

Dose response curve of allergen and histamine in skin prick tests

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Twenty-six *Dermatophagoides pteronyssinus* (D.pt.) sensitive subjects were skin prick tested in duplicate with 15 concentrations of D.pt. ranging from 0.0018 to 17.8 mg/ml, 15 concentrations of the major allergen of D.pt., antigen P₁, ranging from 0.0002 to 1.88 mg/ml and 15 concentrations of histamine dihydrochloride solution ranging from 0.048 to 114.0 mg/ml. Weal areas and concentrations were transformed by taking logs and linear and non-linear regression curves fitted, allowing for confounding variables, such as subject, and interactions. The weal areas over all concentrations fitted "S" shaped curves with essentially straight central portions, parallel between materials, with differences between subjects but parallel within subjects. The dose response curves of P₁ and D.pt. were coincident when the concentrations were adjusted to allow for differences in potency. The concentrations of allergen and histamine commonly used for standardisation purposes will give weals that can be plotted along a straight line, but at higher and lower concentrations the response will tail off. This accounts for previously ambiguous results. Standardisation of allergens using 10 mg/ml histamine is preferable to 1 mg/ml.

Key words: allergens; dose-response relationship; histamine; reference standards; regression analysis; skin tests; statistics.

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In recent years quantitative skin testing with allergen and histamine has become very important in the standardisation of allergens. It is generally assumed that the increase in weal size, on arithmetic or logarithmic scales, is linear against increasing logarithmic concentrations of allergen over the range of interest (3, 8-10, 14, 20) tailing off at the top (6) and bottom (10) to form a shallow "S" shape. More recently, it has been claimed (8, 14) that when the logarithm of weal size is plotted against logarithmic concentration for different allergens the lines that best fit the data are parallel.

Aas et al. (1) proposed a unit of allergenic potency whereby an extract at a potency of 1 HEP (histamine equivalent prick) would cause on average a skin prick test weal with the same mean diameter as when histamine 1 mg/ml was used. Dome/Hollister-Stier (DHS) preferred to standardise using histamine 10 mg/ml. It was

suggested that DHS was therefore measuring 10 HEP. Our data did not support this.

A "simple" allergen, such as *Dermatophagoides pteronyssinus* (D.pt.), contains a number of specific major and minor allergens. Experimental work has identified the major allergen, antigen P₁ (4), of D.pt.; e.g. up to 75% of the serum IgE antibodies to D.pt. are directed against P₁. Patients clinically sensitive to mite are a heterogeneous group, some may be sensitive to only a single allergen, others to groups of allergens in different proportions. Skin dose responses therefore can be to a single component or complex composite responses to various possible combinations of several components. Because of this, subjects will not necessarily respond similarly to D.pt.

In this trial, extracts of D.pt., antigen P₁, and solutions of histamine dihydrochloride were used over a broad range of concentrations to

derive the shape of the dose response curve of each.

The opportunity was taken to see if multiple skin prick testing would effect serum IgE levels.

MATERIAL AND METHODS

Solutions

All solutions used to skin prick test (SPT) were manufactured by Dome/Hollister-Stier Allergen Development Laboratory. The highest concentration of each material was prepared, then diluted to give all other concentrations, as shown in Table 1. All the histamine concentrations, the 10 strongest D.pt. solutions and three strongest P₁ solutions were assayed. Other solutions were assumed to be accurate dilutions of the assayed solutions. The solutions were aseptically dispensed (0.1 ml) into clear neutral glass vials, fitted with screw caps. The concentrations were based around the allergen concentrations known to be approximately equivalent to histamine 10 mg/ml in terms of skin reactivity, i.e. 1 mg/ml D.pt. and 0.1 mg/ml P₁. The concentrations ranged from the upper limits of solubility

to solutions with almost negligible amounts of material. The higher strengths increased in 4/3rd steps, to minimise the risk of excessive reactions.

Subjects

The subjects had previously attended the Allergy Clinic at Leicester General Hospital with clinical histories of respiratory allergy to house dust mite and a RAST score of 2 or more to D.pt. After giving informed consent to take part they were skin tested with Alphatest D.pt. 400 AUR/ml (Dome/Hollister-Stier); negative control solution, glycerol-Coca's; and positive control solution, histamine dihydrochloride 10 mg/ml. If they reacted to the D.pt. and histamine solutions (> 3 mm diameter weals) and did not react to the negative control solution, and fulfilled the other entry criteria such as age (18 to 60 years), clinical history, etc., they were admitted to the study.

