

In vitro diagnosis of cypress pollen allergy by using cytofluorimetric analysis of basophils (Basotest)

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Background: Cupressaceae pollen allergy is a worldwide pollinosis, but its in vitro diagnosis is notoriously difficult. The Basotest is a newly available in vitro test for the detection of allergen-specific IgE based on the level of cellular activation of basophils by using flow cytometry.

Objectives: The Basotest was compared with the measurement of cypress pollen-specific IgE in highly selected patients.

Methods: We analyzed 34 patients allergic to cypress pollen selected on the basis of a suggestive clinical history and positive skin test and nasal challenge responses to cypress pollen extract. We also analyzed 8 patients with positive skin test responses to cypress pollen extract who did not present symptoms during the pollen season (intermediate group) and 33 control subjects. Sensitivity, specificity, and efficiency of the Basotest and serum-specific IgE levels measured by using the CAP System were determined in patients allergic to cypress pollen. Histamine release was studied in a selected group of patients.

Results: The Basotest was more sensitive (91.2%) than the CAP System (76%) for the in vitro diagnosis of cypress pollen allergy. A dose-response curve was observed in basophils obtained from patients allergic to cypress pollen. There were no false-positive results with either test (specificity 100%). The results of the Basotest or those of the CAP System did not correlate with the patients' in vivo threshold sensitivity assessed by skin tests and nasal challenge.

Conclusions: The Basotest was found to be an effective diagnostic test in patients allergic to cypress pollen. (J Allergy Clin Immunol 2000;105:339-45.)

Key words: Basophil, IgE, Basotest, cypress pollen, allergy

Allergy to Cupressaceae pollen is a worldwide pollinosis caused by several species.¹ Members of the Cupressaceae family are important pollen-producing trees in the Mediterranean area (*Cupressus sempervirens* [common cypress], *Cupressus arizonica* [Arizona cypress], and *Juniperus communis* or *Juniperus oxyedrus* [juniper]),²⁻⁷ North America (*Juniperus ashei* [mountain cedar]),^{8,9} and Japan (*Cryptomeria japonica* [Japanese

Abbreviation used

FMLP: N-formyl-methionyl-leucyl-phenylalanine

cedar]).¹⁰ The amounts of Cupressaceae pollens released into the atmosphere are often far greater than those of grass pollens,³ and the prevalence of allergy to Cupressaceae pollens has increased dramatically over the past 3 decades throughout the world.^{1,11,12}

The diagnosis of cypress pollen allergy remains difficult because cypress (*Cupressus sempervirens* and *Cupressus arizonica*) pollen extracts are not yet available in standardized form, and the reactivity of patients to these extracts is usually weak. Thus in some patients skin test responses are weakly positive or even negative, and specific IgE levels are not detectable in serum.¹³⁻¹⁵ The skin sensitivity often increases after the pollen season and then declines until the next season, making the diagnosis difficult.

The Basotest (Becton Dickinson, Pont de Claix, France) is a new basophil activation test based on the occurrence of CD63 (gp53)¹⁶ in the presence of allergens or nonspecific stimuli.¹⁷⁻¹⁹ CD63 is a member of the transmembrane-4 superfamily, which can be detected in the cytoplasm of a wide variety of cell types, including platelets and basophils.²⁰ In this test CD63 is measured by using cytofluorimetry. Although there has been no published study validating the Basotest in the diagnosis of allergic diseases, the test is commercially available. In a previous publication the expression of CD45 on cell surface was used to monitor the basophil physiology by flow cytometry.²¹

In this study we examined the value of a basophil activation test (Basotest) in the diagnosis of allergy to cypress pollen. Comparison of the expression of CD63 with histamine release was performed in control subjects and in patients allergic to cypress pollen. We studied a select group of patients allergic to cypress pollen who had a suggestive history of allergy and positive skin test and positive nasal challenge responses to cypress pollen extracts, an intermediate group of symptom-free patients who showed positive results to skin prick tests with cypress pollen, and 33 control subjects who were not allergic to cypress pollen. The sensitivity and specificity of the Basotest were compared with the measurement of serum-specific IgE by means of the CAP System.

