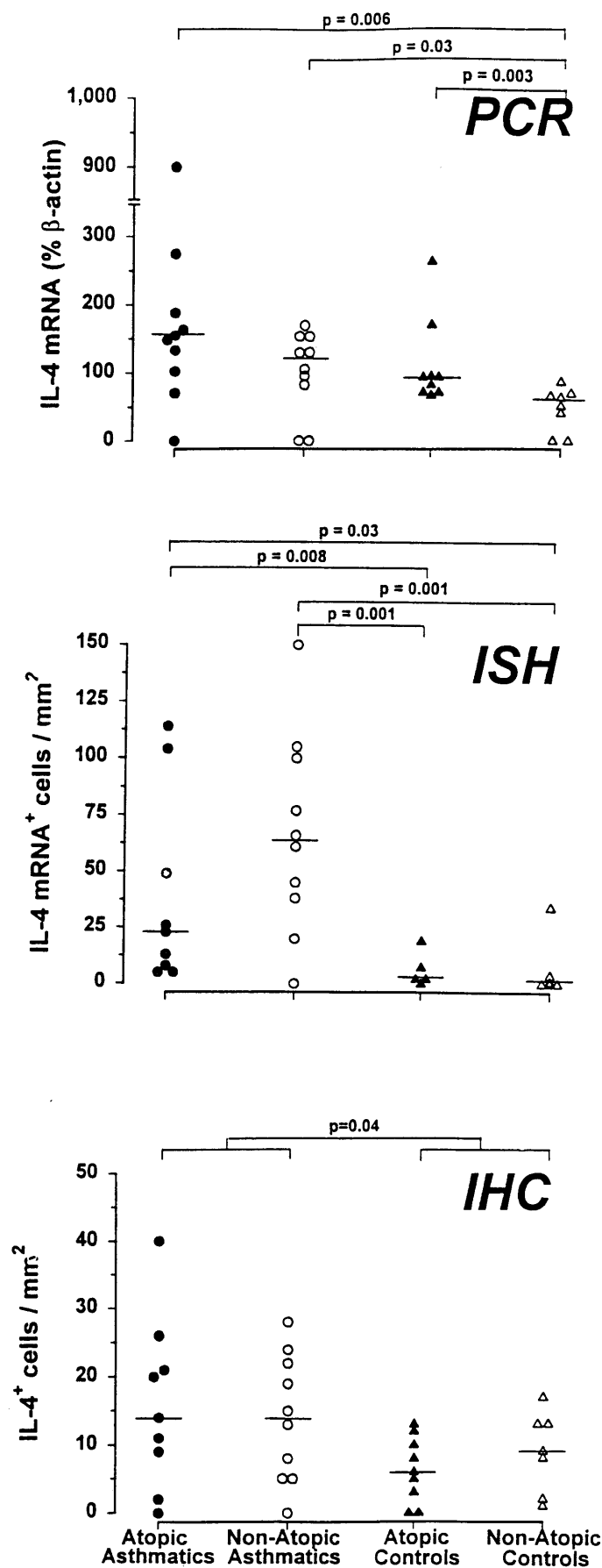


Figure 2. IL-4 mRNA and protein expression in bronchial biopsies from atopic and nonatopic asthmatic subjects, atopic nonasthmatic subjects, and normal controls. IL-4 mRNA expression was measured

frozen bronchial biopsies onto slides coated with 0.1% poly-L-lysine. The sections were then air dried overnight at room temperature, and fixed for 10 min in acetone. Immunohistochemistry was performed with a modified avidin-biotin-complex method. Sections were incubated with PBS containing 1% hydrogen peroxide and 0.02% sodium azide. After two washes, endogenous biotin was quenched with a Vector Laboratories kit (Peterborough, UK). The slides were then washed in PBS containing 0.1% saponin (Sigma) and preincubated with rabbit serum. Following this, the slide-mounted sections were incubated overnight at room temperature with validated (20–22) rat anti-human IL-4 (MP4-25D2; Pharmingen, San Diego, CA) or IL-5 (JES1-39D10; American Type Culture Collection, Rockville, MD) monoclonal antibodies in PBS/saponin at a final concentration of 10 μ g/ml. After washing, the sections were treated with the Vectastain Elite (Vector) kit, which consisted of a secondary biotinylated layer followed by an avidin-biotin-peroxidase complex, as indicated by the manufacturer. Positive cells stained black after development with 3,3'-diaminobenzidine tetrahydrochloride/nickel (Vector). Omission or substitution of the primary antibody with an irrelevant antibody of the same species was used as a negative control. The sections were counted in duplicate, under blind conditions to the patients' clinical status. Results were expressed as the total number of positive cells per square millimeter of submucosa. The CV of the duplicate counts obtained from all slides was less than 5%. No immunoreactivity was observed in sections stained with omission of the primary rat antibody or substitution of this antibody with an irrelevant antibody of the same species.