Skin prick test

SPT were done in two sessions, at least a week apart for each subject. In the first session each subject was tested with 36 solutions, viewed as a 6 × 6 matrix, over most of the back. The 18 solutions coded 1 to 6 in Table 1 were used in duplicate (6 concentrations × 3 materials × 2 replicates = 36 solutions). The locations of the solutions were randomised for each subject using two duplicate 3 × 3 semi-Graeco-Latin squares, so that the top left and bottom right 3 × 3 squares were identical (unknown to the SPT operator), as were the top right and bottom left squares. Thus over the back, each material appeared twice in each row and column and each concentration code once. All testing in the first part was double-blind.

In the second session the positions of testing the various concentrations were the same for all patients but the materials were randomised. The operator, and subject, were blind but an assistant knew the contents of all the vials. The three lowest concentrations and the first of the higher concentrations (codes

Table 1
Solution concentrations (mg/ml)

Code	<i>D. Pteronyssinus</i>	P ₁	Histamine
-2	0.0018	0.0002	0.049
-1	0.0036	0.0005	0.106
0	0.0072	0.0009	0.24
1	0.014	0.0018	0.4
2	0.043	0.0036	1.0
3	0.130	0.011	2.0
4	0.332	0.033	4.4
5	1.03	0.10	10.2
6	3.26	0.33	20.4
7	4.24	0.45	25.3
8	5.26	0.59	36.8
9	7.26	0.78	48.8
10	9.56	1.23	60.5
11	13.29	1.49	84.0
12	17.80	1.88	114.0

-2, -1, 0, 7, in Table 1) of all three extracts were tested initially. If any solution caused a weal greater than 2.5 cm in diameter then testing with that material ceased. Otherwise the next two higher concentrations were used and the process repeated until the top strengths were reached, or all extracts had caused weals greater than 2.5 cm diameter. The unblinded assistant ensured that the correct solutions were used.

One operator (M.A.S.) performed all SPT using a fresh sterile 25 gauge hypodermic needle for each test: puncturing and lifting the skin, without drawing blood, through a drop of each solution. Fifteen minutes later the weals were outlined with a draughtsman's fine tip pen. The markers were transferred by adhesive tape to the correct position on A2 sized case record sheets. The weal areas were measured by a computer image analysis system (Digithurst).

Serology

Each subject had a 5 ml serum sample collected at both sessions and a month after the second session. They were assayed for mite whole cul-

ture and P₁ specific IgE at the Dome/Hollister-Stier Allergen Development Laboratory.

Statistical methods

The weal areas were transformed by adding 1 mm² to the observed area and taking the logarithm. The solution concentrations were also transformed by taking logarithms. For most of the analysis the log concentrations had 4 added for P₁, 3 added for D.pt. and 2 added for histamine. This meant that all values were greater than zero and the concentrations were approximately matched by expected reactivity. Main effects - subjects, materials, etc. - and their interactions were investigated by using dummy variables in linear regression models, removing sets of terms in sequence. For more detailed investigation of the curves, stepwise linear regression was used for linear models and the Marquardt iterative method (17) for non-linear models. When each extract was investigated independently, the responses were standardised within each extract to adjust for differences between subjects, so that each subject's mean log weal area in the first part was equal to the overall mean log weal area in the first part.

Table 2
Weal areas. Log (area mm² + 1)

Strength Code	<i>D. pteronyssinus</i>			P ₁		
	<i>n</i>	Mean	SD	<i>n</i>	Mean	SD
-2	21	0.88	0.32	21	0.59	0.28
-1	20	0.98	0.29	21	0.61	0.34
0	21	1.16	0.32	22	0.71	0.31
1	52	1.15	0.38	52	0.72	0.39
2	52	1.35	0.39	51	0.86	0.35
3	52	1.64	0.37	50	1.07	0.36
4	52	1.91	0.36	51	1.22	0.44
5	52	1.97	0.32	52	1.40	0.40
6	51	2.16	0.34	52	1.77	0.34
7	17	2.25	0.37	23	1.72	0.42
8	15	2.24	0.31	22	1.72	0.37
9	9	2.13	0.36	18	1.83	0.36
10	7	2.15	0.31	17	1.83	0.37
11	6	2.04	0.14	11	1.93	0.19
12	6	2.00	0.29	11	1.93	0.22

Histamine was standardised about its general mean. Linear equations were fitted using linear, quadratic and cubic components. The non-linear Gompertz growth model (7) $y = \alpha \exp [-\beta_0 \exp (-\beta_1 x)]$ was used on all extracts. The statistical computer package SAS (19) was used for all the analyses.

