The basis of the histamine assay in human skin: II wheal formation

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- 1 Subepidermal injection of histamine solutions in isotonic saline in four subjects evoked whealing after a mean delay of 2.6 min, this estimate being derived by regression modelling.
- 2 Wheal growth was better modelled by the logarithm of time than by time, suggesting that wheal size depends upon assisted diffusion of histamine in the dermis.
- 3 Wheal growth was piecemeal, not continuous, consistent with the successive involvement of neighbouring vascular territories.
- 4 Wheal growth was completed by 9 min after injection.

Keywords histamine wheal formation

Introduction

The histamine assay procedure depends usually upon fully developed wheals; in our first paper wheals were measured at 10 min from injection time [1]. During that work it was confirmed that wheals appeared after a delay [2] and that wheal growth in time rose slowly to the maximum. Others have suggested that the delay is 1–2 min, and may involve delay in appearance, not growth, of the wheal. It has also been stated that wheal area coincides with that of the first flare [2–4]. A new experiment was made to extend our earlier observations. Ethical permission was obtained from the Tower Hamlets District Ethics Committee and informed consent was given by each subject.

Methods

Histamine solutions were injected (0.1 ml) subepidermally, with very fine needles, [1] into the palmar aspect of the forearm in the area between 4 and 12 cm below the elbow crease. Four concentrations of histamine acid phosphate in isotonic saline were used, 2.25, 0.56, 0.14, 0.035 mmol l⁻¹ calculated as histamine free base. This natural logarithmic sequence was used in computation as $\ln(\text{dose}) = 3, 2, 1$ and 0, respectively. Four subjects (3M, 1F) aged 26 to 61 years injected one another on the palmar aspect of the forearm between 11.00 h and 12.30 h and measured wheal areas using cellulose acetate sheet, tracing wheal margins including

any 'pseudopodia' at intervals of 1 min from 1–10 min after each injection. Wheal areas were measured by transferring the tracings to squared paper and counting squares by two observers independently; their mean was taken as the area.

Statistical methods

The data were analysed

- (i) as a whole block, by analysis of variance for unbalanced repeated measures, using the BMDP5V programme [5].
- (ii) by regression analysis, for each dose separately. Each analysis was repeated four times using the data from 2, 3 or 4 min to 9 min using either time or the logarithm of time as covariate. The best model fit for the analysis of variance was selected using Akaike's information criterion [6, 7] which is provided by the P5V programme. This is $N \ln R_E + 2p$, where N is the number of data points, p the total of estimated parameters and R_E the sum of squares of error residuals.
- (iii) by summing the absolute values of the differences between estimates from regression for each of the four doses of histamine and the mean slope taking successive steps in the time logarithm of 0.1. The least sum of differences was taken as the best estimate of the start time for wheal formation.

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Results

The wheal areas are given in Table 1. Three were spoiled and are removed from the data. Inspection of the data suggested that wheal growth began between 2 and 3 min from injection, and was completed by 9–10 min, the time selected in previous work for assay measurement [1]. The mean wheal areas at 1 and at 2 min were 47.3 mm² and 51.0 mm² respectively. This may be compared with the area expected beneath a hemisphere of 0.1 ml volume, 41.35 mm², plus epidermal thickness.

The data were analysed in several ways using BMDP5V repeated measures two-way analysis of variance for unbalanced models (Table 2). The highest information criterion, combined with the most significant dependence on time and dose contrasts, accrued from the analysis structured by taking the logarithms of both dose and time, and including data from 2–9 min. This model best fits the data by analysis of variance.

Further analysis by separation of the data into individual dose sets followed by independent regression analysis (Table 3) showed that including the 2 min data gave smaller residual sums of squares than starting the analysis with the 3 min results. In what follows we have therefore taken the best model as regression from 2–9 min with ln (dose) and ln (time) as the independent variates.

Wheals obtained with saline gave a slope against time which did not differ significantly from zero.

