

Biological equilibration of allergen preparations: methodological aspects and reproducibility

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(Received in original form 18 December 1986 and in revised form 26 April 1987; accepted for publication 30 April 1987)

Summary

A method for biological equilibration (BE) of allergen reference preparations using the skin-prick test (SPT) method and histamine HCl 10 mg/ml as reference substance (reference method), was evaluated. The precision was low for weals less than 10 mm². The slope (log weal area/log concentration) of allergen and histamine did not vary significantly between investigators and allergens. The median slopes were 0.39 ($n=384$) and 0.34 ($n=397$), for allergen and histamine, respectively ($P<0.01$). The concentration of allergen eliciting a weal of the same size as that of histamine HCl 1 mg/ml (C_{hi}) in the median sensitive patient, 1000 Biological Units/ml (BU/ml), did not vary significantly between clinics/geographical regions (grasses, mites and moulds). As BE is repeatable between regions, BUs estimated by this method are generally valid. A high correlation ($r=0.91$, $P<0.001$) was found between the median C_{hi} as estimated with histamine 1 and 10 mg/ml as reference substance, respectively. Thus, this reference method for BE is valid. The precision of the SPT method with histamine HCl 1 mg/ml is not as good as with 10 mg/ml, which is therefore recommended as the reference concentration.

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Introduction

Biological standardization of allergen extracts by means of skin-prick testing (SPT) has been proposed by Aas *et al.* [1] and recommended by the regulatory authorities in the Nordic countries [2]. The unit, 1000 biological units/ml (BU/ml), was defined as the concentration of allergen eliciting a weal of the same size as that of histamine HCl 1 mg/ml (C_{h1}), in the median sensitive patient in a sample of patients clinically sensitive to the allergen in question. Recently, the term biological equilibration (BE) has been proposed as more accurate for the purpose [3], as the aim with this procedure is to establish standards of allergenic preparations of different species with biologically equivalent potency. Several methods for BE have been described [4–9], which are either not documented in detail [4–7] or do not ensure reliable results from all patients included [8,9].

In order to improve the method for BE, we studied the dose–response relationship of histamine and allergens (dog and Timothy) and the precision of the SPT method [10]. The precision was higher for concentrations inducing weals larger than, rather than smaller than, 10 mm² (approximately 3.5 mm in diameter). Furthermore, the slope of the allergen dose–response relationship was essentially parallel to that of histamine.

The aim of this communication is to evaluate a new method [9] for BE that allows the use of results from all included patients. Patient inclusion criteria, SPT procedure and statistical method are evaluated.

Definitions

A	The area of a single weal reaction.
$\bar{A}$	The geometric mean of weal areas obtained with quadruplicate SPTs performed with one concentration of test solution.
C_{h1}	The concentration of allergen, most often expressed in μ g dry weight/ml (μ g d.w./ml), estimated to induce an allergen weal response corresponding to that of histamine 1 mg/ml in the same patient.
C_{h10}	The concentration of allergen estimated to induce an allergen weal response corresponding to that of histamine 10 mg/ml in the same patient.
$C_{h10:10}$	The concentration of allergen estimated to induce an allergen weal response corresponding to that of histamine 1 mg/ml in the same patient by first estimating C_{h10} and then dividing by 10.
1000 BU/ml	The median C_{h1} in a sample of patients.

Subjects and methods

Patient material

Patient characteristics are given in Table 1. The main patient inclusion criteria were a positive clinical history and a positive SPT supplemented for moulds and mites by a positive RAST test. One or two trials were performed with each allergen (Table 1, Figs 1 and 2). One or two clinics within the same geographical region participated in each trial. In Table 1 are given the number of patients included in each trial and in Fig. 2 the number of patients fulfilling the statistical criteria set up (see below). In Fig. 3 the number of patients in each clinic with a positive weal response to the histamine references are shown. Trials were divided into clinics as the technique might differ