RESULTS

Twenty-eight subjects were tested but two were later excluded due to low RAST scores. The remaining subjects (12 men and 14 women) had a mean age of 32.9 years (SD 10.9, range 18 to 53); mean duration of disease 19.2 years (SD 14.4, range 4 to 50). Three subjects did not return for the second session. There were only eight missing observations in the first part, out of a possible total of 936 weals. The results are summarized in Table 2. Because of the upper safety limit on weal size, the number of subjects decreased as the concentration increased and the mean area stayed below the limit.

The men had weals about 20 % larger than the women but there was no apparent change with age or duration of disease. Previous workers have reported age differences and larger reactions in males (2, 12).

Analysis of the data from the first part showed that the majority of the variation was explained by: the linear component, i.e. increasing weal area with increasing concentration; difference between subjects; difference between extracts; and difference between extracts within each subject. Some 75 % of the variation was explained by these variables ($R^2 = 0.749$). The interaction between subject and linear component was statistically significant ($P = 0.001$) i.e. the best fit lines through the extracts were not parallel between subjects, but it only accounted for an additional 1.4 % of variation. The lines through the extracts were parallel (extract $\times$ linear component interaction and subject $\times$ extract $\times$ linear component interaction, $P > 0.1$). There was evidence for row and column effects, though this may be an order effect. The tests were always administered and marked starting in the top left corner, working along the rows, down to the bottom right corner. Therefore, position and order effects were confounded and not

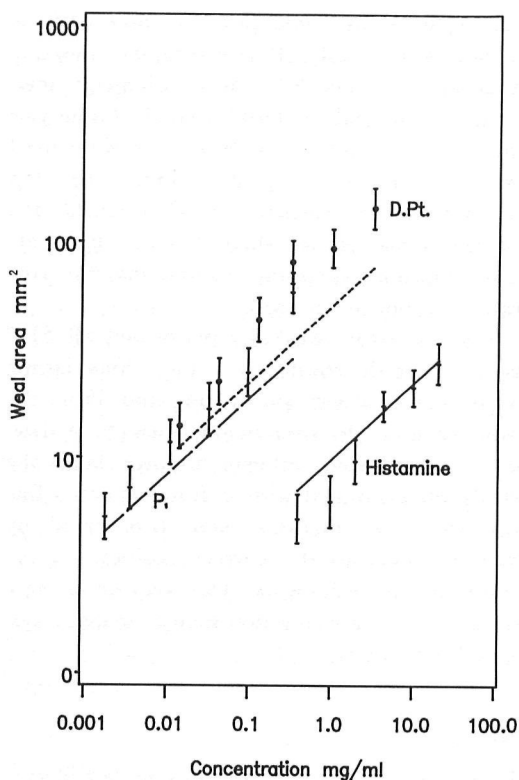


Fig. 1. Best fit regression lines, from model including subjects, extract, subject $\times$ extract interaction, and subject $\times$ concentration interaction effects. This gave the questions:

Histamine: $\log(\text{weal area} + 1) = 0.635 + 0.3547(\log(\text{concentration}) + 1)$

D.pt.: $\log(\text{weal area} + 1) = 0.635 + 0.3547(\log(\text{concentration}) + 2) + 0.3557$

P.: $\log(\text{weal area} + 1) = 0.635 + 0.3547(\log(\text{concentration}) + 3) - 0.0726$

Means and 2 SEM error bars.

separable. There was no evidence of any curvature (square and cubic terms added, $F < 1$, not significant). The three best fit lines from the above are shown in Fig. 1.

The possibility that straight lines could be improved upon was investigated by looking at all the data, from both parts of the experiment, for each extract separately.

