

Isolation and partial characterization of *Cupressus sempervirens* allergens

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SUMMARY. Allergic rhinitis due to cypress pollen is a well-recognized clinical condition that is evermore frequently diagnosed as a winter allergy in the Mediterranean area. Little is known about the allergen composition of its pollen and the dynamics of its immune response. For these reasons IgE-specific antibody distribution was determined and the allergenic pollen characterized. SDS-PAGE analysis of the whole extract revealed more than ten proteic bands ranging from 10 to 90 kD. The major allergen had a molecular weight of 36 kD and the specific IgE antibody was present in >95% of the sera tested by immunoblotting technique. Two minor allergens were observed at the 43 and 49 kD protein bands. The results obtained with the whole extract were then compared to those of five commercially available preparations.

Key words: cypress allergens, immunoblotting, SDS-PAGE.

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INTRODUCTION

Pollen of *Cupressus sempervirens* is an increasing and significant cause of pollinosis in central Italy, where this species — Cupressaceae family (Negrini, 1985, 1987) — is widely distributed (Ariano, 1985, 1988; Ballero, 1986; Bousquet, 1984; Caiaffa, 1987; Rottoli, 1984; Tas, 1965).

The cypress inflorescences usually appear in winter, from December to March (Michel, 1978; Negrini, 1987) and generally cause a seasonal allergic rhinitis or oculorhinitis (Ordman,

1970; Panzani, 1962, 1986), often mistaken as infectious. Because of this confusion, its incidence is certainly underestimated.

The cypress-sensitive patients are usually monosensitive and complain of serious symptoms.

At our Institute, during the last 24 months, we have observed some cases of cypress pollinosis, chiefly in monosensitive patients with winter oculorhinitis. The diagnosis was done on the basis of a significant concordance between case history and skin prick tests. On the other hand, poor correlation was observed between

these outcomes and Rast (Radio Allergo Sorbent Test) results, and the sporadic attempts at specific immunotherapy were rarely successful.

With respect to the standardization of commercial reagents for diagnosis and for sensitization therapy (Budens *et al.*, 1989; Goetz *et al.*, 1989; Gross *et al.*, 1978; Hale and Hutchinson, 1986; Ishizaka, 1984; Löwenstein, 1980, 1983, 1985, 1987; Marsh *et al.*, 1987; Sacchi and Tassi, 1982; Schweitz, 1989; Yunginger, 1984, 1988), we prepared an extract from fresh cypress pollen; then we identified the major and minor allergenic protein fractions and compared them with commercial extracts.

MATERIALS AND METHODS

Cupressus sempervirens pollen collection

The mature inflorescences from *Cupressus sempervirens* trees were collected and brought to our laboratory, from January to March. The released pollen was shaken on glossy brown paper and then purified by sequential sieving. By observation with light microscopy, the obtained pollen seems pure.

Patients and controls

Sera were taken from 10 patients with documented positive clinical histories and positive reactivity to cypress mixtures, as determined by skin prick testing (Bayropharm Italiana srl, Milano, Italy). Four of these patients had been previously diagnosed as cypress sensitive and were receiving immunotherapy, whereas the remaining patients were newly diagnosed. Sera were stored at -20°C in small aliquots, prior to use. All patient's sera were screened by Rast (Pharmacia, Uppsala, Sweden) as previously described (Johansson and Yman, 1988). Ten atopic non cypress-sensitive subjects, with skin reactivity to at least four other aeroallergens were also enrolled in the study.

Fifteen non-atopic control sera were obtained from individuals with no history of allergic disease and no skin reactivity to a panel of 24 seasonal and perennial allergens.

Preparation of the fresh pollen extract

Cupressus sempervirens pollen was extracted in 0.001 M ammonium bicarbonate at a ratio of 1 gm of pollen to 10 ml of solution (1:10 wt/wl). The suspension was kept overnight at 4°C in a Sorvall RC5 Superspeed Refrigerated Centrifuge (Du Pont Instruments). About 0.4% of dry pollen weight was recovered in the supernatant, as determined by Bio-Rad protein Assay (Bio-Rad Laboratories, Richmond, CA, USA). The extract was then concentrated about threefold by polyethylene glycol in dialysis tubing (Bethesda Research Laboratory, Bethesda, MA, USA), and finally stored at -20°C .

