

Selection of patients for biological standardization as exemplified by standardization of mugwort, goosefoot and English plantain pollen allergen extracts/preparations

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The biological activity of a partly purified, biochemically/immunochemically characterized mugwort pollen allergen preparation and crude pollen extracts of mugwort, goosefoot and English plantain was determined by means of skin prick test (SPT). The patient inclusion criteria with mugwort were a well-defined positive clinical history and a positive SPT. Symptoms related to goosefoot/English plantain pollens are difficult to define, as these weeds flower during the grass pollen season. Thus patients tested with these allergens did not fulfill the most important inclusion criterion for so-called biological standardization. To elicit a wheal of the same size as that produced by histamine 1 mg/ml required 100 to 10,000 times more material from these weeds, than from mugwort and other pollen allergen extracts investigated earlier. One thousand Biological Units/ml (BU/ml) corresponded to 8.3 µg dry weight (dw/ml) of the crude and 1.8 µg dw/ml of the purified mugwort pollen allergen preparation. Only 7/22 goosefoot-and English plantain-tested patients were positive at conjunctival or nasal challenge. All three weeds showed a similar composition with 5–10 allergens by CIE/CRIE analysis and 10–13 by immunoblotting analysis. One dominating allergen (approx. 15,000 d), could be identified for each weed species by protein gel blot after separation by SDS g-PAGE. There was no other explanation for the difference in biological activity than the criteria of selection. If there is no obvious clinical history, which is the main patient inclusion criterion in biological standardization, then additional criteria should be used.

Key words: biological standardization; CIE/CRIE; conjunctival provocation test; nasal provocation test; nitrocellulose electroblotting; SDS g-PAGE; skin prick test; RAST.

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CLINICAL ASPECTS

The aim of biological standardization is to establish a standard with a biologically meaningful potency, allowing comparison of the potency of extracts produced from different allergen source materials. Thus, allergen preparations with equal potency should give the same diagnostic information when tested in humans. To achieve this, only patients with proven clinical symptoms from the allergen in question should be included in the biological standardization of different allergen extracts.

Biological standardization (BS) by means of skin prick testing (SPT) has been proposed by Aas et al. (1) and recommended in Nordic Guidelines for the registration of allergen preparations (17). This concept implies that the

biological potency of allergen preparations from different species labelled with the same BU/ml (Biological Unit) should be similar in equally sensitive patients. We have reported on the repeatability of results of BS, provided the

patient inclusion criteria remain constant (7, 9, 12).

Mugwort pollen (*Artemisia vulgaris*) is the main cause of pollinosis in Scandinavia during late summer: August to early September. English plantain (*Plantago lanceolata*) and goosefoot (*Chenopodium album*) flower during the main grass pollen season in Scandinavia: June and July. Because the grass pollen season overlaps it is difficult to establish a clinical history for pollinosis caused by these two weed species.

The purpose of this study was to establish reference preparations of mugwort, goosefoot and English plantain allergen preparations. The references were characterized with respect to antigenic/allergenic composition by biochemical and immunochemical analysis. Further, we aimed at biological standardization of the extracts. The main inclusion criterion for BS, clinical sensitivity to the allergen in question, was not fulfilled by patients tested with goosefoot and English plantain. We will therefore focus on the influence of differences in clinical history on the determination of biological activity.

MATERIAL AND METHODS

Patients

For BS of the two mugwort preparations, 29 patients (10 men and 19 women, mean age 17–67 years), attending the allergy units, County Hos-

pital, Halmstad and Karolinska Hospital, Stockholm, Sweden participated. All suffered from pollinosis during August, which is the main mugwort season in Scandinavia, 15 of them also had asthma during this period. They did not suffer from perennial symptoms or symptoms during late autumn, as would be expected in patients with mite or mould allergy. All patients were SPT positive to a routine mugwort extract (Neohelisen®, 5000 PNU/ml, Allergopharma, Reinbeck, FRG, or Soluprick® ALK, 0.4 HEP, Copenhagen, Denmark). Thus, previously published inclusion criteria were followed (Table 1).

For the BS of goosefoot and English plantain extracts, 22 patients (9 men and 13 women, mean age 28, range 14–56) participated. Patient selection could not be based on clinical history, the main inclusion criterion, as there is no established clinical history for these two weeds in Sweden. Thus, patients with rhinoconjunctivities and in six cases also asthma during the grass pollen season, were included in the determination of biological activity. They all had a positive SPT (2 + or more according to Aas & Belin (2)) to extracts of the two weeds (Neohelisen, Allergopharma, Reinbeck, FRG). In addition, all patients showed positive SPT to various grass pollen extracts.