Statistical Analysis

Data were analyzed nonparametrically with a statistical package (Minitab Release 7; Minitab Inc., State College, PA). Median values are indicated in Figures 1 and 2. When multiple comparisons were made between groups, significant between-group variability was first established using the Kruskal-Wallis test. The Mann-Whitney U test was then used for intergroup comparisons. Correlation coefficients were obtained by Spearman's rank-order method.

RESULTS

Interleukin-4

The median numbers of copies of IL-4 mRNA relative to β -actin mRNA, as detected by semiquantitative RT-PCR, were greater in bronchial biopsies from asthmatic patients, irrespective of their atopic status, than from nonatopic controls ($p < 0.02$) (Figure 2). There was no significant difference in the median numbers of IL-4 mRNA copies in the atopic and nonatopic asthmatic subjects. IL-4 mRNA expression was significantly increased in bronchial biopsies from nonatopic asthmatic subjects as compared with nonatopic controls ($p < 0.05$), and in atopic controls as compared with nonatopic controls ($p < 0.01$). *In situ* hybridization confirmed that IL-4 mRNA expression could be located to discrete cells within the bronchial mucosa. Both atopic and nonatopic asthmatic subjects had increased median numbers of cells expressing IL-4 as compared with the atopic and nonatopic controls (Figure 2). Unlike RT-PCR, *in situ* hybridization did not show a significant difference in IL-4 mRNA expression in the atopic versus the nonatopic controls. The asthmatic subjects also had increased median numbers of IL-4-immunoreactive cells as

by semiquantitative PCR (top), *in situ* hybridization (middle), and protein expression by immunohistochemistry (bottom) in atopic asthmatic subjects (solid circle), nonatopic asthmatic subjects (open circle), atopic controls (solid triangle), and nonatopic controls (open triangle). In the case of IL-4 protein expression there was a significant difference between the asthmatic subjects and controls considered as two groups, as shown. There were no significant differences between the numbers of positive cells from the atopic asthmatic subjects versus the atopic controls, or from the nonatopic asthmatic subjects versus the nonatopic controls.

