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Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma

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*We have studied the phenotype and activation status of leukocytes in the bronchial mucosa in patients with isocyanate-induced asthma. Fiberoptic bronchial biopsy specimens were obtained from nine subjects with occupational (five toluene- and four methylene diisocyanate-sensitive) asthma, 10 subjects with extrinsic asthma, and 12 nonatopic healthy control subjects. Bronchial biopsy specimens were examined by immunohistology with a panel of monoclonal antibodies and the alkaline phosphatase-antialkaline phosphatase method. There was a significant increase in the number of CD25⁺ cells (interleukin-2 receptor-bearing cells, presumed "activated" T-lymphocytes; $p < 0.01$) in isocyanate-induced asthma compared with that of control subjects. There were also significant increases in major basic protein (BMK-13)-positive ($p < 0.02$) and EG2-positive ($p < 0.01$) cells that represent total and "activated" eosinophil cationic protein-secreting eosinophils, respectively. In agreement with our previous findings, CD25⁺ ($p < 0.01$), BMK-13 ($p < 0.03$), and EG2⁺ ($p < 0.01$) cells were also elevated in extrinsic asthma. No significant differences were observed in the numbers of T-lymphocyte phenotypic markers (CD3, CD4, and CD8) between subjects with asthma (isocyanate-induced and extrinsic) and control subjects. Similarly, no significant differences in immunostaining for neutrophil elastase (neutrophils) or CD68 (macrophages) were observed. The results suggest that isocyanate-induced occupational asthma and atopic (extrinsic) asthma have a similar pattern of inflammatory cell infiltrate. The results support the view that T-lymphocyte activation and eosinophil recruitment may be important in asthma of diverse etiology. (*J ALLERGY CLIN IMMUNOL* 1992;89:821-9.)*

Key words: Eosinophils, T-lymphocytes, neutrophils, macrophages, bronchial biopsies, subjects with asthma, isocyanates

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Abbreviations used

MBP:	Major basic protein
MDI:	Methylene diisocyanate
TDI:	Toluene diisocyanate
PC ₂₀ :	Provocation concentration causing a 20% fall in FEV ₁
MAb:	Monoclonal antibody
IL-2R:	Interleukin-2 receptor
BAL:	Bronchoalveolar lavage
LAR:	Late asthmatic response

Bronchial asthma is a common clinically diverse condition with an appreciable inflammatory component. Postmortem studies in fatal asthma have demonstrated the presence of viscid-occluding plugs throughout the large and small airways, epithelial desquamation, and an intense peribronchial inflammatory cell infiltrate in which eosinophils and mononuclear cells are prominent.¹⁻⁴ Considerable airway inflammation is present even in asymptomatic patients with mild disease.⁵ There is evidence that activation of lymphocytes and eosinophil recruitment and secretion in the bronchial mucosa may contribute to epithelial damage and increased bronchial responsiveness in asthma.⁶⁻⁹ In contrast, recent studies of patients with mild atopic asthma have demonstrated no significant changes in mast cell numbers or subtypes in the bronchial mucosa.^{10, 11} Similarly, tissue neutrophil numbers are not increased in stable atopic asthma.⁹⁻¹²

We have extended the aforementioned studies to explore whether there are differences in the pathology of asthma of different etiologies. Studies of BAL suggest that the inflammatory component in isocyanate-induced and extrinsic asthma may be similar, with the exception that the former may demonstrate a more prominent neutrophil component, at least after allergen provocation in the laboratory.^{13, 14}

LARs after exposure to TDI have been associated with transient increases in bronchial hyperresponsiveness and a bronchoalveolar eosinophilia and neutrophilia.¹³ However, the origin of cells obtained in BAL fluid and their relationship to cell types present in the bronchial mucosa is unclear. For this reason we have sampled the bronchial mucosa directly by use of fiberoptic bronchoscopy and biopsy specimens. Biopsy specimens were examined by immunohistology with a panel of MAbs directed against T cells, eosinophils, neutrophils, and macrophages. We have compared the findings in patients with isocyanate-induced asthma, extrinsic allergic asthma, and normal healthy control subjects.