Sodium dodecyl sulphate (SDS)-polyacrylamide gradient gel electrophoresis (PAGE) and western immunoblotting

SDS-PAGE was performed essentially according to the method of Laemmli (Laemmli, 1970), under reducing conditions.

A continuous gradient of 7.5-14% acrylamide was used. The mobility of the bands was measured as described by Weber and Osborn (1969) by comparison with low molecular weight markers (Bio-Rad Laboratories). PAGE was carried out on 0.75 mm thick «minigels», at 12 mA until the dye front had reached the bottom of the gel.

The gels were immediately equilibrated in 0.025 mol/L of Tris HCl (pH 8.3), 0.192 mol/L of glycine, 20% methanol (transfer buffer), and blotted onto 0.22 μm NC paper with a Bio-Rad transblot system. Transfer was carried out for 120 minutes at 400 volts, according to the method of Towbin (1979). Adequate transfer of

proteins to the NC sheets was demonstrated by cutting a strip from the NC sheets and staining it with Amido black. The remainder of the NC sheets were immediately put in a blocking buffer: 10% Goat Serum and 3% Non Fat Dry Milk in Phosphate Buffered Saline (PBS) (pH 7.4). This solution was used to block all the unbound sites on the NC membranes and for the dilution of all sera and alkaline phosphatase-conjugated Goat Anti-Human IgE and IgG (GAHIgE - GAHIgG, Sigma Chemical Company, St. Louis, MO, USA), as described below. To block unbound sites, the NC sheets were incubated in blocking buffer overnight at 4°C. The sheets were then cut into 4 mm strips and transferred to an individual well.

Sera from atopic cypress-sensitive, atopic non cypress-sensitive and non-atopic subjects were diluted 1:2 and 0.5 ml was added to each individual well. The NC strips were incubated overnight at room temperature with constant agitation. The sera were then removed and the strips washed for two hours (four changes of washing buffer: PBS pH 7.4, 0.1% Tween 20). One milliliter of GAHIgE, GAHIgG and four non conjugated mouse monoclonal anti-human IgG subclasses (ICN Immunobiologicals, The Binding site Ltd, UK) were added to each well and incubated for three hours with constant agitation.

All the strips were washed (three changes of 20 minutes). For the IgG subclass detections, a further two-hours incubation with alkaline phosphatase anti-mouse IgG (Sigma) was necessary.

For enzymatic detection of bound IgE, IgG and IgG subclasses, the strips were finally incubated in an alkaline phosphatase substrate. The color reaction was allowed to develop until no further staining was observed, usually 20-30 minutes, and then stopped in distilled/deionized water and air dried.

RESULTS

The collection of cypress pollen was carried out in January 1990, according to the well-demonstrated seasonal (winter) prevalence of this pollen in central Italy (Fig. 1).

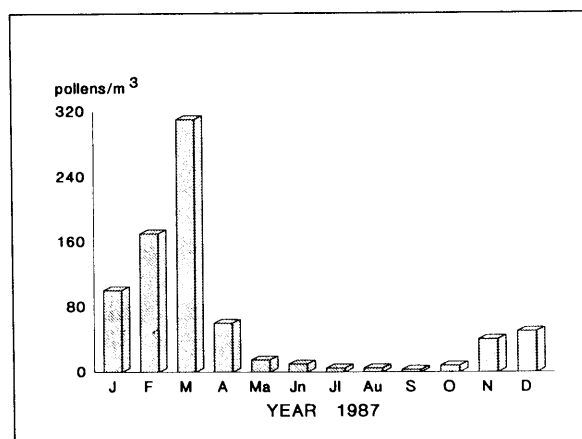


Figure 1. Cypress pollen counts (monthly average particles per cubic meter of sampled air) observed in Perugia, central-Italy, during 1987.

