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Cypress (*Cupressus sempervirens*) Pollen Allergens: Identification by Protein Blotting and Improved Detection of Specific IgE Antibodies

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Abstract. On the basis of results of an investigation of the effects of different treatments employed, a dialysed and reduced extract of *Cupressus sempervirens* was separated electrophoretically on sodium dodecylsulphate-polyacrylamide gels before being transferred and then fixed with glutaraldehyde to nitrocellulose membrane. Probing with sera from 91 subjects allergic to *C. sempervirens* pollen followed by detection of bound IgE antibodies with [¹²⁵I]-labelled anti-human IgE revealed 17 IgE-binding proteins in the molecular weight range 14–96 kilodaltons (kDa). One component, of molecular weight ~42 kDa, reacted with IgE antibodies in the sera of 81.3% of the allergic subjects and, for each of the subjects, this component bound the greatest quantity of IgE. Almost 50% of the sera recognized only the ~42 kDa component, reinforcing the conclusion that this component is the major allergen of *C. sempervirens* pollen. A comparative study employing *C. sempervirens* pollen allergen discs prepared commercially or in the laboratory showed that values of the uptakes of [¹²⁵I]-anti-IgE indicating the presence of pollen-reactive IgE antibodies obtained with the latter discs were consistently higher (means 4.5 vs. 0.88), and that false-negative results were obtained when many sera were used with the commercial discs. The results of this study provide an essential basis for the production of standardized, safe and effective *C. sempervirens* pollen extract applicable to diagnosis and therapy of cypress pollen allergy.

Introduction

Allergies to cypress pollens, although apparently not widespread, are a significant problem in some countries bordering the Mediterranean, principally France and Italy [1–3], and in South Africa [4].

At present, extracts of *Cupressus sempervirens* are used for diagnosis and immunotherapy but, as with many allergen extracts, there is a pressing need for characterization of the active components and standardization of the solutions employed [5–7]. As a first step in achieving this aim, we determined optimum extraction conditions and employed protein blotting [8–11] procedures to provide a qualitative definition of the number and rank order of importance of allergens in a *C. sempervirens* extract. This, we believe, is a necessary prelude to effective standardization of any allergenic extract [8, 12]. In addition, and because of the many negative or equivocal results obtained with

a commercially available radio-allergosorbent test (RAST) for *C. sempervirens* allergen-reactive IgE antibodies, we set out to improve the detection of cypress-pollen-reactive IgE antibodies by obtaining and employing a highly reactive *C. sempervirens* pollen extract.

Materials and Methods

Pollen Extracts

Dry, non-defatted *C. sempervirens* pollen was purchased from Greer Laboratories (Lenoir, N.C., USA; lot 55X241-3). The pollen (20 g) was extracted in distilled water (150 ml) at 4°C overnight, centrifuged (17,300 g × 60 min) and the supernatant freeze-dried. The yield of lyophilized material was 2.3 g. Lyophilized extract (0.5 g) was reconstituted in 20 ml distilled water, dialysed in Spectrapor membrane tubing [Spectrum Medical Industries, Los Angeles, Calif., USA; molecular weight (MW) cut-off 2,000] at 4°C for 24 h and freeze-dried. Using the Pierce BCA[®] protein assay reagent [13], the protein content for the undialysed and dia-