Allergen preparations, albumin diluent and histamine solutions

Pollen from mugwort (*Artemisia vulgaris*), goosefoot (*Chenopodium album*) and English plantain (*Plantago lanceolata*) were obtained from Allergon AB, Vålninge, Sweden (< 1% pollens of other species or impurities). Mugwort pollen was defatted with acetone and methanol prior to the main extraction. The crude mugwort extract was prepared by extraction in 0.125 mol/l ammoniumbicarbonate buffer (extraction ratio 1/10 w/v) in cold for 24 h. The solid phase of the crude extract was removed. Crude goosefoot and English plantain extracts were similarly prepared, but without the initial treatment with acetone and methanol. The partly purified preparation (8) of mugwort was produced by extracting the weed pollen in 0.125 mol/l ammoniumbicarbonate buffer as described above, followed by ultrafiltration (nom-

Table 1
Patient inclusion criteria (7, 8)

1. A positive case history for the allergen to be tested.
2. A mean weal diameter obtained with histamine HCl ≥ 4 mm.
3. No previous immunotherapy with the allergen in question or any cross-reacting allergen.
4. Living in an environment where the allergen investigated is a significant problem.
5. More than 15 years of age.
6. No antihistamines or other anti-allergic drugs for an adequate period.

inal cut-off 2000 d) to remove small non-allergenic molecules. All allergen preparations were lyophilised. BS and biochemical/immunochemical characterization were performed as part of the establishment of an inhouse reference preparation (Pharmacia AB, Uppsala, Sweden). On the day of testing all allergen extracts/preparations were reconstituted and further diluted with Albumin diluent® (Pharmacia) (0.03 % human serum albumin, 0.4 % phenol in saline). Histamine HCl 1 mg/ml (5.43 mg/ml, 0.63 mg/ml histamine base) was obtained from Pharmacia.

Biological standardization by skin prick test

BS was performed according to the Nordic Guidelines for registration of allergen preparations (17) using the method of Dreborg & Grimmer (7). SPT was performed as previously described (7). Inclusion criteria are given in Table 1. With this method at least 20 patients with clinical symptoms should be included. Either consecutive patients attending an allergy clinic or unselected patients from an index at such a clinic should be used. Duplicate SPTs were performed according to Pepys (18), with six 3-fold concentrations of the extracts on the backs of the patients. The highest concentration of mugwort extracts used was 100 µg dw/ml and of goosefoot and English plantain extracts 30 µg dw/ml. Both mugwort extracts were tested simultaneously, as also the goosefoot and English plantain extracts. The longest and the mid-point orthogonal diameters of the weals were measured. Weals with a mean diameter < 2 mm were regarded as negative. The concentration (C_h) eliciting a weal of the same size as that of histamine 1 mg/ml was estimated using the model $\log D = a + b \log (\text{conc})$ where D denotes the mean weal diameter, a the intercept with the Y-axis, b the slope of the dose response relationship and conc the concentration of allergen. The median C_h is defined as 1000 BU/ml.

Nasal and conjunctival provocation test (NPT and CPT)

All patients tested with goosefoot and English plantain extracts were challenged with con-

junctival provocation test and if negative also with nasal provocation tests. Conjunctival provocation tests were performed according to Möller et al. (15). The CPT was started at 300 µg dw/ml and continued with 10-fold increments to the highest concentration. Nasal provocation tests were performed in patients with negative CPT by applying one drop of the highest concentration of allergenic extract to the inferior concha. If repeated (> 3) sneezes, rhinorrhea and/or blockage occurred, then the provocation test was regarded as positive. This is often the routine procedure in Sweden, but the test's precision has not been validated. This method was used, as the aim was to get a yes/no answer, and a high correlation had already been found between CPT and NPT results (19).

Determination of specific serum IgE

Blood was drawn from all patients prior to the skin prick test. Specific IgE-antibodies were determined by duplicate tests with commercially available Phadebas RAST® reagents (Pharmacia AB) according to the procedure prescribed by the manufacturer. The results were expressed in PRU per ml (Phadebas RAST Units). Phadebas RAST class 1 or more (> 0.35 PRU/ml) was regarded as positive. The RAST reactions to crude and purified mugwort allergen preparations were compared by testing sera from patients with known mugwort allergy with different degrees of sensitization as measured by Phadebas RAST. The crude and purified weed discs were coupled analogously to the commercial RAST discs to obtain maximal uptake.

Patient sera pools used for CRIE/immunoblotting experiments consisted of several sera with high levels of specific IgE. Sera with PRU > RAST class 4 were diluted prior to mixing, to decrease the risk of one component dominating the serum pool.

Rabbit antibodies

Antisera to mugwort, goosefoot and English plantain were raised in rabbits essentially

according to Harboe et al. (13), injecting 6 mg dw per rabbit of crude extract in Freund's complete adjuvant subcutaneously; sera from several bleedings were pooled.

