

Activation of CD4+ T cells, increased T_{H2}-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma

Douglas Robinson, MRCP,* Outayba Hamid, PhD,*^{a, b}

Andrew Bentley, MRCP,* Sun Ying, MD,* A. Barry Kay, PhD,* and

Stephen R. Durham, MD* London, England

Background: We have examined whether the local eosinophilia provoked by inhalational allergen challenge of patients with atopic asthma is associated with the appearance, in vivo, of activated T_{H2}-type T helper lymphocytes.

Methods: Fifteen patients with atopic asthma had bronchial wash and bronchoalveolar lavage (BAL) 24 hours after allergen or diluent challenge separated by at least 21 days.

Results: There was an increase in eosinophils in both bronchial wash ($p = 0.01$) and BAL ($p = 0.02$) after allergen challenge but not after diluent challenge. Activation of CD4+ BAL T cells was suggested by an increase in the expression of CD25 shown by flow cytometry after allergen challenge, when compared with diluent ($p = 0.02$). There was no evidence of activation of CD8 T cells. By in situ hybridization after allergen challenge as compared with diluent, increases were shown in the numbers of cells expressing mRNA for interleukin-4 (IL-4) ($p = 0.005$), IL-5 ($p = 0.01$), and granulocyte-macrophage colony-stimulating factor ($p = 0.03$) but not IL-3, IL-2, or interferon- γ . In situ hybridization of BAL cells after immunomagnetic separation of CD2-positive and CD2-negative cell populations showed that IL-4 and IL-5 mRNAs were associated with T lymphocytes after allergen challenge. BAL and bronchial wash eosinophilia closely correlated with maximal late fall in forced expiratory volume in 1 second after allergen challenge.

Conclusion: Cytokines produced by activated T_{H2}-type CD4+ T cells in the airway may contribute to late asthmatic responses by mechanisms that include eosinophil accumulation. (*J ALLERGY CLIN IMMUNOL* 1993;92:313-24.)

Key words: T_{H2} cells, eosinophils, in situ hybridization, flow cytometry, late asthmatic response

Inhalation challenge of subjects with atopic asthma with allergen provokes an immediate or early asthmatic response (EAR), which is detectable within minutes as a decrease in forced expiratory volume in 1 second (FEV₁). This may be

followed by a second fall in FEV₁ between 4 and 12 hours after allergen exposure, which is associated with an increase in nonspecific bronchial responsiveness that may persist for several days.¹⁻³ Although the EAR appears to be dependent on IgE triggering of mediator release from mast cells resulting in bronchoconstriction and airway edema,^{4, 5} the late asthmatic response (LAR) is associated with an influx of inflammatory cells, particularly eosinophils,⁶⁻⁸ into the bronchial mucosa. Eosinophil infiltration of the bronchial mucosa is a feature of the airways of patients who are dying of asthma,⁹ and is present in bronchial biopsy specimens¹⁰ and bronchoalveolar lavage (BAL)^{11, 12} of patients with mild disease. Thus although it does not entirely reproduce the pattern of natural exposure, allergen challenge may

From *the Department of Allergy and Clinical Immunology and ^athe Department of Lung Pathology, National Heart and Lung Institute, London.

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Reprint requests: Stephen R. Durham, Senior Lecturer, Department of Allergy and Clinical Immunology, National Heart and Lung Institute, Dovehouse St., London, SW3 6LY, England.

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Abbreviations used

BAL:	Bronchoalveolar lavage
BW:	Bronchial wash
EAR:	Early asthmatic response
FEV ₁ :	Forced expiratory volume in 1 second
FOB:	Fiberoptic bronchoscopy
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
IFN- γ :	Interferon- γ
IL:	Interleukin
LAR:	Late asthmatic response
mRNA:	Messenger RNA
PC ₂₀ :	Provocative concentration causing a 20% fall in FEV ₁
T _H :	T helper

provide a model for investigating allergen-induced inflammatory events in atopic asthma.¹³

In vitro and animal studies have suggested a role for cytokines in the initiation of eosinophilic inflammation. Interleukin-5 (IL-5) promotes in vitro survival and activation of eosinophils,^{14, 15} enhances adhesion to endothelium,¹⁶ and produces eosinophilia in vivo.¹⁷ Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 may also contribute to eosinophil maturation and activation.^{18, 19} The description of a subset of murine T helper cell clones (termed T_{H2}) that produced IL-5, IL-3 and GM-CSF together with IL-4 but not interferon- γ (IFN- γ)²⁰ raised the possibility that activation of a T_{H2}-like subset of CD4+ T lymphocytes might contribute to eosinophil infiltration and IgE production in human allergic disease.^{21, 22} Allergen-specific CD4+ T cell clones from atopic individuals had a T_{H2} pattern of cytokine production.^{23, 24} A role for T_{H2} cells in atopic asthma in vivo was supported by the demonstration of messenger RNA (mRNA) for IL-4 and IL-5 in T cells in BAL from patients with atopic asthma together with increased numbers of BAL cells expressing mRNA for IL-2, IL-3, IL-4, IL-5, and GM-CSF but not IFN- γ when compared with normal control subjects.²⁵ Cytokine activity for IL-3, IL-5, and GM-CSF was found in serum samples from patients with atopic asthma.²⁶

Participation of T lymphocytes in allergen-induced asthmatic responses was suggested by the finding of lymphocyte infiltration in BAL after local allergen challenge⁷ and by changes in CD4 and CD8 T cells in both peripheral blood and BAL after allergen challenge.²⁷⁻²⁹ We therefore hypothesized that allergen challenge in patients with atopic asthma results in local activation of

T_{H2}-type CD4+ T cells and eosinophils, which in turn contributes to the associated changes in airway calibre and hyperresponsiveness.