MATERIAL AND METHODS**Subjects**

The clinical features of the subjects studied are presented in Table I. Patients with occupational asthma (sensitive to either TDI or MDI) were recruited from the Istituto di Medicina del Lavoro, Padua, Italy. The details of exposure to isocyanates are documented in Table II. Patients with extrinsic asthma were recruited from the Allergy Clinic, Royal Brompton National Heart and Lung Hospital, London, England. Nonatopic healthy control individuals were recruited in London from laboratory staff and students. All participating subjects were nonsmokers, and none had received oral corticosteroids. Patients with asthma had not received inhaled corticosteroids within the preceding 3 months. The protocol for patient recruitment, bronchial biopsy, and processing of samples was standardized for both centers before commencement of the study. The bronchoscopies were performed by one operator at each site.

The diagnosis of asthma was based on (1) a history of variable wheeze, shortness of breath, chest tightness, or cough, and (2) a documented spontaneous or β_2 -agonist-induced airflow reversibility, recorded as a change of 20% in FEV₁, or peak expiratory flow rate, and/or airway hyperresponsiveness, as measured by a methacholine inhalation test. In the patients with asthma, the PC₂₀ was <4 mg/ml in all but two subjects (both in the group with occupational asthma). One had a methacholine PC₂₀ of 6 mg/ml, and the other subject, a PC₂₀ of 13.6 mg/ml. This finding is not unusual in isocyanate-induced asthma after a period without exposure to the offending agent.¹⁵ The methacholine PC₂₀ values for the remaining patients with occupational and extrinsic asthma were similar.

The diagnosis of isocyanate-induced asthma was based on the following: (1) no asthma symptoms before first exposure to isocyanates, (2) a "latent" interval (period of sensitization) of at least several months exposure to isocyanates before onset of symptoms (Table II), (3) work-related asthma symptoms with a history of improvement away from work, and (4) a positive occupational-type challenge test to the relevant isocyanate (exposure concentration <0.02 ppm).^{16, 17}

The diagnosis of extrinsic asthma was based on (1) a history of asthma after exposure to one or more common inhaled allergens, (2) a positive SPT to one or more common inhaled allergens (skin wheal ≥ 2 mm larger than wheal of the control solution), including cat fur, dog hair, grass pollen, house dust, and house dust mite (*Dermatophagoides pteronyssinus*).

Seven of the nine subjects with occupational asthma and all the normal control subjects were nonatopic, as designated by negative SPTs and IgE RAST values. Two subjects with occupational asthma had positive skin reactions to aeroallergens, including cat fur, house dust mite, and grass pollen, but their respiratory symptoms were unrelated to such allergen exposure. Only one of these individuals had a positive RAST, in this case, to grass pollen with a clinical history of mild seasonal allergic rhinitis.

TABLE I

	Asthma		Normal control subjects
	Occupational	Extrinsic	
No.	9	10	12
(M/F)	4/5	2/8	5/7
Age (yr)			
Mean	37.8	24.7	24.3
Range	23-54	21-32	19-33
FEV ₁ (%)			
Mean	104.3	81.8	105
Range	82-126	75-88	95-115
PC ₂₀ MeCH (mg/ml) geometric			
Mean	0.79	0.60	>32
Range	0.15-13.7	0.25-1.45	
Atopic (one or more positive SPT)	2/9	10/10	0/12
Total IgE			
Mean	98	358	32
Range	<32-597	25-1256	2-210

MeCH. Methacholine.

All patients were nonsmokers. None were taking inhaled/oral corticosteroids. β_2 -Agonists were stopped at least 12 hours before bronchoscopy; five subjects with occupational asthma were sensitive to TDI and four were sensitive to MDI.

