# Assessment of skin prick test and serum specific IgE detection in the diagnosis of Cupressaceae pollinosis

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**Background:** *There is increasing evidence for the relevance of Cupressaceae pollinosis among persons living in geographic areas where these species are native or imported.*
**Objective:** *Previously reported problems in obtaining valid allergenic extracts to be used in the diagnosis of this winter pollinosis prompted us to assess the value of available Cupressaceae pollen extracts for in vivo and in vitro diagnosis.*
**Methods:** *Commercial and in-house allergenic extracts from Cupressaceae and Taxodiaceae families were used for skin prick testing and specific IgE detection in six groups of subjects exposed to a high concentration of Cupressaceae pollen.*
**Results:** *Four commercial and two in-house* Cupressus sempervirens *pollen extracts showed low cutaneous reactivity. Positive test results were recorded in 26% of the 713 subjects tested.* C. arizonica *in-house pollen extracts gave rise to larger cutaneous reactions. Furthermore, the skin prick test response was positive in a greater number of subjects (38%) of the same group. Six commercial immunoassays were able to detect specific IgE to* C. sempervirens *in rates ranging from 8.1% to 81.1%. Specific IgE to* C. arizonica *was detected by means of an in-house immunoenzymatic method in 70.3% of 54 patients with suspected "cypress" allergy, and specific IgE to* C. sempervirens *was detected in 75.9% of these patients by using a commercial system. High rates of cross-reactivity within the Cupressaceae family and with species of the Taxodiaceae family were recorded with both in vivo and in vitro tests.*
**Conclusions:** *The use of* C. sempervirens *in vivo diagnostics should be carefully evaluated until better characterized extracts are developed. In-house–characterized extracts of* C. arizonica *seem to be more reliable in the diagnosis of Cupressaceae allergy by means of skin prick testing. The sensitivity of commercially available in vitro methods to detect specific IgE to* C. sempervirens *should be carefully evaluated; nevertheless, valid results can be obtained with some already available immunoassays. (J Allergy Clin Immunol 1996;98:21-31.)*

***Key words:*** *Cupressaceae, cypress, diagnosis, skin prick test, specific IgE, Taxodiaceae, winter pollinosis*

Cupressaceae pollen allergy has been reported as a cause of winter conjunctivitis, rhinitis, and asthma in North America,[1-3] South Africa,[4] Australia,[5,6] and the Mediterranean area.[7-13] Clinical and epidemiologic data on Cupressaceae pollen allergy differ, depending on the species and the region considered. Similarly, data on the nature and characteristics of allergenic structures involved in the hypersensitivity response and on their potential cross-reactivity are not always available.

*Abbreviations used*

| | |
|---|---|
| Ca-IH: | *Cupressus arizonica* in-house extract |
| Cs-DHS: | *C. sempervirens* extract (Dome/Hollister-Stier) |
| Cs-IH: | *C. sempervirens* in-house extract |
| sIgE: | Specific IgE |
| SPT: | skin prick test |

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Recently, characterization of allergenic pollen extracts from *Cupressus* genera has been reported for *Cupressus sempervirens* (Mediterranean cypress)[6, 14] and *C. arizonica* (Arizona cypress),[15] but in geographic regions where aerobiologic studies show a high concentration of pollens from these species, clinical and epidemiologic data vary greatly or are unavailable.[11, 13, 16-18] Clinical studies on "cypress" pollinosis have been carried out with commercial noncharacterized nonstandardized in vivo diagnostic extracts,[12, 13, 18, 19] although authors themselves highlight the low potency of these extracts and the resulting poor cutaneous response.[12, 19]

Specific IgE (sIgE) has been demonstrated by immunoblotting technique in the allergenic characterization of *C. sempervirens*[6, 14] and *C. arizonica,* extracts,[15] but in vitro detection of sIgE with commercial immunoassays for clinical and epidemiologic purposes seems to be problematic because of the reported low sensitivity of available methods.[6, 15, 19, 20]

To assess the usefulness of available tools for Cupressaceae allergy diagnosis, we designed in vivo and in vitro studies. Tests performed with in-house allergenic extracts of *C. arizonica* and *C. sempervirens* obtained from freshly collected and from purchased pollen were used along with commercially available tests to assess how characterized extracts can enhance the diagnosis. A quantitative skin prick test (SPT) was used in comparative studies to evaluate the cutaneous response to allergenic extracts available and possible diagnostic pitfalls when nonstandardized extracts are used. Similarly, to obtain information on sensitivity of available methods, commercial immunoassays for sIgE detection were used along with an in-house immunoenzymatic method.[15] On the basis of data on cross-reactivity reported by several authors,[20-22] commercial extracts of Cupressaceae species, other than *C. arizonica* and *C. sempervirens,* and commercial extracts of Taxodiaceae species were also added to define the possible use of other species in "cypress" hypersensitivity diagnosis.

## METHODS

### Patients and environment

Subjects studied were those attending the allergy outpatient department of the National Health Service, located in Città di Castello, Umbria, 50 km north of Perugia in the central region of peninsular Italy. Patients were not receiving allergen-specific immunotherapy, and their informed consent to participate in the study was obtained.

The subcontinental climate[23] of the apparently unpolluted geographic area selected for the study allows Cupressaceae to pollinate in winter with the highest concentration and for the longest period in Europe.[17] High levels are recorded early in January when Cupressaceae pollen represents up to 95% of all the pollen shed in that period.[17]

### Allergens

The panel used in screening SPTs included commercial extracts (Dome/Hollister-Stier; Bayropharm, Milan, Italy) of *Dactylis glomerata* (orchard grass), *Lolium perenne* (ryegrass, perennial), *Parietaria officinalis* (wall pellitory), *Artemisia vulgaris* (mugwort), *Olea europea* (olive tree), *Betula pendula* (birch), *Corylus avellana* (hazel), *Alnus incana* (adler), *Platanus occidentalis* (sycamore), *Quercus* spp. (oak), *Tilia* spp. (lime), *Dermatophagoides pteronyssinus, D. farinae,* cat, dog, horse, *Alternaria alternata,* and *Aspergillus fumigatus.* A preliminary study was performed with three commercial allergenic *C. sempervirens* extracts available in Italy at the beginning of the study in order to select which *C. sempervirens* extract to use for screening SPT (Mari. Unpublished data). *C. sempervirens* extract from Dome/Hollister-Stier (Cs-DHS) showed the best sensitivity of the extracts tested, providing the highest rate of recognition in a group of 20 patients with suspected cypress allergy.