To test this hypothesis, we examined BAL cell counts, T-cell phenotype and activation status, and the cytokine mRNA profile of BAL cells 24 hours after inhalation challenge with either allergen or diluent control. To determine whether cytokine mRNA detected after allergen challenge was associated with T lymphocytes, in situ hybridization for IL-4 and IL-5 mRNA was performed after immunomagnetic separation of CD2-positive and CD2-negative BAL cell populations.

METHODS

Patients

Fifteen patients with atopic asthma who were sensitive to either grass pollen or house dust mite were recruited from the allergy clinic of the Royal Brompton National Heart and Lung Hospital (Table I). Asthma was defined by (1) a clinical history of intermittent chest tightness, wheeze, cough, or shortness of breath and (2) documented reversible airflow obstruction (20% change in FEV₁ or peak expiratory flow), which occurred either spontaneously or with treatment in the preceding year. All subjects had mild symptoms that required intermittent use of inhaled β_2 -agonists only. None had received orally administered or inhaled corticosteroids in the 3 months preceding the study. Patients with seasonal symptoms were studied out of the United Kingdom's pollen season. None were smokers, and any patient with a history compatible with respiratory infection in the 3 weeks preceding or during the study was excluded. All patients gave written informed consent, and the study was approved by the ethics committee of the hospital.

Study design

In a randomized crossover study, patients underwent either diluent or allergen challenge followed 24 hours later by fiberoptic bronchoscopy (FOB); on a second occasion at least 3 weeks after the first, they underwent the corresponding allergen or diluent challenge followed by FOB.

At an initial assessment, history and examination together with a full explanation of the study were performed. Skin prick tests to a panel of common aeroallergens were performed, and blood was taken for blood count, determination of total IgE, and RAST for specific IgE to grass pollen and dust mite (Phadebas RAST; Pharmacia, Uppsala, Sweden). The allergen used for challenge was selected on the basis of a history of clinical sensitivity supported by a positive skin prick test result (wheal diameter at least 5 mm greater than that produced by control solution). Allergen sensitivity was determined by skin prick tests with 10-fold serial dilutions of allergen, starting from 10,000 BU/ml *Der-matophagoides pteronyssinus* or 30,000 BU/ml timothy

TABLE I. Patient characteristics

Patient No.	Age (yr)	Sex	FEV ₁ (% pred)	Methacholine PC ₂₀ (mg/ml)
1	24	F	108	0.36
2	23	F	87	0.25
3	23	F	100	4.6
4	26	M	96	19.9
5	27	M	116	15.5
6	29	F	109	12.8
7	19	F	103	2.2
8	34	M	84	2.5
9	34	F	106	8.0
10	23	F	84	3.2
11	21	F	100	13.8
12	19	M	129	15.5
13	26	M	104	8.0

Data are for the 13 patients from whom paired samples were analyzed.
pred, Predicted value.

grass extract, *Phleum pratense* (Alutard, ALK, Horsholm, Denmark). At 10:00 AM methacholine provocative concentration causing a 20% fall in FEV₁ (PC₂₀) was measured as previously described.³⁰ At noon allergen challenge was performed, starting with the concentration 1 half-log serial dilution below that which produced a 2 mm diameter skin prick wheal (threshold dose for a response). Increasing half-log concentration increments were given until a 20% fall in FEV₁ was achieved. Patients inhaled allergen solution through a Wright nebulizer by tidal breathing for 2 minutes. FEV₁ and peak expiratory flow rate were recorded before challenge, after administration of nebulized saline solution, every 5 minutes for 30 minutes after allergen challenge, then every 10 minutes for the first hour, and then hourly until FEV₁ had returned to baseline (at least 90% of the initial value), generally at 3 to 4 hours. Patients were then allowed to go home after this initial visit, and they recorded peak flow hourly for the rest of the day and at 9:00 AM on the next day. After an interval of 3 weeks, patients underwent two further challenges, one with allergen and the other with diluent. Challenges were performed in random order with an interval of at least 3 weeks. Allergen challenge, which consisted of three inhalations of the last three allergen dilutions up to the dose that previously, produced a 20% fall in FEV₁ was performed between 9:00 and 10:00 AM. For diluent challenge three inhalations of the diluent vehicle (0.9% saline solution) were used. Patients remained on the ward, and FEV₁ was measured hourly for the rest of the day. FOB was performed between 8:00 and 9:00 AM the next morning; either the left or right lung was sampled in random order.

Fiberoptic bronchoscopy

Each subject was given nebulized albuterol (2.5 mg) and atropine (0.6 mg, intravenously) 15 minutes before

FOB. Thirty milliliters of blood was drawn into a heparinized container for later separation of mononuclear cells. Sedation was achieved with intravenously administered midazolam, and supplemental oxygen was given throughout the procedure. Patient oxygenation was monitored by pulse oximetry. FOB was performed by the same operator in all subjects. After 1% lidocaine spray was applied to the nose and throat of the subject, the Olympus BF P20 bronchoscope (Olympus Corp., Tokyo, Japan) was introduced. Local anesthesia of the larynx was produced with topical 4% lidocaine, and 2% lidocaine was used below the vocal cords. After inspection of the airways, the bronchoscope was wedged in a segmental or subsegmental division of the right middle lobe or lingula. An initial 60 ml aliquot of pH-adjusted, warmed, sterile saline solution was introduced, and returned fluid was collected by gentle machine suction (less than 60 cm water) as a bronchial wash (BW). An additional three 60 ml aliquots of saline solution were then introduced and collected into a sterile siliconized glass bottle (BAL). Endobronchial biopsy specimens were then taken from segmental or subsegmental carinae with Olympus FB 15C forceps (results from these biopsies will be the subject of a separate report). After FOB was completed, patients were given an additional 2.5 mg of albuterol and were observed on the ward for at least 4 hours.