TABLE II

Patient	Isocyanate exposure		
	Total exposure to isocyanates (yr)	Latent interval (yr)	Workplace exposure-bronchoscopy interval (wk)
S. B.	3	2.5	12
B. N.	7	6.0	12
D. F.	0.25	0.25	12
P. A.	9	7	8
B. P.	14	13.5	5
B. A.	2	0.5	5
S. G.	10	9.5	15
B. V.	11	9.0	8
I. A.	10	8.0	5

Study design

The study was performed with the written informed consent of the subjects and with the approval of the ethics committees of both participating centers. All patients (with extrinsic and occupational asthma) had current asthma at the time of bronchoscopy as reflected by symptoms and/or methacholine PC₂₀. In patients with isocyanate-induced asthma, fiberoptic bronchoscopy was performed a minimum of 4 weeks after diagnostic challenge and a maximum of 15 weeks after exposure to isocyanates in the workplace. This timing avoided the possible confounding influence of acute exposure in the laboratory, whereas it allowed the

immunopathology to be related to the patients' previous chronic isocyanate exposure. At an initial visit, all subjects completed a respiratory questionnaire, and SPTs were performed. Baseline spirometry (FEV₁) was recorded, and a methacholine inhalation test was performed at least 48 hours before fiberoptic bronchoscopy. Patients were required to stop medication, other than β_2 -agonists, at least 2 days before bronchoscopy and all β_2 -sympathomimetics 12 hours before the procedure. After the bronchoscopy procedure, all subjects returned to the ward to recover from the effects of sedation, and their peak expiratory flow rate was monitored every half hour for 4 hours before discharge.

Methacholine inhalation test

Airway responsiveness to methacholine was measured with a Wright nebulizer and a tidal breathing method.¹⁸ The nebulizer output was standardized at 0.13 ml/min, and methacholine was inhaled for 2 minutes at each doubling concentration. The results were expressed as the methacholine PC₂₀ compared with the FEV₁ after an initial inhalation challenge with 0.9% sodium chloride.

Fiberoptic bronchoscopy

Fiberoptic bronchoscopy was performed following the guidelines of the American Thoracic Society.¹⁹ Subjects were premedicated with atropine, 0.6 mg intravenously, and nebulized salbutamol, 2.5 mg, was inhaled 30 minutes before the procedure. Immediately before the bronchoscopy, midazolam, 5 to 10 mg, was administered intravenously. The nose and throat were anesthetized with a topical lidocaine spray. Four percent lignocaine was applied through the bronchoscope channel to the vocal cords and 2% lignocaine was used to anesthetize the upper airways. Oxygen, at 4 L/min, was administered via nasal cannula throughout the procedure. Oxygen saturation was monitored continuously. The bronchoscopes used were Olympus models BFP20 or BF1T10 (Olympus Co., Tokyo, Japan) with 2.6 mm width biopsy channels. Olympus alligator forceps (model FB15C) were used to obtain four to eight biopsy specimens from segmental divisions of the major bronchi.

Biopsy samples

Each biopsy specimen was immediately placed in phosphate-buffered saline solution and frozen within 30 minutes. Biopsy specimens were placed in ornithine carbamyl transferase-embedding medium and snap frozen in isopentane precooled in liquid nitrogen and stored at -80° C. Biopsy specimens from Padua, Italy, were transported on dry ice to London and stored at -80° C before cryostat sectioning within 3 months. Six micrometer sections were cut on a Bright cryostat (Bright Instrument Co. Ltd., Huntington, Cambridge, England), and three consecutive sections were placed on microscope slides. The slides were air dried for 1 hour and fixed in equal parts of acetone and methanol for 5 minutes and additionally air dried for 1 hour. The slides were then wrapped in pairs in aluminum foil and stored at -20° C before immunostaining.