Amino acid analysis

The total protein content of the weed preparations was determined by amino acid analysis according to Spackman et al. (22).

Carbohydrate analysis

The total carbohydrate content of the weed preparations was determined according to Sawardeker et al. (20). The qualitative and quantitative composition of the carbohydrate moiety in the weed pollen preparations was determined as methylated alditol acetates by gas liquid chromatography (20).

Crossed immunoelectrophoresis (CIE)/crossed radio immunoelectrophoresis (CRIE)

Crossed immunoelectrophoresis was performed essentially as previously described (24). Samples of 5 µl of weed extract (approximately 5 mg protein/ml) were run in the first dimension electrophoresis at 10 V/cm and 15°C for 40 min (mugwort) and for 30 min (goosefoot and English plantain). The second dimension electrophoresis was performed at 2 V/cm for 18 h using 25 µl rabbit antiserum against mugwort, goosefoot and English plantain proteins per cm² gel (upper and lower gel compartment) with a blank intermediate gel. CRIE analysis was performed according to the previously described procedure (23, 24).

Protein-gel-blot

The electroblotting procedure was also used for establishing the content of allergenic components according to the size of the molecules. The proteins were separated by SDS g-PAGE (sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis) (7.5–20 %) followed by electrophoretic transfer to nitrocellulose strips (5). The incubation of the strips with

allergic patient serum and the probing of specific IgE antibodies bound to the separated protein components were performed as previously described (23).

Statistical analysis

Patients with positive response to less than three consecutive concentrations and patients with a correlation coefficient (*r*) of their allergen dose response relationship < 0.85 or a slope (*b*) < 0.01 according to the model $\log(\text{mean diameter}) = a + b(\text{conc})$, were excluded. The reason for exclusion was that if these criteria were not fulfilled, no precise estimate of the patient sensitivity was possible (7, 8, 9, 12).

C_h was defined as the concentration estimated to elicit a wheal reaction of the same size as that of histamine HCl 1 mg/ml in the individual patient (7).

The Pearson correlation coefficients were calculated, using logarithmically transformed C_h and PRU values. Differences between groups were determined using the Student's *t*-test (two-tailed).

The study was approved by the Swedish Medical Board and the local Ethics Committees. All patients were given information and gave their written informed consent.

RESULTS

Biological standardization of weed allergen preparations

Of the 29 patients initially selected for BS of the crude and purified mugwort preparations, six and nine, respectively, were excluded since they did not fulfill the statistical criteria (Table 2). One patient was excluded due to low reactivity to both extracts.

Individual C_h -values for the crude and purified mugwort extracts, using patients from whom a C_h value was obtained with both preparations (*n* = 16), correlated well (*r* = 0.08, *P* < 0.0001).

The median C_h -value, including all patients, for the crude mugwort extract was estimated to 8.3 µg dw/ml (range 1.09–710 with 95 % con-

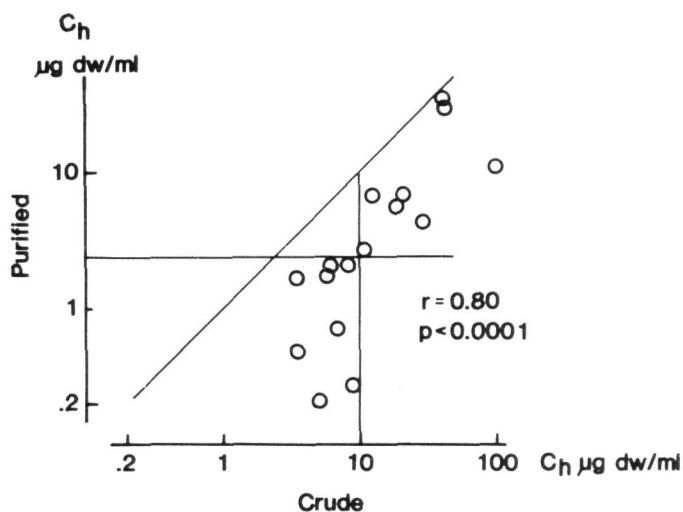


Fig. 1. Concentration of allergen extract in µg dw/ml corresponding to C_h in patients with approved data for the purified as well as the crude mugwort extract (Table 2).

fidence limits 4 and 21.7 µg/ml) (Fig. 2). The corresponding data for the purified extract were 1.8 µg dw/ml (range 0.11–153 with 95 % confidence limits 0.93 and 3.6 µg dw/ml). Obviously, more material of the crude mugwort extract than of the purified ($P < 0.0001$) was required to elicit the same skin response.