Table 1. The allergens tested and the country in which BE was performed

Allergen	Country	n*	M/F†	Mean age	Range
Rye grass	U.K.	22	12/10	26	(18–51)
	Sweden/Norway	26	17/9	23	(19–36)
Timothy	U.K.	22	12/10	26	(18–51)
	Sweden	12	6/6	22	(18–36)
Mugwort	Sweden	17	11/6	32	(15–60)
Wall pellitory					
<i>P. officinalis</i>	Spain‡	36	13/23	31.5	(15–49)
<i>P. judaica</i>	Spain‡	36	13/23	31.5	(15–49)
Birch	Sweden	29	11/18	26	(17–53)
Dog	Sweden	20	8/12	27	(18–51)
Cat	Sweden	22	9/13	33	(23–51)
Horse	Sweden	18	4/14	26	(19–41)
<i>Dermatophagoides pteronyssinus</i>	U.K.	24	13/11	22	(19–44)
	Sweden/Norway	29	9/20	32	(17–45)
<i>Dermatophagoides farinae</i>	U.K.	24	13/11	22	(19–44)
	Sweden/Norway	30	8/22	32	(17–45)
<i>Alternaria alternata</i>	Denmark	31	18/13	24.5	(17–55)
	Sweden/Norway	28	12/16	24	(15–41)
<i>Cladosporium herbarum</i>	Denmark	31	19/12	26.5	(17–55)
	Sweden/Norway	26	11/15	25	(16–41)

* n = number of patients.

† M/F = sex distribution.

‡ Reported in reference 13.

between centres participating in the same trial. Patients were excluded from the calculations if they had taken anti-allergic medication (one patient). Statistical exclusion criteria are given below.

Material for skin-prick test (SPT)

Freeze-dried allergen preparations (in-house references, Pharmacia AB, Sweden) of each species were used. These preparations were highly or partly purified [9]. Their composition was characterized by biochemical and immunochemical methods (Table 2 [12–20]). Albumin diluent (saline 0.9%, albumin human 0.03% and phenol 0.4%), Histamine HCl, 1 and 10 mg/ml (5.43 and 54.3 mmol/l or 0.63 mg/ml and 6.3 mg/ml histamine base) (Pharmacia AB), skin-prick test lancets [21] (uncoated Phazet®, Pharmacia AB), fine filter-tip pens and transparent tape for the registration of weal reactions were used.

The skin-prick test method

The SPT method has been described in detail elsewhere [9]. The stock solution (Table 2) was diluted five times to obtain six ten-fold dilutions. The three lowest concentrations were tested in duplicate on the forearm of the patient together with histamine 1 mg/ml. The concentration of allergen eliciting a weal of approximately the same size as that of histamine HCl 1 mg/ml (C_{h1}) and the next two higher concentrations, were used in the final test. If all three allergen concentrations gave smaller weals than that of histamine HCl 1 mg/ml, then the three highest concentrations were used. The test sites

Table 2. Biochemical and immunochemical characteristics of allergen preparations used for biological equilibration (BE)

	Highest concentration (mg d.w./ml)	Proteins (% of d.w.)	Carbohydrates (% of d.w.)	CIE/CRIE (reference)
Rye grass	0.12	51.0	13.0	*
Timothy grass	0.06	59.4	9.3	*
Mugwort	1.0	31.4	36.8	12
Wall pellitory				
<i>P. judaica</i>	3.0	22.6	25.0	13
<i>P. officinalis</i>	3.0	37.9	24.0	13
Common silver birch	0.5	43.2	6.6	14
Dog dander	10.0	52.7	9.6	15
Cat epithelium	10.0	59.6	8.0	16
Horse dander	10.0	81.8	2.4	*
<i>Dermatophagoides pteronyssinus</i>	10.0	41.9	22.0	17
<i>Dermatophagoides farinae</i>	10.0	44.7	13.7	18
<i>Cladosporium herbarum</i>	10.0	23.2	53.6	19
<i>Alternaria alternata</i>	10.0	14.8	74.6	20

* Not published.