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METHODS

Subjects

Thirty-four patients allergic to cypress pollen (17 men and 17 women, 19 to 64 years [mean \pm SD, 36 ± 11 years]) were enrolled by using the following criteria. All patients had exhibited symptoms of rhinitis between January and March during the peak of the cypress pollen season for at least the past 2 years and demonstrated a positive skin prick test response and a positive nasal challenge response to cypress pollen extract, as described below. Patients were excluded if they had taken antihistamines, cromoglycate, or corticosteroids of any form within the previous 4 weeks. None of the patients had received allergen-specific immunotherapy to cypress pollen extracts.

An intermediate group of 8 patients (5 men and 3 women, 21 to 52 years [mean \pm SD, 37 ± 11 years]) with positive skin test responses to cypress pollen but without a clinical history of cypress pollen allergy was studied. These patients were not undergoing any form of treatment at the time of the investigation.

Nonallergic healthy subjects (13 men and 20 women, 24 to 63 years [mean \pm SD, 38 ± 12 years]) were used as a control group. These subjects had no history of allergic diseases, and skin prick test responses to a battery of 60 common allergens²² were negative.

All subjects gave their written informed consent.

Cypress pollen allergen extract

Nonstandardized extracts of cypress pollen (*Cupressus sempervirens*, 1:10 wt/vol) were prepared according to the guidelines of the Allergen Subcommittee of the International Union of Immunology Societies, as previously described,²³ and prepared by Stallergènes SA (Antony, France). Separate aliquots were lyophilized and reconstituted each test day at a concentration of 90 μ g of specific allergen in 100 μ L of saline solution. The same extract was used for skin tests, nasal challenges, and Basotests.

Skin prick tests

Patients were tested with standardized extracts of a battery of allergens (Stallergènes SA), as previously published,²² and the cypress pollen extract. A negative control solution consisted of a 50% glycerol solution. The positive control solution consisted of 9% codeine phosphate. Skin prick test evaluation was carried out with the pollen cypress extract by using five 3-fold increasing concentrations ranging from 0.014% to 10% (wt/vol), as previously described in detail.²³ The wheal and flare responses were recorded by using the Scotch tape technique 15 minutes after the performance of the prick. Mean wheal and flare sizes were measured by using computed planimetry.

Nasal provocation test

Before inclusion in the study, patients were carefully evaluated for the absence of nasal symptoms (nasal discharge, sneezing, and itching). A new vial of the lyophilized allergen extract was reconstituted every test day. Increasing concentrations of the cypress pollen extract (5-fold increments from 0.003% to 10%) were insufflated into a nostril by using a pump, with the patient refraining from breathing during the insufflation. The first insufflation consisted of a 0.9% saline solution. Increasing concentrations of cypress pollen extracts were then insufflated every 15 minutes until a symptom score of at least 5 was obtained from the sum of scores for 5 consecutive sneezes (score of 3), rhinorrhea (score of 1-3), and nasal blockage (score of 1-3). This threshold score has been previously validated and was shown to correlate with the release of mediators in nasal secretions.^{24,25}

Serum-specific IgE

Serum samples were drawn before nasal challenge and stored at -20°C until measurement of *Cupressus sempervirens*-specific IgE

by using the CAP System (Pharmacia Upjohn, Uppsala, Sweden), following the instructions on the package insert.

Blood samples

Peripheral blood samples from all subjects were drawn into heparinized tubes. Blood samples were processed within 4 hours after the blood was drawn.