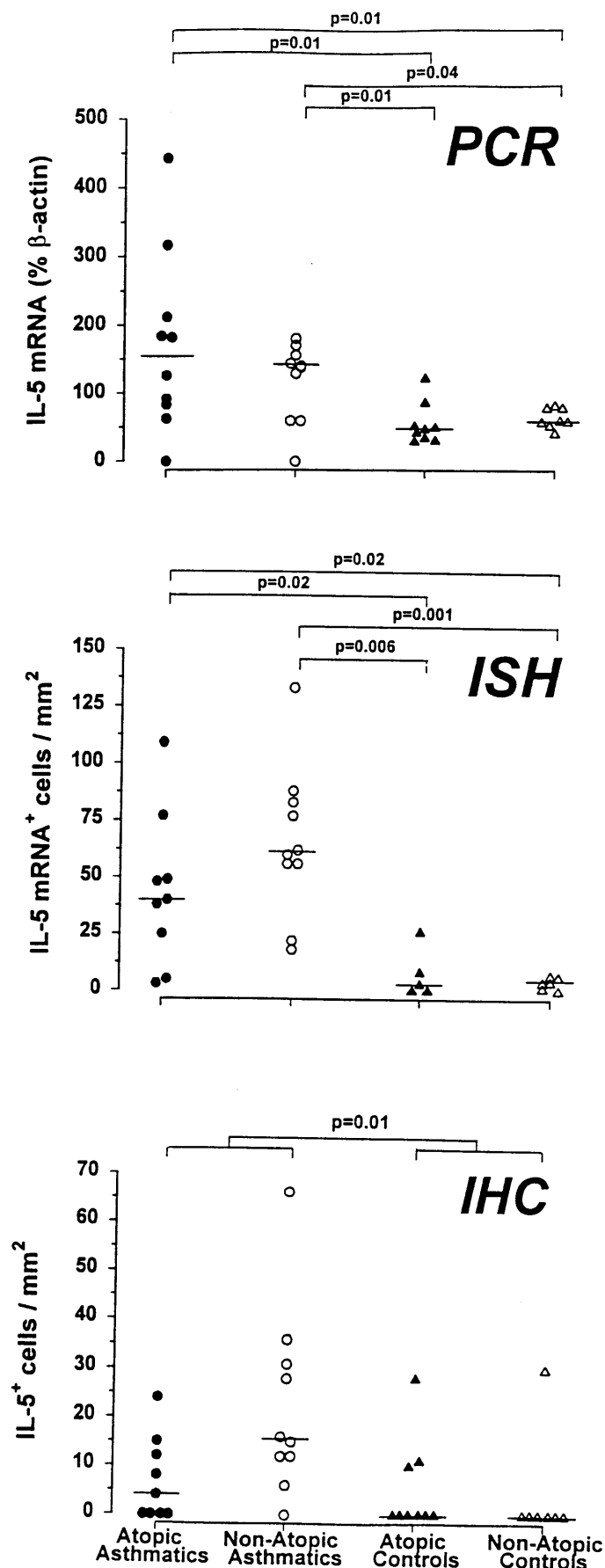


Figure 3. IL-5 mRNA and protein expression in bronchial biopsies from atopic and nonatopic asthmatic subjects, atopic nonasthmatic subjects, and normal controls. IL-5 mRNA expression was measured

compared with the controls, although considerable overlap was observed (Figure 2; atopic and nonatopic asthmatic subjects versus atopic and nonatopic controls; $p < 0.05$). The median numbers of IL-4-immunoreactive cells in the atopic and nonatopic asthmatic subjects were not significantly different.

Interleukin-5

The median numbers of copies of IL-5 mRNA relative to β -actin mRNA, as detected by semiquantitative RT-PCR, were also greater in bronchial biopsies from the asthmatic subjects, irrespective of their atopic status, than in bronchial biopsies from both the atopic and nonatopic controls ($p < 0.001$, Figure 3). As with IL-4, there was no significant difference between the median numbers of IL-5 mRNA copies in the atopic and nonatopic asthmatic subjects. However, in contrast to the case with IL-4, atopic nonasthmatic controls showed no increase in IL-5 mRNA expression as compared with normal controls. The *in situ* hybridization data were similar to those observed with IL-4 (Figure 3). As with IL-4, there were also increased median numbers of IL-5-immunoreactive cells in the asthmatic subjects as compared with the controls (Figure 3) ($p < 0.02$). The numbers of IL-5-immunoreactive bronchial mucosal cells were significantly increased in the nonatopic asthmatic subjects as compared with their atopic counterparts ($p < 0.05$).

In the asthmatic subjects (both atopic and nonatopic), IL-5 mRNA expression (relative to β -actin), but not IL-4 mRNA expression, correlated inversely with the patients' bronchial responsiveness as measured by histamine PC₂₀ ($r = -0.81$, $p < 0.001$), and positively with the patients' asthma severity as assessed by the Aas score ($r = 0.41$, $p < 0.05$).

Examples of *in situ* hybridization of bronchial biopsies with antisense IL-4 and IL-5 riboprobes are shown in Figure 4. No hybridization signals were observed in biopsies hybridized with the control sense probe or biopsies treated with RNase A prior to hybridization with the antisense probe. Figure 5 shows an example of positive immunostaining for IL-4 and IL-5. Sections incubated with irrelevant control antibodies showed no positive cells.