Seven patients included in the biological standardization of English plantain and goosefoot were CPT/NPT positive, 12 were negative and three had an equivocal test response to the highest concentration of goosefoot. The corresponding data for the English plantain extract were; 7 positive, 13 negative, and 2 equivocal tests.

One of the seven patients with positive response to goosefoot provocation test and 2/7 with positive response to English plantain were excluded due to low correlation coefficients and/or a slope (b) < 0.1 of their dose response relationships. The corresponding data for patients with a negative or uncertain response to CPT were 10/15 and 6/15 respectively. In patients with positive/negative CPT or NPT to goosefoot, 6000 µg dw/ml and 26,000 µg/dw/ml respectively, corresponded to the median C_h ($P = 0.057$). The corresponding figures for English plantain were 240 and 3800 µg dw/ml ($P = 0.002$).

A positive RAST score to goosefoot was found in 5/7 patients with a positive response to provocation test, and in 2/15 patients with a doubtful or negative response. The corresponding data for English plantain were 4/7 and 2/15.

Biochemical and immunochemical properties of mugwort, English plantain and goosefoot allergen preparations

The protein content of the lyophilised weed pollen allergen preparations used for biological standardization varied between 9 and 30 % due to species and degree of purification. The total carbohydrate content was approx. 30 % based upon the dry weight for all three weed species (Table 3). A more detailed analysis of the carbohydrate pattern in mugwort is shown in Table 4. One pentose (arabinose) was the domi-

Table 2

Total number of patients and number excluded in the final calculation. Patients with positive CPT/NPT, are given in parenthesis

	Excluded due to regression line				Included	Total
	Approved points < 3	$r < 0.85$	$b < 0.1$	Total excl.		
Mugwort						
purified	2	6	2	9	20	29 ¹
crude	1	5	1	6	23	29 ¹
Goosefoot	0	10	1	11 (1)	11 (6)	22
English plantain	0	7	1	8 (2)	14 (5)	22

¹16 og whom were included in the calculations for both extracts in Fig. 1.

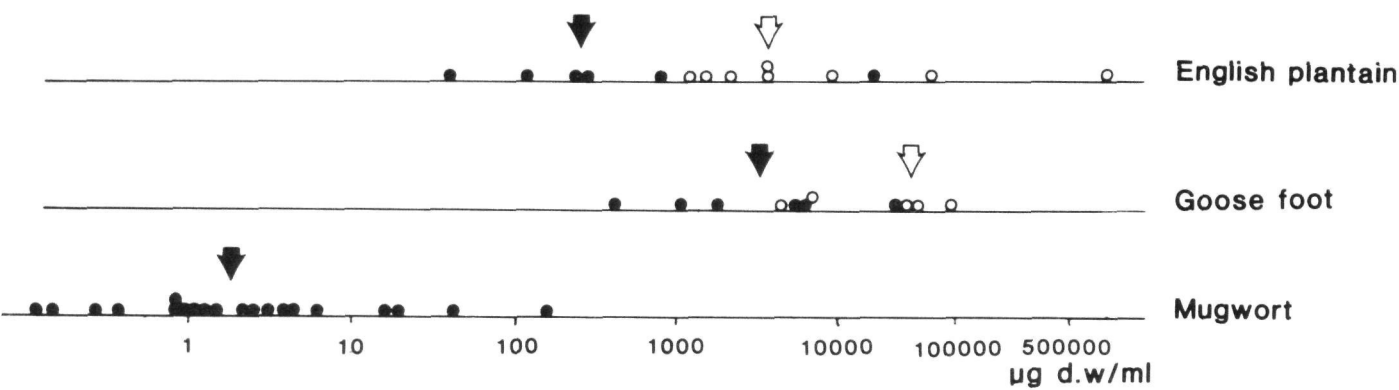


Fig. 2. Concentrations of allergenic extract in µg dw/ml corresponding to C_h in patients with approved data for the purified mugwort, goosefoot and English plantain extracts. Patients with a positive provocation test to goosefoot and English plantain are indicated by filled circles (●), those with a negative challenge are indicated with open circles (○). Correspondingly the median C_h-values are indicated by filled and open arrows.

nating monosaccharide in the mugwort allergen reference preparation. In both English plantain and goosefoot extracts about 20 % of the dw was glucose, whereas the content of arabinose was only 3 %. Acidic monosaccharides, sialic and glucuronic acid, were not detected in any of the chromatograms.

The CIE/CRIE patterns of all three weed allergen preparations showed great similarities, even if the CIE patterns of English plantain and goosefoot were better resolved than that of mugwort according to the experimental conditions used (Figs. 3–5). A majority of the identified antigens moved towards the anode. This was most pronounced for English plantain. In mugwort and goosefoot extracts, also neutral and alkaline antigens were detected after protein staining. After incubation with a pool of patient sera all weed pollen extracts showed several visible radiostained immunoprecipitates.