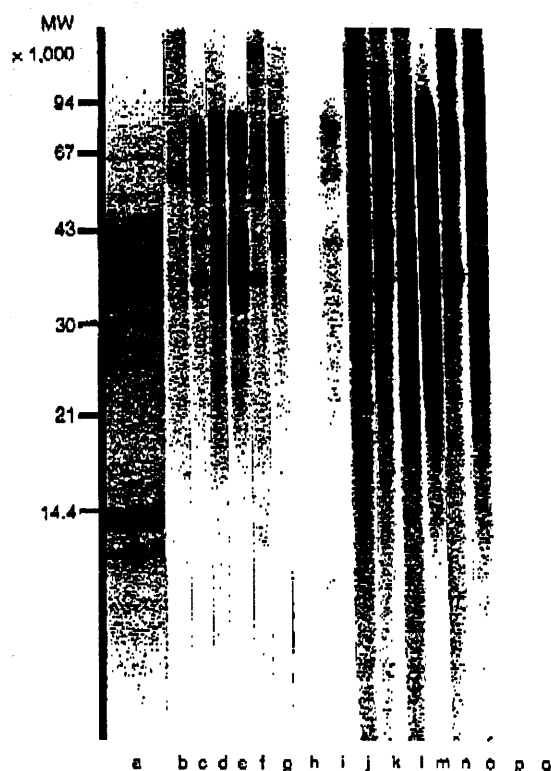


Fig. 1. Comparison of the IgE antibody-binding patterns of *C. sempervirens* pollen extracts prepared by the employment of various treatments detailed in table 1. Strip a - *C. sempervirens* dialysed, reduced pollen extract separated by SDS-PAGE and silver-stained; strips b-i and j-o = NC strips incubated with patients' sera CY-35 and CY-67, respectively, followed by [^{125} I]-anti-human IgE; strip p = NC transfer strip incubated with [^{125} I]-anti-human IgE only; strip q = strip incubated with serum from a subject not allergic to cypress pollen but allergic to house dust mites followed by [^{125} I]-anti-human IgE. The extract samples and quantities applied to gels before silver staining (strip a) or before transfer to strips b-q, and the treatments employed for each, are set out in table 1. Newborn calf serum (25%) was included in the incubation buffer when human serum was incubated with strip h.

lysed extracts was found to be 0.03 and 0.13%, respectively. A second preparation involved the extraction of 12 g dry pollen with 75 ml 0.001 M NH_4HCO_3 , followed by centrifugation. Half of the supernatant was dialysed and freeze-dried whilst the other half was concentrated by Amicon ultrafiltration (MW cut-off 2,000). Both samples were resuspended in 30 ml distilled water and to each was slowly added 21 g $(\text{NH}_4)_2\text{SO}_4$. After 60 min stirring at 4°C, the solutions were centrifuged at 4°C (40,000 g for 20 min) and the resultant pellets resuspended in 20 ml distilled water. Following extensive dialysis (48 h), the dialysates were freeze-dried. All preparations were stored at 4°C.

Subjects and Sera

Serum samples were collected in Provence, France, and in Canberra and Sydney, Australia, from subjects diagnosed on the

Table 1. Different treatments employed for the preparation and comparison of allergenic extracts of *C. sempervirens* pollen

Strip	Treatment employed				Relative protein loading ¹	Number of IgE reactive bands detected
	dialysis	glutaraldehyde	2-ME	$(\text{NH}_4)_2\text{SO}_4$ precipitation		
b					1	4
c	•				2	4
d	•		•		2	6
e	•	•	•		2	8
f	•			•	5	6
g ¹	•			•	11	6
h		•			1	0
i		•			1	4
j					1	5
k	•				2	4
l	•		•		2	9
m	•	•	•		2	10
n	•			•	5	6
o ²	•			•	11	6
p	•	•	•		2	0
q	•	•	•		2	0

For details of the treatment employed, see Materials and Methods. 2-ME = 2-Mercaptoethanol (5%).

¹ Relative to a loading of 5 µg protein per gel (represented by the figure 1 in the table).

² Prior to $(\text{NH}_4)_2\text{SO}_4$ precipitation, allergen extract was subjected to Amicon ultrafiltration (MW cut-off 2000).

basis of case history and skin prick tests as allergic to cypress pollen. Sera were also obtained from atopic subjects attending the Allergy Clinic, Royal North Shore Hospital (RNSH), who were not allergic to cypress pollen. Cord serum was from the Department of Obstetrics, RNSH, and used as an IgE-'free' control.

Tests for Cypress-Pollen-Reactive IgE Antibodies

All sera were tested for cypress-pollen-reactive IgE antibodies in assays using 6-mm nitrocellulose (NC) discs impregnated with 200 µg *C. sempervirens* pollen extract per disc [14] and commercial *C. sempervirens* discs (Pharmacia, Uppsala, Sweden; batch No. 22100). Bound IgE antibodies were detected by the addition of [^{125} I]-labelled anti-human IgE (Pharmacia).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Electrotransfer to NC

