

Allergens, IgE, mediators, inflammatory mechanisms

Immunologic studies of the mechanisms of occupational asthma caused by western red cedar

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Background: Occupational asthma caused by western red cedar (*Thuja plicata*) is a common problem in sawmill industries. The objective of this study was to examine the cellular and immunologic mechanisms of western red cedar asthma (WRCA) more closely.

Methods: Bronchial biopsy specimens, bronchoalveolar lavage (BAL) mast cells and peripheral blood basophils from patients with WRCA, patients with atopic asthma, and nonatopic control subjects were challenged in vitro with plicatic acid (PA), PA-human serum albumin conjugate (PA-HSA), grass pollen, or calcium ionophore.

Results: PA (100 µg/ml) released histamine from the basophils of 9 of 11 patients with WRCA, 1 of 7 patients with atopic asthma, and 2 of 7 normal subjects. PA triggered histamine release from 10 of 11 bronchial biopsy specimens and 8 of 8 BAL samples from patients with WRCA. Interestingly, PA released histamine from BAL cells and bronchial biopsy specimens from 3 of 7 normal subjects but in none of the patients with atopic asthma. PA-HSA-induced histamine release from basophils and biopsy specimens was confined to patients with WRCA. PA-specific IgE was not detectable in serum from most patients with WRCA, and their serum did not transfer PA sensitivity to human lung fragments or lactate-stripped basophils. After pretreatment with anti-IgE in the absence of calcium, basophils from 14 subjects with WRCA still responded to PA (mean 64% to 67% of pretreatment response), whereas responses to grass pollen or anti-IgE were abolished.

Conclusions: This study confirms that PA releases histamine from bronchial mast cells of most patients with WRCA but not from those of patients with atopic asthma. The PA response of some normal subjects suggests that PA may have both specific and nonspecific actions on mast cells and basophils, whereas the serologic studies indicate histamine release in WRCA cannot simply be attributed to PA-specific IgE. (*J ALLERGY CLIN IMMUNOL* 1993;92:466-78.)

Key words: Asthma, basophils, mast cells, occupational asthma, histamine, IgE, serology, cedar, immediate hypersensitivity

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Supported by grants from the Medical Research Council of Canada, the British Columbia Lung Association, the Scadding Morriston-Davies Trust, and Wellcome Trust (UK).

Received for publication Mar. 10, 1992; revised Mar. 8, 1993; accepted for publication Mar. 8, 1993.

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0091-6749/93 \$1.00 + .10 1/1/47120

Abbreviations used

BAL:	Bronchoalveolar lavage
FEV ₁ :	Forced expiratory volume in 1 second
HSA:	Human serum albumin
HMW:	High molecular weight
LMW:	Low molecular weight
PA:	Plicatic acid
PC ₂₀ :	Provocative concentration causing a 20% fall in FEV ₁
TB:	Tyrodé's buffer
WRCA:	Western red cedar asthma

Occupational asthma caused by western red cedar (*Thuja plicata*) is a common problem in the sawmill industries of the Pacific Northwest. Western red cedar asthma (WRCA) is thought to be caused by sensitization to plicatic acid (PA), a low molecular weight organic compound in cedar dust.¹ In sensitive subjects inhalation of PA induces bronchoconstriction and elicits immediate and late-phase responses, analogous to those observed in other forms of allergic asthma.¹⁻³ Mast cells and their chemical mediators have been implicated in WRCA, because histamine and leukotrienes can be detected in bronchoalveolar lavage (BAL) fluid after inhalation of PA.⁴ Similarly, peripheral blood basophils from patients with WRCA release histamine when exposed in vitro to PA.¹ Antigen-induced basophil histamine release has traditionally been regarded as evidence of antigen-specific IgE,⁵ but specific IgE to PA is only detectable by serologic testing in a proportion of patients with WRCA,^{6, 7} which raises some questions about whether IgE is involved in mast cell mediator release in WRCA.

Although IgE-dependent degranulation is the best understood mechanism for histamine release in hypersensitivity responses, recent evidence indicates that mast cells and basophils can respond to a wide variety of stimuli, including neuropeptides, platelet products, and several cytokines.⁸⁻¹¹ On the other hand, it has been suggested that WRCA and other forms of occupational asthma may be due to the presence of very low concentrations of allergen-specific IgE, which cannot readily be detected by existing serologic methods.⁵ Allergen-specific IgE can be detected by several in vitro and in vivo methods. These include serologic testing (RAST), heat lability of cytophilic antibody, passive sensitization of human mast cells and basophils, and cross-desensitization of basophils by pretreatment with anti-IgE in the absence of calcium.^{12, 13} To examine the cellular and immunologic mechanisms of WRCA more closely, we have obtained bronchial mast cells by BAL and bronchial biopsy from patients with WRCA and have compared their responses to PA with those of peripheral blood basophils. In addition, the disease specificity of these in vitro responses has been assessed by challenging similar samples from patients with atopic asthma and from non-atopic nonasthmatic subjects. To supplement these direct challenge experiments, we have also performed serologic studies and desensitization studies to assess the IgE dependence of PA-induced basophil degranulation.

METHODS

Chemicals

Unless otherwise stated, all chemicals were purchased from Fisher Scientific Ltd., Vancouver, B.C., Canada. Calcium ionophore A23187, human serum albumin (HSA), Ficoll, and antibiotics were purchased from Sigma Chemical Co., St Louis, Mo. PA was prepared from western red cedar dust by Forintek, Vancouver, B.C., Canada. PA-HSA conjugate was prepared as previously described^{6, 7} with approximately 25 PA molecules per albumin molecule (PA:albumin ratio 13% wt/wt). Mixed grass pollen extract (Grass Pollen mix no. 5 containing equal parts of orchard, timothy, Kentucky blue, sweet vernal, and red top grass pollens) was purchased from Hollister-Stier Laboratories (Elkhart, Ind.). Goat anti-human IgE was purchased from Sigma Chemical Co., reconstituted with sterile water, passed through a Millipore filter (Millipore Corp., Bedford, Mass.), aliquotted, and frozen at -20°C until it was needed.