In all, nine different allergens were identified in the purified mugwort allergen preparation by CRIE analysis using a panel of patient sera. At least five different allergens could be identified in the English plantain and goosefoot preparations. One of the allergens in each of the weed allergen preparations fulfilled the criterion for a major allergen (3).

Fig. 6 demonstrates the binding of specific IgE antibodies to electrophoretically separated mugwort, English plantain and goosefoot antigens. Many well-resolved protein bands with a high degree of specific IgE binding could be identified between 10,000 and 90,000 daltons. The blotting patterns were similar for all weed preparations: mugwort (10 visible radiostained bands), goosefoot (11 visible radiostained bands), and English plantain (13 visible radiostained bands). A distinct protein band with a high degree of specific IgE-binding, molecular weight approx. 15,000 d, could be identified in

Table 3

Protein and carbohydrate content in mugwort, goosefoot and English plantain allergen preparations analysed by amino acid analysis and gas liquid chromatography

Species	Per cent of monosaccharides	
	Protein (% dw)	Carbohydrate (% .dw)
Mugwort		
purified	31.4	36.8
crude	22.0	26.0
Goosefoot, crude	11.3	30.6
English plantain, crude	8.9	34.3

Table 4

Carbohydrate composition of crude and partially purified mugwort preparations analysed by gas liquid chromatography

	Per cent of monosaccharides				
	Ara	Rha	Man	Gal	Glu
Mugwort					
crude	16.0	0.4	1.2	3.9	5.4
purified	25.1	0.8	0.8	7.8	2.3

Ara = Arabinose; Rha = Rhamnose; Man = Mannose; Gal = Galactose; Glu = Glucose.

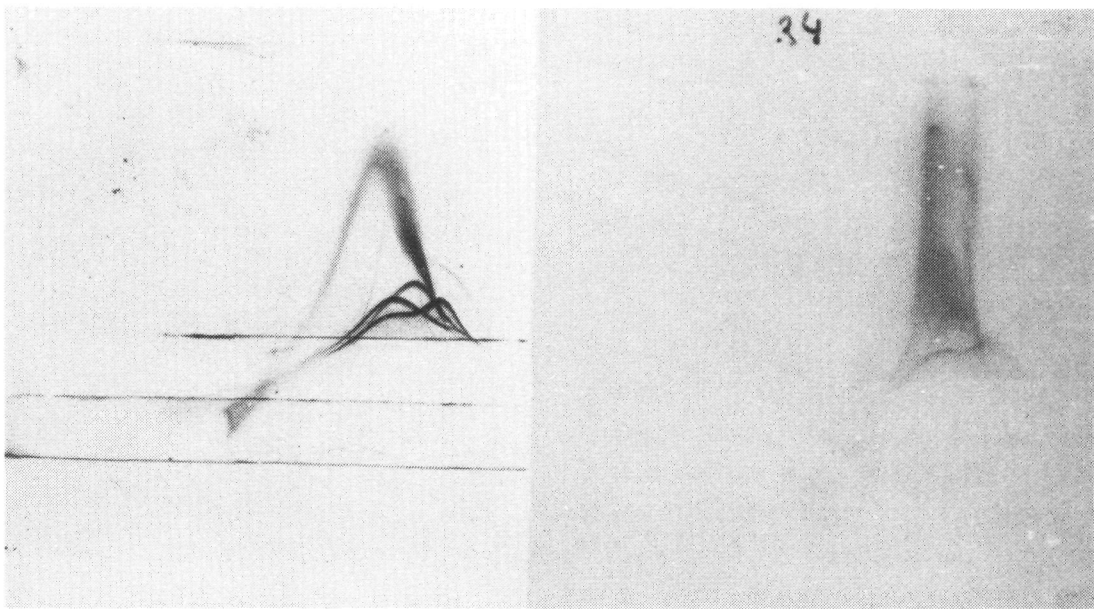


Fig. 3. Crossed immunoelectrophoresis and crossed radio immunoelectrophoresis of the partly purified (8) mugwort pollen preparation against hyperimmune rabbit antiserum. Anode to the right in the first dimension and at top in the second (a). X-ray film from the CRIE analysis of mugwort after incubation with a patient serum pool (b).

