

Results of biological standardization with standardized allergen preparations

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The aim of biological standardization (BS) is to equilibrate the activity (potency) of allergen extracts from different source materials. This was done by performing skin prick tests (SPT) on patients who were sensitive to one of the following 10 allergens: Birch, alder, hazel, timothy, rye grass, velvet grass, cultivated rye, mugwort, *D. farinae* and *Cladosporium herbarum*. Patient sensitivity varied within a range of three to four powers of ten for each allergen investigated. The weal size in each patient corresponding to that elicited by histamine 1 mg/ml was calculated using the model $\log(\text{mean weal diameter}) = a + b \log(\text{concentration})$. The correlation coefficients of the regression lines of the allergen dose response relationship were found to be > 0.85 in most cases. The median slope for all extracts was 0.24. The slope for *Cladosporium* was significantly steeper than that for pollens. The amount of material in μg dry weight (d.w./ml) equal to 1000 biological units/ml (BU/ml) varied within a factor of three between species for all tested purified allergen preparations but *Cladosporium*. For *Cladosporium*, about 30 times more material was needed than for *D. farinae*. When using crude rather than purified material, it was necessary to use five to ten times more to elicit a reaction corresponding to 1000 BU/ml, but the difference was significant only for *Cladosporium*. The narrow range of allergen concentrations used by us as well as other investigators does not assure positive skin prick test results in all patients with clinical symptoms due to the allergen in question. Skin prick testing should therefore be done over a wide range of concentrations to improve the methods for BS.

Key words: allergen; biological standardization; biological units/ml; histamine; HEP (Histamine Equivalent in skin Prick testing); skin prick test.

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CLINICAL ASPECTS

Biological standardization of allergen extracts aims at establishing a unit indicating a similar allergenic activity for all species. However, the individual sensitivity in patients with clinical allergy to the allergen in question differs considerably. In addition, for technical reasons the weal size resulting from testing with a given concentration of allergen differs considerably between centres. Thus a reference substance such as histamine should be used in parallel with allergen skin prick tests to adjust for differences in skin prick test technique, at least if the results are to be compared between investigators or be used to analyse changes in patient sensitivity over time by the same investigator.

The aim of biological standardization (BS) of allergen extracts is to equilibrate the activity (potency) of allergen extracts from different source materials (1). A unit, 1 HEP (Histamine Equivalent in skin Prick testing) has been proposed (1). The Nordic guidelines for registration of allergen extracts proposed the more convenient biological units/ml (BU/ml), 1000 BU/ml defined as the concentration of allergen extract/preparation eliciting a weal of the same size as that of histamine 1 mg/ml (5.43 mmol/l) in the median sensitive patient in a sample of patients sensitive to the allergen in question (16).

Different methods for BS using the skin prick test (SPT) method have been described, methods which differ with regard to patient inclusion criteria, test technique (clinical method) and the statistical evaluation of the results. Results from BS have recently been reported (2, 3, 5-9, 15, 19, 20).

The aim of this paper is to report on results obtained with various types of allergens using a method (6) which was developed to fulfil the criteria for BS as defined by the Nordic Council (16).

MATERIAL AND METHODS

Patients

All patients were allergic to the allergen in question and fulfilled the other inclusion criteria given in Table 1.

To identify mite-sensitive patients for BS of *D. farinae* extracts a positive skin prick test (SPT) with routine mite allergen extracts was added as

an inclusion criterium (9). To identify patients sensitive to *Cladosporium* screening was performed (11). Since the highest spore counts outdoors are registered from July to October in Scandinavia, patients with increased symptoms of their asthma or rhinitis during this period, as well as patients with symptoms in a humid indoor environment, were included. All were skin prick tested with three commercially available *Cladosporium herbarum* extracts and a prototype of a purified *Cladosporium herbarum* preparation (11). Specific circulating IgE against *Cladosporium* was determined with Phadebas RAST® (Pharmacia Diagnostics AB, Sweden). Patients with a positive clinical history and/or a positive skin test to at least one of the allergen extracts and/or a positive RAST test were included in the BS.

Sixteen to 42 patients were included in the different BS trials. Patient numbers and characteristics are given in Table 2.