Basotest

We used the Basotest for the quantitative determination of in vitro basophil activation. Heparinized blood (100 μ L) was first incubated with a stimulation buffer for 10 minutes at 37°C and then with 100 μ L of allergen solution (*Cupressus Sempervirens*) for 20 minutes at 37°C . A concentration dose-response curve was performed with 90 μ g, 9 μ g, 900 ng, 90 ng, 9 ng, and 0.9 ng of allergenic extract diluted in saline solution (100 μ L) in 30 allergic patients, 8 intermediate patients, and 23 control subjects. The chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) was used as a positive control,¹⁸ and PBS solution served as a negative control. The activation process was stopped by incubating the samples at 4°C for 10 minutes. The samples were then incubated for 20 minutes at 4°C with 20 μ L of phycoerythrin-conjugated anti-IgE and FITC-conjugated anti-gp53. Erythrocytes were subsequently removed by the addition of 2 mL of lysing solution (Becton Dickinson). Cells were washed twice with PBS solution, resuspended in 200 μ L of PBS solution, and analyzed within 1 hour by means of cytofluorimetry (FASCalibur, Becton Dickinson). The basophil population was gated (Fig 1) by the presence of phycoerythrin-conjugated anti-IgE, and the expression of gp53 (CD63) was analyzed on this gated cell population. Acquisition was performed on 1000 cells for each sample, and results are given as the percentage of basophils expressing gp53 (Fig 2).

Histamine release

Histamine release from peripheral blood basophils was partially purified over dextran from 6 control subjects and 6 patients allergic to cypress pollen in PBS buffer containing Ca^{2+} , Mg^{2+} , and IL-3, as previously described in detail.²⁶ Activation was carried out by using 200 μ L of cell suspension and either 50 μ L of stimulation buffer (spontaneous and total release) or 50 μ L of cypress allergen extract solution (final concentrations 0.18, 1.8, and 18 μ g/mL). Histamine was measured by using a highly specific and sensitive enzyme immunoassay (Immunotech, Luminy, France) with an mAb against acylated histamine.²⁷ Results are expressed as the net percentage of histamine release.

Design of the study and statistical analysis of the data

All subjects were investigated 7 to 9 months after the cypress pollen season. The same investigator (L. P.) performed the whole clinical study. Initially, the reproducibility of the Basotest was assessed by performing the test in triplicate on cells obtained from 7 allergic subjects by using an extract of *Cupressus sempervirens*. We then determined, for 33 control subjects and 34 allergic patients, the specificity, sensitivity, and efficiency of the CAP System and Basotest by using the method of Galen and Gambino.²⁸ Intergroup comparisons on the Basotest were performed by using the ANOVA test with the Bonferroni-Dunn correction. In addition, we compared the threshold dose inducing a positive skin test or positive nasal challenge response with the results of the CAP System or Basotest by using the Kendall test to assess whether results of in vitro tests were correlated with those of in vivo tests. Sensitivity, specificity, and efficiency were calculated for the Basotest and CAP system as follows:

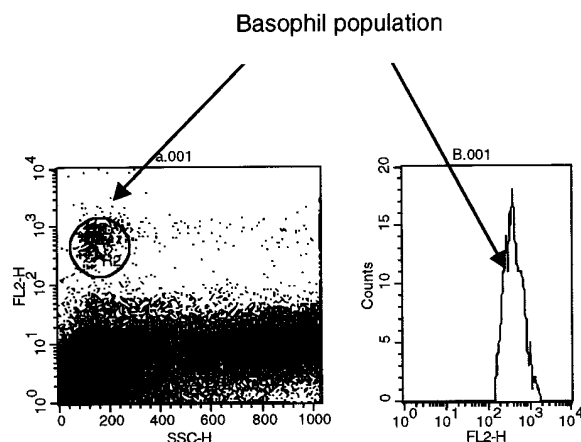


FIG 1. Basophil selection by phycoerythrin-conjugated anti-IgE detection as determined by means of cytofluorimetry.

Sensitivity = true-positive test response/true-positive test response + false-negative test response
Specificity = true-negative test response/true-negative test response + true-negative test response + false-negative test response
Efficiency = sensitivity + specificity/2.

Correlations between Basotest, the CAP system, and histamine release were analyzed by using the Spearman rank test. All analyses were conducted by using the statistical package Statview 4.5. Results are given as medians and 25th and 75th percentiles, except for normally distributed demographic data.

RESULTS

Clinical characteristics of the subjects

All 34 patients allergic to cypress pollen presented nasal symptoms during the cypress pollen season. Conjunctivitis was present in 23 allergic patients, and 1 patient had asthma. Five patients were monosensitized (Table I).