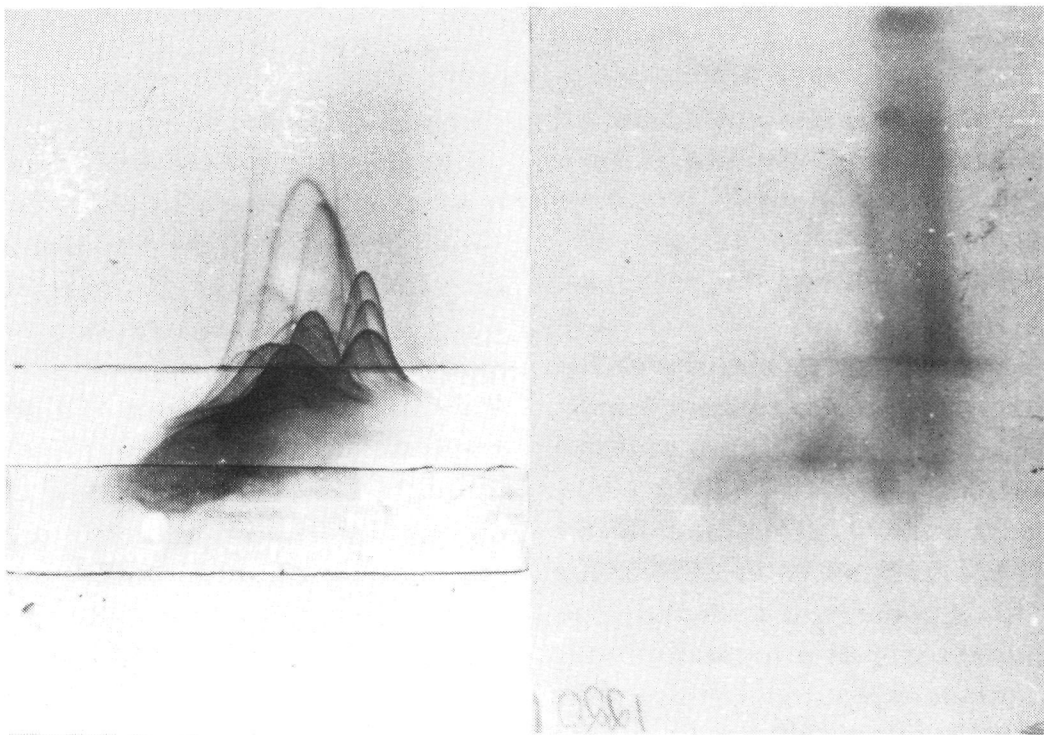


Fig. 4. Crossed immunoelectrophoresis and crossed radio immunoelectrophoresis of English plantain pollen extract against hyperimmune rabbit antisera. Anode to the right in the first dimension and at top in the second (a). X-ray film from the CRIE analysis of English plantain after incubation with a patient serum pool (b).

all three weeds. Furthermore, the blotting patterns were characterized by many IgE-binding components between 30,000 and 90,000 daltons.

The relation between the allergic composition

of crude and purified mugwort allergen preparations was also analysed by comparative RAST. The individual mugwort patient sera were analysed with discs coupled with crude and purified mugwort extract, respectively. The