Tyrod's buffer (TB) was prepared as a total of 8 gm/L sodium chloride, 1 gm/L glucose, 2.4 gm/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.06 gm/L disodium hydrogen phosphate, and 0.2 gm/L potassium chloride, adjusted to pH 7.4. TB was used for all washing steps. For in vitro challenge experiments, a challenge buffer was prepared, which consisted of TB with added calcium chloride (0.2 gm/L), 0.1 gm/L magnesium chloride, 0.1% wt/vol HSA, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 0.25 $\mu\text{g/ml}$ amphotericin. For anti-IgE desensitization studies, a desensitization buffer was prepared; it consisted of TB without calcium or magnesium but with 0.1% HSA and antibiotics as described above.

Histamine assay

Histamine was detected by a single isotope radioczymatic assay as previously described.^{4, 14} The sensitivity of this assay is 20 pg histamine, and the coefficient of variation of repeat measurement is less than 2.5%. In control experiments we have confirmed that this histamine assay is not affected by any of the antibiotics or challenge materials used in this study.

Subjects

Patients with occupational WRCA were recruited from the University of British Columbia Respiratory Clinic, Vancouver General Hospital. All had experienced symptomatic asthma while working with western red cedar. PA sensitivity was confirmed by formal bronchoprovocation testing with PA as previously described.^{2, 3} Patients were asked to discontinue all medication overnight and underwent a sham challenge with saline diluent. Provided that their forced expiratory volume in 1 second (FEV_1) remained stable $\pm 10\%$ over the next 24 hours, they then underwent challenge with serial dilutions of PA. A positive response was defined as a 20% or greater fall in FEV_1 within 10

TABLE I. Demographic characteristics, baseline spirometry, medication usage, and skin test results in the three study groups used in the direct challenge experiments

Variable	WRCA (n = 11)		Atopic asthma (n = 7)		Normal (n = 7)	
	Mean	SEM	Mean	SEM	Mean	SEM
Age	42.5	2.5	28.8	4.5	30.8	3.0
FEV ₁ (% predicted)	86.6	2.8	98.8	0.8	106.0	4.0
FVC (% predicted)	89.5	2.5	95.3	1.3	99.9	4.0
PC ₂₀ (mg/ml)	1.41	0.58	2.65	1.69	29.94	1.56
Duration of asthma (yr)	3.2	1.0	12.3	3.0	-	-
Geometric mean IgE concentrations						
Total IgE (IU/ml)	58.1		322.2		21.9	
GP-IgE (PRU/ml)	0.38		10.6		0.58	
PA-IgE (Ratio)	1.25		2.62		1.19	
Sex						
Male	11		1		4	
Female	0		6		4	
Race						
Caucasian	5		6		5	
East Indian	5		0		2	
Oriental	1		1		1	
Smoke						
Never	8		5		6	
Ex-	3		1		2	
Current	0		1		0	
Response to PA inhalation challenge	7 Late response only 3 Dual response 1 Immediate response		N/A		N/A	
Grass pollen skin test results						
Positive	4		5		0	
Negative	7		2		8	
Drugs						
B ₂ agonist	8		6		-	
Inhaled steroids	9		1		-	
Theophylline	1		1		-	
Ipratropium	1		0		-	

N/A, Not applicable.

hours of challenge. Patients with atopic asthma were recruited from a local allergy clinic and by advertising for volunteers. The diagnosis of atopic asthma was confirmed by a compatible history, positive immediate skin test results to grass pollen or house dust mite, and methacholine provocative concentration causing a 20% fall in FEV₁ (PC₂₀) less than 4 mg/ml. Nonatopic nonasthmatic volunteers were recruited from laboratory personnel and hospital staff: none had any symptoms of asthma, methacholine PC₂₀ was 20 mg/ml or greater, and immediate skin test results were negative to 14 common airborne allergens. Atopic nonasthmatic volunteers (used only in the serologic studies) met the criteria for nonatopic nonasthmatic subjects but had

strongly positive skin test results to grass pollen (wheal diameter greater than 5 mm). Further details of the subject groups used in the direct challenge studies are given in Table I.

Ethical approval

These studies were approved by the Ethics Committee of the University of British Columbia.

Direct challenge studies

Granulocytes were prepared from heparinized peripheral venous blood by dextran sedimentation and density gradient separation over Ficoll. The pelleted

cells were washed twice in TB and adjusted to 10 million cells/ml. Aliquots of 10 million cells were placed in round-bottomed polypropylene tubes (Sarstedt, Nurnbrecht, Germany), centrifuged, and then challenged as described below. Fiberoptic bronchoscopy was performed by the standard protocol in use at Vancouver General Hospital. Subjects fasted overnight and were then premedicated with intramuscular atropine 0.6 mg and nebulized 2% lidocaine solution. Intravenously administered diazepam (7.5 to 10 mg) was given immediately before insertion of the bronchoscope. Continuous oxygen (2 L/min) given via nasal cannulas, and electrocardiogram, blood pressure, and oxygen saturation were monitored throughout the procedure. The bronchoscope (Olympus BF20D, Olympus Corp., Lake Success, N.Y.) was inserted by the oral route, and additional 2% lidocaine was instilled as required. The bronchoscope was wedged in the medial segment of the right middle lobe, and BAL was performed with two 50 ml aliquots of warmed isotonic saline solution. The aspirated fluid was passed through nylon gauze to remove mucus, and two cytopsin preparations were made. The remaining cells were washed twice in TB and then split into five equal aliquots for challenge studies. Viability of BAL cells was checked by trypan blue exclusion and averaged 70%, with similar viability in each of the three subject groups. Five bronchial mucosal biopsy specimens were obtained from the segmental bronchi of the right lower lobe. Each individual specimen was placed in a round-bottomed tube, washed twice in TB, and then challenged as described below.