Serum-specific IgE

None of the control subjects had a positive serum IgE level. Twenty-six allergic patients had positive serum IgE levels at a low level (median, 2.1 kU/L; 25th-75th percentile, 0.4-4.2 kU/L). No patient had a level of specific IgE over 20 kU/L. There was no difference between monosensitized and polysensitized patients. Three patients with undetectable levels of serum-specific IgE had high or very high Basotest positivity.

Basotest

The intra-assay variation of the Basotest was very low in cells from patients allergic to cypress pollen (coefficient of variation, 5.4%) after stimulation by cypress pollen extracts, demonstrating the reproducibility of the test.

The percentage of expression of gp53 after saline solution incubation was 3.7% (25th-75th percentile, 2.1%-5.1%) in control subjects, 3.7% (25th-75th percentile, 2.3%-9.7%) in the intermediate group, and 2.7% (25th-75th percentile, 2.0%-4.4%) in allergic patients. Thus as suggested in the package insert, a 15% cutoff

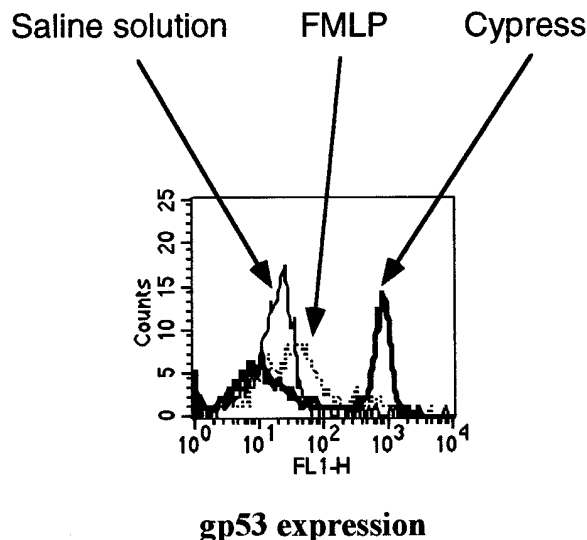


FIG 2. CD63 (gp53) expression after challenge with a saline solution, FMLP, or *Cupressus sempervirens* allergen extract.

limit could be set for a positive value of the Basotest with cypress pollen extracts.

The expression of gp53 after FMLP stimulation is shown in Fig 3. There was no significant difference ($P = .2$, ANOVA) between control subjects and intermediate group and allergic patients after this nonspecific challenge.

All control subjects had a negative Basotest result after cypress pollen extract challenge (gp53 expression, 3%; 25th-75th percentile, 3.1%-11.9%). In the intermediate group gp53 was expressed by 20.8% of basophils (25th-75th percentile, 12.6%-41.1%) after cypress pollen extract challenge. On the other hand, 31 of the 34 patients allergic to cypress pollen (sensitivity, 91.2%) had a positive Basotest result (gp53 expression, 52.1%; 25th-75th percentile, 28.0%-73.4%). Monosensitized patients all had positive test results. Interestingly, 2 of the 3 patients with a negative Basotest result also had undetectable levels of serum-specific IgE. Patients allergic to cypress pollen exhibited a significant difference ($P < .0001$, Mann-Whitney U test) compared with those of the intermediate group, as well as with control subjects (Fig 3). No significant difference was found between the symptom-free patients (intermediate group) and the control subjects.

The expression of gp53 was observed, after challenge with cypress allergen at concentrations ranging from 90 μ g to 9 ng, in basophils from patients allergic to cypress pollen. The dose of 0.9 ng of allergen is unable to induce the expression of gp53. Allergen-induced gp53 expression was not observed on the basophils obtained from patients in the intermediate group nor by basophils from the control subjects (Fig 4).