Processing of BW and BAL

The volumes of BW and BAL fluid returned were recorded. The BAL was passed through sterile gauze to remove any mucus. The BW was not filtered. Cells were pelleted by centrifugation at 300 g for 7 minutes at 4° C followed by a washing step in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland), then resuspended in 1 ml (BW) or 1.5 ml (BAL) of RPMI 1640 with N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer. A total cell count was performed with a

Neubauer hemocytometer (Neubauer GMBH, Neuss, Germany). BW cells were used to make cytocentrifuge preparations with a Shandon 2 cytospin (Shandon Southern Products Ltd., Runcorn, Cheshire, England) at 800 rpm for 5 minutes. Slides were air-dried and either stored at -20°C or stained immediately with May-Grünwald Giemsa stain for cell differential or alcian blue for mast cells. BAL cells were used for flow cytometry after removal of an aliquot adjusted to 5×10^5 cells/ml for cytospin preparations for both cell counts and for in situ hybridization. Peripheral blood mononuclear cells were separated from heparinized blood by density centrifugation on Ficoll-Paque (Pharmacia).

Immunomagnetic separation of BAL cells

To determine whether mRNA for IL-4 and IL-5 was associated with T lymphocytes, aliquots of BAL cells obtained 24 hours after allergen challenge from five subjects were separated into CD2-positive and CD2-negative cell populations as previously described.²⁵ Briefly, BAL cells were mixed with anti-CD2-linked magnetic particles (Dynabeads, Dynal, Wirral, England) in a ratio of three beads to each lymphocyte at 4°C for 20 minutes. Cells were then separated in a magnetic particle separator (MPS, Dynal), CD2-negative cells were removed by pipette, and CD2-positive cells were washed four times in phosphate-buffered saline with 0.1% bovine serum albumin (Sigma Chemicals, Poole, England). Cytocentrifuge preparations were made from both cell populations.

Flow cytometry

BAL cells or peripheral blood mononuclear cells were used in a concentration of 5 to 10×10^6 per milliliter. Fifty micrometers of cell suspension was incubated for 10 minutes at 4°C with two 10 μl aliquots of monoclonal antibody for T-cell phenotype (CD4, CD8; Becton Dickinson, Cowley, England) and activation marker (CD25; Dako, High Wycombe, England) directly conjugated to phycoerythrin or fluorescein isothiocyanate, respectively. Isotype controls were from Becton Dickinson. After two washing steps, cells were resuspended in Isoton II balanced electrolyte solution (Coulter Electronics, Luton, England) before analysis with a Becton Dickinson FACS Analyzer with a Becton Dickinson FACSlite laser. BAL lymphocytes were defined on the basis of volume and 90-degree light scatter of peripheral blood lymphocytes and 10,000 events collected in a live gate. After gating on lymphocytes expressing phenotypic markers (CD4 or CD8), CD25 expression relative to the isotype control antibody was noted as percent positive.

In situ hybridization

Riboprobes (labeled complementary RNA) labeled with sulfur 35 were prepared from cDNAs for IL-2, IL-3, IL-4, IL-5, GM-CSF, and IFN- γ (from Glaxo Biogen, Geneva, Switzerland) as previously de-

scribed.^{31, 32} All reagents for in situ hybridization were from Sigma Chemicals unless otherwise indicated. Cytospins of BAL cells were prepared on poly-L-lysine-coated slides, air-dried for 5 minutes, and fixed in 4% paraformaldehyde (BDH Laboratory Supplies, Poole, Dorset, England) in phosphate-buffered saline, pH 7.4, for 30 minutes followed by 15% sucrose in phosphate-buffered saline twice for 1 hour. Permeabilization and prehybridization and the hybridization protocol were as previously described.³¹ Incubation in N-ethylmaleimide, iodoacetamide, and triethanolamine reduced nonspecific binding of the ^{35}S probes. Negative controls included use of the same probe (identical nucleotide sequence to target mRNA) and pretreatment of slides with RNase A (Promega, Southampton, England). Positive controls were phytohemagglutinin stimulated peripheral blood mononuclear cells (for IL-2 and IFN- γ) and a T-cell clone known to produce IL-3, IL-4, IL-5, and GM-CSF (Dr. Diana Quint, personal communication, 1991). Dense deposits of silver grains on autoradiographs were present over cells expressing cytokine mRNA. Quantification of silver grains was not possible, and to allow a semiquantitative analysis, the number of cells expressing mRNA for each cytokine per 1000 total cells on the cytospin was enumerated as previously described.²⁵ At least two slides were counted for each cytokine mRNA for each BAL by an observer who was blinded to the nature of the challenge (antigen or diluent). Percentages of cells expressing mRNA signals for IL-4 and IL-5 and percentages of lymphocytes present in cytocentrifuge preparations were determined for CD2-positive and CD2-negative BAL cells, as previously described.²⁵

Statistical analysis

Paired data (allergen vs diluent) for cell differential, flow cytometry, and in situ hybridization cell counts were analyzed by Wilcoxon signed-rank test for paired data with Minitab PC software (Minitab Inc., State College, Pa.). Correlation was by nonparametric analysis to give Spearman's rank correlation coefficient. A *p* value of less than 0.05 was taken as significant.