The electrophoretic transfer of cypress pollen extract separated by SDS-PAGE was satisfactory over the entire molecular weight range: there was little or no residual protein remaining in the SDS gel after transfer. As illustrated in Figs. 2 and 3, the banding pattern of the Coomassie blue-stained SDS polyacrilamide gel and the Amido black-stained immunoblot were the same, indicating that significant pollen allergens would not be missed because of ineffective transfer from SDS gel to NC sheets. Both the SDS polyacrilamide gel (Fig. 2) and NC sheets (Fig. 3) showed eleven protein bands, fractionated from fresh pollen extract: M.V. 97, 66, 63, 57, 49, 36, 22, 14 and d12 kD.

Some of these were very intense (36 kD, 43 kD: Fig. 3) while others were not as distinct, but nonetheless observable.

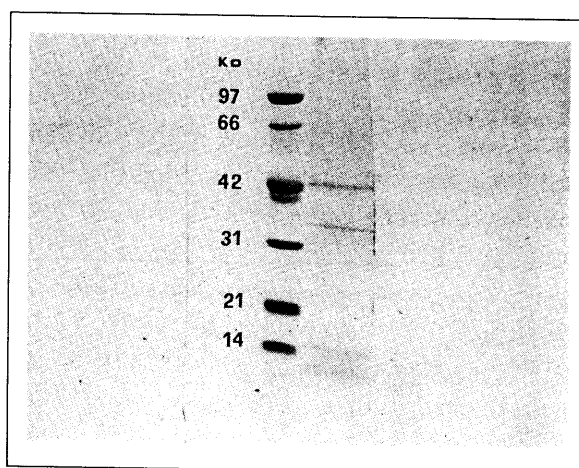


Figure 2. Coomassie-blue staining of SDS gel of cypress pollen extract. On the left, protein molecular weights standards are, from the top: 97.400 kD (rabbit muscle phosphorylase b), 66.200 kD (bovine serum albumin), 42.699 kD (Henn egg albumin), 31.000 (bovine carbonic anhydrase), 21.500 kED (soybean trypsin inhibitor), 14.400 kD (hen egg white lysozyme).

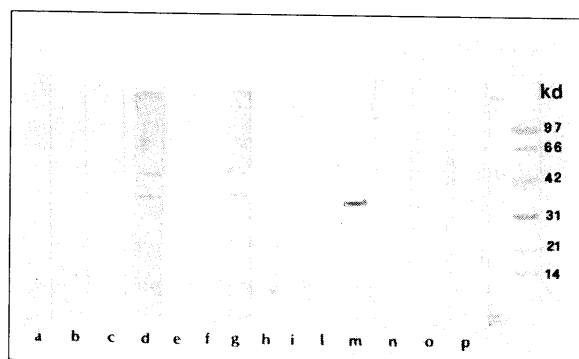


Figure 3. Cypress pollen immunoblots with alkaline-phosphatase-conjugated anti-human IgE. Lanes a and b: sera from non-atopic subjects; lane c: serum from atopic non-cypress sensitive control subjects. Lanes from d to p: sera from atopic cypress-sensitive patients.

In lines a to c three of the total control sera are represented (10 atopic and 15 non-atopic) studied.

On the other hand, none of the five commercially available cypress extracts showed evident Coomassie-blue-stained proteic bands on SDS-PAGE, neither specific IgE binding (data not shown).

The results of immunoblot analysis indicated that many of electrophoretically resolvable polypeptides were able to induce IgE and IgG antibodies in susceptible persons; otherwise, not all subjects were able to produce antibodies to all these polipeptides.

None of the sera from non atopic and atopic non cypress sensitive controls were reactive with GAHIgE (Fig. 3).

All the 11 cypress-sensitive subjects displayed one or more positive bands, as detected by GAHIgE. It is evident from Fig. 3 that cypress sensitive patients generate IgE that are directed against a limited spectrum of pollen proteins, the most common of which had a M.W. of 36 kD (100% sera). The same Fig. shows that for three patients exclusively this band was detected.

The 43 kD protein was detected in seven sera (Fig. 3: sera d, e, g, i, n, o, p), with a percentage of reactivity of 63.6%. These two bands (36 and 43 kD) were, therefore, the major allergen detected by immunoblotting.

On the other hand, four patients had serum IgE (Fig. 3): e, h, o, p) that bound the 49 kD (36.3% of reactivity) and two (Fig. 3: d and h) that recognized higher molecular weight proteins: 66, 63 and 57 kD (18% of reactivity).