Histamine release

Net histamine release of over 10% was considered positive. Histamine release was significantly different

TABLE I. Individual data of in vivo and in vitro test in allergic patients

Patient number	Age (y)	Time (mo)	MS	PS	NPT*	Skin prick test*	Specific IgE (kUa/L)	Basotest (%)
No. 1	59	3	—	+	3	2	0.97	50.27
No. 2	21	3	+	—	3	1	9.34	59.35
No. 3	35	3	—	+	5	2	<0.35	1.33
No. 4	27	6	—	+	5	3	<0.35	85.88
No. 5	34	12	—	+	4	3	<0.35	15.44
No. 6	46	12	—	+	4	4	4.16	75.67
No. 7	45	3	—	+	3	4	<0.35	3.37
No. 8	41	6	+	—	2	1	1.60	59.75
No. 9	39	3	—	+	4	4	1.72	77.59
No. 10	31	9	—	+	5	5	14.70	17.58
No. 11	46	3	—	+	3	4	<0.35	63.80
No. 12	41	3	+	—	4	2	0.53	28.06
No. 13	22	6	—	+	3	1	2.20	27.04
No. 14	23	6	+	—	3	2	2.10	51.51
No. 15	24	12	—	+	4	1	3.92	91.73
No. 16	48	3	—	+	4	2	1.29	4.26
No. 17	52	3	+	—	5	1	<0.35	46.93
No. 18	42	3	—	+	4	6	4.46	65.36
No. 19	28	12	—	+	4	3	<0.35	50.5
No. 20	24	12	—	+	3	3	0.73	65.92
No. 21	27	12	—	+	4	2	6.24	64.34
No. 22	31	12	—	+	3	1	6.82	87.92
No. 23	29	12	—	+	1	4	5.46	45.26
No. 24	31	12	—	+	4	5	0.43	73.45
No. 25	31	6	—	+	5	2	3.02	82.07
No. 26	60	12	—	+	3	1	16.20	46.61
No. 27	23	12	—	+	4	5	3.07	71.48
No. 28	41	6	—	+	4	1	3.81	22
No. 29	41	12	—	+	5	6	2.56	65.24
No. 30	20	3	—	+	5	5	3.54	74.39
No. 31	36	12	—	+	5	4	<0.35	22.27
No. 32	25	6	—	+	5	3	7.94	60.39
No. 33	46	6	—	+	3	1	1.37	80.8
No. 34	64	3	—	+	4	1	2.38	34.33

MS, Monosensitized; PS, polysensitized; NPT, nasal provocation test.

*Values represent the dilution of cypress pollen extract (lowest to highest, 1–6).

between the group allergic to cypress pollen and the control group ($P < .003$, Mann-Whitney U test) as follows: 46.15% (25th–75th percentile, 35.5%–80.1%) and 5.00% (25th–75th percentile, 4.31%–7.20%), respectively.

Specificity, sensitivity, and efficiency of serum-specific IgE and Basotest

Specificity, sensitivity, and efficiency of Basotest and serum-specific IgE levels measured by the CAP System are shown in Table II.

Correlations between in vivo and in vitro test responses

No significant correlations were observed between the different in vivo and in vitro tests. There was a positive correlation between skin prick test and nasal provocation test responses (Table III; $P < .05$, Kendall test). There was no significant correlation between the results of the Basotest and serum-specific IgE levels (Fig 5).

DISCUSSION

In this study a selected group of patients with a positive skin prick test response and clinical history of cypress allergy were studied, and it was found that the Basotest was more efficient than the CAP System for the in vitro diagnosis of cypress pollen allergy. However, neither the results of the Basotest nor those of the CAP System were correlated with the threshold in vivo patient sensitivity, as assessed by skin tests or nasal challenge.