*C. arizonica* and *C. sempervirens* in-house allergenic extracts (Ca-IH-1 and Cs-IH-1) were prepared with pollens collected in February 1992 and March 1992, respectively. Pollen extracts were prepared within 1 month of their collection. Criteria and methods were those previously described for *C. arizonica.*[15] Briefly, for each species, pollen was defatted with ether and then extracted in 1 mmol/L aqueous bicarbonate buffer at 4° C for 48 hours. After centrifugation, a second extraction step was likewise performed on the pollen pellet. The two extracts were pooled, dialyzed, and precipitated with 80% saturated $(NH_4)_2SO_4$. The precipitate was redissolved, extensively dialyzed, lyophilyzed, and stored at 4° C under vacuum. Protein content of the freeze-dried extracts was assayed according to the method of Bradford,[24] providing 3% and 3.2% values, respectively for Ca-IH-1 and Cs-IH-1. *C. arizonica* and *C. sempervirens* extracts were also prepared in-house (Ca-IH-2 and Cs-IH-2) from pollen purchased from Allergon (Engelholm, Sweden) with the same procedure. The extraction of dry material was performed about 12 months after pollen collection. Protein contents were 2% for Ca-IH-2 and 2.8% for Cs-IH-2.

Allergenic solutions of the four in-house extracts to be used in SPTs were prepared by dissolving freeze-dried extracts in water-glycerol 50% vol/vol, at a concentration of 500 μg/ml, corresponding to a concentration of 10,000 protein nitrogen units of the commercial diagnostic

extracts. Four 10-fold dilutions in the same diluent were made, for Ca-IH-1 and Cs-IH-1, from this starting solution up to 0.05 μg/ml.

All of the in-house extracts were tested according to the European Guidelines and the European Pharmacopoeia draft monographs before they were used on human beings.[25]

Commercial nonstandardized *C. sempervirens* allergenic extracts for SPTs were supplied (fall 1993) by four European companies: Abello S.A., Madrid, Spain (1:20 wt/vol) (Cs-Abello); Dome/Hollister-Stier (10,000 protein units) (Cs-DHS); Lofarma Allergeni, Milan, Italy (1:25 wt/vol) (Cs-Lofarma); Stallergenes, Fresnes, France (1:20 wt/vol) (Cs-Stallergenes). Extracts from *C. arizonica* (Ca-Center), *Juniperus californica* (Western juniper), *J. sabinoides* (*J. asheii,* mountain cedar), *J. virginiana* (Eastern red cedar), *Taxodium distichum* (bald cypress) (all 1:20 wt/vol) were supplied by Center Laboratories, Port Washington, N.Y., and extract from *Cryptomeria japonica* (Japanese cedar, Sugi) (1:20 wt/vol) was supplied by Lofarma Allergeni. None of the subjects included in the study had ever been exposed to pollen of *Juniperus* species *(J. californica, J. sabinoides, J. virginiana)* and Taxodiaceae species *(Cryptomeria japonica, T. distichum)* chosen for testing. Positive and negative controls were represented by a histamine solution (10 mg/ml) and by a 50% glycerinated solution in water used as diluent in all SPT extracts. All allergenic extract solutions were stored at 4° C until used in SPTs.

## Skin prick tests

SPTs were performed according to standard procedure.[26] Patients were asked to avoid antihistamine intake for at least 7 days before testing (8 weeks for astemizole). Test evaluation was done after 15 minutes.

The SPT evaluation was carried out in three independent experiments in order to define the quality of the commercially available or in-house prepared allergenic extracts.

*Experiment 1.* To compare cypress allergenic extracts and to evaluate differences in the prevalence of cypress allergy, SPT results in the screening test with Ca-IH-1, Cs-IH-1, and Cs-DHS and with the panel of screening allergens were recorded in 713 unselected consecutive subjects. Patients included were those complaining of symptoms related to upper or lower respiratory tract disorders or conjunctival disease, all suspected of being allergic in nature. The age ranged between 2 and 82 years (mean ± SD, 28.5 ± 17.1; median value 26), with a male-to-female ratio of 1.05. Test responses with a wheal diameter greater than 3 mm were scored as positive.[26]

*Experiment 2.* To compare the cutaneous response to the cypress allergenic extracts used in experiment 1 and to evaluate the reactivity to these nonstandardized preparations, 46 patients who were selected on the basis of clear-cut clinical symptoms for Cupressaceae allergy (respiratory or conjunctival symptoms and signs present during the Cupressaceae pollen season with at least one episode of a strong relationship between heavy exposure and the appearance or worsening of symptoms) were tested in duplicate with commercial Cs-DHS and with Ca-IH-1 and Cs-IH-1 and their dilutions. Wheal areas, transferred on a record sheet by means of adhesive tape, were calculated by means of a computer-aided design software (Autocad 12; Autodesk, Pratteln, Switzerland). The mean value of a duplicate test was considered. Statistical analyses were performed to compare rates of positive test results and the mean of wheal areas for each SPT group.

*Experiment 3.* To compare the cutaneous response to Ca-IH-1 with responses to other Cupressaceae and Taxodiaceae pollen extracts from different sources, 53 consecutive patients selected on the basis of positive SPT response to Ca-IH-1 were tested with commercial and in-house *C. arizonica* and *C. sempervirens* extracts listed above, with extracts of *Juniperus* spp. (*J. californica, J. sabinoides, J. virginiana*) and Taxodiaceae spp. *(Cryptomeria japonica, T. distichum)*. Measurement and analysis of the results were obtained as described for experiment 2.