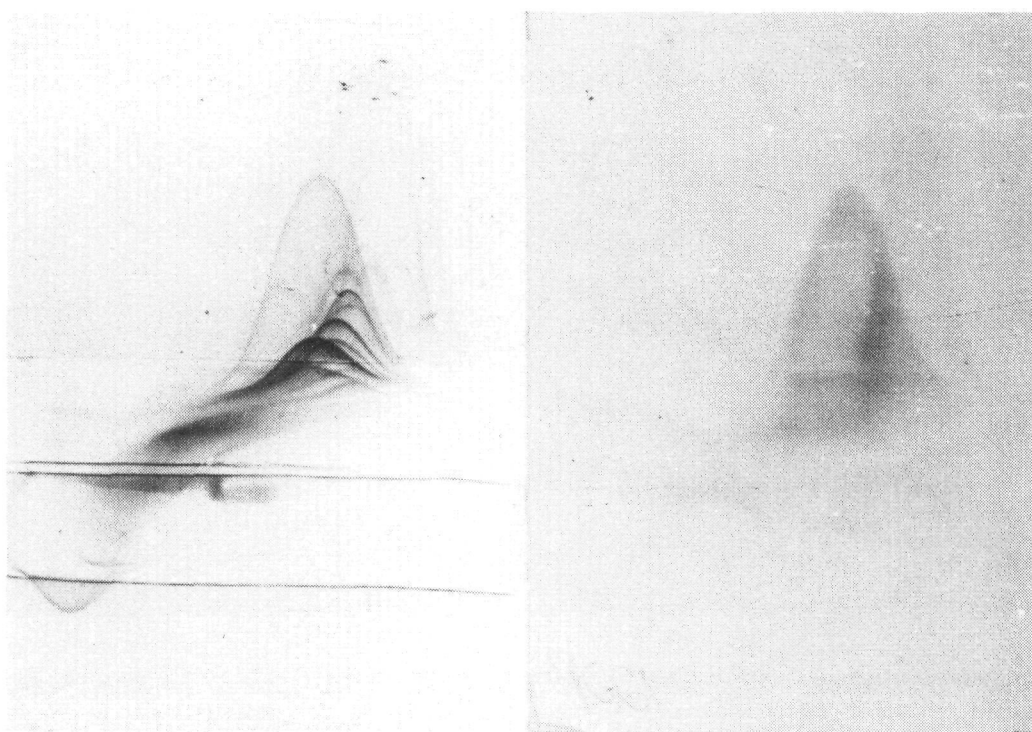


Fig. 5. Crossed immunoelectrophoresis and crossed radio immunoelectrophoresis of goosefoot pollen extract against hyperimmune rabbit antisera. Anode to the right in the first dimension and at top in the second (a). X-ray film from the CRIE analysis of goosefoot after incubation with a patient serum pool (b).

radioactive uptake was similar with crude and purified mugwort coupled to the solid phase ($r = 0.98$; $P < 0.0001$).

DISCUSSION

To establish reference preparations, the biological activity, protein and carbohydrate content and antigenic/allergic composition of three weed pollen preparations were studied.

The biological activity BU/ μ g dw of the purified and lyophilised mugwort preparation was found to be about the same as that of other purified, standardized pollen extracts (7-12) and very close to values reported for three other weed pollen allergen preparations known to cause severe pollinosis: short ragweed (10) and two wall pellitories (*Parietaria judaica* and *officinalis*) (11).

In a previous paper (14) the biological activity of a crude mugwort pollen reference extract, corresponding to an activity of 1000 BU/ml, was found to be 17.4 μ g dw/ml. Their method for BS differed (7, 9) regarding SPT technique, patient inclusion criteria and method for statistical analysis. An explanation for the

observed difference in biological potency of the crude mugwort extracts would be differences in clinical sensitivity of the mugwort allergic patients tested by us and by Ipsen et al. (14), as the antigenic/allergic composition of the mugwort preparations used appeared to be similar. Other possible explanations, although less likely, include difference in SPT technique and statistical method (7, 9, 14). The 4.5-fold difference in specific allergenic activity between the purified and crude mugwort preparations (μ g dw/1000 BU) reflects the removal of non-allergic low molecular weight components by purification. A similar ratio in specific allergenic activity between the purified and crude allergen preparations has previously been reported for different pollen allergens (7, 9).

All patients tested with goosefoot and English plantain extracts had a clinical history of pollinosis during June and July, when mainly grasses but also weeds are flowering. Despite a positive SPT, most individuals were negative to both provocation tests and RAST. This indicates that they were not clinically sensitive to these weeds (19) and thus underlines the importance of establishing additional criteria for