Biopsy specimens, BAL cells, or granulocytes were resuspended in 1 ml of challenge buffer containing either 1 μ g/ml calcium ionophore, 100 μ g/ml PA, 100 μ g/ml PA-HSA, or 100 PNU/ml grass pollen extract. These concentrations were selected on the basis of results of preliminary experiments with WRCA granulocytes, which showed dose-dependent histamine release in response to PA; most patients with WRCA responded to 100 μ g/ml. The pH of these solutions was confirmed to be 7.4 (i.e., the PA was adequately buffered in TB). All tubes were incubated for 30 minutes at 37° C in a water bath with continuous shaking. To assess spontaneous release, control samples were incubated with challenge buffer alone. At the end of incubation, the tubes were centrifuged, and the supernatants were removed and stored at -80° C until assayed. Cell pellets were resuspended in 1 ml TB and then frozen and thawed twice to release residual histamine. After the final thaw, tubes were centrifuged and supernatants were then aspirated and stored at -80° C until assayed. Histamine content of the supernatants and corresponding pellets was assayed in the same assay run, and all histamine measurements were performed in duplicate. Histamine release into supernatants was then expressed as a percentage of the total histamine content in the sample (pellet + supernatant). Net histamine release was calculated by sub-

tracting the percentage release from control samples incubated with challenge buffer alone. Peripheral blood mononuclear cell viability was unchanged after incubation with PA or PA-HSA (>95% before and after challenge), and there was no cell lysis as determined by lactate dehydrogenase measurements in the supernatants (samples assayed in routine biochemistry laboratory of Vancouver General Hospital). Within individuals, positive responses were defined as histamine release 10% greater than that of the negative control sample.

PA binding assay

Granulocytes were prepared from heparinized peripheral venous blood by dextran sedimentation and low-speed centrifugation (to reduce platelet contamination). Erythrocytes were lysed with Tris-ammonium chloride buffer.¹⁵ Granulocytes were resuspended in TB with calcium and incubated at 37° C with 0.1 μ g/ml tritiated PA (147 Ci/mmol; Du Pont Company, Wilmington, Del.), in the presence or absence of 50 μ g/ml unlabeled PA. After 30 minutes, the reaction mixture was filtered through prewetted Whatman GF/C glass fiber filters (Whatman, Inc., Clifton, N.J.) under vacuum. Filters were washed with 5 ml TB to remove unbound tritiated PA. The filters were then dried and counted in a scintillation counter.¹⁶

Serologic studies

Total serum IgE and grass pollen-specific IgE were determined with commercial kits (Pharmacia, Uppsala, Sweden). PA-specific IgE was detected by radioimmunoassay, as previously described,^{7, 17} and results were expressed as a ratio to the amount of PA-specific IgE in pooled normal human serum.

Passive sensitization studies

Human lung samples were obtained from uninvolved portions of resection specimens from patients undergoing surgery for suspected lung cancer ($n = 4$). Samples were obtained within 60 minutes of operation, minced in TB, washed twice, and then aliquotted in round-bottomed tubes. Lung fragments were sensitized by incubation with 0.5 ml of serum from patients with WRCA or control subjects. After 2 hours of incubation at 37° C, the lung fragments were washed twice in TB and then challenged as described above for direct challenge studies.

For human basophil passive sensitization, cells were obtained from a single atopic donor who had positive skin test results to grass, house dust, and cat dander but had no atopic symptoms and was receiving no medication. This donor was selected from a group of four potential donors, after pilot experiments that showed a reliable basophil yield and a low level of intrinsic responsiveness to PA were performed. Donor (endogenous) IgE was stripped by the method of Pruzansky et al.¹² Briefly, leukocytes were prepared by dextran sedi-

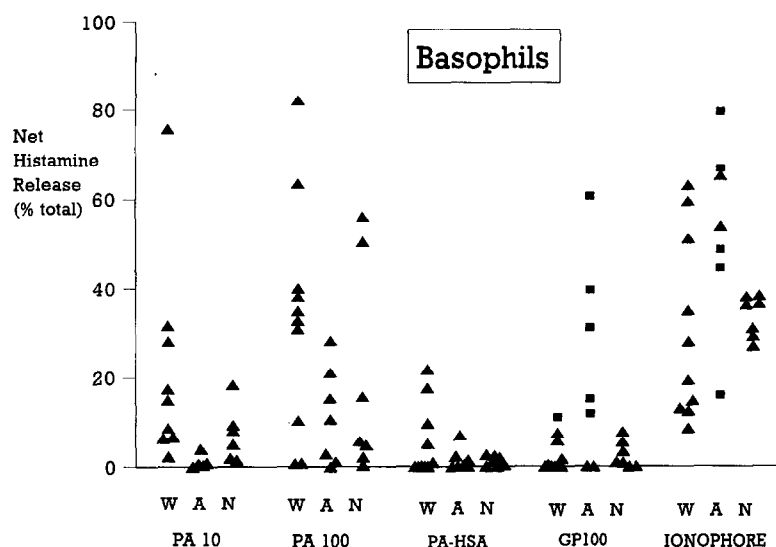


FIG. 1. Net percent release of histamine from granulocytes challenged with 10 $\mu\text{g/ml}$ PA, 100 $\mu\text{g/ml}$ PA, 100 $\mu\text{g/ml}$ PA-HSA conjugate, 100 PNU/ml grass pollen, 1 $\mu\text{g/ml}$ calcium ionophore, or buffer alone. W, Patients with WRCA; A, atopic asthmatic subjects; N, nonatopic nonasthmatic subjects. Spontaneous release has been subtracted (means were 8.3%, 7.0%, and 5.5% for W, A, and N, respectively). Data from subjects with positive grass pollen skin test results are indicated by squares for grass pollen and ionophore responses only.

mentation of heparinized peripheral venous blood, washed twice in TB, and then aliquotted into round-bottomed tubes; each tube received the cells from 0.5 ml of blood. Leukocytes were then pelleted, resuspended in 0.01 mol/L lactate buffer (pH 3.9) and incubated for 10 minutes on ice. An equal volume of cold Tris-HCl buffer 0.025 mol/L (pH 7.6) was added at the end of the incubation to neutralize the lactate and was followed immediately by centrifugation and two washes in TB. This procedure consistently reduced the responses of basophils to grass pollen and to anti-IgE (mean grass pollen response reduced from 35.7% to 3.5% release—90% inhibition; mean anti-IgE response reduced from 43.9% to 27.8% release—37% inhibition: mean of four experiments).