DISCUSSION

This study represents the first systematic comparison of the molecular immunopathology of atopic (extrinsic) and nonatopic (intrinsic) asthma. We have demonstrated increased expression of mRNA encoding IL-4 and IL-5 and increased numbers of IL-4- and IL-5-immunoreactive cells within the bronchial mucosa of both symptomatic atopic and nonatopic asthmatic subjects as compared with atopic and nonatopic nonasthmatic controls. Together with our previous findings of a similar inflammatory cell infiltrate in the bronchial mucosa of subjects with intrinsic and extrinsic asthma (14, 15), these data suggest considerable mechanistic similarities in the pathogenesis of both diseases despite their distinct clinical attributes. Although we have not in-

by semiquantitative PCR (top), *in situ* hybridization (middle) and protein expression by immunohistochemistry (bottom) in atopic asthmatic subjects (solid circle), nonatopic asthmatic subjects (open circle), atopic controls (solid triangle), and nonatopic controls (open triangle). In the case of IL-5 protein expression there was a significant difference between the asthmatic subjects and controls considered as two groups, as shown. In addition, the numbers of positive cells were increased in the nonatopic asthmatic subjects as compared with the atopic asthmatic subjects and both control groups ($p < 0.05$ in each case).

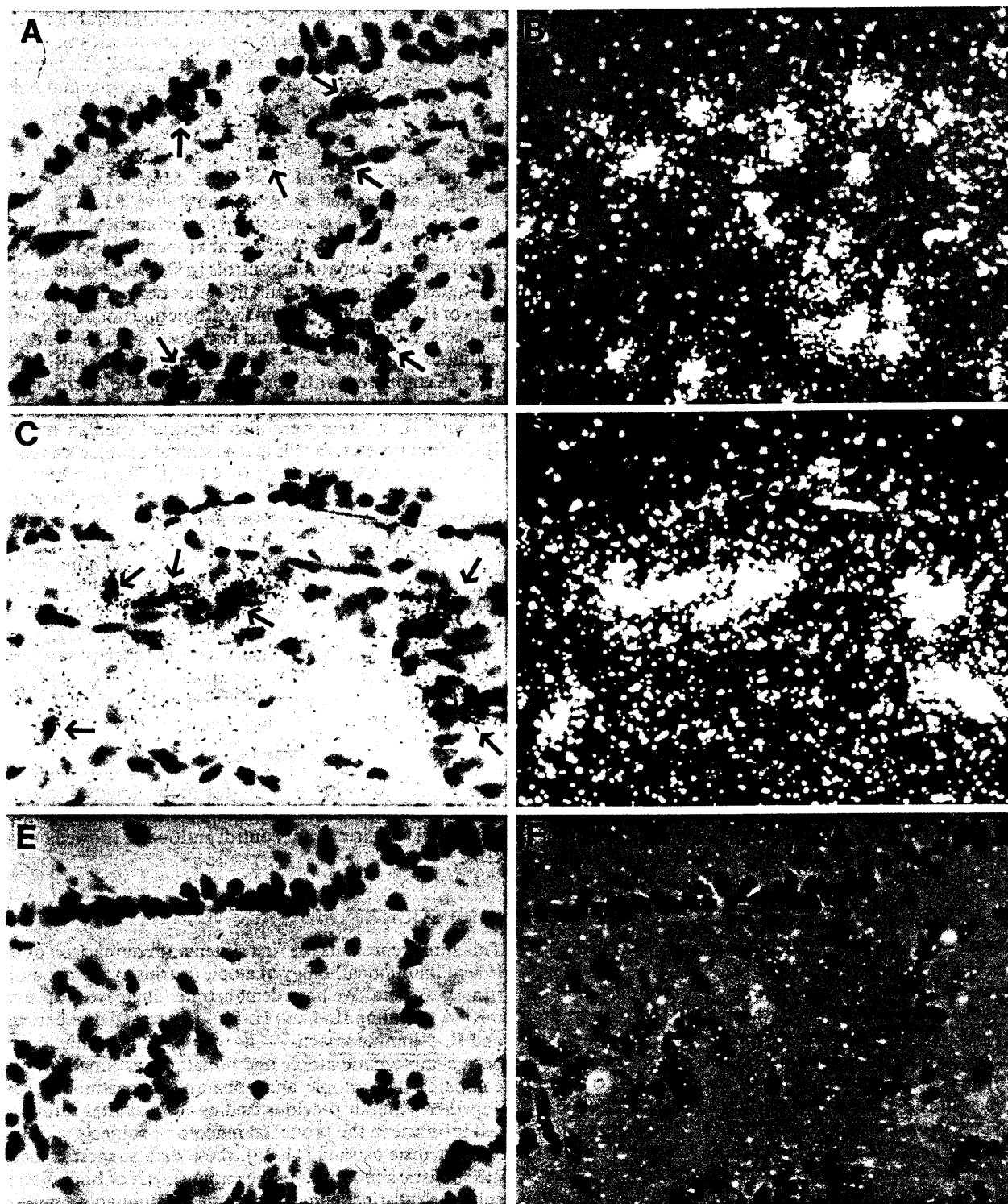