SDS-PAGE [15] was carried out as previously described [16]. Cypress pollen proteins in polyacrylamide gels were stained with silver nitrate [17]. For electroblotting of the different extracts, a range of protein loadings (5.0–55 µg protein/150 µl sample buffer) was applied to each gel (see legend to fig. 1 for further details). For electroblotting and subsequent probing with a panel of 91 individual atopic sera, a total volume of 300 µl (including 5% 2-mercaptoethanol) containing 96 µg protein (dialysed, reduced extract)

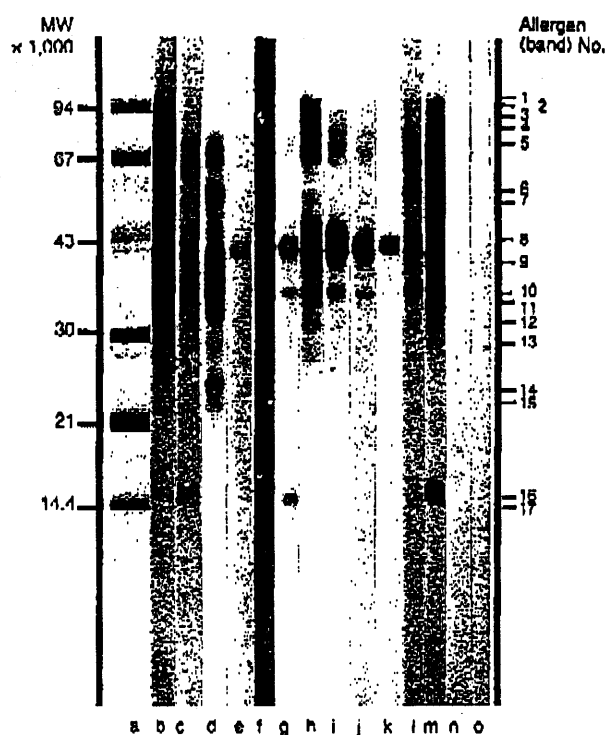


Fig. 2. Identification by autoradiography of the IgE-binding components transferred to nitrocellulose after SDS-PAGE of a *C. sempervirens* pollen extract (refer to strips e and m, table 1 and fig. 1). Strip a = Low-MW markers stained with Coomassie brilliant blue; strips b-m = NC transfers incubated with individual human sera from *C. sempervirens*-allergic subjects followed by [^{125} I]-anti-human IgE; strip n = strip incubated with serum from a subject not allergic to cypress pollen but allergic to house dust mites followed by [^{125} I]-anti-human IgE; strip o = strip incubated with [^{125} I]-anti-human IgE only. Exposure times for autoradiographs were 7-11 days.

was loaded onto each gel. To ensure reliability of the MW estimations, low-MW markers (Pharmacia) were included in each gel. Materials and methods for electrotransfer of separated proteins to NC were as previously described [10, 18], except that total transfer time was extended to 2 h and the voltage employed was 60 V. After transfer, NC membranes were washed for 5 min in 10% methanol in phosphate-buffered saline (PBS) with rocking followed by immersion in 0.1% Tween 20 in PBS for 2 h at room temperature to block unbound membrane sites.

Probing of NC Membrane Strips and Autoradiography

The materials and methods for the identification, by autoradiography, of the IgE-binding proteins were as previously described [19]. Prior to probing with sera, some NC strips were treated with 0.5% glutaraldehyde (1:50 of 25% solution) in PBS for 15 min, followed by a 30-second wash with PBS, and then incubation in 1% ethanolamine in PBS for 1 h. With some other strips, 25% newborn calf serum was included in the incubation buffer instead of 0.5% bovine serum albumin.

Results

Selection of a *C. sempervirens* Extract for Allergen Analysis

Silver staining of an SDS-PAGE-separated post-dialysis, reduced extract of *C. sempervirens* pollen revealed 27 bands in the MW range 5-100 kilodaltons (kDa; fig. 1, strip a).