Basophils were resensitized by incubating aliquots of leukocytes with 0.5 ml of a 1 in 4 dilution of serum from patients with WRCA or from control subjects. After 2 hours of incubation at 37° C, the cells were washed twice in TB and then challenged as described above.

Basophil desensitization studies

Preliminary experiments were conducted to establish optimum conditions for basophil desensitization by anti-IgE in the absence of calcium, based on the methods of MacGlashan and Lichtenstein.¹³ These pilot experiments showed that after desensitization, basophils from atopic donors ($n = 4$) were unable to respond to anti-IgE or grass pollen when challenged subsequently in the presence of calcium (mean inhibition, 92% and 95% respectively; $p < 0.01$ for both). Desensitization was found to depend on the concentration of anti-IgE and could be consistently induced by preincubation

with 10 $\mu\text{g/ml}$ anti-IgE. Next, peripheral blood leukocytes were obtained by dextran sedimentation of heparinized venous blood from patients with WRCA, washed twice in TB, and aliquotted into round-bottomed polypropylene tubes with leukocytes from approximately 1 ml of blood in each tube. Basophils were desensitized by incubation for 30 minutes at 37° C in the desensitizing buffer that contained 10 $\mu\text{g/ml}$ goat anti-human IgE. A parallel set of tubes was sham-desensitized in desensitizing buffer without anti-IgE. Leukocytes were then washed twice, resuspended in challenge buffer, and challenged with 1 $\mu\text{g/ml}$ calcium ionophore, 100 $\mu\text{g/ml}$ 100 PA-HSA, 10 $\mu\text{g/ml}$ anti-IgE, 100 PNU/ml grass pollen extract, and with PA in a range of concentrations between 1 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$. After 30 minutes of incubation at 37° C, the percent histamine release was assessed as described above.

Statistical analysis

Comparisons within groups between spontaneous histamine release and release induced by another agent were performed by paired t tests. Comparisons between groups of net release induced by each agent were performed by t test or single-factor analysis of variance as appropriate. Probability (p) values less than 0.05 were regarded as statistically significant.

RESULTS

Granulocytes from most patients with WRCA released histamine when challenged with PA (mean response to 100 $\mu\text{g/ml}$ PA, 41.7% vs 8.3% spontaneous release; $p < 0.05$) (Fig. 1). Basophils

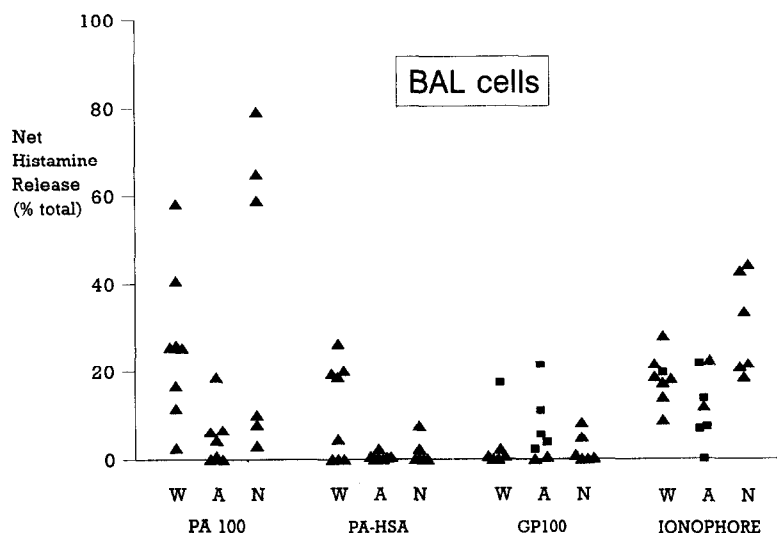


FIG. 2. Net percent release of histamine from bronchoalveolar cells challenged with 100 $\mu\text{g/ml}$ PA, 100 $\mu\text{g/ml}$ PA-HSA conjugate, 100 PNU/ml grass pollen, or 1 $\mu\text{g/ml}$ calcium ionophore. *W*, Patients with WRCA; *A*, atopic asthmatic subjects; *N*, nonatopic nonasthmatic subjects. Spontaneous release was 10.6%, 5.5%, and 14% for *W*, *A*, and *N*, respectively. *Squares* denote data from subjects with positive grass pollen skin test results (for grass pollen and ionophore responses only). Mean histamine content was 2.1 ng per aliquot. See text for statistical analyses.

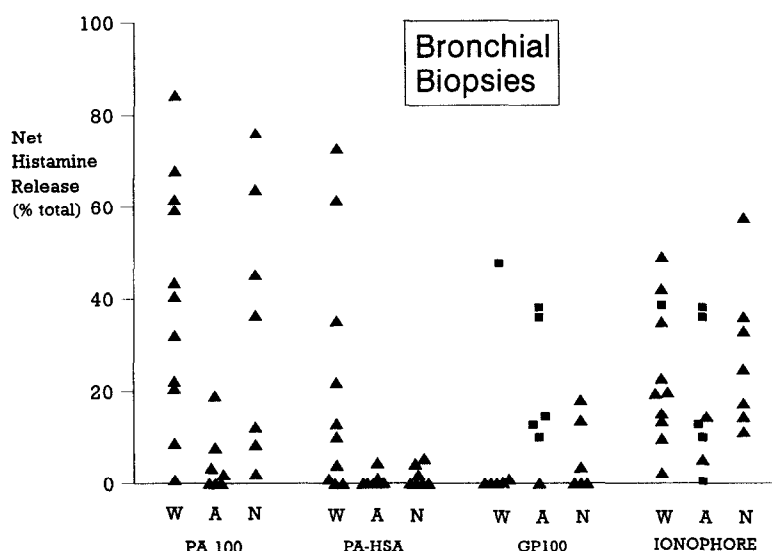


FIG. 3. Net percent release of histamine from bronchial biopsy specimens challenged with 100 $\mu\text{g/ml}$ PA, 100 $\mu\text{g/ml}$ PA-HSA conjugate, 100 PNU/ml grass pollen, or 1 $\mu\text{g/ml}$ calcium ionophore. *W*, Patients with WRCA; *A*, atopic asthmatic subjects; *N*, nonatopic nonasthmatic subjects. Spontaneous release was 9.7%, 4.7%, and 13.1% for *W*, *A*, and *N*, respectively. *Squares* denote data from subjects with positive grass pollen skin test results (shown for grass pollen and ionophore responses only). Mean histamine content of biopsy specimens was 2.35 ng. See text for statistical analyses.