None of the tested subjects showed an appreciable reactivity against the two low-molecular weight (14 and 12 kD) bands.

CONCLUSIONS

The three most frequent specific patterns of immune response to cypress allergens were represented by different IgE-binding activity, corresponding to: a) 36-43 kD bands, b) 36-43-49 kD bands and c) 36 kD band only.

As expected after immunotherapy the IgG4-specific response in the four cypress-sensitive patients was weak. Interestingly, there was a

strong reactivity against two low MV polypeptides (12 and 14 kD) that probably represent the antigenic composition and/or a contamination of the commercial extracts.

From a clinical point of view it may be relevant to investigate whether specific patterns of response are predictive of the outcome of therapy and/or correlated with symptoms. To this purpose the number of studied sera is too exiguous and doesn't allow to draw general conclusions.

However, there was a relationship between the degree of sensitivity, as confirmed by symptoms and skin test reactivity, and the number and strength of positive IgE binding proteins (Fig. 3: the sera d, g and m were from three patients with very serious symptoms and strong skin reactivity).

The present study on the characterization of cypress pollens, like all studies on allergenic extracts with western blotting methods (Gross, *et al.*, 1978; Hale and Hutchinson, 1986; Löwenstein, 1980; Schweitz *et al.*, 1989), reveals that SDS-PAGE-containing polypeptides binds to IgE patients known to be cypress-sensitive. Clearly it does not reveal which of these bands contains the proteins that are responsible for the allergic disease. Nonetheless, it is unreasonable to assume that all or a fraction of the bands detected contain true allergens. Thus the western blotting provides an ideal starting point for the purification and characterization of allergens and a simple, reproducible method for the comparison of pollen extracts (Löwenstein, 1985; Marsh *et al.*, 1987; Sacchi and Tassi, 1982).

Further investigations will be needed for biochemical and immunological characterization of these allergens, that will allow a future use of purified extracts for both diagnostic and therapeutic approach.

REFERENCES

- ARIANO R., (1985) — *Allergia al polline del Cipresso in Liguria*. Atti del 17° Congr. Naz. Soc. It. Allerg. Immunol. Clin., Milano.
- ARIANO R., (1988) — *Allergia respiratoria al polline di «Cupressaceae»*. Foglia Allerg. Immunol. Clin.
- BALLERO M., PIU G., SECHI P., (1986) — *Ricerche sulle concentrazioni aeropolliniche di «Cupressaceae» e «Pinaceae» nell'atmosfera di Cagliari e considerazioni sul loro potere allergizzante*. Aero-biologia, **2**:41.
- BOUSQUET J., COUR P., GUERIN B., (1984) — *Allergy in Mediterranean area. I. Pollen counts and pollinosis of Montpellier*. Clin. Allergy, **14**:233.
- BUDENS R.D., STASTNEY P., SULLIVAN T.J., (1989) — *Studies of the immunochemistry and immunogenetics of mountain Cedar allergy*. Abstracts - Forty-fifth Annual Meeting of the American Academy of Allergy and Immunology. San Antonio, Texas.
- CAIAFFA M.F., BASILETTO G., CARBONARA A.M., GATTI E., STRADA S., TURSI A., (1987) — *Le pollinosi da «Cupressaceae» in Puglia: aspetti aerobiologici e clinici*. Atti del corso di Aerobiologia. Bisceglie.
- GOETZ D.W., VAUGHN M.P., PATTERSON W.R., REID M.J., (1989) — *Monoclonal antibody of epitopes shared by the major allergen and other glycoproteins of Mountain Cedar (MC) pollen*. Abstract - Forty-fifth Annual Meeting of the American Academy of Allergy and Immunology. San Antonio, Texas.
- GROSS G.N., ZIMBUREAN J.M., CAPRA J.D., (1978) — *Isolation and partial characterization of the allergen in mountain cedar pollen*. Scand. J. Immunol., **8**:437.
- HALE R., HUTCHINSON K., (1986) — *Standardization of allergenic extracts*. Annals of Allergy, **56**:103.
- ISHIZAKA T., (1984) — *Japanese cedar pollinosis*. 40th Annual Congress American College of Allergists. Abstracts, **52**:25.
- JOHANSSON S.G.O., YMAN L., (1988) — *In vitro assays for immunoglobulin E. Methodology, indications and interpretation*. Clinical Reviews in Allergy, **6**:93-139.
- LAEMLI U.K., (1970) — *Cleavage of structural proteins during the assembly of the head of the bacteriophage T4*. Nature, Lond., **227**:580-685.
- LÖWENSTEIN H., (1980) — *Physico-chemical and immunochemical methods for the control of potency and quality of allergenic extracts*. Regulatory control and standardization of allergenic extracts. Brede Going Ed., Fisher Verlag, New York.