Immunohistochemistry

The slides were allowed to defrost in their foil covering. MAb staining was detected by a modification of the alkaline-phosphatase-antialkaline-phosphatase method of Mason and Sammons.²⁰ This technique has been described in detail previously.²¹ Twenty percent normal human serum was used to prevent nonspecific binding of the second and third layer antibodies. System and specificity controls were included in each staining run with human tonsil obtained at routine tonsillectomy operations and a mouse IgG2a myeloma protein as a negative control. Cytospins of mitogen-stimulated (phytohemagglutinin) peripheral blood mononuclear cells were used as the positive control for CD25 staining. The

MAbs used were CD45 (Hle-1), CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), and CD25 (anti-IL-2R), obtained from Becton Dickinson, Ltd., Cowley, Oxfordshire, England), EG2, recognizing the cleaved form of eosinophil cationic protein and representing actively secreting eosinophils^{22, 23} (obtained from Bradsure Biologicals, Loughbrough, Leicestershire, England), DAKO elastase (antineutrophil elastase) and macrophage (anti-CD68) obtained from DAKO Ltd., Highwycombe, Buckinghamshire, England. BMK-13, a newly identified MAb, recognizing MBP, was developed in our hybridoma laboratory.²⁴ It does not cross-react with other eosinophil cationic proteins and has been demonstrated to stain significantly more tissue eosinophils in bronchial mucosal biopsy specimens than does EG2.

Quantitation

Biopsy specimens were coded and sections were counted blind. The number of positively stained cells was counted in a zone 115 µm deep (as defined by a squared eyepiece graticule) along the length of the epithelial basement membrane. A calibrated and computerized (Apple IIe, Apple Computer, Inc., Cupertino, Calif.) graphics tablet was used to determine the length of the basement membrane. The cell counts were expressed as the number per unit length (1 mm) of basement membrane. A minimum of two sections, 36 µm apart, were stained and counted for each antibody and each biopsy specimen, and the mean value was obtained. The coefficient of variation for three repeat cell counts by the same observer (A. M. B.) of CD3 staining was 4%; CD4, 11%; CD8, 11%; CD45, 10%; EG2, 8%; and for CD25, 7%.

Statistical analysis

Cell counts in the bronchial mucosa were expressed per 1 mm length of basement membrane. Data demonstrated a positive skew. Therefore, nonparametric statistical tests were used in the analysis, and median values are presented on the accompanying figures. Cell counts from biopsy specimens from the subjects with occupational and extrinsic allergic asthma were compared with counts of the normal healthy control subjects with the Mann-Whitney U test (with Bonferroni's correction).²⁵ Correlations were performed with Spearman's rank-order method.

RESULTS

Immunohistochemical staining of bronchial mucosal sections revealed a cellular infiltrate in all subjects studied. There were no differences in total leukocyte numbers (CD45⁺ cells) in the biopsy specimens from the subjects with asthma compared with that of normal healthy control subjects (Fig. 1). Similarly, no differences in the numbers of CD3-, CD4-, and CD8-positive T-lymphocytes were observed (Fig. 2). CD3-positive lymphocytes formed the largest proportion of the leukocyte population in each of the groups studied (occupational asthma, 61%; extrinsic asthma, 49%; normal control subjects, 65%).