Test preparations

The first batch of each species of highly purified (6) allergen preparation, characterized regarding allergen content and freeze-dried, was delivered by the manufacturer (the inhouse standard, Pharmalgen®, Pharmacia AB, Uppsala, Sweden). In some trials (Nos. 10-12) a crude extract (10a, 11a and 12a) obtained from the same primary extraction procedure was tested simultaneously with the purified material (Table 2). The preparations used for standardization of timothy in different regions were all produced from the same intermediate product (6) (batch No. 403/151-1, NYCO, Oslo, Norway). The allergen preparations were delivered freeze-dried in six three-fold concentrations or in most cases in one concentration, and then diluted in Albumin Diluent® to obtain six three-fold concentrations (1/2-12243). Data on the highest concentration of these preparations are given in Table 2.

Reagents for determination of circulating specific IgE (Phadebas RAST®) were obtained from the manufacturer (Pharmacia, Sweden).

Albumin Diluent (0.9 % saline, 0.03 % albumin human, 0.4 % phenol) was used for reconstitution, dilution on the day of the test and as a negative control. Histamine HCl, 1 mg/ml (5.43

Table 1
Patient inclusion criteria

1. A positive case history for the allergen to be tested.
2. A mean diameter > 4 mm using histamine HCl 1 mg/ml.
3. No previous immunotherapy with the offending allergen or any allergen cross-reacting with this.
4. Living in an environment where the allergen in question is an adequate problem.
5. More than 15 years of age.
6. No treatment with anti-allergic drugs for 15 days prior to the test.

mmol/l; 0.63 mg/ml histamine base) was used as a positive reference (Pharmacia AB, Sweden).

Skin prick test method

The SPT was essentially performed as previously reported (5). In essence six three-fold concentrations of the extract/preparation were tested in duplicate on the back of the patient. When one crude and one purified extract from the same allergen source material were tested at the same time, the tests with the crude extract and with the purified preparation were allocated on the back of the patient in a mirrored way (5).

Determination of specific IgE in serum

Specific IgE was determined using Phadebas RAST (Pharmacia AB, Sweden). Sera were

analysed undiluted and diluted 5, 25 and 125 times. Duplicate tests were performed with each dilution. Results were given in Phadebas RAST Units (PRU/ml). Results obtained between the “B” and “C” points of the reference curve (RAST class 2, 0.7–3.5 PRU/ml) multiplied by the dilution factor were used.

Statistical analysis

The concentration of allergen extract/preparation eliciting a weal of the same size as histamine, 1 mg/ml (C_h), was calculated according to the model $\log D = a + b \log (\text{conc})$, where D is the mean diameter of allergen weals obtained with the same concentration, “a” the intercept and “b” the slope. Only mean weal diameters ≥ 2 mm were considered, as weals < 2 mm in diameter can be provoked by the trauma of the

Table 2
Basic data on trials and patients. The trial number given is used in the text and Figs. 1 and 3

Trial No.	Species	C/P	μg d.w./ml	Pat. n	Age (mean SD)	Sex M/F	Excluded patients		
							n < 3	r < 0.85	b < 0.1
1.	Birch <i>Betula ver.</i>	P	150.0	38	–	–	–	–	9
2.	Alder <i>Alnus glut.</i>	P	8.0	16	25 $\pm$ 7	13/6	1	2	–
3.	Hazel <i>Corylus av.</i>	P	6.4	16	29 $\pm$ 9	13/3	–	3	–
4.	Timothy <i>Phleum prat.</i>	P	225.0	42	–	–	–	3	–
5.	<i>Phleum prat.</i>	P	31.5	16	25 $\pm$ 7	11/5	1	1	–
6.	<i>Phleum prat.</i>	P	23.8	22	–	–	1	8	–
6a.	<i>Phleum prat.</i>	P	23.8	23	–	–	1	7	–
7.	Rye grass <i>Lolium perenne</i>	P	2.9	20	26 $\pm$ 6	16/4	–	2	–
8.	Cultivated rye <i>Secale cereale</i>	P	46.0	18	23 $\pm$ 6	10/8	1	1	–
9.	Velvet grass <i>Holcus lanatus</i>	P	11.0	16	24 $\pm$ 5	15/1	1	2	1
10.	Mugwort <i>Artemisia vulg.</i>	P	100.0	20	27 $\pm$ 15	13/7	2	5	1
10a.	<i>Artemisia vulg.</i>	C	100.0	23	27 $\pm$ 15	11/12	1	3	1
11.	Mite <i>D.farinae</i>	P	750.0	24	23 $\pm$ 15	16/8	–	2	–
11a.	<i>D.farinae</i>	C	1140.0	23	23 $\pm$ 15	15/8	1	2	–
12.	Mould <i>Cladosporium</i>	P	729.0	26	25 $\pm$ 8	18/8	4	–	–
12a.	<i>Cladosporium</i>	C	7290.0	25	29 $\pm$ 20	18/7	4	1	–