from the patients with atopic asthma did not respond significantly to 10 $\mu\text{g/ml}$ PA or to PA-HSA but did release histamine when they were challenged with calcium ionophore (58.1% vs 7.0% spontaneous release; $p < 0.01$) (Fig. 1). Most of the nonatopic, nonasthmatic control sub-

jects released negligible amounts of histamine in response to PA-HSA or grass pollen, but basophils from three of seven normal subjects responded to 100 $\mu\text{g/ml}$ PA (Fig. 1). Among the subjects who responded to PA, histamine release to PA was dose-dependent, with a greater re-

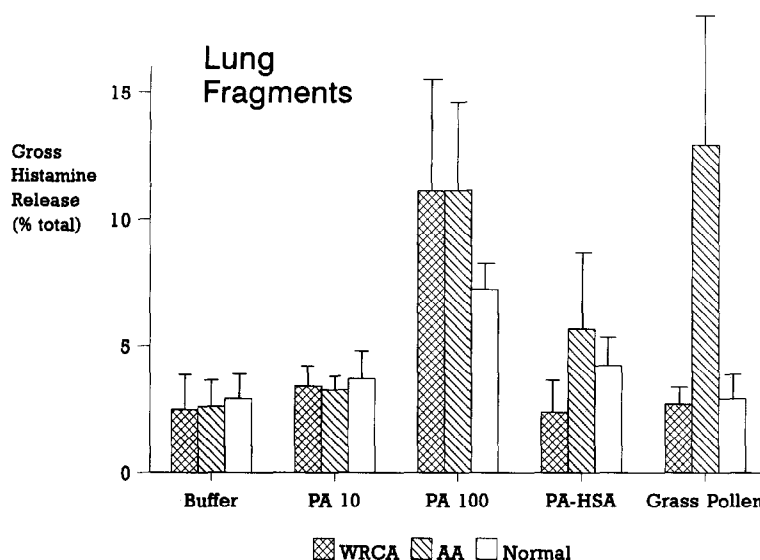


FIG. 4. Gross percent release of histamine from human lung fragments sensitized with sera from patients with WRCA (W: $n = 5$), patients with atopic asthma (A: $n = 5$), or nonatopic nonasthmatic control subjects (N: $n = 5$) and challenged with buffer, 100 $\mu\text{g/ml}$ PA, 100 $\mu\text{g/ml}$ PA-HSA, or 100 PNU/ml grass pollen. Bars show means $\pm$ SEM.

sponse to 100 $\mu\text{g/ml}$ PA than to 10 $\mu\text{g/ml}$ PA (for patients with WRCA mean release was 41.7% and 29.5%, respectively; $p < 0.05$). Grass pollen was used as a method control and induced histamine release from basophils of grass-allergic subjects but not from those of the nonatopic control subjects (mean release for atopic and nonatopic subjects was 38.7% and 10.2%, respectively; $p < 0.001$) (Fig. 1).

When challenged under similar conditions, BAL cells released a lesser proportion of total cellular histamine as compared with the response of basophils (Fig. 2). This was particularly evident for the atopic asthmatic group, in which calcium ionophore induced net release of only 11% of BAL cell histamine, as compared with 51% net histamine release from basophils ($p < 0.01$; spontaneous release 5.5% and 7%, respectively). Among the patients with asthma with positive skin test results to grass pollen, net release to grass pollen from BAL cells averaged 12% (i.e., equivalent to the level of histamine release induced by calcium ionophore). This compared with mean 31.6% net release when basophils from the same individuals were challenged with grass pollen. BAL cells from patients with WRCA responded to 100 $\mu\text{g/ml}$ PA with 25.8% mean net release, compared with mean net release of 33.4% from their basophils (spontaneous release was 10.6% and 8.3%, respectively). Of the various stimuli used, only PA-HSA was more active on BAL cells

than on basophils (mean net release was 9.9% and 4% for BAL cells and basophils of subjects with WRCA, respectively).

The pattern of histamine release from bronchial biopsy specimens was similar to that observed with the BAL cells, except a greater proportion of histamine was released from the bronchial biopsy specimens (Fig. 3). PA (100 $\mu\text{g/ml}$) induced significant histamine release from bronchial biopsy specimens from 9 of 11 patients with WRCA as compared with control-challenged specimens (mean, 49.9% vs 9.7% spontaneous release; $p < 0.01$). PA induced 10% or more net histamine release from biopsy specimens from 1 of 7 patients with atopic asthma but also from specimens from 4 of 7 normal control subjects. As with BAL cells and basophils, PA-HSA-induced histamine release from bronchial biopsy specimens was more disease-specific; 6 of 11 patients with WRCA, but none of the control subjects, showed positive responses ($p < 0.05$). Similarly, histamine release to grass pollen from bronchial biopsy specimens was largely restricted to those subjects with positive skin test results to grass pollen (mean net release was 23.5% in atopic subjects vs 3.1% in nonatopic subjects; $p < 0.01$). As with BAL cells, calcium ionophore released a lesser fraction of histamine from bronchial biopsy specimens from patients with atopic asthma as compared with granulocytes from the same subjects (mean 16.7% vs 51%; $p < 0.05$). No such