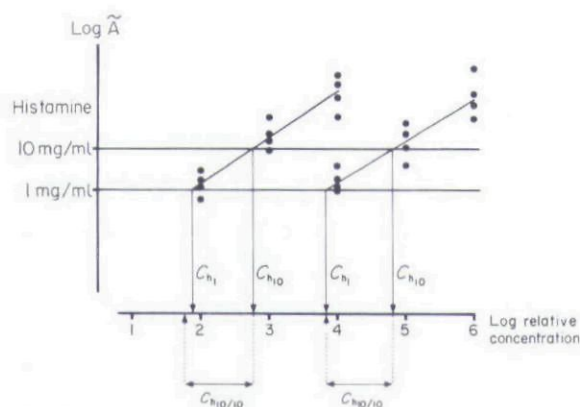


Fig. 1. The principles of the skin-prick test method. The concentration eliciting a weal of approximately the same size as that of histamine 1 mg/ml is chosen, in a pre-test on the forearm, in the examples shown, relative concentrations 2 and 4. The area of weals obtained with these concentrations of allergen, the pre-selected and two 10 and 100 times higher concentrations, in two patients are shown. The geometric means of the weal areas obtained with histamine HCl 1 and 10 mg/ml are indicated by horizontal bars. According to the method, the concentration of allergen eliciting a weal area of the same size as that of histamine 10 mg/ml is estimated by regression-line analysis and the result divided by 10 to obtain the C_{h1} concentration for that patient. For evaluation of the method, the C_{h1} value was also estimated directly using histamine HCl 1 mg/ml as reference.

were allocated in a mirrored way on the back of the patient. The final test consisted of quadruplicate tests with the three allergen concentrations chosen according to the pre-test, histamine HCl 1 and 10 mg/ml. All patients were thus tested at the same part of the dose-response curve (Fig. 1). After 15 min, the weals were encircled with a fine filter-tip pen and the contours transferred by means of a translucent tape to a record sheet. The area of each weal was estimated with a digitizer connected to a Vax computer.

Carbohydrate content

The total carbohydrate content was determined by gas liquid chromatography according to Sawardeker *et al.* [22].

Protein determination

The protein content was determined by amino acid analysis according to Spackman *et al.* [23].

Statistical analysis

The model $\log \hat{A} = a + b \log (\text{concentration})$, best fitted to the allergen and histamine dose-response relationships was used in all calculations [10].

Three main areas were evaluated: the selection of patients, the SPT procedure and the statistical method.

The criteria for selection of patients. These were evaluated by studying the repeatability of the 1000 BU/ml concentration between different clinics/geographical regions.

The SPT method. This was evaluated by estimation of the precision at different response levels and the variability of the weal response to histamine HCl 1 and 10 mg/ml between centres. The precision of the SPT method was studied in terms of the coefficient of variation (c.v.). For this purpose, weal areas $< 3 \text{ mm}^2$ were regarded as negative. Geometric mean weal areas $< 3 \text{ mm}^2$ were excluded. Due to the high c.v. at low response levels [10, 11], a higher cut-off limit (7 mm^2) was used when estimating the C_{h1} (see below) than for estimation of the precision.

The variability of the response to histamine was studied by estimating the median, 95% confidence limits and range for the geometric mean of weal areas obtained with each of the two concentrations of histamine used.

The statistical method. This was evaluated by estimation of the possible difference in slopes ($\log \text{weal area} / \log \text{concentration}$) between allergen (three concentrations) and histamine (only two concentrations) and the correlation between C_{h1} obtained by direct and indirect calculation as described below (C_{h1} and $C_{h10}:10$).

The statistical method that was applied has been described elsewhere [9]. For each patient, linear regression analysis was performed using the model $\log (\hat{A}) = a + b \log (\text{concentration})$. The 'slope' (b) of the histamine dose-response relationship was estimated from the geometric mean of weal areas of only two ten-fold concentrations of histamine HCl, 1 and 10 mg/ml, that of allergen using three ten-fold concentrations.

Results obtained from patients who showed a correlation coefficient < 0.85 and/or slope < 0.1 were excluded. This position has been taken in earlier studies [8-11] since such dose-response relationships will give non-precise results. Data from patients with a geometric mean of weal areas $< 7 \text{ mm}^2$, with all concentrations of allergen or the histamine HCl concentration used for estimation of C_{h1} (1 or 10 mg/ml), were excluded in this part of the study. If only two concentrations of allergen elicited a positive response (geometric mean of weal areas $> 7 \text{ mm}^2$), then these were used for estimation of the C_{h1} .

For testing of the difference in slope between allergen and histamine, only patients who gave data with both allergen and histamine were considered.