C: crude, P: purified material. μg d.w./ml: amount of allergen material in the highest concentration tested. Reason for exclusion, according to the statistical criteria set up, and the number of patients excluded because of these criteria, are given.

needle itself. Data from patients showing weals ≥ 2 mm in mean diameter to less than three concentrations of allergen were excluded from the final calculations. Patients giving responses resulting in regression lines with $b < 0.1$ or $r < 0.85$, were also excluded (statistical exclusion criteria), as too flat (b) or undefined (r) regression lines may influence the final result in an unpredictable way. The C_h concentration was calculated for each patient. C_h from patients fulfilling the inclusion and the statistical criteria were used for the calculation of the median C_h concentration, 1000 BU/ml (earlier also called 1 HEP), the range of C_h and the 95 % confidence intervals of the C_h -values. The median C_h was preferred to arithmetic or geometric mean values, as the median is not influenced by single extreme results (5). All slopes with a coefficient of correlation ≥ 0.85 were included when calculating the variation of slopes within and between allergen preparations. Differences in biological activity between extracts were tested with the Mann-Whitney test.

Ethics

All trials were approved by the respective authorities in the different countries and by the local ethics committees. All patients gave their informed consent.

RESULTS

In the final calculation 16–42 patients were included in the BS of each allergen (trial). This corresponds to 70–94 % of the patients initially included in each trial. In one trial (No. 6 and 6a), using two preparations of timothy (4), results from about 30 % of the patients were not possible to use for statistical reasons, despite the fact that they had initially complied with the inclusion criteria. The reasons (statistical) for exclusion of patients are given in Table 2. In most cases this was due to a combination of a low coefficient of correlation (r) and a flat dose response relationship.

The same concentrations of timothy allergen preparation from the same batch and histamine 1 mg/ml were used in two trials (trial Nos. 5

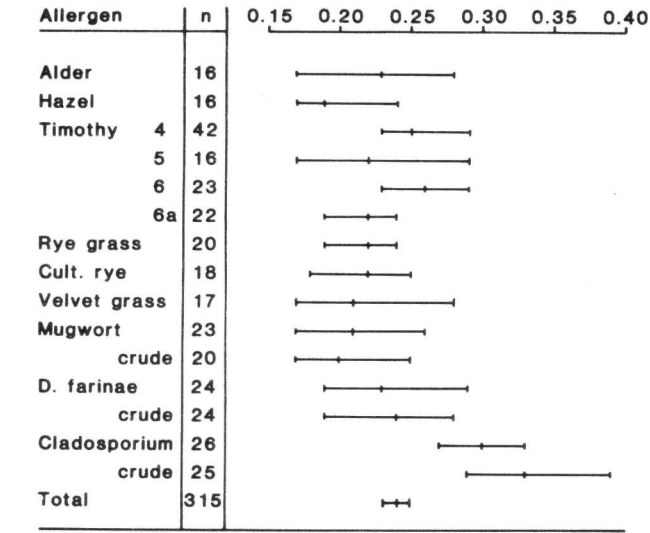


Fig. 1. The slope of the allergen dose response relationship in SPT for a number of highly purified allergen preparations (1–12) and crude extracts of mugwort, *Cladosporium* and *D. farinae* (10a–12a). n denotes the number of patients, 4 to 6a are different trials with timothy allergen preparations produced from the same purified intermediate product (IMP) (6) (Table 2). Trials 6 and 6a were performed simultaneously in the same patients using two different batches from that IMP. The median b and the 95 % confidence limits are shown. At the bottom is shown the median for all 315 patients investigated with different allergen preparations.

and 6a). The response to histamine was higher in trial No. 5 than in trial No. 6a ($P < 0.05$). In trial No. 6 nine of 31 patients, primarily included (30 %) on the basis of a positive clinical history of grass pollinosis, showed negative or only slightly positive responses to approximately 10,000 BU/ml. On the other hand, in the *D. farinae* trial the concentrations chosen induced weals larger than those by histamine 1 mg/ml in more than 50 % of the patients (9).