Table 1 Wheal area (mm²) for various times and doses in four subjects

	Time from injection (min)								
	1	2	3	4	5	6	7	8	9
<i>Histamine dose (mmol l⁻¹)</i>									
2.25	48	48	48	72	140	192	248	308	312
	24	32	60	76	100	100	112	116	120
	48	52	60	88	124	120	132	156	144
	28	40	84	100	136	144	152	148	168
Mean	37	43	63	84	125	139	161	182	186
0.56	42	32	60	108	176	184	168	200	192
	*	32	76	88	84	96	104	108	116
	44	76	108	120	120	128	124	140	136
	76	80	84	112	116	128	148	128	144
Mean	54	55	82	107	124	134	136	144	147
0.14	34	32	44	60	76	72	84	72	84
	26	40	68	76	76	84	76	96	88
	84	88	100	108	116	112	112	128	128
	64	56	76	100	100	96	96	*	*
Mean	52	54	72	86	92	91	92	98.67	100
0.035	44	52	56	88	108	96	120	160	180
	20	20	20	24	24	28	24	16	20
	80	72	84	88	96	104	104	108	108
	48	64	64	76	68	72	80	68	72
Mean	48	52	56	69	74	75	82	88	95

*: Missing data.

Table 2 Repeated measures two-way analysis of variance with ln (time) and ln (dose) as contrasts

Data analysed			Parameter estimates	SE of estimate	Two sided P value	Wald χ^2	P value
Times included	Model contrasts	Negative Akaike information criterion					
Linear time 2–9 min	Time, ln (dose) CS	437.2 <i>n</i> = 32	C	13.45	3.67	0.0003	
			LD	−3.02	1.51	0.045	4.00
			LT	0.896	0.48	0.062	3.48
			DT	1.35	0.25	<0.00001	28.23
ln (time) 2–9 min	Unstructured	992.54 <i>n</i> = 32	C	11.33	6.46	0.08	
			LD	−5.9	3.45	0.086	2.95
			LT	0.0044	0.003	0.256	1.29
			DT	0.0065	0.002	0.002	9.78
ln (time) 4–9 min	ln (time) ln (dose) CS	328.73 <i>n</i> = 24	C	15.3	9.04	0.09	
			LD	−8.5	4.53	0.06	3.54
			LT	0.0023	0.004	0.62	0.25
			DT	0.0079	0.002	0.0013	10.31
ln (time) 3–9 min	ln (time) ln (dose) CS	383.62 <i>n</i> = 28	C	12.7	6.28	0.044	
			LD	−7.61	3.05	0.013	6.20
			LT	0.0037	0.003	0.26	1.26
			DT	0.007	0.002	<0.00001	18.14
ln (time) 2–9 min	ln (time) ln (dose) CS	435.05 <i>n</i> = 32	C	11.31	4.386	0.0099	
			LD	−5.93	1.9775	0.0027	8.99
			LT	0.0044	0.0022	0.049	3.89
			DT	0.0064	0.0012	<0.00001	29.75

Key: C, General mean; LD, ln (dose) = 3, 2, 1 or 0; LT, ln (time) $\times$ 1000; DT, dose $\times$ time interaction; CS, compound symmetry.

Table 3 Regression calculations for individual doses

Histamine dose (mmol l ⁻¹)	Linear regression				SE of estimate	Wheal area estimate at 2 min		
	A	SE(A)	B	SE(B)		CI	1-β	P value
2.25	122.9	12.292	89.96	3.444	9.396	± 19.86	0.05	NS
0.56	116.1	7.388	62.64	1.80	4.912	± 10.032	0.74	NS
0.14	84.8	4.460	29.08	1.428	3.608	± 7.652	0.68	0.05> P> 0.025
0.035	73.88	7.240	28.04	2.49	6.808	± 13.900	0.035	NS

A, mean area; SE, standard error; B, regression co-efficient. CI, 95% confidence intervals for the area estimate at 2 min. (1-β), power of test for 2 min mean against estimate from mean slope. P value, for the estimated residual at 2 min for each dose, calculated from the mean slope for all doses, *t*-test with 30 degrees of freedom (28 in the 0.14 mmol l⁻¹ case).