Estimation of the C_{h1} concentration was done in two different ways: the concentration eliciting a weal of the same size as histamine 10 mg/ml (C_{h10}) was

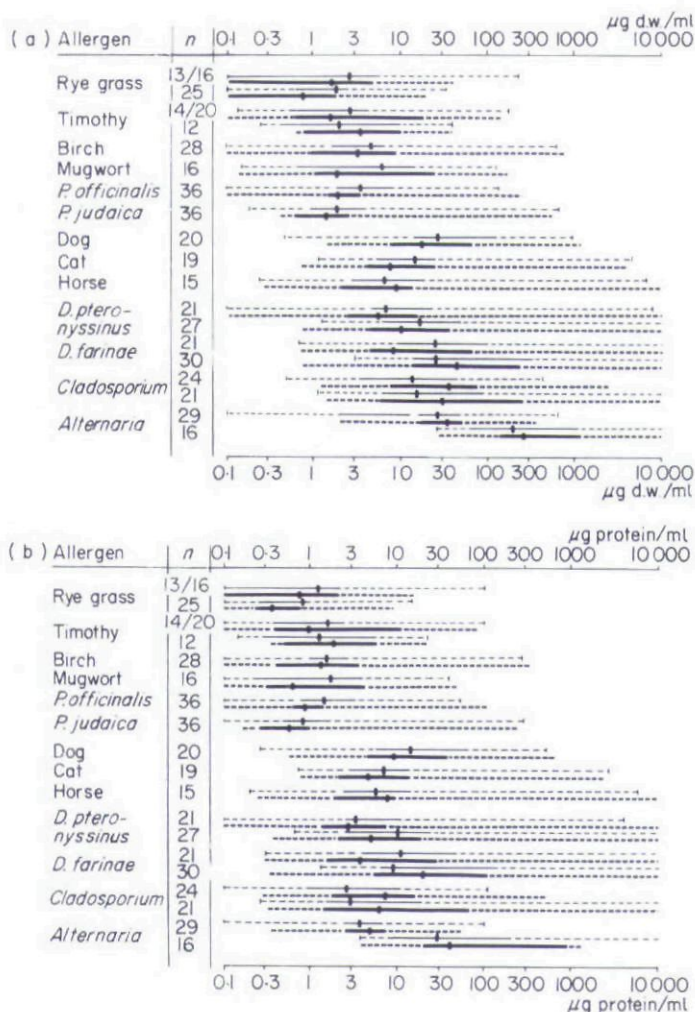


Fig. 2. The median, 95% confidence intervals and the range for C_{h1} (upper, thin line) and $C_{h10:10}$ (lower thick line) for all trials. The amount of allergenic material in $\mu\text{g d.w./ml}$ ($\mu\text{g dry weight/ml}$) (a) and in $\mu\text{g protein/ml}$ (b). For most allergens data from two BE trials in different clinics in different geographical regions are given (Table 1).

estimated and the result divided by 10 (Fig. 1) [9]; the concentration C_{h1} was also estimated directly using histamine HCl 1 mg/ml as reference [8].

The relation between C_{h1} concentrations obtained according to these two methods was studied estimating the Pearson correlation coefficient.

Ethics

All patients got full information about the aim of the trials, written or oral, and gave their oral or written informed consent, in accordance with the regulations in the different countries.