The median slope of the allergen dose response relationship calculated from 315 slopes with a correlation coefficient ≥ 0.85 was 0.24 (Fig. 1), with 95 % confidence limits from 0.23 to 0.25. For all allergens but *Cladosporium* the 95 % confidence interval of the median slope overlapped, at least partly, this interval. The median slopes of pollen preparations varied between 0.19 and 0.25, whereas the median slopes of the regression lines of purified and crude *Cladosporium herbarum* extracts were significantly steeper, or 0.30 and 0.33 for the purified and the crude preparations respectively

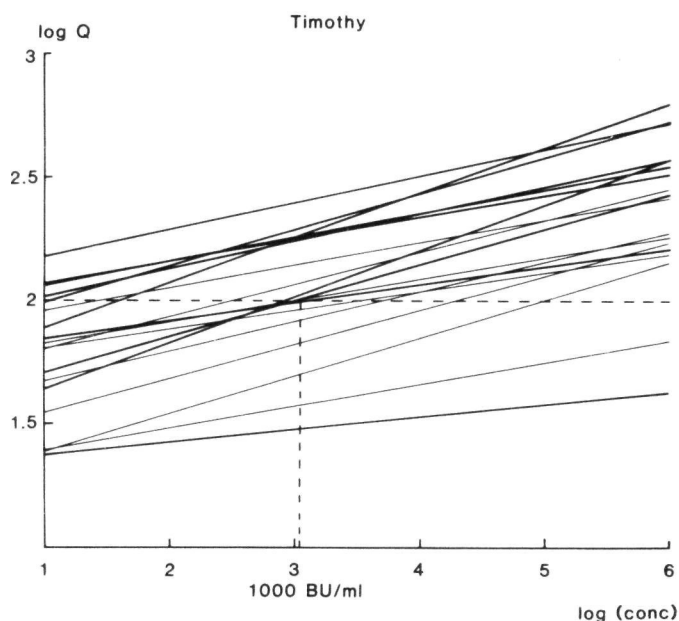


Fig. 2. The calculated regression lines of the allergen dose response relationships in one trial with 18 patients (trial No. 5), two patients with $b < 0.1$ are included. The relative concentrations given on the X-axis are the three-fold concentrations used for SPT. Q indicates the mean diameter of allergen weals in % of the histamine weal. Thus 2 indicates 100% i.e. the histamine response in the individual patient, the crossing point between the regression lines and the horizontal dotted line indicates the individual C_h concentrations and the vertical dotted line the median C_h or 1000 BU/ml.

(95 % confidence limits 0.27 to 0.33 and 0.29 to 0.39, respectively). A typical example of regression lines in individual patients is shown in Fig. 2 (timothy, trial No. 5).

A hook (10), i.e. an unexpected low response to one intermediate concentration causing a hook on the plotted dose response curve, was found in many patients. These hooks varied from scarcely detectable to pronounced. A negative response to one of the duplicate tests resulted in pronounced deviation of the plotted dose response relationship. Data not shown as the classification was difficult.

The median C_h , the range of C_h and the 95 % confidence limits of the median C_h for all allergens are given in Fig. 3. Timothy allergen preparation was tested in four trials (4, 5, 6 and 6a) in different regions, all with material deriving from the same purified batch. The amount of material needed to elicit a C_h reaction in the median sensitive patient, i.e. 1000 BU/ml, varied within a factor two. The median value

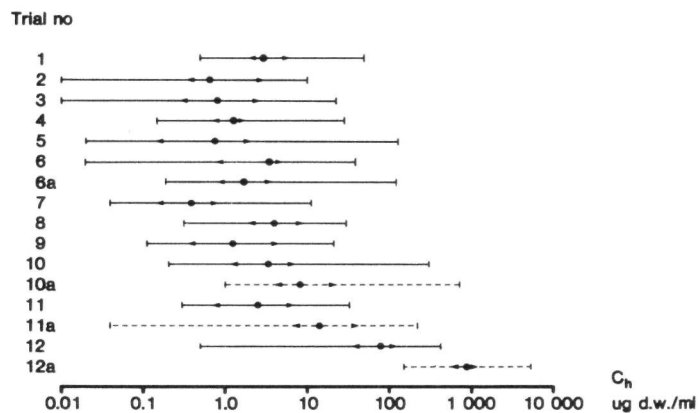


Fig. 3. The amount of allergen eliciting a weal of the same size as histamine 1 mg/ml (C_h), in the sample of patients used for standardization of different allergens. Trial numbers are given in Table 2. The median values (closed circles), the 95 % confidence intervals (arrows) and the range (end of horizontal bars) are indicated. Broken lines indicate crude extracts and unbroken lines denote purified preparations.

varied within a factor three within and between all other species of purified allergens except *Cladosporium*. The purified *Cladosporium* preparation required 60 times more material than the timothy. The crude extracts required five to ten times more material than the purified preparation to obtain an activity corresponding to 1 HEP, demonstrating that the allergens are concentrated in purified preparations. The individual sensitivity of patients to most preparations varied, up to four powers of ten.