## Specific IgE detection

Five commercially available immunoassays for *C. sempervirens, J. sabinoides,* and *Cryptomeria japonica* serum sIgE were used: AlaSTAT system (Diagnostic Product Corp., Los Angeles, Calif.), CAP system (Pharmacia, Uppsala, Sweden), CARLA system (RADIM, Pomezia, Italy), Magic Lite SQ Allergy system (ALK Laboratories, Horsholm, Denmark), and RAB system (RADIM, Pomezia, Italy). The RAST system (Pharmacia) was used for detection of *C. sempervirens* sIgE only. Companies were invited to participate in the in vitro studies on the basis of availability of Cupressaceae or Taxodiaceae allergens on their list and acceptance of study design. All tests were performed according to the instructions of the manufacturer. Features of methods used are extensively reported in the literature.[27-31]

An ELISA for *C. arizonica* sIgE detection, developed in our laboratory, was performed as previously reported (in-house ELISA).[15] Briefly, plates were coated with *C. arizonica* extract dissolved in 0.05 mol/L carbonate buffer, pH 9.6. After washing and blocking with 3% gelatin, 1:6 diluted individual sera were added and incubated for 3 hours. After additional washings, 1:2000 diluted peroxidase-labeled rabbit anti-human IgE (KPL, Gaithersburg, Md.) was added and left overnight. After washings, the colorimetric reaction was developed, and a quantitative reading was done at 495 nm in a Microplate Reader (Bio-Rad, Richmond, Calif.). On each plate a *C. arizonica*–positive reference serum, which was assigned 10,000 arbitrary units, was tested at four dilutions (1:3, 1:6, 1:12, and 1:24). This reference serum and its working dilutions were chosen on the basis of results of preliminary experiments. Arbitrary units in the various samples were calculated by interpolation on the stan-

dard curve. The value of the mean arbitrary units plus 3 standard deviations of the mean, calculated for a group of seven normal subjects, tested as control subjects, was regarded as the negative cutoff.

Three independent experiments were set up by using immunoassays for serum sIgE detection to evaluate the sensitivity of methods and their relevance in diagnosis of Cupressaceae allergy when compared with SPT and clinical data. Serum samples were taken from patients who gave informed consent. Collected sera were stored at −20° C until they were used.

*Experiment 4.* An evaluation of the capability of systems available to recognize patients allergic to Cupressaceae was carried out in this experiment. Sera from 37 consecutive patients selected on the basis of class [++][26] or higher SPT positivity to commercial *C. sempervirens* (Cs-DHS) and positive clinical history of Cupressaceae allergy were tested for sIgE with six different methods for *C. sempervirens* and five methods for *J. sabinoides* and *Cryptomeria japonica.* Control sera were represented by 15 subjects randomly selected among those classified as having a nonatopic respiratory disorder. The nonatopic condition was verified by a negative response in screening SPTs (including Ca-IH-1, Cs-IH-1, and Cs-DHS). Eight healthy nonatopic volunteers were added. Coded sera were randomized and tested by experienced operators of each company. Class I sera or higher were considered positive for allergen sIgE.

*Experiment 5.* To evaluate differences between *C. arizonica* and *C. sempervirens* in identifying suspected allergy to Cupressaceae, sera from 54 patients were selected on the basis of clear-cut clinical symptoms for Cupressaceae allergy, as described in experiment 2. Sera were tested for sIgE with an in-house ELISA for *C. arizonica* and with the CARLA system for *C. sempervirens.* Serum samples from seven healthy subjects were used as controls. Tests were performed on coded randomized sera by a trained operator from the company and by one of the authors. Results are expressed as rates of positive test results, and comparison between the two groups was performed. *C. arizonica* and *C. sempervirens* concordance and the coefficient of correlation were also evaluated. Furthermore, patients were skin prick tested with Ca-IH-1 and Cs-IH-1 to evaluate SPT response to both allergens. SPT results were expressed as positive or negative according to criteria established earlier and were compared with IgE results.

*Experiment 6.* To further compare diagnostic results by using in vivo or in vitro tests in a large number of subjects and on the basis of the results recorded in experiment 4, the RAB system was used to detect sIgE for *C. sempervirens, J. sabinoides,* and *Cryptomeria japonica* in 606 patients, a large representative sample of the 713 subjects examined in experiment 1. The 606 subjects matched with the whole population previously examined for age, sex, and distribution of positivity to the panel of allergens tested. Cypress pollen–related symptoms were assessed on the basis of a detailed questionnaire on clinical history and by physical examination during the pollen season. Those patients who had positive elements were recorded as suspected of having a Cupressaceae pollinosis. Patients who were also sensitive to mites or pollens from hazel or alder (both species pollinating approximately in the same period as Cupressaceae) and having symptoms with a doubtful correlation to Cupressaceae pollens were excluded from the experiment. Patients were divided into four groups according to their Cupressaceae SPT response (negative response to the three cypress extracts Ca-IH-1, Cs-IH-1, and Cs-DHS [Cupressaceae $SPT^-$] or positive response to at least one of them [Cupressaceae $SPT^+$]), and to the presence of suspected allergy-related signs and symptoms in the Cupressaceae pollinating period (suspected Cupressaceae related symptoms positive or negative). A patient with RAB sIgE class I or higher for at least one of the three allergens tested in vitro was recorded as having Cupressaceae-positive sIgE.

## Statistics

Comparison of the rates of positive test results between groups with a low cumulative number of observations (<200) was performed by means of Fisher's exact test. The chi square test was used to compare rates of positive test results between groups with a higher cumulative number of observations (>200). Comparison of the mean values and distributions was performed by using the paired and unpaired Student's *t* test. Concordance between test results was evaluated by means of McNemar's test. Linear correlation between in vitro tests was evaluated by means of Pearson's correlation test. Probability *(p)* values less than 0.01 were considered statistically significant.