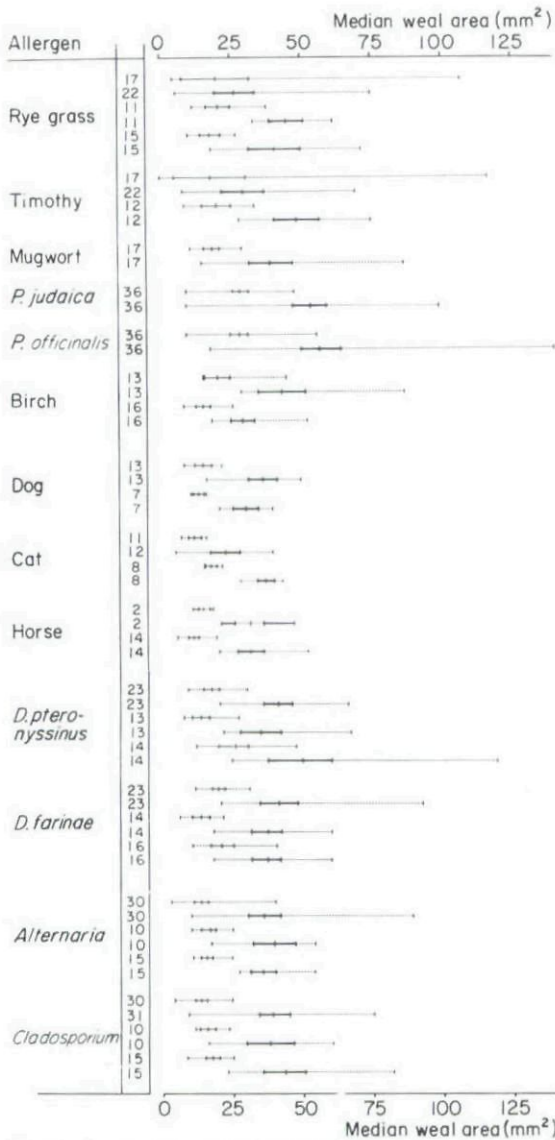


Fig. 3. The area of weals obtained with histamine HCl 1 (upper bar) and 10 (lower bar) mg/ml in one to two centres/trial are given (mean, 2 s.e.m. and the range). n denotes the number of patients with a mean weal area more than 7 mm² (3 mm in diameter).

Results

Patient selection criteria

The 95% confidence interval of the C_{h1} concentration, obtained with the same test material, the same test and the same method for estimation of the median C_{h1} in different clinics/geographical regions overlapped. Thus, there was no significant difference in the median C_{h1} value between regions (Fig. 2). Only one of the *Alternaria* trials gave a higher median C_{h1} than expected.

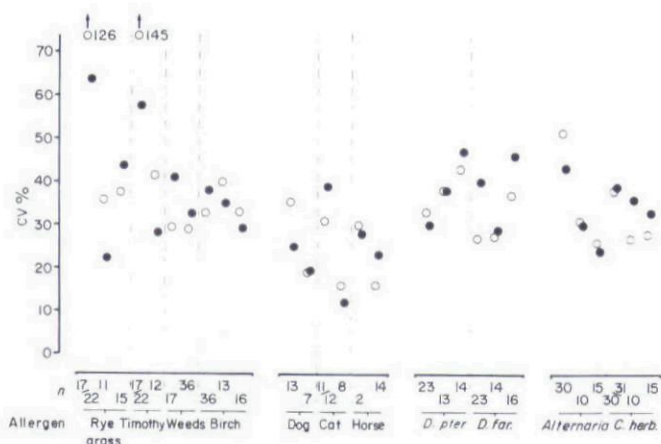


Fig. 4. The precision (c.v. %) of the SPT method calculated on tests with a geometric mean weal area $> 3 \text{ mm}^2$ with allergens (O) and histamine (●) in different trials. If two centres were involved in one trial, this is indicated by two numbers i.e. 17/22. n denotes the number of patients.

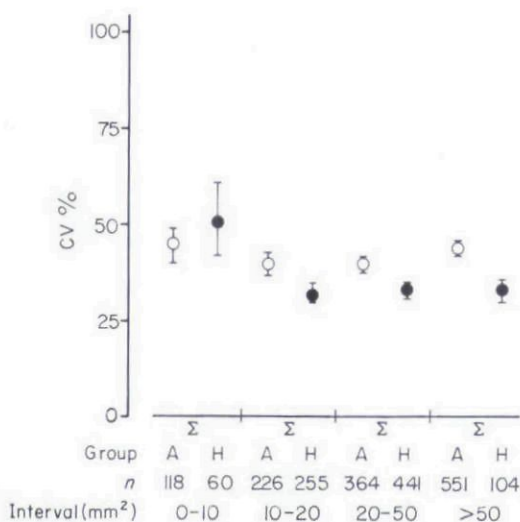


Fig. 5. The precision (c.v. %) of the SPT calculated on all tests with a geometric mean weal area $> 3 \text{ mm}^2$. Allergen (A, O) and histamine (H, ●) data are given separately. n denotes the number of quadruplicate tests with a geometric mean weal area within the respective interval (for explanation see statistical analyses).