Histamine

No subject's reaction to histamine reached the 2.5 diameter upper limit, though some subjects

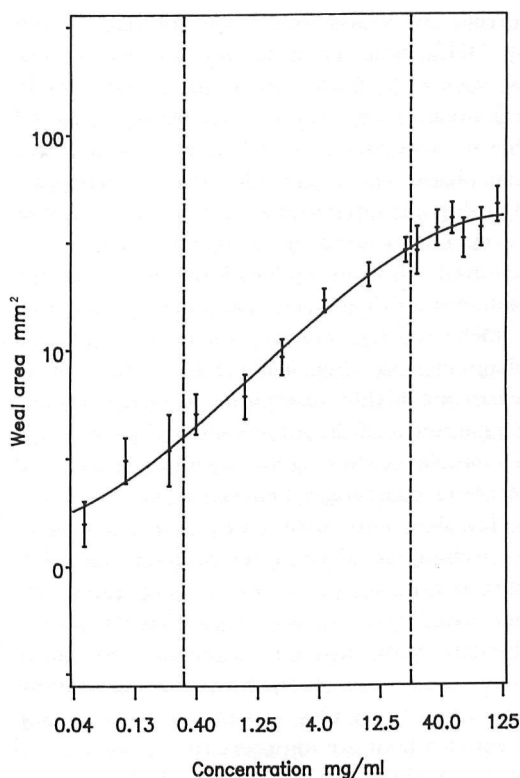


Fig. 2. Histamine. Weal areas standardised about the general mean of the first part. Means and 2 SEM error bars. First part within dotted lines. Cubic curve shown.

did not receive the highest concentrations due to large reactions to the other solutions. Two observations appeared to be outliers, distant from the body of the data, and were omitted from the analysis. A straight line explained 69 % of the variation and though the inclusion of a cubic only improved the $100R^2$ by 0.5 % it had $P = 0.004$ (Fig. 2). Fitting the Gompertz gave an almost identical curve to the cubic over the concentrations used.

Very small weals are often unreliable. Histamine caused fairly small weals: 4.4 mg/ml was the lowest concentration for which all SPT were greater than zero, and at 0.049 mg/ml less than half the SPT caused reactions. However, when curves were fitted to the data without the three lowest concentrations the equations of the best fit curves were practically unchanged.

P_1

P_1 caused larger weals than histamine over the concentrations used, with some zero area weals at the low concentrations but no evidence of plateauing at the high concentrations. Two observations appeared to be outliers and were omitted from the analysis. When a straight line was fitted there was evidence for lack of fit ($P = 0.035$), and the residual plots were clearly "U" shaped, implying that a quadratic was a better fit. Inclusion of a square term solved both problems. The Gompertz failed to converge because the term for the upper asymptote increased with each iteration. The simple exponential growth model $y = \beta_0 \exp(\beta_1 x)$ gave as good a fit as the quadratic.

D. pteronyssinus

D.pt. caused the largest weals over the concentrations used: only one SPT at the lowest concentration did not cause a reaction and only eight subjects reached the highest concentration. Two observations appeared to be outliers and were omitted from the analysis. As with histamine, the data were close to a straight line but inclusion of a cubic term improved the fit and gave a curve very similar to that fitted using a Gompertz.

P_1 and *D.pt.*

The above analysis of the first part showed that *D.pt.* and P_1 were parallel. Therefore, the log concentration of one was adjusted to match the other and the data re-examined. There was no evidence of any difference between extracts ($P > 0.1$). The lack of an upper turning point for P_1 was not surprising as it was comparable to the lower part of the *D.pt.* curve. The best fit curve to P_1 and *D.pt.* combined was clearly "S" shaped (Fig. 3).

IgE

Nine subjects showed an increase in *D.pt.* specific IgE and 10 subjects an increase in P_1 specific IgE between the first and the second

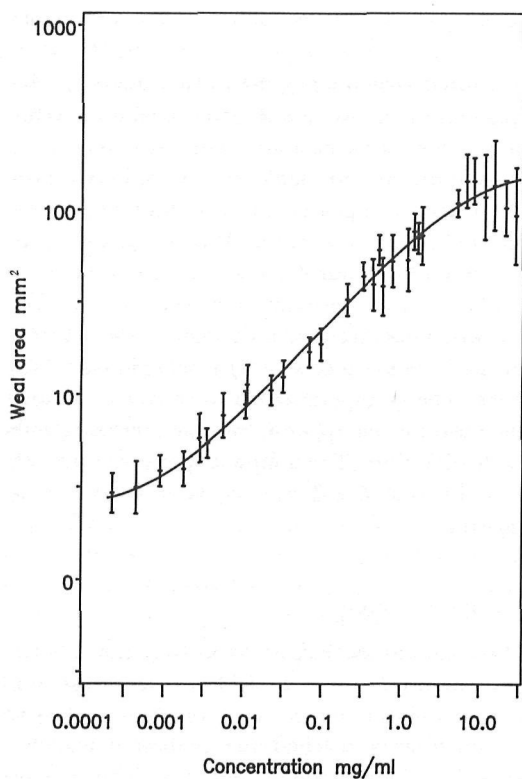


Fig. 3. D.pt. and P_1 . Weal areas after fitting terms for patient effects. Concentration of D.pt. increased by 0.2073 on log scale. Means and 2 SEM error bars. Cubic curve shown.