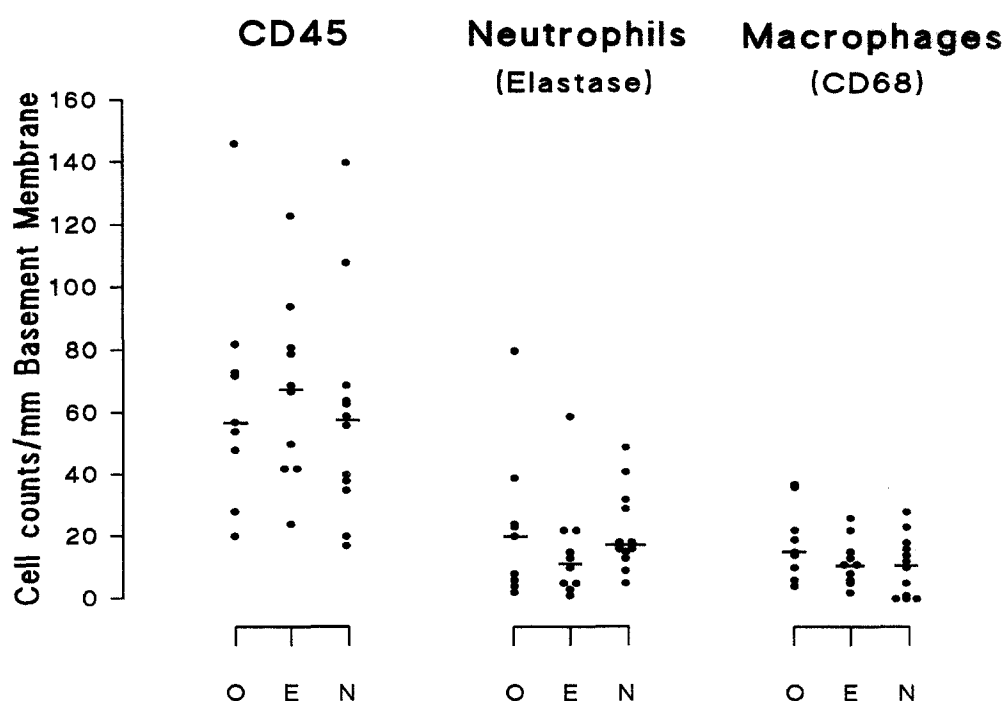


FIG. 1. Individual cell counts expressing CD45 (total leukocytes), neutrophil elastase (neutrophils), and CD68 (macrophages) in the bronchial mucosa, expressed as the number of positive cells per millimeter of basement membrane. Median values are represented by *horizontal bars*; *O*, occupational asthma; *E*, extrinsic asthma; *N*, normal nonatopic control subjects.

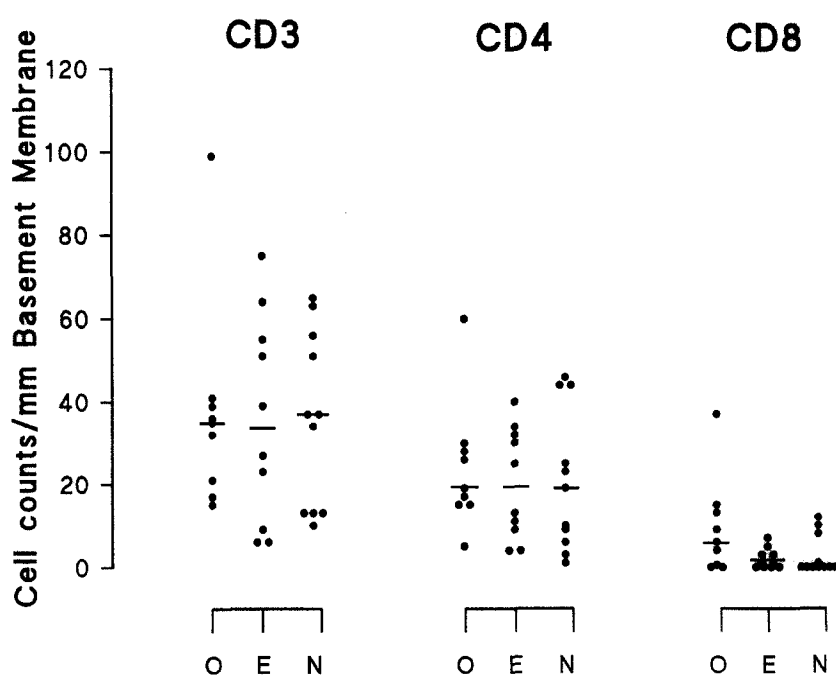


FIG. 2. Individual T-lymphocyte counts (CD3, CD4, and CD8) in the bronchial mucosa, expressed as positive cells per millimeter of basement membrane. Median values are represented by *horizontal bars*; *O*, occupational asthma; *E*, extrinsic asthma; *N*, normal nonatopic control subjects.