The skin-prick test method

Positive reactions were obtained with the concentrations of allergen chosen from the pre-test, in all but two grass pollen trials, those with the lowest precision. The areas obtained with histamine HCl 1 mg/ml, varied considerably. Individual geometric means were less than 7 mm^2 in seven of twenty-nine centres and the median varied from 11 to 28 mm^2 in different centres (Fig. 3). In one centre/trial (rye grass U.K.) the C_{hi} value was not possible to estimate in nine of twenty-two patients due to the fact that the geometric mean of weal areas, with allergen and/or histamine, was less than 7 mm^2 . In

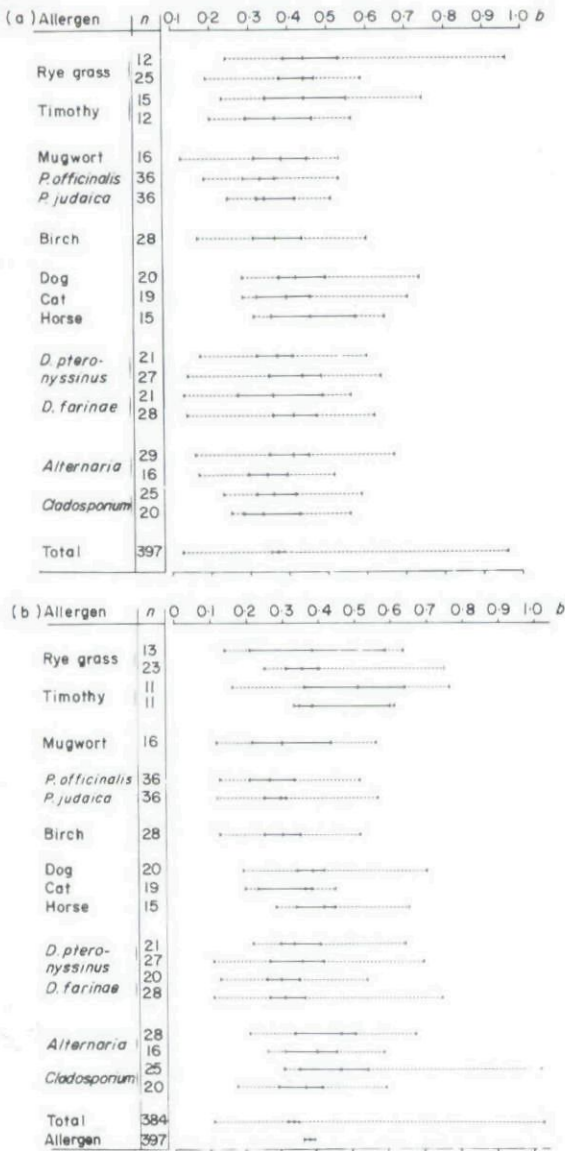


Fig. 6. The slope (b) (log weal area/log concentration) of the allergen (a) and histamine (b) dose-response relationships (median, 2 s.e.m. and range). *n* denotes the number of patients. At the bottom the median and 95% confidence limits of all allergen and histamine dose-response relationships, respectively. At the very bottom of Fig. 6b, the confidence limits for the allergen slope (Fig. 6a) are given for comparison.

all but this trial, weal areas obtained with histamine HCl 10 mg/ml were significantly larger than those elicited by histamine HCl 1 mg/ml.

The precision of the SPT method was similar for the different allergens (Fig. 4). However, in two grass pollen trials, the c.v. was high and in some of the animal allergen trials it was lower than in most other trials.

The precision estimated on all tests with all allergens using tests with geometric mean of weal areas $> 3 \text{ mm}^2$, is shown in Fig. 5. The precision was lower at low

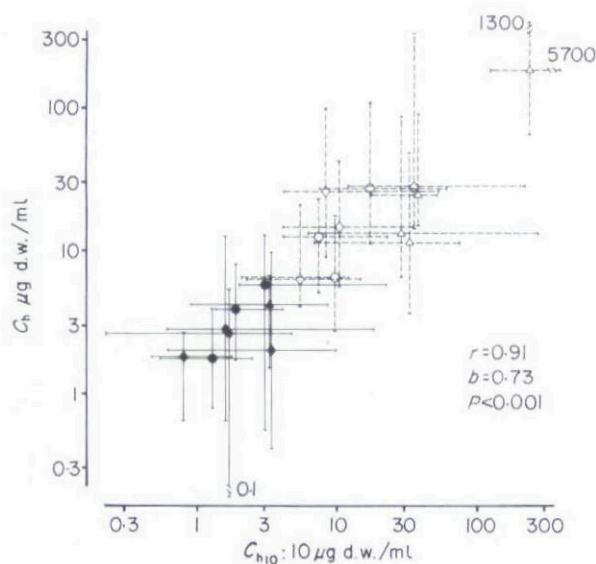


Fig. 7. The correlation between the median C_{h1} and $C_{h10:10}$ concentrations in $\mu\text{g d.w./ml}$ (crossing point). The 95% confidence limits are indicated by the vertical and the horizontal bars, respectively. (—▲—) Trees, (—◆—) grasses, (—●—) weeds, (—△—) moulds, (—◇—) mites, (—○—) animals.