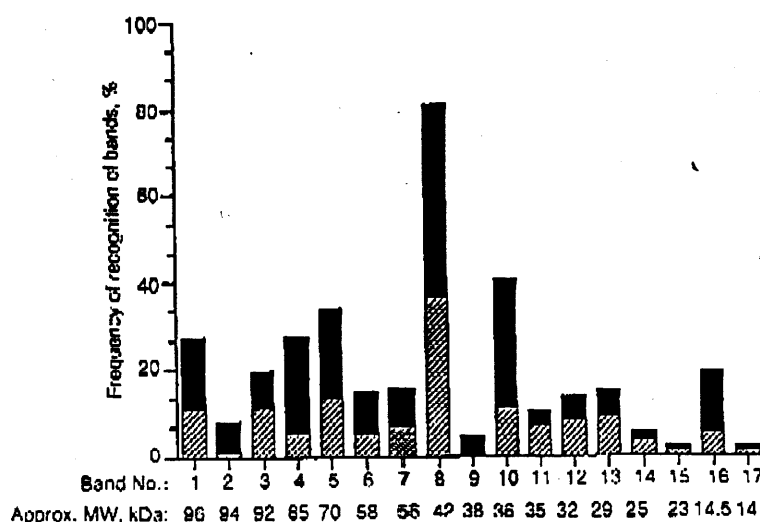
Table 1, which complements figure 1, lists the various treatment combinations used in the preparation of samples of *C. sempervirens* pollen extracts and NC transfers. The procedure that resulted in the greatest number of IgE-binding components involved dialysis, reduction with 2-mercaptoethanol and treatment of NC transfers with glutaraldehyde (fig. 1, strips c, m). No IgE binding occurred when this blotted extract was probed with [^{125} I]-anti-human IgE alone, or with sera from subjects allergic to house dust mite and/or pollens (but not cypress pollen; fig. 1, strips p, q). Non-employment of 2-mercaptoethanol (fig. 1, strips b, c, f-k, n, o), glutaraldehyde (fig. 1, strips b-d, f, g, j-l, n, o) and dialysis (fig. 1, strips b, h-j) yielded inferior patterns both in terms of the number of bands identified and the intensity of bands visualized on the autoradiographs. Although ammonium sulphate precipitation (with or without ultrafiltration) increased the protein concentration of the extract, the IgE-binding profiles obtained (fig. 1, strips f and g, n and o) were inferior to the non-precipitated samples. When newborn calf serum was included in the incubation buffer instead of Tween 20, no IgE binding bands were seen (fig. 1, strip h).

In summary, the results of allergen analysis experiments led us to select the methodology used in the preparation of strips e and m (table 1, fig. 1), that is, the employment of dialysis, reduction and glutaraldehyde fixation.

Identification of IgE-Binding Components

Sera from 106 different patients diagnosed as clinically allergic to cypress pollen were examined for IgE binding to cypress pollen proteins electroblotted onto NC. Of the transferred proteins, 17 components in the MW range 14-96 kDa bound IgE antibodies (fig. 2, strips b-m). Ninety-one of the sera reacted with at least one of the pollen components. Sera from subjects allergic to house dust mite and/or pollens (but not cypress pollen) and [^{125} I]-anti-human IgE alone, also did not bind to any of the transferred proteins (fig. 2, strips n, o). The two most frequently recog-

Fig. 3. Frequency of recognition of the IgE-binding components detected in *C. sempervirens* pollen extract. Hatched areas of bars show proportions of reactions that were weak.



nized components were bands 8 (MW ~42 kDa) and 10 (MW ~36 kDa) which bound IgE from 81.3 and 40.7% of sera tested, respectively (fig. 3), and for the majority of sera these components also bound the greatest quantity of IgE as evidenced by the depth of staining on the autoradiographs (see for example fig. 2, strips h, i, l, m). Although broad IgE-binding patterns were seen on some autoradiographs (fig. 2, strips b, h, i, m), exposure of autoradiographs for 40 h and 3, 7, 10 and 11 days failed to clearly reveal more than 3 IgE-binding bands in the 36- to 50 kDa region. A further band, or bands, obscured by the 42 kDa component cannot be ruled out, however. Bands 1, 4 and 5 of approximate MW 96, 85 and 70 kDa, respectively, were the only other components recognized by >20% of subjects' sera (fig. 3). Binding of IgE by components 3, 11, 12, 13, 14, 15 and 17 was not only low in frequency (fig. 3) but also weak for the majority of the reactive sera.