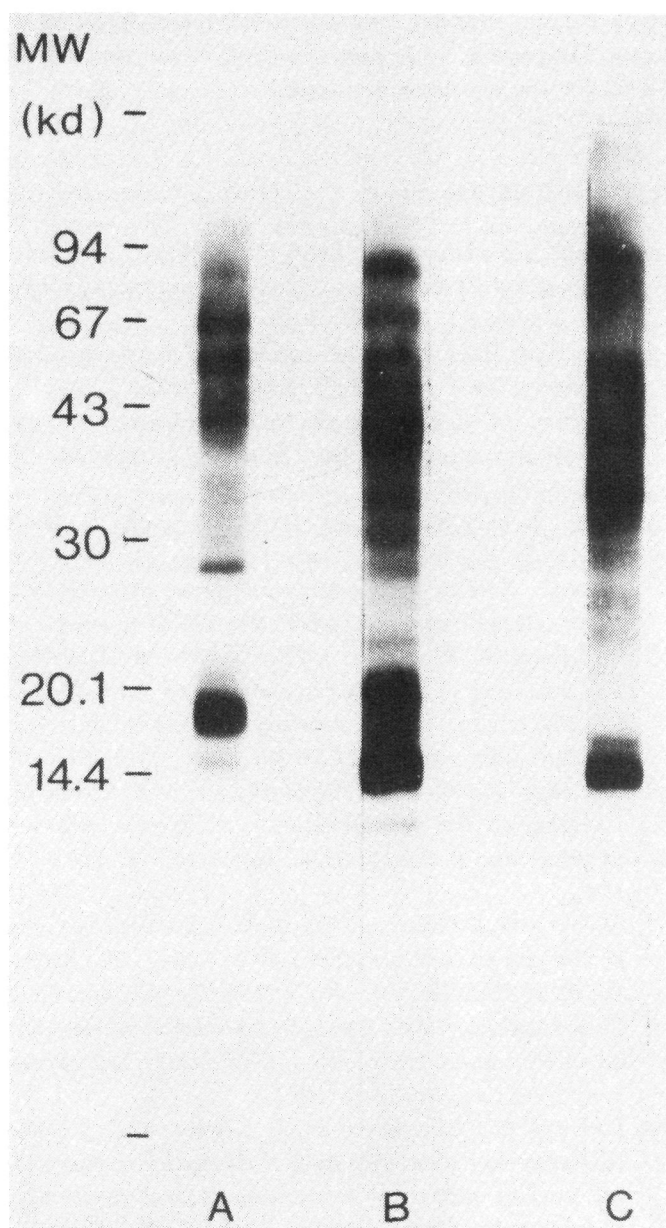


Fig. 6. Screening of specific IgE antibody binding components on nitrocellulose strips of mugwort (a); English plantain (b) and goosefoot (c) weed pollen extracts using a patient serum pool and isotope detection. The weed pollen proteins were separated according to size by SDS-gPAGE.

patient selection in biological standardization of allergens with seasonal overlap. The biological activity of these two weeds should not be regarded as biological standardization data, possible to use for comparing the activity with other allergen species, as the patients tested did not fulfill the inclusion criteria.

To obtain the median C_h for goosefoot and English plantain extract, 100 to 10,000 times higher concentrations were required than for such well-known weed allergens as mugwort, ragweed and wall pellitory. This finding illustrates the impor-

tance of including patients in BS trials with obvious clinical symptoms to the allergen tested.

Several papers have discussed the possible importance of English plantain pollen as an allergen in other regions (4, 16, 25). We have been in contact with several European allergy centers and tried to gather patients with hay fever symptoms correlated to goosefoot and English plantain pollen peaks. Only a few patients with obvious clinical symptoms related to these two pollen species were found. This indicates that allergy to these weeds is uncommon in Europe.

The total protein and carbohydrate contents of these weeds were of the same order as those of purified wall pellitory allergen preparations (11). In two such preparations (11) and in English plantain and goosefoot extracts, the hexoses were the dominating monosaccharides indicating a similar composition of carbohydrates. In the mugwort preparations, the pentoses dominated.

We found a lower protein and carbohydrate content (30 and 37 %, respectively) in the analysed mugwort extracts compared to that found by Smedstad-Paulsen et al. (21) (56 % and 71 %, respectively). The discrepancy might be due to the less reliable assay methods used by them.

Protein-gel-blot data based on SDS g-PAGE separation and electrophoretic transfer to nitrocellulose demonstrate several allergens in the weeds with molecular weights in the region of 10,000–90,000 d. The molecular weight of individual allergen components in these weeds has not been studied previously. The major allergenic activity in all the weeds studied was located around 15,000–20,000 d. Many bands outside this region with specific IgE-binding capacity were revealed in all three weed extracts. Mugwort is a common weed all over Scandinavia. Goosefoot is common in gardens, whereas English Plantain mainly grows wild. A relative to English plantain, *Plantago major* is, however, common on lawns. Consequently, mugwort pollens are the dominating weed pollen in pollen traps. Thus, sensitization may mainly occur to the dominating allergen present in mugwort pollen.

The CIE/CRIE analysis by Ipsen et al. (14) of a crude mugwort extract supports our data. Compared to our findings, they found a higher number of identified antigens (CIE), but an equal number of allergens (CRIE). However, these authors analysed a crude mugwort extract while the mugwort preparation used in our CIE/CRIE-study and in biological standardization was partly purified (8). The weed pollen preparations used in this study were prepared in a similar way, known to assure high potency (7, 9, 11, 12) and relevant composition (8, 11). They contained a similar number of allergens, as determined by immunochemical methods, using sera with known high level of specific IgE. Thus, the difference between results obtained by estimating the activity of mugwort, English plantain and goosefoot allergens, is probably due to the criteria used for patient selection.

Difference in skin sensitization to common allergens between patients with clinical allergy and skin positive individuals in cohort studies (6) have been documented. It is therefore important that patient inclusion criteria for BS of different species remain constant. The most suitable criterion is clinical symptoms at exposure and can be used for many allergens. If experienced clinicians select consecutive patients or take patients from their register, sensitive to the allergen in question, then reproducible results (median C_h value) are obtained between regions (7, 9, 12). The present study, on the other hand, demonstrates the need for additional criteria for allergens with seasonal overlap. Provocation tests using allergen source material (e.g. pollen) would be suitable.

These results in combination with our previous results point to the need for consistent patient inclusion criteria in BS trials (7, 8, 9, 12), with documented clinical allergy as the major criterion. The data obtained from the experiments with goosefoot and English plantain extracts can not be regarded as biological standardization.

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