response levels, i.e. geometric mean weal areas less than 10 mm^2 (3.5 mm diameter). However, this tendency was significant only for histamine. In the trials with low precision mentioned above, the tendency towards lower precision was most marked at low response levels (data not shown).

Statistical method

The slope of the allergen dose-response relationship varied considerably between patients. However, no statistically significant difference in slope was found between species, trials or regions (Fig. 6). If all data were pooled, the 'slope' of histamine was significantly flatter than that of allergen ($P < 0.01$) (histamine: median 0.343, 95% confidence limits 0.332–0.357, range 0.109–1.028; allergen: median 0.387, 95% confidence limits 0.371–0.403, range 0.126–0.962).

The C_{h1} value varied at least 10^4 between individual patients tested with the same allergen preparation (Fig. 2). The activity, expressed by the median C_{h1} given in $\mu\text{g d.w./ml}$, varied within a factor of two between allergens from the same group of allergen source materials, i.e. grass pollen, weed pollen, animal, mite and mould allergens. Actually, the median C_{h1} varied between $0.8 \mu\text{g d.w./ml}$ and $6 \mu\text{g d.w./ml}$, i.e. a factor of 2.2 for all pollens and between $6 \mu\text{g d.w./ml}$ and $45 \mu\text{g d.w./ml}$, i.e. a factor of 2.8 for all non-pollen allergen preparations (Fig. 2a). There was only one exception, *Alternaria*, which was significantly less potent than pollen allergens in one of the trials.

The variation in activity between different source materials related to protein content/ml was slightly less than that related to d.w./ml (pollens 0.35–1.2, i.e. a factor of 1.7, and non-pollens 3–20 $\mu\text{g protein/ml}$, i.e. a factor 2.6) (Fig. 2b).

There was a good agreement between the 1000 BU/ml concentrations estimated as median C_{h1} for all tested species and the median $C_{h10:10}$ for the same species (Fig. 2)

and a tendency towards lower values for $C_{h10:10}$ than for C_{h1} , especially for pollens. The opposite seemed to be true for some non-pollen allergens, e.g. the moulds. A high correlation was found ($r=0.91$, $P<0.001$) between C_{h1} and $C_{h10:10}$ values (Fig. 7).

Discussion

Several other methods for estimation of the biological activity of allergen preparations have been described. The activity has been expressed in HEP (histamine equivalent in skin-prick testing) [24], SARA [6,25] or BUs [4]. These different units are not comparable to the BUs described in this communication, as most of them differ from our method regarding patient selection criteria, SPT procedure and statistical evaluation. Their relevance is discussed elsewhere [3,26].

The median C_{h1} concentration (1000 BU/ml) was repeatable between different geographical regions (Fig. 2) [8,11]. The allergenic material and the methods used for estimation of biological activity were the same. The fact that the median C_{h1} did not differ between clinics/geographical regions indicates that the patient selection criteria used are relevant for the determination of biological units.

In a previous study [8,11] we found repeatable median C_{h1} results for Timothy in Sweden, Norway and Germany. In this study the reproducibility was extended to rye grass, mite and mould allergen preparations, tested in Sweden, Norway, Denmark and the U.K. The high concentration corresponding to 1000 BU/ml in one of the *Alternaria* trials illustrates the difficulty in defining patient inclusion criteria for allergens with less known clinical symptomatology.

These data are supported by results obtained by two other groups who used methods similar to ours [8,9] and with the same patient selection criteria. Maasch *et al.* [27] investigated the biological activity of Timothy allergen extracts in two groups of patients with grass pollinosis and found a similar activity. Constable *et al.* [25] found that 11 and 13 $\mu\text{g d.w./ml}$ of a *Dermatophagoides pteronyssinus* allergen preparation corresponded to an activity of 1000 BU/ml, when using histamine HCl 1 and 10 mg/ml, respectively, as reference substance compared to 6–26 μg in our trials using different evaluation methods. These results were similar to those found by us (Fig. 2), thus confirming the repeatability of the median C_{h1} between patient samples.