The regression slopes were used to find predicted values for each slope at 2 min, and the standard errors of the regression coefficients and of the estimates (Table 3). Three estimates of the predicted mean at 2 min do not differ significantly from the observed mean. The estimate which did reach significance arose from the dose with two missing data at the end of the slope. The variance between individuals is large, hence the power of the test is low against the false null hypothesis for two of the four doses.

The regression coefficients ln (dose) or ln (time) differ strongly significantly, both from zero and from one another, by analysis of variance and by regression (Tables 2 and 3).

It was concluded that whereas the dose difference was present, there was no evidence to support a claim that the intercepts for the various dose slopes with the mean 0.1 ml wheal area (51 mm²) at 2 min were different. Since the means at 1 min did not differ significantly from those at 2 min, this evidence is consistent with a delay in the onset of wheal growth of between 2 and 3 min. To discover more clearly what the delay was, sums of differences from the mean slope for the four dose slopes, were calculated at 0.1 min intervals from time zero, using the regression data. These showed a clear minimum at 2.6 min (Figure 1).

During the experiments described in this paper and in previous work [1] there were many opportunities to note the pattern of wheal growth. This was not gradual in time, but piecemeal (Table 4, Figure 2), both for first flare (rapid) for wheal growth (slow). Wheal area could at times exceed, or be slightly less than, the areas of first flare.

Discussion

Lewis [3] repeatedly stated that the first, or immediate, flare response to injected histamine demarcated the later area of the wheal response precisely, though a few exceptions to this were noted. Others, later have re-emphasised the coincidence of wheal area with first flare [2, 4]. If true, this would suggest that histamine

Figure 1 Wheal area related to ln (time) for four different four-fold dilutions of histamine (x ln (dose) 3, ● ln (dose) 2, ▲ ln (dose) 1 and ■ ln (dose) 0; see text for dose molarities). The line *—* represents the sum of absolute differences between the four slopes and the mean slope.

wheal area is not determined by the diffusion of histamine into the dermis to the point (the wheal edge) where its concentration reaches the lower end of tissue response. Since first flare occurs almost immediately, no time for such diffusion could have elapsed, and the

Figure 2 Successive boundaries for 'first flare', wheal and 'second flare' traced from two lesions evoked by standard histamine solution diluted 1 in 8. The associated events are listed in Table 4.

Table 4 Successive events associated with the boundaries shown in Figure 2

<i>Time (s)</i>	<i>Lesion A</i>	<i>Time (s)</i>	<i>Lesion B</i>
4	injection	4	injection
25	'first flare' appeared	22	first flare to area 2
54	first flare covered area 1	205	first flare to area 3
216	first flare covered area 2	284	first flare to area 4
301	wheal extended to area 1	300	wheal to line 2
472	'second flare' appeared to area 4	404	'second flare' to line 5
592	wheal to area 2	524	wheal to line 3 in places
1402	wheal to area 3, its maximum	944	wheal to line 3 almost entirely; no further wheal extension

wheal edge must therefore represent the limit of some irritant vascular response which pre-conditions skin to wheal formation. However, Lewis [3] clearly discussed histamine diffusion in skin, and described it as such; he also showed that the vascular response of first flare was not neurally mediated; it occurs in denervated skin. It may be inferred that Lewis envisaged the histamine as diffusing more slowly, evoking a wheal response until it reached the first flare margin.

Our findings suggest that the response is less simple; though first flare and eventual wheal nearly coincide, the latter may exceed the former, or at times may not quite attain to it. Both flare and wheal accrue piecemeal, the first rapidly and the second more slowly, though first flare does begin within an area far too large to be accounted for by diffusion, whether unassisted or assisted by fluid flows within the dermis.