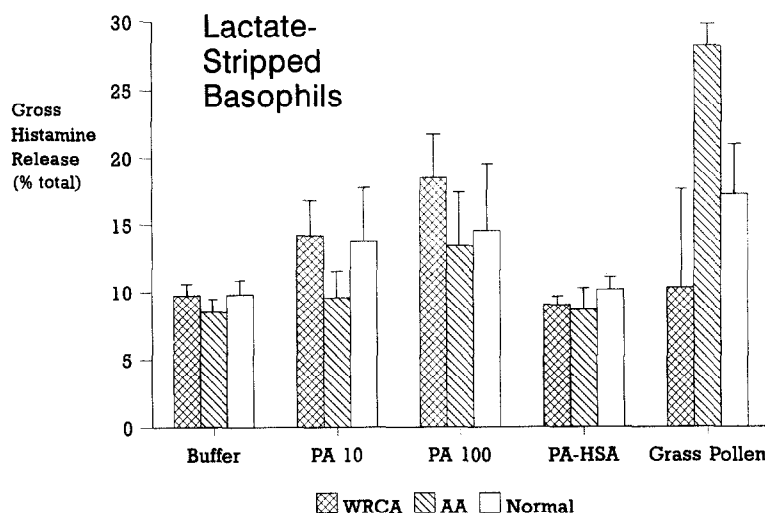


FIG. 5. Gross percent release of histamine from lactate-stripped human basophils sensitized with sera from patients with WRCA (W: $n = 11$), patients with atopic asthma (A: $n = 5$), or nonatopic nonasthmatic control subjects (N: $n = 4$) and challenged with buffer, 100 $\mu\text{g/ml}$ PA, 100 $\mu\text{g/ml}$ PA-HSA, or 100 PNU/ml grass pollen. Bars show means $\pm$ SEM.

difference was observed for the corresponding cells of patients with WRCA or those of normal control subjects.

IgE measurements

As expected, serum total IgE concentrations were higher in members of the atopic group than in the patients with WRCA or control subjects (Table I). Serum PA-specific IgE, measured by radioimmunoassay, was not significantly elevated in patients with WRCA as compared with the control groups. In fact, there was a trend for slightly higher levels of PA-IgE in the atopic group than in the WRCA group (geometric means, 2.62 and 2.11, respectively; $p = \text{NS}$). On the other hand, grass pollen-specific IgE was clearly demonstrated in all those atopic subjects with positive skin test results to grass pollen. There was no relationship between the concentrations of PA-specific IgE and total IgE in the same sera ($r = -0.054$; $p = \text{NS}$) (correlation performed after log-transformation of total IgE values).

Passive sensitization of human lung fragments

To address the possibility that our PA-IgE methods were insufficiently sensitive to detect biologically relevant small quantities of PA-IgE, we proceeded to conduct a series of passive sensitization studies and basophil desensitization experiments. Serum from grass pollen-allergic subjects was able to passively sensitize human lung

fragments and induce histamine release on subsequent challenge with grass pollen (100 PNU/ml) (mean release, 12.3% vs 2.9% for buffer-challenged fragments; $n = 7$; $p < 0.05$) (Fig. 4). On the other hand, it was not possible to demonstrate increased histamine release from human lung fragments that had been sensitized with serum from patients with WRCA and then challenged with 10 $\mu\text{g/ml}$ PA or 100 $\mu\text{g/ml}$ PA-HSA. PA (100 $\mu\text{g/ml}$) (the concentration used in the direct challenge studies) released histamine from lung fragments sensitized with sera from patients with WRCA (mean, 11.0%; $n = 5$), patients with atopic asthma (mean, 11.1%; $n = 5$), or nonasthmatic subjects (mean, 7.6%; $n = 8$).

Passive sensitization of basophils

Sera from atopic subjects were able to transfer grass pollen sensitivity to donor basophils: basophils resensitized with sera known to contain grass pollen IgE responded to grass pollen on subsequent challenge (mean histamine release increased from 9.5% to 27.9%; $n = 8$; $p < 0.01$). On the other hand, sera from patients with WRCA ($n = 11$) failed to transfer PA sensitivity to basophils (mean histamine release to 100 $\mu\text{g/ml}$ PA was 18.4% compared with 13.4% for asthmatic sera [$n = 5$] and 14.6% for nonatopic sera [$n = 4$]; $p = \text{NS}$) (Fig. 5). Neither 100 $\mu\text{g/ml}$ PA-HSA nor 10 $\mu\text{g/ml}$ PA induced increased response in basophils resensitized with sera from any group as compared with background release (mean background release in these studies was 9.8%).

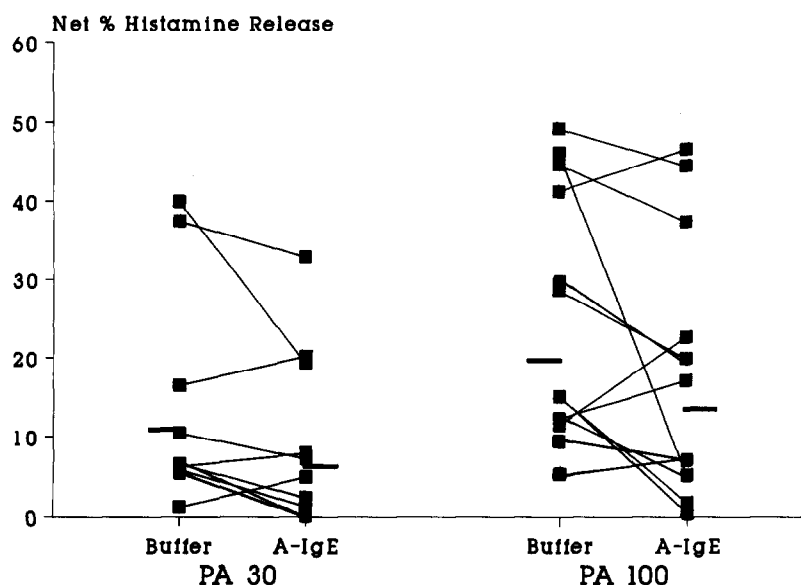


FIG. 6. Net percent release of histamine from basophils of patients with WRCA, pretreated with anti-IgE without calcium (*A-IgE*) or sham-desensitized with TB without calcium and then challenged with 30 $\mu\text{g/ml}$ PA ($n = 11$) or 100 $\mu\text{g/ml}$ PA ($n = 14$). Linked data points compare pretreatment with buffer or anti-IgE. Bars show arithmetic means.