# RESULTS

## Experiment 1

Two hundred three patients (28.4%) had negative SPT responses to all common inhalant allergens and to the three cypress extracts. Prevalence of allergens among examined patients is shown in Fig. 1 by homogeneously grouping single allergenic extracts used, excluding cypresses. Ranking the overall value of cypress allergy prevalence among other inhalant allergens varies, depending on the positive SPT response rates of each cypress allergenic extract tested. For instance, about 31.8% of cypress positive SPT responses could be missed by using Cs-DHS.

Thirty-six patients who had positive SPT responses to cypress (5% of 713) had negative SPT responses to other allergens; 35 were identified by Ca-IH-1, 29 by Cs-IH-1, and 25 by Cs-DHS, confirming that about 30% of the SPT diagnoses could also be missed among monospecific subjects by using Cs-DHS.

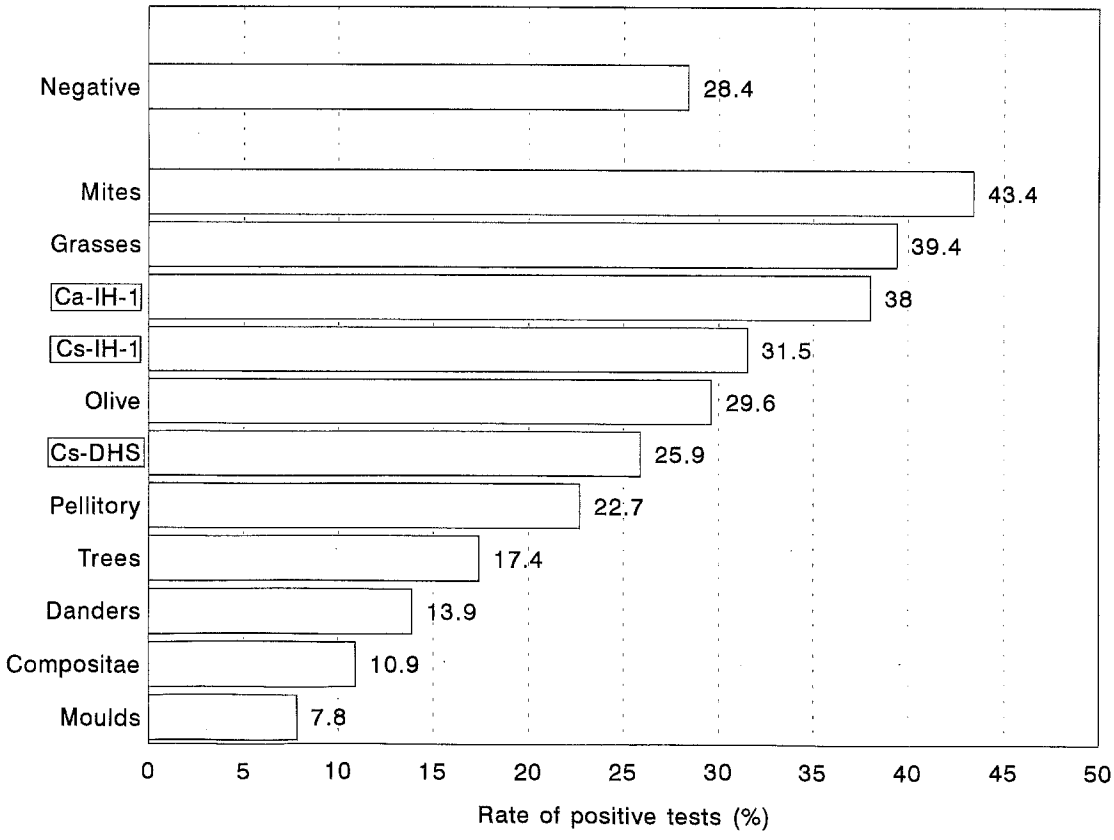


**FIG. 1.** Rate of distribution of SPT results in 713 patients with screening allergens and cypress extracts.

## Experiment 2

The number of positive SPT responses and mean values (± SD) of the wheal areas obtained with Ca-IH-1, Cs-IH-1, and Cs-DHS in 46 subjects are reported in Table I. The number of positive SPT responses did not show significant reductions in response to the 5 μg/ml dilution of Ca-IH-1, whereas there was a significant difference at 50 μg/ml for Cs-IH-1. The mean wheal area obtained with the highest concentration of Ca-IH-1 (500 μg/ml) was 3 and 4.5 times greater than those obtained with the same concentration of Cs-IH-1 and Cs-DHS, respectively (Table I). A significant reduction in the mean wheal areas of the positive SPT response was recorded at different dilutions for Ca-IH-1 and Cs-IH-1.

## Experiment 3

The number of positive SPT responses and mean values (± SD) of the wheal areas obtained in 53 consecutive subjects who had positive SPT responses to Ca-IH-1 are reported in Table II. All 53 subjects had positive SPT responses to all *C. arizonica* and *Juniperus* spp. extracts and to only one of six *C. sempervirens* extracts. Wheal areas from *C. sempervirens* extracts were the lowest obtained among Cupressaceae pollens, and their mean values, which were almost comparable among the six extracts, were confirmed as being three to four times lower than that of Ca-IH-1. Unexpected intense cutaneous reactions were recorded for commercial extracts of *J. californica, J. sabinoides, J. virginiana;* and the wheal areas were greater than those produced by Ca-IH-1 in two, eight, and seven subjects, respectively. *Cryptomeria japonica* and *T. distichum* demonstrated a quite different rate of positivity and very low cutaneous response.