Figure 4. Cryostat sections of a representative bronchial biopsy from a nonatopic asthmatic subject (magnification $\times 400$). (A) *In situ* hybridization with ^{35}S -labeled IL-4 antisense riboprobe. Several IL-4 mRNA $^{+}$ cells are observed (arrows). (B) The same section shown in darkfield. (C) *In situ* hybridization with ^{35}S -labeled antisense riboprobe for IL-5. Several IL-5 mRNA $^{+}$ cells are observed (arrows). (D) The same section shown in darkfield. (E) No positive hybridization signals were observed when the sections were hybridized with ^{35}S -labeled sense riboprobe for IL-4. (F) The same section shown in darkfield.

investigated the cellular sources of the IL-4 and IL-5 mRNA in this study, our previous studies with atopic asthmatic subjects showed that these mRNA species are predominantly T-cell derived, with a small but significant contribution from mast cells and eosinophils (7). Since IL-4 promotes IgE switching in B-lym-

phocytes (11), it is interesting to speculate that inappropriate IgE synthesis may also be a feature of nonatopic asthma, as assumed in atopic asthma. The reason why the nonatopic asthmatic subjects had undetectable systemic concentrations of allergen-specific IgE as shown by skin prick and/or RAST testing is not, how-

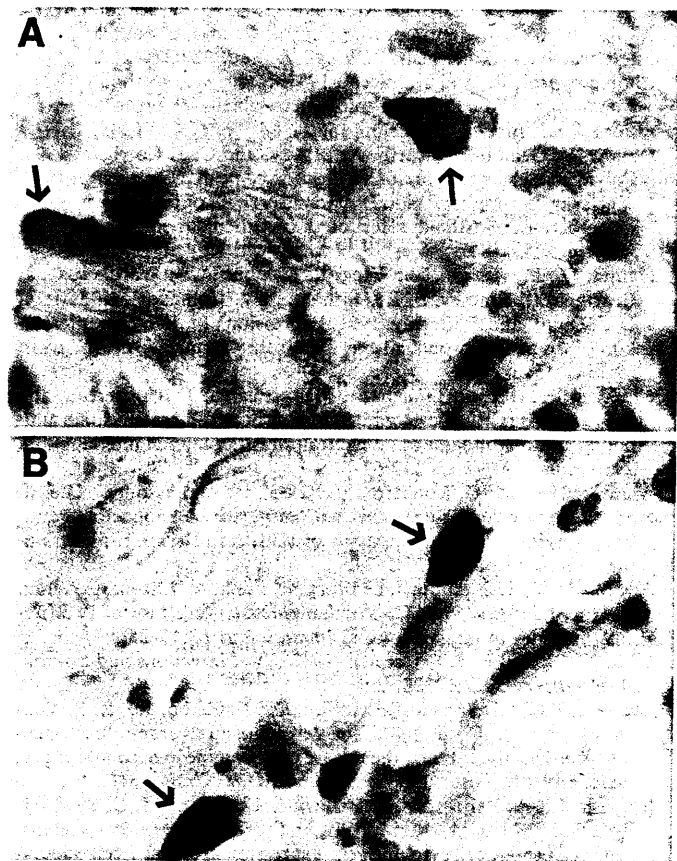


Figure 5. Cryostat sections of a representative bronchial biopsy from a nonatopic asthmatic subject (magnification $\times 1,000$). (A) Immunohistochemistry with IL-4-specific monoclonal antibody. Several IL-4+ cells are observed (arrows). (B) Immunohistochemistry with IL-5-specific monoclonal antibody. Several IL-5+ cells are observed (arrows).