The median C_{h1} concentrations found in these studies had wide confidence limits. However, when data from eighty-seven patients tested with the same Timothy allergen preparations as used in this investigation [8,11] were utilized, then the confidence limits were 0.8 and 1.8 $\mu\text{g d.w./ml}$. Thus, more precise estimates of the biological activity can be obtained by increasing the number of patients included. The fact that the confidence limits narrowed when data from several trials were pooled indicates that the results in these different trials are similar. By including more patients a better estimate of the possibly small difference in sensitization between samples of patients may be obtained. For this purpose a simplified method has been developed [26].

Based on results from a previous study [10] we chose histamine 10 mg/ml as reference. We also included histamine HCl 1 mg/ml to investigate the possibility of changing the reference, without changing the unit. In this report, patients in some centres did not respond with weals of measurable size ($> 7 \text{ mm}^2$) to histamine HCl 1 mg/ml (Fig. 3). This finding stresses the need for investigators to practice the SPT technique and document their precision, prior to the start of BE, or any other type of

trial using the SPT method, and to use histamine HCl 10 mg/ml rather than 1 mg/ml as reference substance, as earlier proposed [3,28,29].

In a preliminary study [10], the c.v.% was similar at response levels more than 10 mm². This finding was confirmed in the present report (c.v. 15–50% using the weal area, corresponding to 7–25 using the diameter). There was no difference in precision with different groups of allergens. In previous reports the c.v. has varied between 8% and 25% (diameter) [10,28–33], which is similar to our data. The best precision [33] has been found with a metallic device [21]. A c.v. as high as 60% [31], based on the weal area, was found in one report using a plastic device [30]. The present study indicates that a low c.v. is possible to obtain, but also that c.v. varies with investigator [33].

In patients with response to all allergen concentrations, a high coefficient of correlation of the dose–response relationship was found. Although the slope of the dose–response relationship varied considerably between patients there was no significant difference between allergen preparations from different groups of source materials. In a previous study [11] *Cladosporium* had a significantly steeper slope than pollen allergens. This observation could not be confirmed by Malling [28] or by the present study. Malling [28], who used the same *Cladosporium* and *Alternaria* allergen preparations as used in the present report, found the slope of the allergen dose–response relationship to be 0.32 with the 95% confidence limits overlapping our median value (0.39).

The slope of allergen was significantly steeper than that of histamine, if all data in this study were pooled. In contrast, in a previous study [10], the slopes of the Timothy allergen and histamine dose–response relationships were found parallel over a wide range of concentrations. However, the slope of dog allergen and histamine were not parallel in that study. As there were histamine slopes both steeper and flatter than those of dog allergen, we concluded that the allergen and histamine slopes were essentially parallel. In the present study more patients were included. Therefore, the confidence intervals were lessened, which explains the significant difference between slopes. Also the use of only two concentrations of histamine might have contributed to the difference between this and the previous study [10].

In order to check our method, which utilizes histamine HCl 10 mg/ml instead of 1 mg/ml as reference substance [9], we correlated the median C_{h1} to the median $C_{h10:10}$ (Fig. 7). A high correlation was found and for all allergens tested the 95% confidence interval overlapped the ideal slope ($x=y$). A less pronounced, but significant correlation between the individual C_{h1} and $C_{h10:10}$ has been found for *Alternaria* and *Cladosporium* by Malling [32]. This indicates that the reference can be changed without changing the unit prescribed by the Nordic Council on Medicines [2].

The most important finding was that the result of BE was repeatable between clinics/regions, provided the patient selection criteria were kept constant. Thus, the BU values obtained are generally valid. Furthermore, using biologically equilibrated allergens, grading of the skin sensitivity to different types of allergens is possible.

Acknowledgments

The authors wish to express their sincere thanks to all nurses who performed the skin-prick tests, Brigitta Juneskans who co-ordinated some of the trials, Birgitta Dahlström

and Anders Hansson who did the computer work, Leif Hammarström, Johan Björkstén and Hannu Ukkonen who prepared the figures and Hannu Ukkonen who took all the photographs.

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