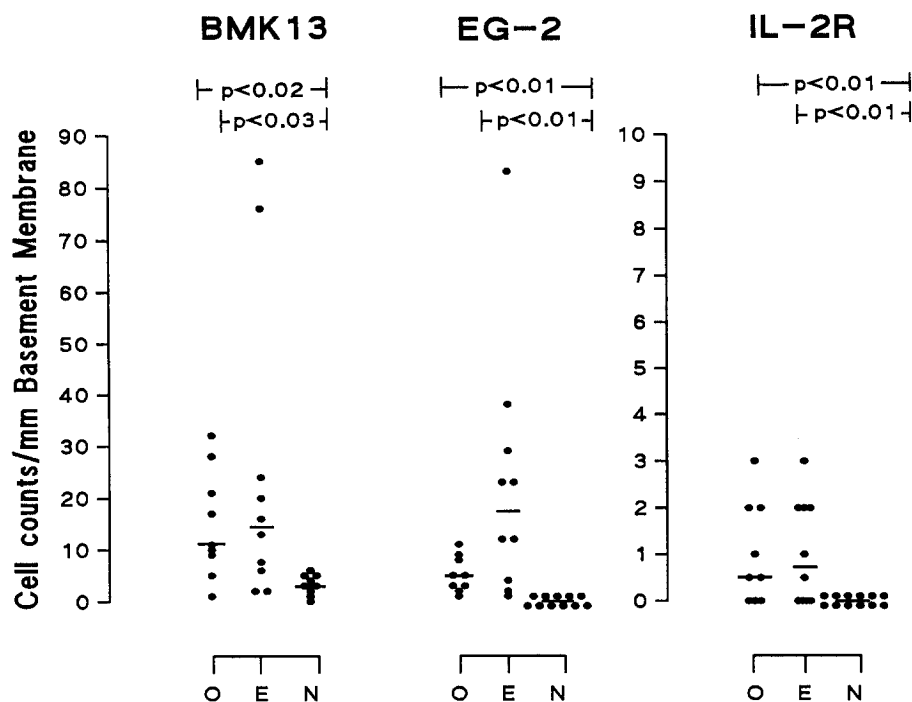


FIG. 3. Individual counts for cells expressing MBP, EG2 (eosinophils), and CD25 (IL-2R-bearing cells) in the bronchial mucosa, expressed as number of positive cells per millimeter of basement membrane. Median values are represented by horizontal bars; O, occupational asthma; E, extrinsic asthma; N, normal nonatopic control subjects.

CD4-positive cells were identified in all subjects, whereas CD8-positive cells were present in low numbers throughout.

CD25-positive cells (IL-2R-bearing cells) were increased in biopsy specimens from subjects with occupational asthma ($p < 0.01$) and in patients with extrinsic asthma ($p < 0.01$) compared with that of control subjects (Fig. 3). No CD25⁺ cells were observed in any of the biopsy specimens from the 12 normal control subjects. A positive correlation was observed between CD25- and CD4-positive cells ($r = 0.52$; $p < 0.03$) but not between CD25⁺ and CD68⁺ cells (macrophages) ($r = 0.40$; $p = 0.09$). Total (MBP⁺) eosinophils were increased in both occupational asthma ($p < 0.02$) and extrinsic asthma ($p < 0.03$) (Fig. 3). Similarly, actively secreting EG2⁺ eosinophils were also significantly increased in both occupational ($p < 0.01$) and extrinsic asthma ($p < 0.01$). No differences in neutrophil counts (neutrophil elastase-positive cells) or macrophage counts (anti-CD68-positive cells) were observed in either group with asthma compared with that of normal control subjects (Fig. 1). For all MAbs used, the findings for subjects with occupational and extrinsic asthma were comparable. Examples of sections from bronchial biopsy specimens from subjects with asthma stained by the alkaline phosphatase-antialkaline