## Experiment 4

Results of detection of sIgE to *C. sempervirens, J. sabinoides,* and *Cryptomeria japonica* are reported in Table III. *C. sempervirens*–positive sera ranged from 8.1% (Magic Lite) to 81.1% (CARLA). Higher positive values were recorded with all methods when *J. sabinoides* was used; the lowest

**TABLE I.** Skin prick tests with *C. arizonica* and *C. sempervirens* and their dilutions in 46 patients selected on the basis of clear-cut clinical symptoms for Cupressaceae allergy: Data and statistics

| | SPT+ | Mean wheal area ($mm^2$) ± SD | SPT+ (Fisher's exact test) | Wheal area (Student's *t* test) |
|---|---|---|---|---|
| Ca-IH-1 | | | | |
| 500 μg/ml | 46 | 89.1 ± 67.8 | vs Cs-IH-1 (500 μg/ml) NS<br>vs Cs-DHS NS<br>vs Ca-IH-1 (50 μg/ml) NS<br>vs Ca-IH-1 (5 μg/ml) NS | vs Cs-IH-1 (500 μg/ml) $p < 10^{-6}$<br>vs Cs-DHS $p < 10^{-6}$<br>vs Ca-IH-1 (50 μg/ml) $p < 10^{-5}$ |
| 50 μg/ml | 45 | 40.2 ± 20.5 | vs Cs-IH-1 (50 μg/ml) NS<br>vs Ca-IH-1 (5 μg/ml) NS | vs Cs-IH-1 (50 μg/ml) $p < 10^{-6}$<br>vs Ca-IH-1 (5 μg/ml) $p < 0.0001$ |
| 5 μg/ml | 41 | 25.8 ± 11.4 | vs Cs-IH-1 (5 μg/ml) $p < 10^{-8}$ | vs Cs-IH-1 (5 μg/ml) $p < 0.008$<br>vs Ca-IH-1 (0.5 μg/ml) $p < 0.005$ |
| 0.5 μg/ml | 27 | 18.4 ± 9.2 | vs Cs-IH-1 (0.5 μg/ml) $p < 10^{-8}$ | vs Ca-IH-1 (0.05 μg/ml) NS |
| 0.05 μg/ml | 7 | 16.2 ± 5.6 | vs Cs-IH-1 (0.05 μg/ml) $p < 0.005$ | |
| Cs-IH-1 | | | | |
| 500 μg/ml | 45 | 31.0 ± 16.2 | vs Cs-DHS NS<br>vs Cs-IH-1 (50 μg/ml) $p < 0.002$ | vs Cs-DHS $p < 0.0002$<br>vs Cs-IH-1 (50 μg/ml) $p < 10^{-5}$ |
| 50 μg/ml | 39 | 18.8 ± 7.4 | | vs Cs-IH-1 (5 μg/ml) NS |
| 5 μg/ml | 10 | 15.5 ± 5.7 | | |
| 0.5 μg/ml | 1 | 14.7 | | |
| 0.05 μg/ml | — | — | | |
| Cs-DHS (10,000 PU) | 45 | 20.2 ± 9.4 | | |
| Histamine | 46 | 51.3 ± 32.3 | | |

*NS,* Not significant; *PU,* protein units.

was 64.9% (Magic Lite), whereas CARLA showed positivity in all 37 sera (100%). *Cryptomeria japonica* sIgE was measured in 35.1% of the subjects tested with Magic Lite, and RAB showed positivity in 89.2% of the sera.

Unexpected results were obtained in two of 15 subjects with generic symptoms suspected of being allergic, who had negative SPT responses, and were therefore classified as having a nonatopic respiratory disorder. The first patient had class II positive responses to *J. sabinoides* when the sera were tested with the CARLA method and class I responses to the same allergen when AlaSTAT and CAP were used. The second patient had class I responses to *J. sabinoides* and *Cryptomeria japonica* when the CARLA method was used and to *C. sempervirens* when AlaSTAT was used. Both patients were recalled and tested by SPT with Cs-DHS. Negative SPT responses were recorded again. A clinical follow-up visit during the subsequent cypress pollen season allowed us to record a worsening of symptoms, previously reported as a fall-to-spring rhinitis. These findings prompted us to set up experiment 6 to evaluate the presence of sIgE to *C. sempervirens, J. sabinoides,* and *Cryptomeria japonica* in a larger number of subjects with negative SPT responses to cypress extracts.

### Experiment 5

Thirty-eight of 54 (70.3%) sera were positive to *C. arizonica* according to in-house ELISA, whereas 41 of 54 (75.9%) were positive to *C. sempervirens* with CARLA. Differences in positive rates were not statistically significant. McNemar's test for concordance of positive results did not demonstrate any differences. Fig. 2 shows the distribution of values of sIgE to *C. arizonica* and *C. sempervirens* and their linear correlation. Table IV shows distribution of sera tested in this experiment according to SPT response to Ca-IH-1 and Cs-IH-1 and to the in vitro responses to the same pollen species. Comparison of in vitro and in vivo test results for *C. arizonica* and *C. sempervirens* provided a complex pattern of positive and negative responses to the allergenic extracts tested, showing differences in in vitro and in vivo responses to the allergens of these two species. Some patients with negative sIgE to one of the two cypresses tested had positive SPT responses when the same allergen was used.

### Experiment 6

The choice of the method to be used in this part of the study was based on detection of positive sIgE to at least one of the three allergens (*C. sempervirens, J. sabinoides,* or *Cryptomeria ja-*