RESULTS

Clinical response

Fifteen patients were studied. One patient experienced localized bronchospasm during FOB, and BAL was not performed. One subject had a chest infection after the first challenge. Thus paired data were obtained from 13 subjects. Allergen used for challenge, allergen dose producing a 20% fall in FEV₁ from baseline (PC₂₀), and size of EARs and LARs (maximum fall in FEV₁ from post-saline value) for the remaining 13 subjects are shown in Table II, together with the timing of the late response (hours after challenge) and the difference between FEV₁ at 24 hours on the allergen day and on the diluent day. Eight of the

TABLE II. Clinical response to allergen challenge

Patient No.	Allergen	Allergen PC ₂₀ (BU)	EAR (% FEV ₁)	LAR (% FEV ₁)	tmax (hr)	FEV ₁ d-a at 24 hr
1	GP	160	39	6	10	0
2	HDM	460	39	4	6	4.0
3	GP	7,000	21	13	9	2.5
4	GP	10,000	21	23	10	8.0
5	HDM	15,000	30	25	9	0
6	GP	15,000	20	11	9	2.5
7	HDM	4,000	49	44	9	28.0
8	HDM	2,500	29	2	10	0
9	HDM	600	32	19	10	5.0
10	HDM	700	24	55	10	40.0
11	HDM	6,500	30	17	9	7.5
12	HDM	6,000	28	58	24	62.0
13	GP	7,300	26	29	6	0

Allergen refers to that used for challenge; early (EAR) and late (LAR) asthmatic responses are expressed as maximal fall in FEV₁ (percent predicted from baseline). Also shown are timing of the LAR in hours after challenge (tmax) and fall in FEV₁ at 24 hours after challenge relative to the value on the diluent challenge day (FEV₁ d-a).

BU, Biological units; GP, grass pollen; HDM, house dust mite.

TABLE III. Details of BW and BAL cells

	BW			BAL		
	Diluent	Allergen	p Value	Diluent	Allergen	p Value
Fluid return (%)	50 (42-58)	50 (42-58)	NS	67 (55-72)	72 (58-82)	NS
Total cells/ml*	1.8 (1.1-2.0)	1.7 (0.8-2.6)	NS	1.0 (0.8-1.5)	0.9 (0.7-1.2)	NS
Macrophages (%)	83 (71-93)	64 (41-83)	0.014	83 (76-87)	75 (68-86)	NS
Lymphocytes (%)	5.5 (4.5-6.8)	6.0 (5.0-10.0)	NS	12.2 (10.8-16.6)	12.7 (9.3-17.3)	NS
Eosinophils (%)	3.5 (0.8-17.3)	9.0 (3.8-37.3)	0.013	3.0 (0.6-7.2)	3.6 (1.3-11.3)	0.023
Neutrophils (%)	0.5 (0.1-1.25)	1.0 (0.5-5.5)	0.043	0.4 (0.2-0.9)	0.6 (0.2-2.1)	NS
Epithelial cells (%)	1.0 (0.3-3.0)	2.5 (0.5-4.8)	NS	0 (0-0.1)	0.2 (0-0.85)	NS
Mast cells (%)	0.3 (0.2-0.5)	0.15 (0-0.38)	NS	0 (0-0.1)	0 (0-0.1)	NS

Numbers represent median percentage of cell types in BW ($n = 9$; four were too mucoid for analysis) and BAL ($n = 13$) with interquartile ranges (in parentheses).

NS, Not significant.

*Number of cells per milliliter of fluid is $\times 10^5$.

13 patients were late responders as defined by a fall in FEV₁ of 15% or greater during the LAR. Nine of the subjects showed a fall in FEV₁ in response to diluent challenge. The median timing of maximal late fall in FEV₁ was 9.5 hours after challenge (range, 6 to 24 hours), and three subjects showed a fall in FEV₁ from baseline that was greater than 20% at 24 hours. All subjects tolerated FOB well, and none (including the patient with localized bronchospasm) required additional medication.

BW and BAL cell yield

There were no significant differences in the volume of BAL fluid or total cell yield between diluent and allergen bronchoscopy in either BW

or BAL (Table III). There were significant increases in both the percentages and the absolute numbers of eosinophils in allergen obtained from both BW and BAL fluid compared with that obtained after diluent challenge (Fig. 1), an increase in percent neutrophils in BW ($p = 0.043$), and a decrease in percent macrophages in BW (though not in cell number) ($p = 0.179$).