phosphatase method have been demonstrated previously.^{5, 10}

DISCUSSION

We have demonstrated, in patients with occupational asthma caused by the low-molecular-weight agents, TDI and MDI, that there is an increase in CD25⁺ (IL-2R-positive) cells and total and actively secreting eosinophils in the bronchial mucosa. These results are comparable to our previous findings in mild atopic asthma.⁵ These results suggest that T-lymphocyte activation and eosinophil recruitment and activation may be important in asthma of diverse etiology.

The pathologic features of patients dying of status asthmaticus are well documented.^{1, 3, 26} Fiberoptic bronchial biopsy specimen studies in patients with mild to moderate disease have identified epithelial and submucosal changes⁷⁻⁹ and have further suggested that the degree of the inflammatory response correlates with the level of airway responsiveness. A significant bronchoalveolar eosinophilia is well recognized in stable atopic asthma.^{27, 28} Numerous studies have identified increased numbers of epithelial cells and mast cells in BAL in subjects with mild asthma that have been demonstrated to correlate with the degree of airway responsiveness.^{9, 27, 29-31}

Asthma caused by occupational exposure has long

been recognized.^{32, 33} Low-molecular-weight compounds, including TDI and MDI (molecular weight, <1000 daltons), are among the most common causative agents. The mechanisms are largely unknown, but it has been suggested that agents, such as TDI, may act as haptens, combining with protein-carrier molecules to provoke an immune response, documented in asthma caused by acid anhydrides.³⁴ Elevated serum-specific IgE antibodies have been detected in only a small proportion of subjects with asthma caused by isocyanates or plicatic acid and may reflect simple exposure and not clinical expression of the disease.^{33, 35} The possible role of T-lymphocytes in isocyanate-induced asthma has not previously been assessed. Low-molecular-weight haptens may possibly be recognized by T cells in the same way as an allergen in atopic asthma.

LARs induced by allergen have been associated with increased numbers of neutrophils and eosinophils in BAL.^{36, 37} LARs induced by occupational sensitizing agents are similarly associated with increases in the number of leukocytes in BAL, mainly eosinophils after plicatic acid (western red cedar) challenge and neutrophils after isocyanate exposure.^{13, 38} In addition, corticosteroids have been demonstrated to inhibit the LAR and the associated increase in airway responsiveness after TDI challenge.¹⁴ An autopsy study of a patient dying of TDI-induced asthma demonstrated histologic changes similar to other fatal causes of asthma.³⁹ We propose that the inflammatory response in the bronchial mucosa of patients with isocyanate-induced and atopic asthma is similar.

In the present study, findings in occupational asthma were compared with findings in extrinsic (atopic) asthma and in normal subjects. Total leukocyte numbers and CD3⁺, CD4⁺, and CD8⁺ T-lymphocytes were prominent in the bronchial mucosa of both normal subjects and subjects with asthma with no differences between the groups. In contrast, there were increases in IL-2R-positive cells in both occupational and extrinsic asthma. On no occasion were CD25⁺ cells observed in biopsy specimens from normal healthy control subjects (Fig. 3). In view of the potent regulatory properties of T cells, large numbers may not be required to play a significant role in the inflammatory response in asthma. We cannot be sure that these CD25⁺ "activated" IL-2R-bearing cells represent T cells rather than macrophages⁴⁰ or even basophils.⁴¹ However, the concentration of the CD25⁺ MAb used had previously been titrated by us to stain preferentially T-lymphocytes on mitogen-stimulated peripheral blood mononuclear cell cytopins. In addition, the number of CD25⁺ cells correlated with CD4⁺ cells but not CD68⁺ tissue macrophages. Furthermore, double immunostaining (CD4⁺/CD25⁺) by

flow cytometry of BAL from patients with asthma supports this view.⁴² Confirmation that these "activated" CD25⁺ cells are indeed T cells will require application of double immunostaining methods.