**TABLE II.** Skin prick tests with Cupressaceae and Taxodiaceae pollen extracts in 53 consecutive patients with positive SPT responses to the in-house extract of *C. arizonica:* Data and statistics

| | SPT+ | Mean wheal area ($mm^2$) ± SD | SPT+ (Fisher's exact test) | Wheal area (Student's *t* test) |
|---|---|---|---|---|
| Ca-IH-1 | 53 | 59.5 ± 33 | vs Cs-DHS $p < 0.003$<br>vs Cs-Lofarma $p < 0.0005$<br>vs *Cryptomeria japonica* $p < 10^{-6}$<br>vs *Taxodium distichum* $p < 10^{-6}$ | vs Ca-IH-2 $p < 0.003$<br>vs Ca-Center $p < 10^{-6}$<br>vs Cs-IH-1, Cs-IH-2, Cs-Abello, Cs-DHS, Cs-Lofarma, Cs-Stallergenes $p < 10^{-6}$<br>vs *Juniperus californica, Juniperus virginiana* $p < 0.0001$<br>vs *Juniperus sabinoides* $p < 0.01$<br>vs *Cryptomeria japonica, Taxodium distichum* $p < 10^{-6}$ |
| Ca-IH-2 | 53 | 35.7 ± 13.5 | (see Ca-IH-1) | vs Ca-Center $p < 0.00001$<br>vs Cs-IH-1, Cs-IH-2, Cs-Abello, Cs-DHS, Cs-Lofarma, Cs-Stallergenes $p < 10^{-6}$ |
| Ca-Center | 53 | 21.5 ± 10.2 | (see Ca-IH-1) | vs Cs-IH-1, Cs-Abello, Cs-DHS, Cs-Lofarma $p < 0.005$ |
| Cs-IH-1 | 51 (96.2%) | 15.4 ± 7.7 | | |
| Cs-IH-2 | 53 | 19.8 ± 5.8 | (see Ca-IH-1) | vs Cs-Lofarma $p < 0.001$ |
| Cs-Abello | 48 (90.6%) | 15.9 ± 8.7 | | |
| Cs-DHS | 45 (84.9%) | 15.1 ± 6.6 | | |
| Cs-Lofarma | 40 (75.5%) | 13.4 ± 6.1 | | |
| Cs-Stallergenes | 52 (98.1%) | 18.9 ± 8.4 | | |
| *Juniperus californica* | 53 | 34.5 ± 23.8 | (see Ca-IH-1) | vs Ca-Center $p < 0.0005$ |
| *Juniperus sabinoides* | 53 | 44.5 ± 25.8 | (see Ca-IH-1) | vs Ca-Center $p < 10^{-6}$ |
| *Juniperus virginiana* | 53 | 38.9 ± 18 | (see Ca-IH-1) | vs Ca-Center $p < 10^{-6}$ |
| *Cryptomeria japonica* | 38 (71.1%) | 15.7 ± 8.4 | | |
| *Taxodium distichum* | 20 (37.7%) | 11.7 ± 5 | | |
| Histamine | 53 | 42.1 ± 15.4 | | |

*ponica*) tested in experiment 4. The following results were recorded: 37 of 37 (100%) subjects had positive results with CARLA, 34 of 37 (91.9%) with RAB, 33 of 37 (89.2%) with AlaSTAT and CAP, and 26 of 37 (70.2%) with Magic Lite. Although the highest sensitivity was obtained with the CARLA immunoassay, its commercial unavailability at the time of the study prevented the testing of a large number of sera and forced us to choose the second method, RAB, to detect serum sIgE to *C. sempervirens, J. sabinoides,* and *Cryptomeria japonica* in experiment 6. Table V shows rates of detection of positive sIgE to at least one of the three allergens tested in the four selected groups. The difference between positive sIgE rates of patients with positive SPT responses to Cupressaceae, with or without suspected cypress-related symptoms, was not statistically significant, whereas rates of in vitro test positivity in patients with negative SPT responses to Cupressaceae, with or without a suspected clinical history of Cupressaceae allergy, were significantly different ($p < 10^{-6}$). Nineteen of 30 patients with negative Cupressaceae SPT responses, positive Cupressaceae sIgE, and suspected Cupressaceae-related allergy were recalled and skin prick

**TABLE III.** Rate of positivity of 37 sera tested for specific IgE to *C. sempervirens, J. sabinoides,* and *Cryptomeria japonica* by means of six different methods

| Methods | Allergens | | |
|---|---|---|---|
| | *C. sempervirens* | *J. sabinoides* | *Cryptomeria japonica* |
| AlaSTAT | 46 | 83.8 | 78.4 |
| CAP | 70.3 | 89.2 | 59.5 |
| CARLA | 81.1 | 100 | 81.1 |
| Magic Lite | 8.1 | 64.9 | 35.1 |
| RAB | 19.1 | 70.3 | 89.2 |
| RAST | 10.8 | NT | NT |

Sera were selected on the basis of class [++] or higher SPT positivity to *C. sempervirens* (Cs-DHS) and positive clinical history. Results equal to or greater than class I were considered sIgE-positive.
*NT,* Not tested.

tested again with Ca-IH-1, Cs-IH-1, and Cs-DHS. In all of these patients, negative SPT results were confirmed.

## DISCUSSION

At present, *C. arizonica* and *C. sempervirens* are the most common Cupressaceae species in Italy and other countries.[4, 7, 9, 13]

The hypothetical underestimation of the real prevalence of cypress allergy, which might be due to the low potency of available allergenic extracts,[12, 19] is demonstrated in experiment 1. In fact, cypress hypersensitivity prevalence, among the population studied, differed when commercial *C. sempervirens* extract versus Cs-IH was used (+5.6% patients identified, +18% cypress-positive) or even more when commercial *C. sempervirens* extract versus Ca-IH was used (+12.1% patients identified, +32% cypress-positive). The cutaneous response to *C. sempervirens* extracts measured in selected patients (experiments 2 and 3) was always low when compared with those obtained with *C. arizonica* or with juniper extracts and comparable to that reported by other authors.[19, 32] Because both *C. arizonica* and *C. sempervirens* in-house extracts contained very low protein concentrations, a rather low or incomplete presence of allergenic components in the *C. sempervirens* extracts may account for the poor SPT response. This feature is common to all *C. sempervirens* extracts tested here, suggesting that the production of an effective in vivo diagnostic tool for detection of cypress allergy, starting from *C. sempervirens* pollen, may be problematic, as reported by Bousquet et al.[19] Moreover, the significant loss of potency observed after the first dilution of the Cs-IH makes this allergenic extract unsuitable for clinical studies in which SPT end-point titration is needed.