Flow cytometry

There was no difference between CD4/CD8 lymphocyte ratio in BAL or peripheral blood after allergen or diluent challenge (median 1.9 vs 1.7 for diluent and allergen challenge, respectively; $p = 0.9$). When T-cell activation markers after allergen challenge were compared with those af-

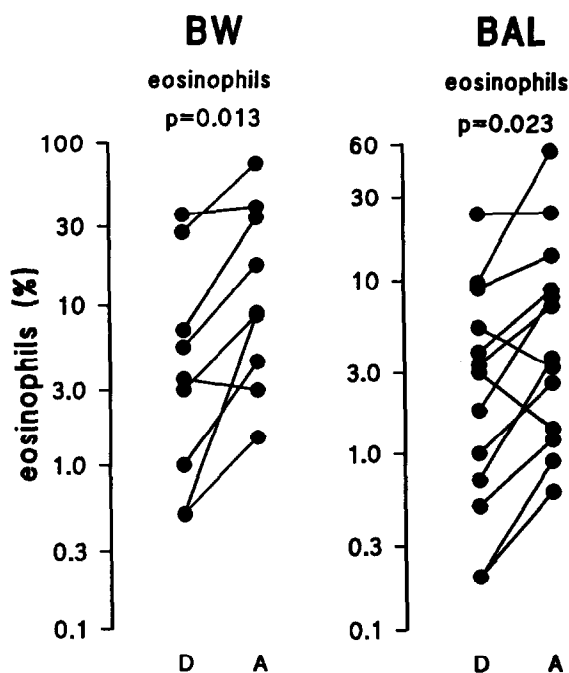


FIG. 1. Percentages of eosinophils in BAL and BW 24 hours after allergen inhalation challenge (A) and after inhalation of diluent (control) solution (D).

ter diluent challenge, there was a significant increase in expression of CD25 by CD4-positive BAL T cells ($p = 0.02$) (Fig. 2) but not by CD8-positive BAL T cells (3.6% vs 3.7% positive; $p = 0.13$). There were no significant changes in CD25 expression by either CD4+ (14.1% vs 16.4% positive; $p = 0.9$) or CD8+ (3.6% vs 3.6% positive; $p = 0.7$) T cells in peripheral blood.

In situ hybridization

There were significant increases in the numbers of BAL cells per 1000 expressing positive hybridization signals for IL-4 ($p = 0.005$), IL-5 ($p = 0.01$), and GM-CSF ($p = 0.003$) mRNA on the allergen day compared with the diluent day, but there was no significant change in IL-2 ($p = 0.10$), IL-3 ($p = 0.16$), or IFN- γ ($p = 1.0$) (Fig. 3). Positive hybridization signals were detected for all cytokines in both diluent and allergen BAL. Negative controls of slides pretreated with RNase or hybridized with a sense probe produced no specific hybridization signal.

Association of IL-4 and IL-5 mRNA with CD2-positive cells in BAL after allergen challenge ($n = 5$)

By use of immunomagnetic particles, BAL lymphocytes were enriched from 10% to a median of 70% of cells in cytocentrifuge preparations or

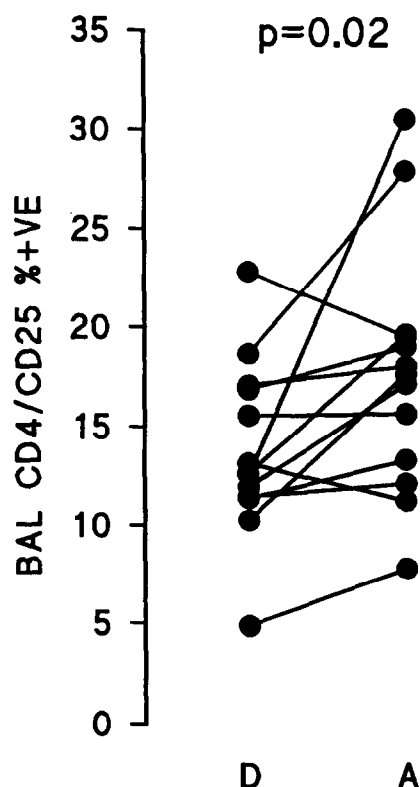


FIG. 2. CD25 expression by CD4-positive T cells in BAL after allergen inhalation challenge (A) and after inhalation of allergen diluent (D, control) solution (detected by flow cytometry). CD25 is represented as percent positive relative to an isotype control.

depleted to 4% (Table IV). In situ hybridization showed that a median of 45% of the CD2-positive cell population produced signals for IL-4 mRNA and 59% were positive for IL-5 mRNA, whereas only 1.5% of the CD2-depleted BAL samples were IL-4 mRNA-positive and 1.7% gave signals for IL-5 mRNA (Table IV). The close association of mRNA-positive cells with CD2-linked magnetic particles confirmed the T-cell source of the mRNA signal (Fig. 4).

Relationship between clinical response to allergen and cells in BAL and BW

A significant correlation was observed between the percentages of eosinophils in both BW and BAL (Fig. 5, C) after allergen challenge and the maximal fall in FEV₁ from baseline during the LAR ($r = 0.87$, $p < 0.0001$ and $r = 0.73$, $p = 0.005$, respectively). An association was also observed between the difference in percent eosinophils in both BW and BAL on the allergen day compared with the diluent day and the size of the LAR ($r = 0.85$, $p = 0.004$ and $r = 0.66$, $p = 0.02$, respectively). There was also a significant corre-