Previous studies have demonstrated a relationship between asthma severity and eosinophil numbers in blood,^{43, 44} sputum,⁴⁵ and BAL.^{27, 31, 46} In this study, we have demonstrated increased numbers of total (MBP⁺) and activated eosinophils (EG2⁺) in isocyanate-induced, as well as in extrinsic, asthma. There was evidence of extracellular staining for both EG2 and MBP within the submucosa in subjects with asthma, but not in normal control subjects. In view of the known toxic effects in vitro of MBP on the bronchial epithelium,⁴⁷ this finding further supports a causal role for eosinophils in inducing epithelial damage.

No significant differences in neutrophil or macrophage numbers were observed between the subjects with asthma and normal control subjects. This finding is in contrast to previous findings in BAL from patients with occupational asthma in which neutrophils were prominent 8 hours after TDI exposure.¹³ However, in the present study, the subjects with occupational asthma had not been exposed to the sensitizing agent for at least 4 weeks. The effect of cessation of exposure to isocyanates on inflammatory cell numbers in both BAL and mucosal biopsy specimens has been studied recently.⁴⁸ Persisting bronchial hyperresponsiveness appeared to be related to the magnitude of the eosinophil infiltrate. In agreement with our findings, neutrophils were not prominent in the bronchial mucosa.

In conclusion, this study provides evidence for T cell and eosinophil activation in occupational asthma caused by isocyanates comparable to that observed in extrinsic asthma. These observations support a degree of uniformity in the bronchial inflammatory response in patients with asthma, whatever the nature of the provoking agent.

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Effect of dietary fish oil supplementation on the antigen-induced late-phase response in the skin

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*We have studied the effect of dietary fish oil supplementation (3.2 gm/day of eicosapentaenoic acid and 2.2 gm/day of docosahexaenoic acid) for 10 weeks on the antigen-induced, cutaneous late-phase response (LPR) in 16 atopic individuals. All subjects developed an LPR to intradermal allergen injection. The mean $\pm$ SEM area of induration at 6 hours was 1840 ± 472 mm². A biopsy was performed at the LPR site at 6 hours. Subjects were then randomized to receive fish oil or placebo olive oil in a double-blind, parallel fashion, for 10 weeks. After the diet, there were no significant differences between fish oil and placebo treatment in the size of the immediate wheal-and-flare response or the extent of induration at 4 and 6 hours, as compared with prediet values in each group. Histologically, at 6 hours, there was a significant infiltrate into the allergen site compared with that of the control diluent site of total inflammatory cells ($p < 0.002$) as well as of eosinophils ($p < 0.001$). However, there were no significant differences when pre- and postdiet values were compared. Hence, we conclude that dietary fish oil supplementation does not inhibit the cutaneous LPR clinically or histologically. (*J ALLERGY CLIN IMMUNOL* 1992;89:829-35.)*

Key words: Allergens, late-phase response, eicosapentaenoic acid, fish oils, eosinophils

On exposure to relevant allergen, atopic individuals mount a well-characterized, IgE-mediated immediate reaction. In addition, an LPR occurs some hours later that is more complex than the early reaction and in-

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Abbreviations used

EPA:	Eicosapentaenoic acid
DCHA:	Docosahexaenoic acid
LPR:	Late-phase response
BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
SQ:	Standardized quality
SPT:	Skin prick test
LTC ₄ , LTB ₄ :	Leukotrienes C ₄ and B ₄

volves recruitment of inflammatory cells from the circulation. This LPR has been described with allergen reactions in skin, nose, and lung¹⁻³ and may be a more relevant model of chronic allergic inflammation.