The clinical diagnosis of cypress pollen allergy is not easy to make in the Northern Mediterranean region during the pollen season area because pollinization occurs in these trees during the winter at a time when ash and *Phillyrea* (Oleaceae) species pollinate.²⁹ The methods used for skin tests and the scoring system for symptoms used in this study have been widely implemented in our clinic. However, for a number of reasons, cypress pollen extracts are not available in a standardized form. First, it

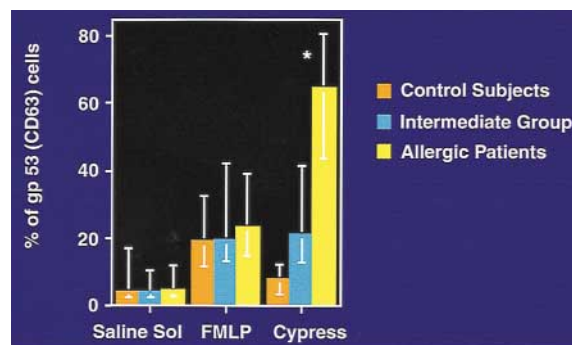


FIG 3. Percentage of expression of gp53 (CD63) after challenge with a saline solution, FMLP, or *Cupressus sempervirens* allergen extract (correlation by Wilcoxon W test between saline and challenge test, * $P < .0001$).

TABLE II. Sensitivity, specificity, and efficiency of in vitro tests calculated on 34 patients allergic to cypress pollen and 31 healthy control subjects

	Basotest	CAP System
Sensitivity	91.2%	76%
Specificity	100%	100%
Efficiency	95.5%	88%

TABLE III. Correlations between the different diagnostics tests

	Basotest	CAP System	Skin test
Basotest	—	—	—
CAP System	$P = .15$	—	—
Skin test	$P = .5$	$P = .5$	—
Nasal challenge	$P = .61$	$P = .4$	$P = .02^*$

Basotest and the CAP System were compared by using the Spearman test.

The other tests were compared by using the Kendall test.

*Correlation was significant at a P value of less than .05.

is difficult to find appropriate sera with high levels of specific IgE, making performance of RAST-inhibition experiments difficult. Second, the amount of protein released by the pollen grains are low. Third, it is difficult to increase the strength of the extract because of the physicochemical properties of the cypress pollen grain.¹³ Thus *Cupressus sempervirens* pollen extracts are usually of low potency. Moreover, in our area many patients with clear-cut cypress pollen allergy have low or nondetectable levels of cypress pollen IgE in their serum, and the level of specific IgE decreases at time points distant from the pollen season.³ These considerations do not apply to *Cryptomeria japonica* pollen because serum-specific IgE titers are usually very high, and IgE immunoprints can be easily performed.³⁰ However, in the Mediterranean area there is a need for a new in vitro test that is more accurate than the measurement of cypress pollen-specific IgE.

In the present study the sensitivity and specificity of the CAP System in patients allergic to cypress pollen

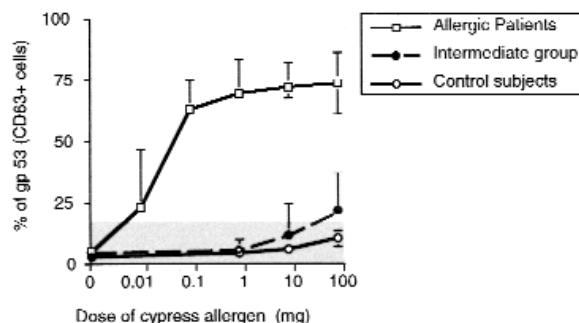


FIG 4. Expression of CD63 after a dose-response curve to cypress allergen challenge by basophils from allergic patients ($n = 34$), the intermediate group ($n = 8$), and control subjects ($n = 31$). Data are expressed in median with 25th and 75th percentiles. Shaded area, Negative Basotest response area.

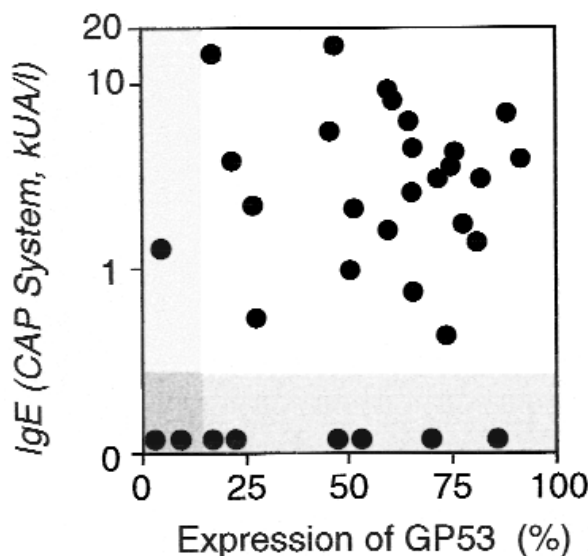


FIG 5. Correlation between serum *Cupressus sempervirens*-specific IgE and the Basotest (correlation by Spearman rank test). Shaded area, Undetectable IgE level or negative Basotest result.