Figure 4 summarizes the frequencies of recognition of one or more *C. sempervirens* allergens by the panel of subjects. By far the most commonly recognized pattern was reactivity with only a single component – band 8 of MW ~42 kDa. A recognition frequency of 46.7% was seen with this single component, reinforcing the conclusion that it is the dominant allergenic protein in *C. sempervirens* pollen. Combinations of IgE-binding components 2 and 6 were the only other patterns to show recognition frequencies of 10% or greater (fig. 4).

Detection of *C. sempervirens*-Reactive IgE

Antibodies: Comparison with the Pharmacia RAST

Figure 5 shows a side-by-side comparison of uptakes of [¹²⁵I]-anti-IgE obtained with the in-house

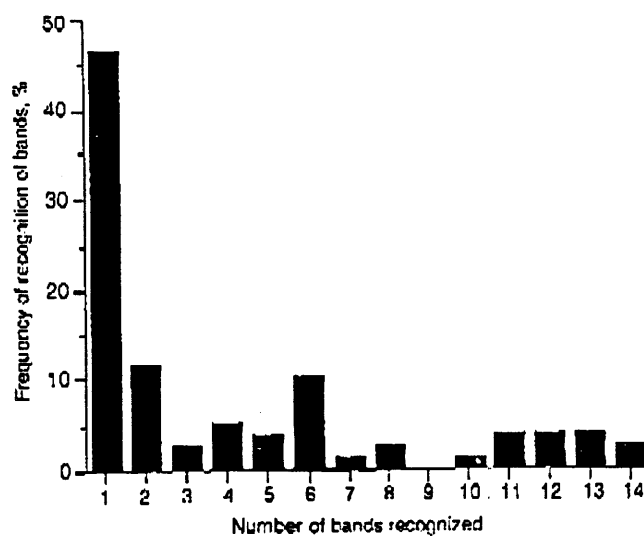


Fig. 4. Frequency of recognition of 1 or more *C. sempervirens* pollen IgE-binding components by sera from cypress-pollen-allergic subjects.

C. sempervirens pollen allergen discs (fig. 5a) and the commercial allergen discs (fig. 5b). The means of the results, that is, percent uptakes of [¹²⁵I]-anti-IgE, obtained with the Pharmacia *C. sempervirens* allergen discs and the discs prepared in the laboratory were 0.88 and 4.50, respectively. Analysis by the Student t test revealed a statistically significant difference ($p = 0.0005$). In the RAST assay using the commercial discs (fig. 5b), cord serum and sera from subjects not allergic to cypress pollen gave radioactive uptakes of 0.3 and 0.5%, respectively. When the laboratory-prepared discs were used (fig. 5a), the corresponding radioactive uptakes were 0.8 and 1.0%, respectively.