bleeds. Eleven and 10 subjects, respectively, showed a decrease. For the change between the first and third bleed, for samples available, the respective figures were 9 and 13 increased and 6 and 3 decreased. This provided no convincing evidence that multiple skin testing with mite extracts results in any consistent change in specific IgE levels.

DISCUSSION

Skin prick testing is a method with a long history in the diagnosis of allergy. In recent years, with the biological standardisation of test solutions and improvements in materials and techniques, the method has become more precise and reproducible. In this study, the solutions of D.pt. and P_1 were manufactured by

current techniques from mites cultured on site by DHS. Skin prick testing was by a very experienced operator with a highly reproducible technique. The experimental design ensured that the trial was balanced, free from any location biases, and totally blind in the first part. By using a computer analysis system all sources of operator-caused measurement error were removed. Previous workers have used either the diameter, or diameters, of the weals or the area - measured by counting squares, or using a planimeter or digitalising tablet (18), all of which are highly susceptible to operator bias. Replacement of these methods by image analysis would reduce error within trials and increases consistency between trials.

The data were analysed by thorough statistical techniques, allowing for confounding variables, non-linearity, etc. Previous analyses have not gone beyond fitting simple straight lines to the data, sometimes after screening the data to remove unsatisfactory subjects or observations according to certain criteria (8-10, 16, 21). These methods are unnecessarily restrictive and do not make full use of the available information. There were clear differences between subjects but this was allowed for in the analyses so that all the data were used. Some investigators excluded small weals from the curve fitting (8-10, 14) but this causes increasing bias with decreasing concentration. Genuine negative, or small, responses are as important and valid as any others.

The study could not estimate any variation in sensitivity between different sites on the back because it was confounded with order of treatment. However, a report (11) of variations in sensitivity between different sites on the back may have been an order of treatment effect. Over the range of concentrations of D.pt. extract (0.014 to 3.26 mg/ml), P_1 (0.0018 to 0.33 mg/ml) and histamine (0.4 to 20.4 mg/ml), the relationships between skin prick weal and concentration, both on log scales, were linear and parallel for the three extracts. There is no reason to believe that other allergen extracts would be parallel. There was no evidence for any curvature in the lines. There were clear differences in the size of weals caused by dif-

ferent extracts, and in reactivity of different subjects. The responses within a subject to the three solutions were parallel but they were not parallel between subjects. However, this was a small effect compared to the others. On the transformed scales, the lines had a gradient of 0.355, that is for every 10-fold increase in concentration the weal area increased by about 225 % (95 % confidence interval 167 % to 300 %). At 10 mg/ml histamine caused weals about 21 mm². D.pt. at 0.1 mg/ml and P₁ at 0.16 mg/ml caused weals the same size as 10 mg/ml histamine, i.e. D.pt. was 100 times as potent as histamine. When a similar model to the above was fitted to all the concentrations, from both study sessions, there was much the same result but with evidence for curvature in the best fit lines. There was no unexpected increase in area at very low concentrations (13).

In vitro work showed that D.pt. was about twice as potent as P₁ (by quantitative immunoelectrophoresis). This is similar to the *in vivo* result. From the first study session, 61 % more P₁ was needed to cause the same size weals as D.pt. In terms of SPT, the P₁ extract fitted the same "S" shaped curve as the D.pt. extract i.e. non P₁ active components of D.pt. did not differentiate SPT reactions to D.pt. from P₁.

It is not surprising that previously both straight lines and curves have been fitted to weal area plotted against concentration. The curvature at the ends is only apparent at very high and low concentrations. Also because the variation between subjects is large, the plateauing will be hidden unless subject effects are allowed for.

Seven of the 52 SPT (13.5 %) did not respond to histamine 1 mg/ml, thus confirmig the unsatisfactory nature of the HEP unit. In the patients who did respond to histamine 1 mg/ml, the weal may be equivalent to that part of the mite response curve which is non-linear. Standardisation based on 10 mg/ml histamine is preferred (8, 15) as it is well away from either tail.

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