were similar to that previously published.^{3,7,13,31,32} However, it appears that the test is less accurate for the diagnosis of cypress pollen allergy than for the diagnosis of other allergies for which standardized allergen extracts are available.³³⁻³⁹ The lower sensitivity of the CAP System in the diagnosis of cypress pollen allergy may be related to the IgE titers, which were usually low. Alternatively, the higher sensitivity of the Basotest may be related to the allergens used. Because it has been suggested that the cypress pollen extract used for the CAP System is not standardized, there may be some epitopes that are lacking and that were present in the extract used for the Basotest. However, this is not the case in this study because the same extract was used for skin tests, nasal challenges, and Basotests. However, in our clinical practice skin tests appear to be more sensitive than specific IgE in the diagnosis of cypress pollen allergy, and in

a previous study we confirmed our clinical impression.³ The Basotest concentration-response curve to cypress allergen stimulation in the allergic group proves the very high sensitivity of the test. Moreover, the lack of differences between the control group and the intermediate group is another demonstration of the high selectivity of this in vitro test.

The sensitivity of the Basotest was found to be superior to that of the CAP System. The percentage of activated basophils was higher than 59% for most patients, and there was a large difference between the cutoff limit set at 15% of activated cells and the positive responses observed. The variability of the test is low. We did not try to correlate the Basotest with histamine release because of the limited number of subjects analyzed. However, we found that the group of subjects allergic to cypress pollen generally exhibited positive test responses (Basotest and histamine release), whereas in the control subjects negative test results (Basotest and histamine release) were observed.¹⁷ It was found that the kinetics of CD63 upregulation and histamine release were identical, and a strong correlation was found between the percentage of mAb 435-binding basophils and the extent of histamine release.¹⁷ Thus the Basotest appears to be an effective in vitro diagnostic test for this difficult to diagnose allergy.¹³ It has not been determined in the present study whether cells stored for 24 or 48 hours have the same reactivity as freshly recovered cells.

Previous studies with the Basotest have been published. Activation of human basophilic granulocytes with anti-IgE or with the chemotactic peptide FMLP leads to increased expression of the CD63 antigen on the cell surface, as detected by mAb 435.¹⁷ It therefore appears that CD63 (gp53, mAb 435) could be an interesting new tool for investigating the activation of human basophils in addition to the measurement of mediator release. This marker may be useful for the detection of basophil activation in vivo.¹⁸

No correlation between thresholds of sensitivity of in vivo and in vitro tests were observed in this study. First, there may be differences between the patient's reactivity during challenge and symptoms during the season. However, in the case of grass pollen allergy, we observed that the results of nasal challenge with a method similar to that used in the present study were significantly correlated with symptoms during the pollen season.²⁶ On the other hand, there is usually little correlation between skin test responses and symptoms during the pollen season.²⁶ Second, it is not completely surprising that there was no significant correlation between in vivo and in vitro parameters because experiments with various concentrations of anti-IgE demonstrated that the binding of mAb 435 to basophilic granulocytes follows an all-or-nothing-like response per cell.¹⁷ Third, there have been no clear-cut studies showing a significant correlation between in vivo and in vitro parameters when threshold levels of sensitivity were compared or when a threshold level has been reached,³⁹ although a comparison of IgE measured by the CAP System and serial dilution titration skin testing

by receiver-operating curve analysis showed a significant correlation between IgE levels and skin test responses.⁴⁰

Our study shows that the Basotest may be useful in the diagnosis of cypress pollen allergy and is more efficient than the measurement of allergen-specific IgE by using the best available method, the CAP System.

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