Basophil desensitization

Optimal conditions for anti-IgE desensitization were established in preliminary experiments. With this protocol, desensitization of basophils from four atopic subjects reduced their net basophil histamine release to anti-IgE from 37.8% to 2.0% (95% inhibition; $p < 0.01$), whereas the net response to grass pollen was reduced from 44.8% to 9.8% (92% inhibition; $p < 0.01$).

Basophils from 14 patients with WRCA were desensitized and then rechallenged with varying concentrations of PA and PA-HSA. There was minimal histamine release during the preincubation (mean release 6.5% of total histamine, compared with 3.8% release from sham-desensitized basophils). Overall, the response to PA was slightly decreased by pretreatment with anti-IgE: 100 $\mu\text{g/ml}$ PA mean response fell from 31.4% to 27.1% ($n = 14$; $p = \text{NS}$), whereas the 30 $\mu\text{g/ml}$ PA mean response fell from 18.9% to 16.5% ($n = 10$; $p = \text{NS}$) (Fig. 6). In some instances, PA-induced histamine release increased after desensitization, as compared with the response of sham-desensitized cells from the same subject. In half of the subjects, the response to PA was not altered significantly by desensitization, and in only one third of the patients with WRCA was there any significant reduction in histamine release after desensitization (Fig. 6). Similar patterns of responses were observed whether desensitized

cells were challenged with PA in concentrations of 10, 30, or 100 $\mu\text{g/ml}$. In these experiments PA-HSA induced low levels of response in both desensitized and sham-desensitized cells (data not shown). Activation of mast cells and basophils by calcium ionophore bypasses surface receptors, so pretreatment with anti-IgE should not affect basophil responses to calcium ionophore.¹³ In fact, the mean response of WRCA basophils to ionophore was slightly increased after desensitization (mean response rising from 47.3% to 60.0% release; $n = 17$; $p = \text{NS}$). Background release in these studies was 9.7% for desensitized basophils and 6.9% for sham-desensitized cells.

PA binding studies

To address the possibility that PA might bind directly to a receptor on human leukocytes, binding assays were performed with tritiated PA by means of a standard protocol.¹⁶ Granulocytes from five patients with WRCA and three non-atopic normal control subjects were studied. None of these cell preparations showed any evidence of specific PA binding. In view of the initial negative results, several alternative conditions were tried, but no specific binding was found when Hank's balanced salt solution or TB without calcium was used, whether tested at 37° C, at room temperature, or at 4° C. As a positive control for this study, parallel experiments were performed in guinea

pigs sensitized to PA or to ovalbumin by serial intraperitoneal injection. Leukocytes from PA-sensitized animals were able to bind tritiated PA in a dose-dependent manner, which could be competitively inhibited by addition of excess unlabeled PA. In contrast, leukocytes from ovalbumin-sensitized animals did not bind tritiated PA to any greater extent than did leukocytes from unsensitized animals, and in both of these cases the amount of bound radioactivity was unaffected by the addition of unlabeled PA ($n = 5$ in each group; data not shown).

DISCUSSION

This study has shown that PA and PA-HSA can induce histamine release in vitro from the basophils and bronchial mast cells of patients with WRCA. At concentrations of 10 $\mu\text{g/ml}$ PA or 100 $\mu\text{g/ml}$ PA-HSA (PA content = 13 $\mu\text{g/ml}$), this process is relatively disease-specific. However, bronchial mast cells from some normal subjects (but not from subjects with atopic asthma) released to 100 $\mu\text{g/ml}$ PA, suggesting that at this concentration PA may have a direct action on basophils and bronchial mast cells in at least a subset of normal individuals.

As compared with basophils, BAL cells and bronchial biopsy specimens released a lesser proportion of their histamine in response to specific and nonspecific challenge. This was particularly evident from the atopic asthmatic group, whose basophils released nearly five times as much histamine as BAL cells when exposed to the same concentration of calcium ionophore. At a mean of 12%, net specific histamine release to grass pollen from BAL mast cells may appear unimpressive, but in fact, this is equal to the release seen with calcium ionophore (net, 11%). This may represent the maximum level of calcium-dependent release possible from these cells and may thus mean that mast cells in BAL and in the superficial bronchial mucosa are preactivated in atopic asthma and therefore less able to release their residual histamine. This would be consistent with other observations of chronic degranulation of mast cells in asthma,¹⁸ which has been proposed as a possible mechanism for the perpetuation of inflammation in asthmatic airways.¹⁹⁻²¹ In normal subjects bronchial mast cells would be expected to be replete with histamine and might respond more readily to a nonspecific challenge. Measurements of total histamine content of the biopsy specimens and BAL cells did not reveal any significant differences among the three groups,

but obviously these values cannot distinguish between a given number of replete mast cells and a larger number of partially replete cells and should not be overinterpreted. Downregulation of histamine release in the airways of patients with atopic asthma could represent a useful homeostatic mechanism; that is, mast cells that degranulate frequently could become relatively refractory to subsequent stimuli and thus protect against the overresponse to environmental allergens. This might partly explain the unexpected nonspecific response to PA in the nonatopic nonasthmatic group, but if correct, it remains unclear why only half of the normal subjects should have responded.

Allergens that induce occupational asthma can be divided into two broad groups: high molecular weight (HMW) compounds (plant and animal proteins, enzymes, glycoproteins) and low molecular weight (LMW) compounds (e.g., isocyanates, acid anhydrides, and PA). HMW and LMW agents appear to cause asthma through different mechanisms: HMW allergens readily induce specific IgE and behave similarly to other aeroallergens that induce atopic asthma (e.g., pollen, mites, danders, molds).^{22, 23} LMW occupational agents that cause asthma are more heterogeneous in that some (acid anhydrides, platinum salts) induce specific IgE, whereas others (isocyanates and PA) do not.^{24, 25} In bronchoprovocation tests LMW agents often induce late asthmatic responses and increased nonspecific bronchial responsiveness, without inducing an immediate asthmatic response.^{2, 3, 22, 23} These observations suggest that LMW and HMW agents induce asthma through different mechanisms.