Second flare greatly exceeded the wheal area [8].

Responses to large molecules (tuberculin) injected intradermally are best fitted by a model based on diameter measurements [9]. In our previous work we showed that the histamine assay depends on wheal area measurement [1]. Its value depends on a clear cut wheal edge. The problem of analysing data where there is a delayed response is uncertainty about the time of onset of the response. Though it seemed clear from simple observation that the wheal response began between 2–3 min from injection, we did not make that assumption, except in removing the 1 min data from the slope estimations, since their mean did not differ significantly from those at 2 min; the 10 min data were also withdrawn, since they did not differ from the 9 min data. Repeated analysis from 2 min onwards, removing successive data sets minute by minute from analysis and using both logarithmic and linear measures for time, led to the finding that data from 2–9 min with logarithmic time gave the best fitting model with least residuals and most information in analysis of variance.

The most likely reason for the formation of a sharp wheal edge is that histamine diffuses outwards from the circular base of the subepidermal injection bleb into the dermis, the wheal forming wherever the concentration of histamine reaches an effective level in the tissue. Hence the clearcut logarithmic dose relationship to

wheal area response represents a true pharmacological effect, whether directly or in response to the release of some second mediator [8]. A relationship between concentration and $\ln$ (time), rather than to time, is consistent with a diffusion mechanism [10]. The precise equations which represent diffusion are highly complex [11, 12]. They are inapplicable to a tissue with the complexity of skin, since they can only be used for isotropic media or granulated mixtures with a degree of anisotropy which is simply describable [13]. However, 'self-similar' groups of equations can be used to describe diffusion. The simplest point about them [14] is that they relate to $\ln$ (time). Even so, our observations cannot be taken to imply simple diffusion in skin substance; that has been measured in dead dermis [15] and occurs at a rate of about two decibels slower than the measured growth rate of histamine wheals (0.1 mm min^{-1}). This suggests that assisted diffusion, (e.g. by fluid flow) may be the best explanation.

The piecemeal accrual of wheal area also cannot be accounted for by simple diffusion. Further work is needed to establish the reason for this. However, histamine is known to dilate capillaries and to cause retraction of capillary endothelial cells so as to open their loose junctions, and in some circumstances to constrict venules [2] especially if H_2 -receptors are blocked selectively [16]. Bradykinin has similar dilating effects on the smaller vessels [17]. It may be that oedema forms only when histamine has diffused in sufficient concentration to not only open capillaries but also to constrict the principal venules draining an area of dermis so raising intracapillary pressure to the oedema formation level. Whether this is due to histamine alone or to a second substance released by histamine is unknown [8]. The time needed for diffusion to the venular layer in dermis, or the release of a second substance or the differences in response times to H_1 - and H_2 -receptor stimulation could explain the long delay in onset of the histamine wheal. But the evidence for venular constriction is not corroborated by experiments in rat mesenteric vessels [18–21], and Lewis [3] showed clearly that the sufficient conditions for wheal formation were increased microvascular permeability and local blood flow, not an increase in vessel distension due to raised internal pressure. The explanation cannot be synchronous absorption of the injected bleb and oedema formation together, since wheal area approximates to saline injection volume as the histamine dose reduces, and does not fall even with saline alone over 9 min.

There is a difference in response time for activation of H_1 - and H_2 -receptors in the microcirculation [21]. H_1 -receptor responses are mediated at once following histamine injection or release in both dog and man, whereas H_2 -effects appear later but within 5 min. These H_1 - and H_2 -receptor mediated effects are linked to vasodilator responses, but their time of onset differs. Whether this difference relates to the delay in histamine wheal formation or not remains to be explored.

Professor Stephen Evans and Jonathan Deeks gave us much help with the statistical analyses and their interpretation. Histamine solutions were prepared by the staff of the Pharmacy, The Royal London Hospital.

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(Received 22 October 1992,
accepted 31 March 1993)