On the other hand, pollen extracts obtained from *C. arizonica* gave rise to a more effective SPT response. Ca-IH-1 was able to identify a much higher number of subjects in the unselected population of experiment 1, almost all showing a clear-cut cutaneous response. Comparison of the SPT wheal areas showed that the greatest cutaneous reactions were recorded by using Ca-IH-1, with no statistically significant variation in the rate of positive SPT responses from 500 μg/ml down to the 5 μg/ml concentration, indicating the high sensitivity of the extract and suggesting a considerable safety margin until batch to batch standardization is available. Ca-IH-2, obtained by means of the same method as Ca-IH-1,[15] but with a 1-year interval between pollen collection and extraction, showed an intermediate SPT activity between Ca-IH-1 (extracted soon after collection) and Ca-Center, the latter not being characterized in terms of protein contents and profile. These differences could be related to spontaneous degradation of allergenic components when the pollen is stored for a long period before extraction, even if dried.[33] In any case, the activity of Ca-IH-2 in SPT was greater than that of *C. sempervirens* extracts.

Comparison of data obtained with six commercially available methods showed variable rates of subjects with undetectable sIgE to *C. sempervirens* pollen extract. Three of six immunoassays (Magic Lite, RAB, RAST) provided positive results in a number of sera lower than 20%, whereas two methods (CAP and CARLA) provided useful results similar to those available for other allergens.[27, 28] These results indicate that some in vitro systems can be successfully used as a complementary tool in cypress allergy diagnosis and can partially change the common view of the "unusefulness" of detection of sIgE for *C. sempervirens* pollen.

Interestingly, in CAP and CARLA systems the same allergenic extracts of RAST and RAB, respectively, are used (Dr. Daniela Zelaschi, Pharmacia, and Dr. Virgilio Olivieri, RADIM, personal communications). Thus the remarkable enhancement of the results between CAP and RAST and between CARLA and RAB could be mainly due to the increased availability of allergenic epitopes for IgE binding and/or the consequent reduction of specific IgG[15] competition. A similar enhancement

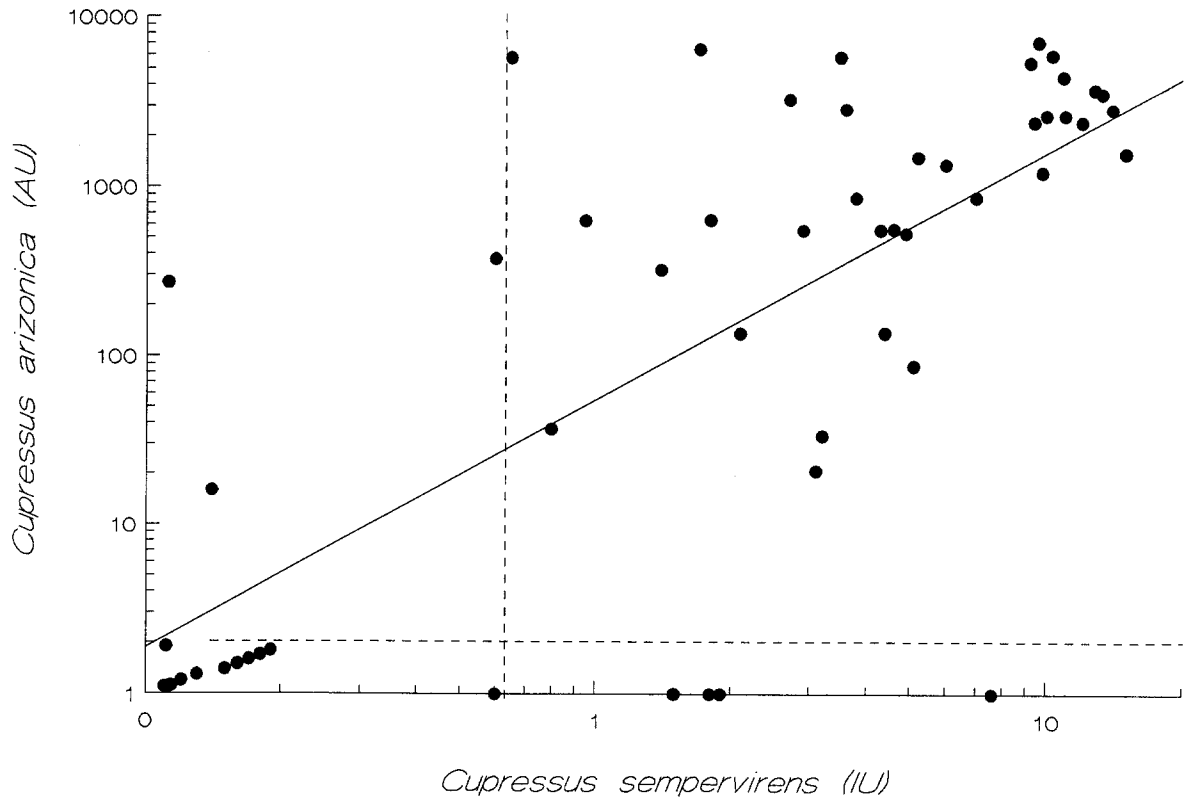


**FIG. 2.** Linear correlation of 54 sera tested for specific IgE to *C. arizonica* with an in-house ELISA and specific IgE to *C. sempervirens* with a commercial immunoassay (CARLA). A positive correlation was found with a Pearson's correlation coefficient of 0.52 ($p < 0.0005$). Sera were selected on the basis of clear-cut cypress-related symptoms. *Dashed lines* indicate negative cutoff limits. *AU,* Arbitrary units; *IU,* international units.

was obtained by Ford et al.[6] by coupling their in-house prepared and characterized *C. sempervirens* extracts to nitrocellulose disks.