Ten times more material was needed to elicit an activity of 1 HEP with cultivated rye than with rye grass (Fig. 4). The difference was statistically significant if all patients were included ($P < 0.05$), as well as when results from the 16 patients included in the BS of both preparations were compared using paired data ($P < 0.01$).

Also the uptake on the RAST-discs differed significantly ($P < 0.05$) between these two species for the eight patients who were tested with both types of discs (Fig. 5).

No correlation was found between the C_h -values and the PRU-values as exemplified by timothy in Fig. 6. However, there was a correlation ($r = 0.94$, NS) between the median C_h -value (1000 BU/ml or 1 HEP) and the median PRU-value for grasses. If all preparations were included no correlation was found.

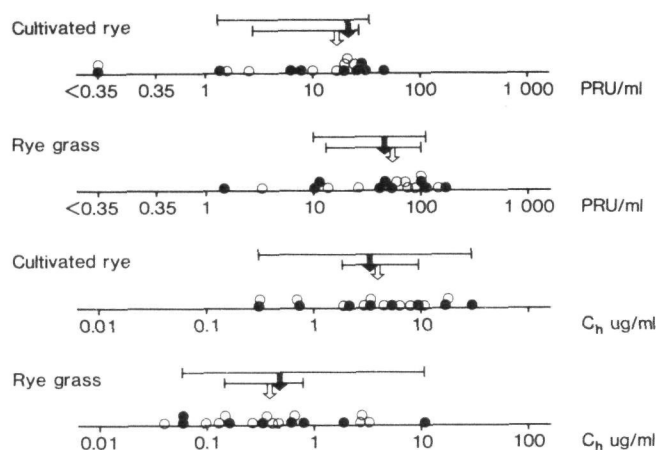


Fig. 4. The median PRU/ml (top) and C_h for patients tested with both cultivated rye and rye grass (filled symbols) and for patients treated with only one of these allergens (open symbols). The C_h concentration (circles), the median C_h (arrows) and 95% confidence intervals (bars) are shown.

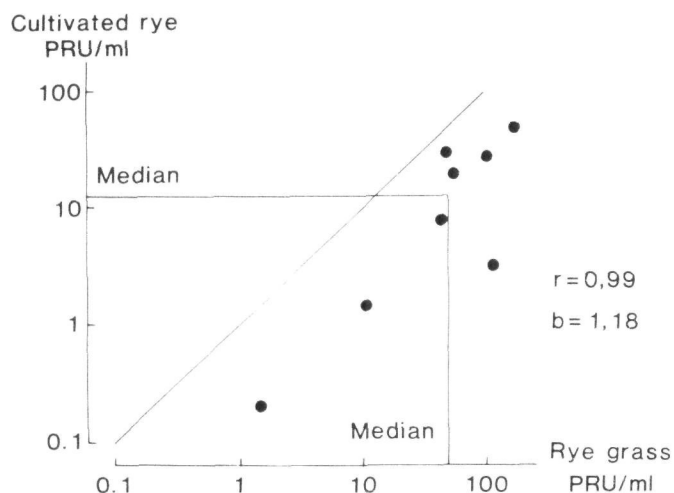


Fig. 5. The correlation between PRU-values to cultivated rye and rye grass in 8 patients included in the BS of both allergens. The ideal correlation $x = y$ and the median PRU-values for both preparations are shown.

DISCUSSION

Biological standardization was performed with 10 different species of highly purified allergen preparations (6). Our method, as well as most other techniques described in previous studies, make use of six three-fold concentrations of allergen (1/1–1/243) (3, 5, 8, 9, 19, 20) or a single concentration (2). The median C_h (1000 BU/ml) was lower than the lowest concentration chosen for BS in the *D. farinae* trial. Therefore,