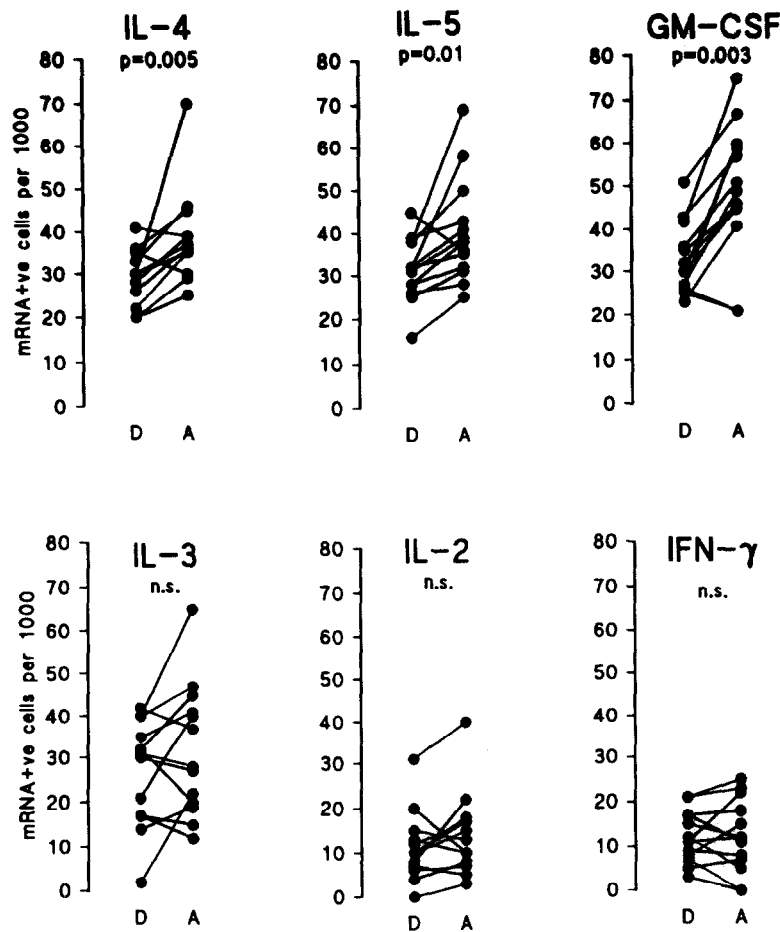


FIG. 3. Positive in situ hybridization signals for mRNA for IL-2, IL-3, IL-4, IL-5, GM-CSF, and IFN- γ in cytopins of BAL cells after diluent (D) and allergen (A) challenge in 13 patients with atopic asthma. Positive cells per 1000 were those over which dense deposits of silver grains were seen on autoradiography after application of ^{35}S cRNA probes for cytokine mRNA.

TABLE IV. Immunomagnetic separation of BAL cells after allergen challenge into CD2-enriched and CD2-depleted aliquots ($n = 5$)

	Percent lymphocytes (May Grunwald Giemsa)	Percent IL-4 mRNA-positive	Percent IL-5 mRNA-positive
Unseparated BAL cells	10 (8-14)	6.7 (4.0-7.5)	6.0 (4.3-6.9)
CD2-positive BAL cells	70 (62.5-93)	45 (39-57)	59 (38-62)
CD2-negative BAL cells	4 (2-5)	1.5 (1.0-2.5)	1.7 (0.9-2.5)

Numbers represent median percentages and ranges (in parentheses) of lymphocytes, IL-4 mRNA-positive cells, and IL-5-positive cells in cytocentrifuge preparations.

lation between the percentage of eosinophils in BAL and reduction in FEV₁ at 24 hours after allergen challenge ($r = 0.63$, $p = 0.02$). No significant relationship was observed between the clinical response to allergen and BAL T-cell activation (percent CD4-positive for CD25, $r = 0.291$) or between clinical response and the numbers of cells expressing cytokine mRNA.

Relationships among BAL T-cell activation, eosinophils, and cells expressing cytokine mRNA

There was a correlation between CD25 expression by BAL T cells and both percent BAL eosinophils ($r = 0.62$, $p = 0.02$) (Fig. 5, B) and the number of cells per 1000 expressing IL-4 ($r = 0.63$, $p = 0.02$) and IL-5 ($r = 0.70$,

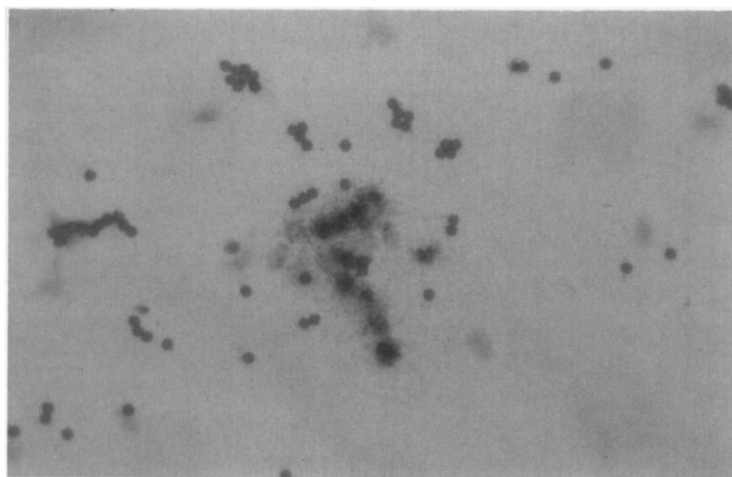


FIG. 4. Autoradiograph showing in situ hybridization signals obtained with an antisense probe to IL-5 mRNA on CD2-positive BAL cells obtained 24 hours after allergen challenge. Dense collections of silver grains over mRNA-positive cells are closely associated with CD2-linked magnetic particles.

$p = 0.008$) (Fig. 5, A) after allergen challenge. An association was also observed between the increase in T-cell activation in BAL (change in percent CD25-positive CD4+ cells) on the allergen day, when compared with that on the diluent day, and the change in numbers of cells expressing IL-5 mRNA ($r = 0.79$, $p = 0.001$) but not other cytokines. After allergen challenge, the number of BAL cells expressing mRNA for IL-4 ($r = 0.70$, $p = 0.008$) and IL-5 ($r = 0.60$, $p = 0.03$) correlated with the percentage of eosinophils.