In experiment 5 in vitro *C. arizonica* sIgE detection confirmed the rate of positive test results reported in a previous article,[15] whereas results recorded for *C. sempervirens* did not differ statistically from those obtained with *C. arizonica* in-house ELISA. Comparison of in vitro and in vivo tests for *C. arizonica* and *C. sempervirens* provided a complex pattern of positive and negative responses to the allergenic extracts tested, showing differences in in vitro and in vivo responses to the allergens of these two species. Some patients with negative sIgE to one of the two cypresses tested had positive SPT responses when the same allergen was used. Because the population examined was exposed to both *C. arizonica* and *C. sempervirens,* this result could be explained either by an almost complete cross-reactivity with different patterns of IgE and SPT reactivity in each subject or by a distinct response to different allergenic determinants present in both pollens. This aspect is currently under investigation.

**TABLE IV.** Skin prick tests with *C. arizonica* and *C. sempervirens* in 54 subjects selected on the basis of clear-cut clinical symptoms for Cupressaceae allergy and tested for specific IgE to both cypress allergens

| | Specific IgE | | | |
|---|---|---|---|---|
| **SPT response** | **$Ca^+/Cs^+$ ($n = 36$)** | **$Ca^+/Cs^-$ ($n = 2$)** | **$Ca^-/Cs^+$ ($n = 5$)** | **$Ca^-/Cs^-$ ($n = 11$)** |
| $Ca^+/Cs^+$ ($n = 43$) | 32 | 1 | 5 | 5 |
| $Ca^+/Cs^-$ ($n = 7$) | 2 | 1 | — | 4 |
| $Ca^-/Cs^+$ ($n = 4$) | 2 | — | — | 2 |

*Ca, C. arizonica; Cs, C. sempervirens.*

*Juniperus* species, which are entirely absent in the area studied,[34] produced SPT results compara-

**TABLE V.** Specific IgE for *C. sempervirens, J. sabinoides,* and *Cryptomeria japonica* in 606 subjects grouped for SPT responses and clinical symptoms

| Cupressaceae SPT response | Suspected Cupressaceae-related symptoms | Subjects | Cupressaceae sIgE$^+$ |
|---|---|---|---|
| 1. Positive | Positive | 201 (33.2%) | 132 (65.7%) |
| 2. Positive | Negative | 43 (7.1%) | 29 (67.4%) |
| Total (1 + 2) | | 244 (40.3%) | 161 (66%) |
| 3. Negative | Positive | 75 (12.4%) | 30 (40%) |
| 4. Negative | Negative | 287 (47.3%) | 22 (7.6%) |
| Total (3 + 4) | | 362 (59.7%) | 52 (14.4%) |

Cupressaceae positive SPT indicates positive cutaneous test response to at least one of the three allergenic extracts used in the screening SPT; Cupressaceae positive sIgE indicates specific IgE for at least one of the three allergens tested. Percentages in brackets refer to all subjects tested (column subjects) and to the number of subjects in the group (column Cupressaceae sIgE$^+$).

ble to *C. arizonica* and greater than *C. sempervirens.* Specific IgE detection for *J. sabinoides* produced results greater than those for *C. sempervirens,* ranging from 64.9% to 100%. These results are in agreement with some previously published data obtained from skin testing,[35] immunodiffusion with rabbit polyclonal antibodies,[35] sIgE detection,[22] which accounted for a high degree of cross-reactivity among Cupressaceae pollens. No or few negative results obtained with *J. sabinoides* in experiment 4 by means of the five commercial methods used, together with the high cutaneous response, suggest that this allergen should be used, in addition to *C. sempervirens* and *C. arizonica,* to complete the panel of extracts required for the diagnosis of Cupressaceae allergy.

Taxodiaceae pollen extracts *(Cryptomeria japonica* and *T. distichum)* were also included in this study because of the clear documented cross-reactivity between *Cryptomeria japonica* and *C. sempervirens.*[21, 22] None of the subjects tested had ever been exposed to the pollen of those two trees, and positive SPT and sIgE results confirm cross-reactivity between species classified by botanists as belonging to two different families.

The results obtained in the group of 15 control subjects with nonatopic respiratory disorders in experiment 4 allowed us to identify two patients with negative SPT responses to all the allergens tested (including Ca-IH-1, Cs-IH-1, and Cs-DHS) and with IgE specific for *C. sempervirens* and/or *J. sabinoides* and/or *Cryptomeria japonica.* Detection of sIgE to at least one of the three allergens used in experiment 6 showed that when a large number of patients with positive SPT responses to Cupressaceae are screened, a lower rate of positive results is recorded (66% of 244) when compared with results from highly selected patients recorded in experiment 4 in which the same method was used (91.9% of 37). Nevertheless, the use of detection of serum sIgE to all Cupressaceae and Taxodiaceae available allergens is of great value in subjects with suspected clinical allergy to Cupressaceae but with negative SPT responses to these pollens (12.4% of the population examined in our study). Forty percent of these patients, who have signs and symptoms of suspected cypress allergy but have negative SPT responses to Cupressaceae, were identified as having Cupressaceae hypersensitivity by the positivity against at least one of the allergens tested in vitro *(C. sempervirens, J. sabinoides, Cryptomeria japonica).*

Cupressaceae pollen monitoring in the atmosphere should always be carried out, and patients allergic to Cupressaceae or Taxodiaceae species should be carefully screened when the presence of these pollens is recorded, even when in low concentrations.[12] A higher overall prevalence of cypress positive SPT responses (about 40%) has been reported in the population of this study by using a better characterized allergenic extract of *C. arizonica. C. sempervirens* extracts for in vivo use require further purification and characterization studies.

Clinical characteristics, mostly when evaluating monosensitized patients,[10] and a low sensitivity of most of the in vivo diagnostics available could be the major causes of the underestimation of this winter pollinosis. Epidemiologic studies should be carried out to clearly define the prevalence of Cupressaceae allergy in the whole population by using validated diagnostic tools. An integrated use of broad spectrum–characterized diagnostic allergenic extracts for SPTs and serum sIgE detection will facilitate the diagnosis of Cupressaceae allergy.

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