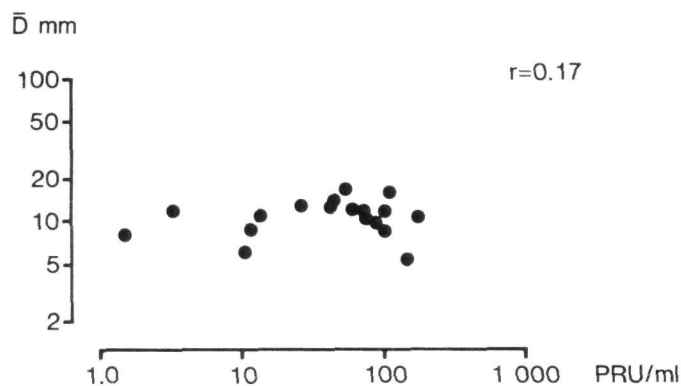


Fig. 6. The correlation between the diameter of the weal induced by the highest concentration of timothy and the corresponding PRU-values (trial No. 5).

most patients reacted with larger weals in SPT to all concentrations of *D. farinae* than to histamine HCl 1 mg/ml. On the other hand, if the chosen concentrations were too low in relation to patient sensitivity, then many patients gave negative results of a low coefficient of correlation as in trial No. 6 and 6a, most often in combination with a slope < 0.1 (Table 2). This was probably due to the low precision of the skin prick test method at low response levels (17).

We have confirmed the presence in SPT of a hook on the plotted allergen dose response curve, earlier described by Huggins & Brostoff (10). We propose that this hook is due to the flat dose response curve of allergen in skin prick testing in combination with the limited precision of the SPT method (7, 13, 14). Single outliers can cause a hook even if the mean of duplicate tests is used for the calculations. To avoid this error, triplicate or even quadruplicate SPTs with each concentration of test solution should be used in BS. If multiple tests are used, this will probably also decrease the number of patients with a bad correlation, which will result in C_h -values in a higher proportion of patients, i.e. more representative results.

Not only our method for BS, but also those of several others use one (2) or a few concentrations (3, 20, 21) of allergen, within a limited range of concentrations. Björkstén et al. (2), as well as Turner et al. (18), excluded negative results and Rastogi et al. (15) included only patients reacting in SPT with erythema

diameters of at least 40 mm to a biologically undefined concentration of allergen preparation, which might exclude clinically sensitive patients and thereby skew the results.

The individual C_h -values were distributed over three to four powers of ten in all patient groups. Therefore, a wide range of allergen concentrations should be used for BS.

Despite the fact that a pronounced cross-reactivity between different grasses has been shown (12), 10 times more (NS) purified cultivated rye material than perennial rye was needed to obtain a response corresponding to 1000 BU/ml. Cultivated rye behaves as a dilution of rye grass in SPT ($r = 0.99$, $b = 1.18$) (Fig. 6). The cultivated rye and the rye grass preparations have been purified by the same procedure (6). The specific biological activity (BU/ μ g d.w.) of both species varies between batches within a factor < 2 (5). It seems likely that cultivated rye contains a higher proportion of nonallergenic material within the molecular range 3000–70,000 d present in highly purified allergen preparations (6).

Crude extracts and the corresponding purified preparations were tested in three trials, (trial Nos. 10, 11, 12). The amount of allergen needed to obtain an activity of 1 HEP with crude extracts and purified preparations differed most for *Cladosporium herbarum* which is the most complicated source material.

Standardization of the timothy pollen allergen preparation was done in three different regions on three different patient populations. The results indicate approximately the same patient sensitivity in these regions (4). In one of the trials with timothy pollen allergen preparation (No. 6a), 30% of the patients did not deliver reliable results. This was mainly because the SPT technique used by that investigator induced smaller weals than others. Thus, many patients had to be excluded because of small weals induced by the allergen concentration used, resulting in bad precision due to a low correlation coefficient in combination with a flat slope. The same concentrations were found suitable in another trial (No. 5). The difference in technique was demonstrated by the difference in weal sizes obtained with histamine 1

mg/ml in these two trials. It is essential that all patients fulfilling the inclusion criteria are included also in the final calculation of the median C_h -value, i.e. the 1000 BU/ml or the 1 HEP value. Otherwise, the results may be skewed, e.g. by excluding patients with low sensitivity.

No correlation was found between the individual PRU-values and C_h -values. This finding can be explained by the fact that the skin reactivity depends not only on the level of specific IgE, but also on other factors such as the releasability of mast cells/basophils as proposed by Conroy et al. (4).

Based on the present results, we suggest that for BS a broader range of allergen concentrations should be used, and the concentrations used in the individual patient should be individually chosen to assure positive test results in all patients.

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