ever, clear, although synthesis of IgE specific for antigens other than allergens, such as viral antigens, has been described in asthmatic individuals (23, 24). In this regard it is of interest that the nonatopic asthmatic subjects in the present study, like the atopic asthmatic subjects, had increased total serum IgE concentrations as compared with the nonatopic, nonasthmatic controls, even though these were within the laboratory "normal range." These data are also in accord with the epidemiologic study by Burrows and colleagues showing that the incidence of self-reported asthma correlated with total serum IgE concentrations regardless of atopic status (16). This same study (16) also showed that serum IgE concentrations (and possibly therefore IL-4 expression) decline with age. The nonatopic asthmatic subjects in our study were significantly older than the atopic asthmatic subjects, a clinical difference that has previously been noted (3). Notwithstanding, these patients showed increased bronchial mucosal IL-4 expression and serum IgE concentrations as compared with controls, suggesting that these differences did not reflect age-related effects.

Our present findings showing correlations of bronchial mucosal IL-5 mRNA expression with asthma severity (histamine PC_{20}) and symptoms (Aas score), irrespective of the patients' atopic status, confirm previous findings in atopic asthma (12, 19) and firmly implicate IL-5 in the pathogenesis of asthma. This is consistent with the activity of IL-5 in promoting specific eosinophil recruitment to the bronchial mucosa through its activities in promoting the maturation of committed eosinophil precursors and the priming, hyperadherence, and survival of ma-

ture eosinophils (4, 10). In contrast, expression of mucosal IL-4 mRNA relative to β -actin in both the atopic and nonatopic asthmatic subjects in the present study did not correlate with bronchial responsiveness. Furthermore, increased bronchial mucosal IL-4 mRNA expression was not specific for asthma in this study, since it was also observed in the atopic, nonasthmatic controls with RT-PCR (although not using the less sensitive sensitive technique of *in situ* hybridization). Taken together, these data are compatible with the hypothesis that increased bronchial mucosal expression of IL-4 is necessary but not sufficient for the development of both atopic and nonatopic asthma. Additional factors, resulting at least partly in locally increased IL-5 synthesis, must be critical for the development of the bronchial mucosal inflammation and eosinophil activation that characterize symptomatic asthma. Alternatively, it is possible that increased IL-4 synthesis in the bronchial mucosa of nonatopic asthmatic individuals reflects the activation of IL-5-secreting cells that elaborate IL-4 coordinately but coincidentally. On the other hand, it has recently been demonstrated that the expression of IL-4 and IL-5 may be regulated independently, at least in T cells (25, 26).

Our present data are at variance with those of Walker and colleagues, who found that IL-4 protein concentrations were increased in concentrated bronchoalveolar lavage fluid from atopic but not nonatopic asthmatics subjects as compared with controls (9). The reasons for this apparent discrepancy are unclear, but it may reflect methodologic problems (variable protein recovery following concentration of bronchoalveolar lavage fluid, or the possible presence of inhibitors that interfere with the protein assay). We believe that our present study, which confirms the presence of IL-4 and IL-5 mRNA using RT-PCR and *in situ* hybridization, as well as the presence of the translated products by immunohistochemistry, clearly demonstrates the potential for synthesis and storage of both of these cytokines in intrinsic asthma. The lack of precise concordance in this study between the numbers of cells expressing IL-4 and IL-5 mRNA and protein probably reflects the differing capacities of various cells for cytokine storage. Unlike T cells, mast cells (8) and eosinophils (27) store various cytokines such as IL-4 and IL-5 as preformed mediators within their granules, from which these cytokines can be rapidly released by exocytosis. Thus, cytokine mRNA and protein synthesis may not always equate temporally.

In summary, we have provided evidence for transcription and translation of the IL-4 and IL-5 genes within the bronchial mucosa of subjects with nonatopic (intrinsic) as well as atopic asthma, supporting the concept that these subtypes of asthma, despite showing some distinct clinical features, share common immunopathologic mechanisms.

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