DISCUSSION

In this study, by use of BAL in patients with asthma 24 hours after allergen challenge, we have provided direct evidence for local activation of CD4+ T lymphocytes, increased expression of mRNA for T_H2-type cytokines, and accumulation of eosinophils. Local eosinophilia was related to the degree of T-cell activation, the level of cytokine mRNA expression, and the magnitude of the observed late-phase asthmatic response.

Although BW may be more representative of proximal airways than BAL, mucus contamination together with the low cell viability and yield precluded flow cytometric analysis and in situ hybridization studies of BW cells. A contribution from small airways to airflow obstruction in asthma is suggested,³³ and the application of BAL has demonstrated a characteristic cellular pattern in both steady-state asthma and after allergen challenge.⁴⁻⁸ For these reasons we believe our results are representative of inflammatory events that

occur in the airway. A 24-hour time point for BAL was chosen because we wanted to avoid the possible risks of the procedure in patients with substantially reduced FEV₁ and because we were principally concerned with markers of T-cell activation which, on the basis of in vitro data and previous studies of late cutaneous responses, we expected to be maximal at 24 hours.^{34, 35} Although we did not obtain BAL at the peak of the fall in FEV₁ during the late response, a number of patients in our study showed falls that persisted to 24 hours, in agreement with a previous report of prolonged bronchoconstriction after allergen provocation.³⁶ BAL and BW eosinophils correlated closely with the maximal falls in FEV₁ at 6 to 11 hours, and a significant correlation was also observed between BAL eosinophils and FEV₁ at 24 hours. Although these findings do not prove a causal association, they support the concept that local eosinophilia may contribute to airway narrowing during late responses.

Eosinophils may contribute to the development of allergen-induced LARs and the associated bronchial hyperresponsiveness by a number of possible mechanisms, which include the release of granule-derived basic proteins and membrane-derived mediators, which include lipooxygenase products (particularly leukotriene C₄) and platelet activating factor.^{37, 38} Changes in peripheral blood eosinophils have been shown to correlate with the magnitude of the LAR.^{39, 40} Our finding of increased eosinophils in BW and BAL after allergen challenge is in agreement with several

previous reports.^{6, 8} In one report no significant changes in cell counts were observed,⁴¹ although this involved a 50 ml BW in only five subjects. Inhalation challenge with toluene 2,4-diisocyanate was also associated with BAL eosinophilia (and neutrophilia) at 8 hours after challenge in subjects who had late responses.⁴² The local instillation of allergen into the airways of patients with atopic asthma was also associated with an influx of eosinophils, neutrophils, basophils, and lymphocytes, which was detectable by BAL at 19 hours.⁴ The eosinophilia persisted to 48 hours and 96 hours and was accompanied by a sustained increase in T cells.⁷

In this study we have demonstrated that CD4+ T lymphocytes were activated 24 hours after allergen challenge and correlated with the accompanying local eosinophilia. These observations confirm and extend results of previous studies, which have provided evidence for CD4+ T-cell activation in peripheral blood from patients with acute exacerbations of asthma,⁴³ and in BAL^{44, 45} and bronchial biopsy specimens¹⁰ from patients with mild, stable asthma. A link between T-lymphocyte activation and eosinophil recruitment and degranulation in the bronchial mucosa may be the local production of cytokines. In particular, IL-3, IL-5, and GM-CSF may contribute to maturation and activation of eosinophils.^{14, 15, 18, 19} In BAL obtained from patients with stable atopic asthma we have previously demonstrated an increase in expression of cytokine mRNA for IL-3, IL-4, IL-5, and GM-CSF. By means of mechanical cell separation with the use of magnetic beads and by dual immunofluorescence we confirmed that mRNA for IL-4 and IL-5 was localized to BAL T cells.²⁵ Using the same techniques, we now demonstrate increased numbers of BAL cells expressing mRNA for IL-4, IL-5, and GM-CSF 24 hours after allergen inhalation challenge and localization of IL-4 and IL-5 mRNA to BAL T cells. The findings are comparable to our previous results in biopsy specimens from cutaneous late-phase reactions obtained 24 hours after allergen injection.³² The limited number of cells available from BAL precluded a more accurate quantification of mRNA, and the increases in numbers of cells transcribing sufficient copy number to be detected by in situ hybridization may not mean an overall increase in cytokine mRNA, and still less, an increased production of cytokines.

Nonetheless, these findings are compatible with gene activation for IL-4, IL-5, and GM-CSF. They are in keeping with the recent demonstra-