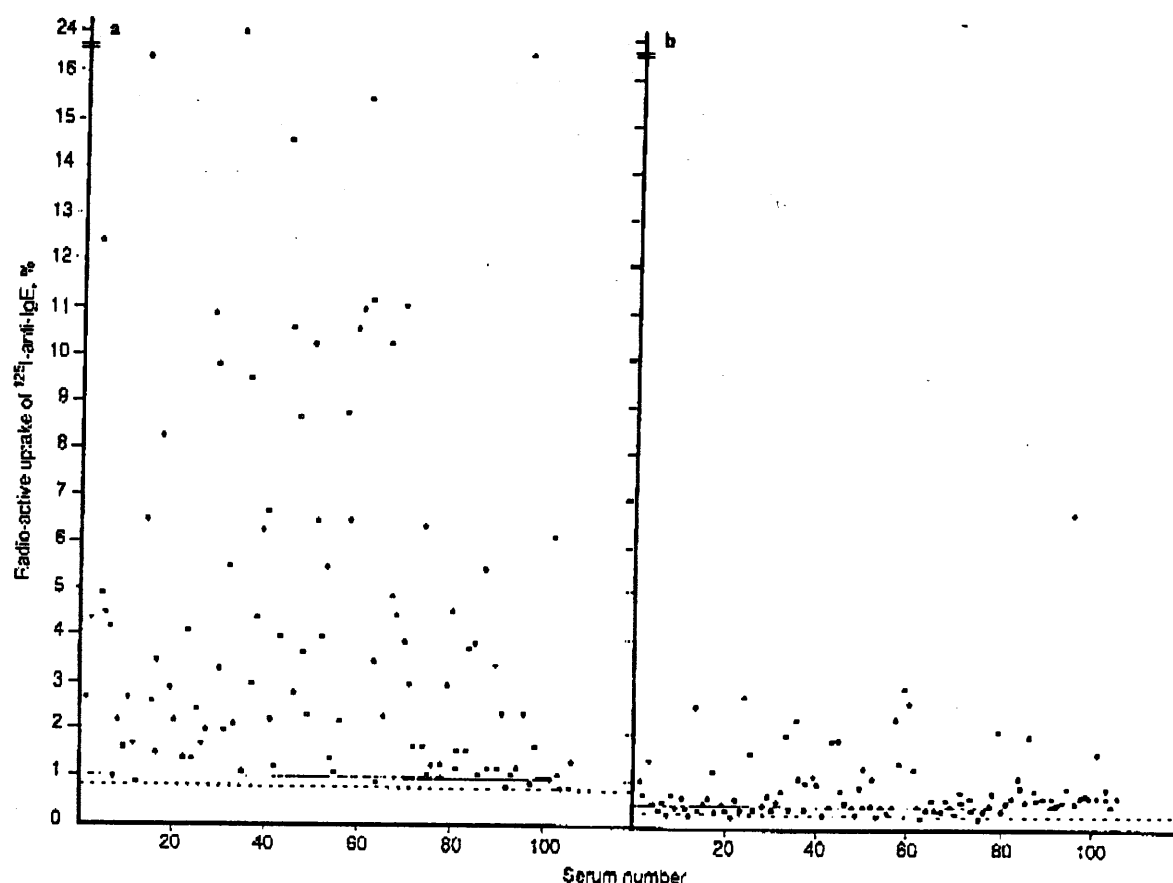


Fig. 5. Results of radioimmunoassay tests for the presence of *C. sempervirens* pollen-reactive IgE antibodies in individual patients' sera using *C. sempervirens* pollen allergen discs prepared in the laboratory (a) and Pharmacia *C. sempervirens* RAST allergen discs (b). Unbroken and dashed lines in a and b indicate radioactive uptakes of [^{125} I]-anti-IgE obtained with sera from allergic subjects who were not allergic to cypress pollen and from cord blood, respectively.

Discussion

An analysis of *C. sempervirens* pollen allergens proved difficult due to the viscous nature and low protein content of aqueous extracts. These problems were overcome in a systematic study in which a variety of extraction treatments were used alone or in various combinations. Results demonstrated that dialysis, reduction and pre-treatment of NC blots with glutaraldehyde improved allergen resolution and IgE binding. Even with the low protein content of the extracts, however, ammonium sulphate precipitation did not improve the quality of the resultant reconstituted solutions.

One *C. sempervirens* pollen component of MW ~42 kDa was prominent both on stained polyacrylamide gels and on the autoradiographs. This component bound IgE antibodies in 81.3% of the atopic sera

screened and, both in terms of frequency of recognition and relative quantities of IgE bound, this component must be considered the major *C. sempervirens* pollen allergen. In fact, a high proportion (47%) of the sera from the allergic subjects reacted only with this allergen. The simplicity of this IgE antibody recognition pattern contrasts with the recognition patterns seen with grass pollens such as *Dactylis glomerata* [18], *Lolium perenne* [20] and *Cynodon dactylon* [21], but not with pollen from the dicot *Parietaria judaica* [22] or, in the case of Scandinavian subjects, pollen from the birch tree *Betula pendula* [23]. The recognition of only a single allergen is an important advantage with respect to the relative simplicity of purification and standardization of *C. sempervirens* pollen extract and for subsequent immunotherapy of allergic subjects.

The need for a reappraisal of the currently avail-

able commercial RAST *C. sempervirens* pollen disc was demonstrated by our findings with allergen discs prepared in the laboratory. Radioactive uptakes of [¹²⁵I]-anti-IgE with the latter discs were generally higher than values obtained with the commercially available discs. In fact, the mean value obtained with the Pharmacia discs did not even fall in what we took as the positive range, demonstrating an unacceptably high incidence of false negatives.

The definition of *C. sempervirens* pollen allergen provided here represents the first important step in the production of a safe, effective and standardized allergen extract suitable for diagnosis and therapy.

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