- LÖWENSTEIN H., (1983) — *Report of behalf of the International Union of Immunological Societies (IUIS)*. Arbeiten aus dem Paul Ehrlich Institut, F. Verlag, Ed. Stuttgart-New York.
- LÖWENSTEIN H., (1985) — *Allergen standardization for clinical investigation*. Allergy Today, **2**:58.
- LÖWENSTEIN H., (1987) — *Selection of reference preparation. IUIS reference preparation criteria*. International Union of Immunological Societies, Arb. Paul Ehrlich Inst., **80**:75-78.
- MARSH D.G., GOODFRIEND L., PIAO KING T., LÖWENSTEIN H., PLATTS-MILLS T.A.E., (1987) — *Allergen nomenclature*. J. Allergy Clin. Immunol., **80**:639.
- MICHEL F.B., DHIVET H., MARTY J.P., (1978) — *Polinoses invernales*. Rev. Fr. Allergol., **18**:83.
- NEGRINI A.C., EBBLI A., TROISE C., VOLTOLINI S., (1987) — *La sensibilizzazione al polline delle piante arboree. I. Note botaniche e rassegna dalla letteratura*. Folia Allergol. Immunol., **34**:57.
- ORDMAN D., (1970) — *Cypress pollinosis in South Africa. Study of seasonal hayfever and allergic conjunctivitis occurring in winter-spring period*. South Africa Med., **19**:739.
- PANZANI R., (1962) — *L'allergie respiratorie aux pollens de Conifères*. Rev. Fran. «Allergie», **2**:164.
- PANZANI R., CENTANNI G., BRUNNEL M., (1986) — *Increase of respiratory allergy to the pollens of cypresses in the south of France*. Annals of Allergy, **56**:460.
- ROTTOLI P., ROTTOLI R., BELTRAMI V., REFINI R.M., ZERBONI R., CAMPO P., MANFREDI M., (1984) — *Allergy to cypress pollen in Tuscany*. Proc. International Symposium on prevention of Allergic Disease, Florence, Abstracts Book, p. 102.
- SACCHI G., TASSI G.C., (1982) — *Caratterizzazione e standardizzazione degli estratti allergenici*. Bol. Ist. Sieroter. Milan, **61**:4.
- SCHWEITZ L.A., HYLANDER R.D., WHISMAN B.A., WAKEFIELD K.J., GOETZ D.W., REID M.J., (1989) — *Cross-reactivity conifer pollens defined by immunoblot and monoclonal antibodies*. Abstract. Forty-fifth Annual Meeting of the American Academy of Allergy and Immunology, San Antonio, Texas.
- TAS J., (1965) — *Hayfever due to the pollen of Cupressus sempervirens*. Italian Mediterranean cypress. Acta Allergol., **20**:405.
- TOWBIN H., STEHELIN T., GORDON J., (1979) — *Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications*. Proc. Natl. Acad. Sci. U.S.A., **76**:4350.
- WEBER K., OSBORN M., (1969) — *The reliability of molecular weight determinations by sodium dodecyl sulphate-polyacrilamide gel electrophoresis*. J. Biol. Chem., **244**:4406-4412.
- YUNGINGER J.W., (1984) — *Allergen standardization: 1984*. J. Allergy Clin. Immunol., **73**:316.
- YUNGINGER J.W., (1988) — *IUIS criteria for reference preparations*. Arb. Paul Ehrlich Inst., **80**:75-78.