This study has confirmed previous observations that serum from most patients with WRCA does not contain measurable quantities of IgE directed against PA or PA-HSA conjugates, as determined by radioimmunoassay.^{7, 17} Serum from patients with WRCA was unable to transfer PA sensitivity to human lung fragments or to human basophils under conditions in which grass pollen sensitivity was clearly transferable. Finally, desensitization by pretreatment with anti-IgE in the absence of calcium did not affect subsequent release to PA. Taken together, these observations suggest that PA-specific IgE is not important in PA-induced histamine release.

Basophils from patients with atopic asthma or atopic rhinitis degranulate when exposed to appropriate allergens, and the mechanism of this response is generally accepted as being IgE-

driven and calcium-dependent.⁵ It is clear that these patients with WRCA did not have significant concentrations of serologically detectable specific IgE. Moreover, in the present study sera from a sample of these same patients with WRCA were unable to transfer PA sensitivity to human lung fragments or human basophils.

Desensitization of basophils by preincubation with anti-IgE without calcium is an effective means of attenuating IgE-dependent responses,¹³ and in the present study such desensitization reduced basophil responses to anti-IgE and to grass pollen by 92% to 95%. Anti-IgE desensitization had a variable effect on basophil responses to PA and PA-HSA. In most cases PA responses were not significantly affected by pretreatment with anti-IgE. A minority of subjects showed some reduction in PA response, but in none did desensitization affect the PA response as much as it affected the grass pollen response in atopic subjects. Interestingly, there was no relationship between serum PA-specific IgE concentrations and histamine release from sham-desensitized basophils. Similarly, there was no association whatsoever between the concentration of PA-specific IgE detected by radioimmunoassay and the degree of desensitization that could be achieved by preincubation with anti-IgE. These desensitization studies leave open the possibility that there may be some heterogeneity of response among patients with WRCA; that is, PA-specific IgE may participate in the response in a minority of patients, but to explain PA-induced histamine release in all patients with WRCA, it is necessary to consider alternative or additional mechanisms.

Apart from IgE-dependent degranulation, mast cells can also release histamine in response to a wide variety of other stimuli including neuropeptides,⁸ platelet products,^{9, 26} complement component C5a,¹⁰ and several mononuclear cell products including well-characterized cytokines (e.g., IL-1, IL-3, IL-6),¹¹ a number of less well-defined leukocyte-derived factors that release histamine from basophils,²⁷⁻³¹ and mast cells.^{27, 32} Furthermore, several cytokines, although unable to release histamine directly, can prime basophils for enhanced response to other stimuli.³³⁻³⁶ Given that PA-induced histamine release at lower concentrations is immunologically specific, the antigen must be recognized by antibody or by the T-cell antigen receptor. The present studies have mainly addressed antibody recognition, but it will be important to address the possible T-cell recognition of PA and PA hapten-conjugate complexes in future

work. It is conceivable that cytokines or factors are released *in vivo* and prime basophils or mast cells for enhanced histamine release when challenged *in vitro*. Such "histamine release enhancing factors" would be a potential T-cell-dependent mechanism for IgE-independent, antigen-specific upregulation of mast cell histamine releasability. Alternatively, there may be a pharmacologic component to WRCA, with PA binding to a specific cell surface receptor and releasing histamine, without requiring recognition by either immunoglobulin or T cells. On the other hand, we have found no evidence that PA binds to a specific receptor on human leukocytes, and the natural history of WRCA strongly suggests an immunologic process rather than a direct pharmacologic effect confined to a subpopulation of workers (the mean latent interval between first exposure to cedar and the onset of WRCA symptoms is 3.6 years³⁷ and many other individuals are exposed to cedar but never develop WRCA^{1, 4}).

It appears that 100 $\mu\text{g/ml}$ PA has a nonspecific effect on basophils and mast cells (at least in the normal group). PA-HSA (100 $\mu\text{g/ml}$) contains 13 $\mu\text{g/ml}$ PA, and both this agent and the 10 $\mu\text{g/ml}$ concentration of PA produced disease-specific patterns of response. It is most unlikely that the cellular responses of our normal subjects to 100 $\mu\text{g/ml}$ PA are due to occult PA sensitization from environmental exposure to cedar. Unlike allergy to tree pollens, sensitivity to red cedar occurs only in individuals who are exposed to high concentrations of red cedar dust, conditions that rarely occur outside the timber industry.¹ Moreover, none of the patients with atopic asthma in this study responded to PA. It also seems unlikely that this difference between WRCA and atopic asthma is due to inhaled medication because the patients with WRCA were, if anything, receiving more medication than subjects in the atopic group, and there was no suggestion of reduced histamine releasability to PA or to nonspecific agents in the subjects with WRCA.

In conclusion, this study has demonstrated that basophils and bronchial mucosal mast cells from patients with WRCA release histamine when challenged *in vitro* with PA or PA-HSA. This response is relatively specific in that at the 10 $\mu\text{g/ml}$ concentration, PA-induced release was essentially confined to cells from patients with WRCA. Moreover, even at a concentration of 100 $\mu\text{g/ml}$, cells from subjects with atopic asthma release much less histamine when challenged under the same conditions. Bronchial cells from

some normal subjects also released histamine in response to 100 µg/ml PA, for reasons that remain unclear. These findings are consistent with the view that WRCA is indeed due to specific sensitization to PA and cannot be explained as a simple pharmacologic response to PA. Using four separate approaches to evaluate the possible presence of biologically significant amounts of PA-specific IgE in WRCA, we have effectively excluded any role for cytophilic IgE in this disorder. The time course of development of WRCA strongly suggests that WRCA is an immunologically mediated disorder, but further research is needed to clarify the reasons for response in normal subjects and to define the process by which immunocompetent cells recognize PA in vivo.

We thank Dr K. S. Tse for kindly assisting in the recruitment of patients for this study. We also thank Ann Chadderton and Mervyn Low for expert technical assistance.

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