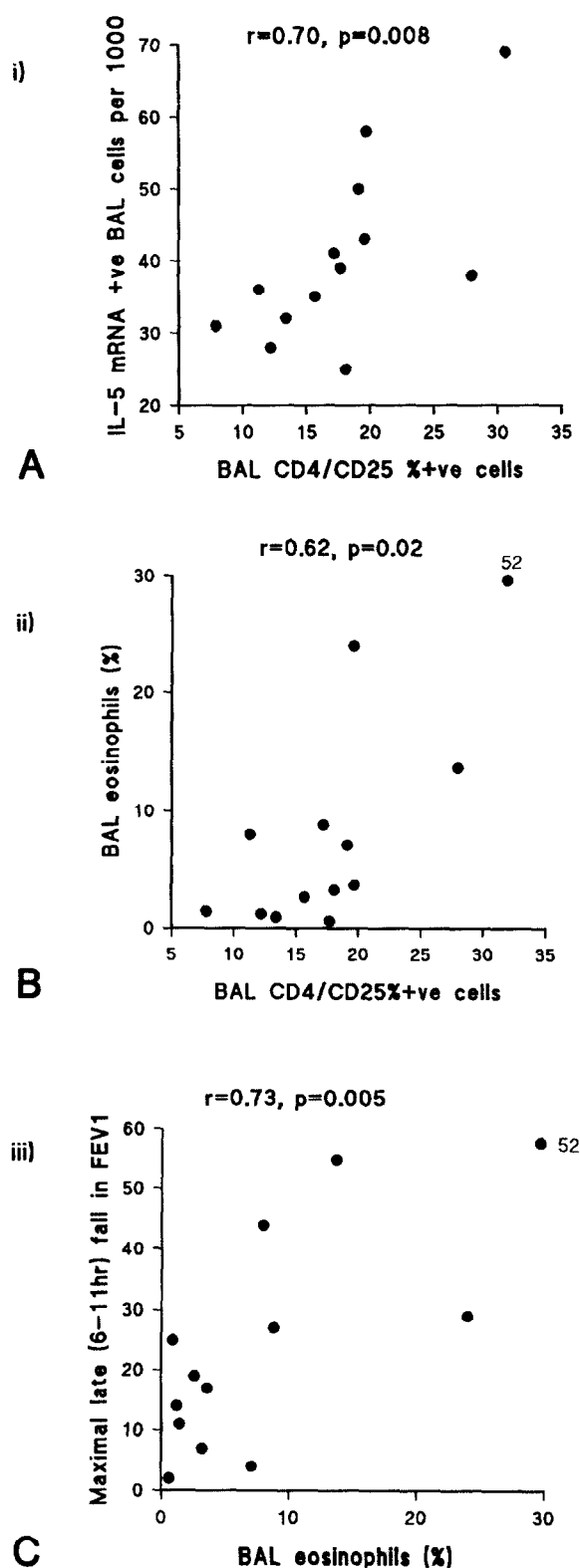


FIG. 5. Relationships among bronchoalveolar CD25+/CD4+ T cells, cells expressing cytokine mRNA, and eosinophils at 24 hours after allergen challenge and the magnitude of the late fall in FEV₁: **A**, CD25+/CD4+ cells and cells expressing IL-5 mRNA; **B**, CD25+/CD4+ cells and eosinophils; **C**, eosinophils and LAR.

tion of increased GM-CSF mRNA in BAL after local allergen challenge, predominantly in lymphocytes,⁴⁶ and the demonstration of IL-5 in BAL 48 hours after local allergen challenge of atopic nonasthmatic subjects.⁴⁷ IL-4 and IL-5 protein have been detected in concentrated BAL fluid from atopic asthmatic subjects,⁴⁸ although IL-4 was not detected in unconcentrated BAL fluid from subjects with acute exacerbations of asthma.⁴⁹ We did not concentrate BAL fluid here because we believed variable loss of protein would make comparison of values after diluent and allergen challenges difficult to interpret. Secretion of IL-4 and IL-5 was localized to points of T-cell-B-cell contact in experimental cell conjugates,⁵⁰ and such discrete circumscribed effects might be difficult to detect in BAL fluid. Nonetheless, the increasing sensitivity of immunoassays for cytokines may allow confirmation that cytokine protein is secreted during late-phase asthmatic responses, and this will be the subject of future studies. Allergen-specific T-cell clones from atopic individuals transcribe mRNA for T_{H2} cytokines when stimulated with allergen in vitro.^{23, 24} It seems likely that our findings may represent the same process occurring in vivo.

It is noteworthy that a high percentage of CD2-positive BAL cells expressed mRNA for IL-4 and IL-5 (Table IV). This may represent a majority of CD4 T cells, because eosinophil-active cytokines were produced by the CD4+ (but not CD8+) population of peripheral blood T cells from patients with asthma.⁵¹

Alternatively, CD8-positive cells may also contribute T_{H2}-type cytokines.⁵² The percentage of BAL CD4+ T cells detected as activated by CD25 expression on flow cytometry appears to be lower than the percentage of those expressing cytokine mRNA. The value from flow cytometry is a relative value from comparison with a control antibody, not an absolute figure, and the time course of CD25 expression and cytokine gene activation after allergen challenge may differ. Nonetheless, separation of T-cell proliferation (and presumably CD25 expression) and cytokine production is described in vitro,⁵³ and this may also occur in vivo.

Mast cell lines have been shown to produce IL-3, IL-4, IL-5, and GM-CSF in response to IgE cross-linking.^{54, 55} Macrophages and eosinophils can produce IL-3 and GM-CSF.⁵⁶⁻⁵⁸ Thus induction of cytokine synthesis after allergen challenge in atopic subjects may occur in a variety of cell types. Because mast cells have been shown to

release preformed cytokines on stimulation in vitro⁵⁹ and because IL-4 favors development of T_{H2} clones,⁶⁰ it is possible that early release of cytokines from mast cells might influence the subsequent T-cell response. However, the majority of cytokine mRNA 24 hours after allergen challenge appears to originate from BAL T lymphocytes. Activation of T_{H2}-type T helper cells may contribute to eosinophil infiltration and activation and hence to the clinical late-phase response to allergen challenge in atopic asthma.

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