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EVALUATION OF THE ROLE OF HISTAMINE H_1- AND H_2-RECEPTORS IN CUTANEOUS INFLAMMATION IN THE GUINEA-PIG PRODUCED BY HISTAMINE AND MAST CELL DEGRANULATION

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with a statistical appendix by D. DANIEL

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1 The role of histamine H_1- and H_2-receptors in mediating the cutaneous inflammatory response produced by exogenous histamine and the release of endogenous histamine from mast cells has been investigated by a method which permits simultaneous, quantitative measurement of vasodilatation, vascular permeability and oedema formation.

2 Histamine and the selective H_1-receptor agonist, 2-(2-aminoethyl) pyridine, both produced vasodilatation, increased vascular permeability and oedema formation whereas the selective H_2-receptor agonist, dimaprit, produced only vasodilatation.

3 Mepyramine and cimetidine both reduced the vasodilatation response to histamine, the combination of antagonists being superior to either antagonist alone. Mepyramine (but not cimetidine) virtually abolished extravascular albumin accumulation and oedema formation.

4 Mepyramine and cimetidine both reduced the vasodilatation response produced by active cutaneous anaphylaxis and compound 48/80. However, mepyramine was less effective in reducing the vascular permeability response to mast cell degranulation than to histamine.

5 In conclusion, the vasodilator response to histamine is mediated by both H_1- and H_2-receptors; the permeability response to histamine is mediated solely by H_1-receptors. A combination of H_1- and H_2-receptor antagonists appears to be more effective than either antagonist alone in reducing cutaneous inflammatory reactions involving histamine.

Introduction

In 1927 Sir Thomas Lewis enunciated the hypothesis that a histamine-like substance was responsible for the phenomena which accompany cutaneous anaphylaxis. The histamine hypothesis for the mediation of anaphylaxis soon gained further support (Dale, 1929; Bartosch, Feldberg & Nagel, 1932; Dragstedt & Gebauer-Fuelnegg, 1932) and wide acceptance. The subsequent development of potent histamine antagonists, such as mepyramine, appeared to provide a means of effectively inhibiting the actions of histamine. However, it was discovered that only certain responses to histamine were blocked by mepyramine and these were defined as being mediated by H_1-receptors (Ash & Schild, 1966). The receptors involved in mepyramine-insensitive responses were defined as H_2-receptors and were characterized by Black, Duncan, Durant, Ganellin & Parsons, (1972). These authors also showed that histamine-induced hypotension, which was only partially inhibited by mepyramine, could be abolished by the additional presence of the H_2-receptor antagonist, burimamide. Furthermore, they indicated, citing the work of Bain (1949), that the effectiveness of the antagonists in combination should be examined against intradermally injected histamine since this represented another situation where mepyramine alone did not totally suppress the response to histamine.

The purpose of this study was to determine accurately the contribution of H_1- and H_2-receptors to the cutaneous inflammatory responses produced by exogenous histamine and the liberation of endogenous histamine. This has been achieved, firstly by examining the effects of the selective H_1-receptor agonist 2-(2-aminoethyl) pyridine (Durant, Ganellin & Parsons, 1975) and the selective H_2-receptor agonist dimaprit (Parsons, Owen, Durant & Ganellin, 1977) on the cutaneous vasculature and secondly by examining the effect of mepyramine and the selective H_2-receptor antagonist cimetidine (Brimblecombe, Duncan, Durant, Emmett, Ganellin & Parsons, 1975) on the inflammatory response to histamine, compound 48/80 and active cutaneous anaphylaxis.

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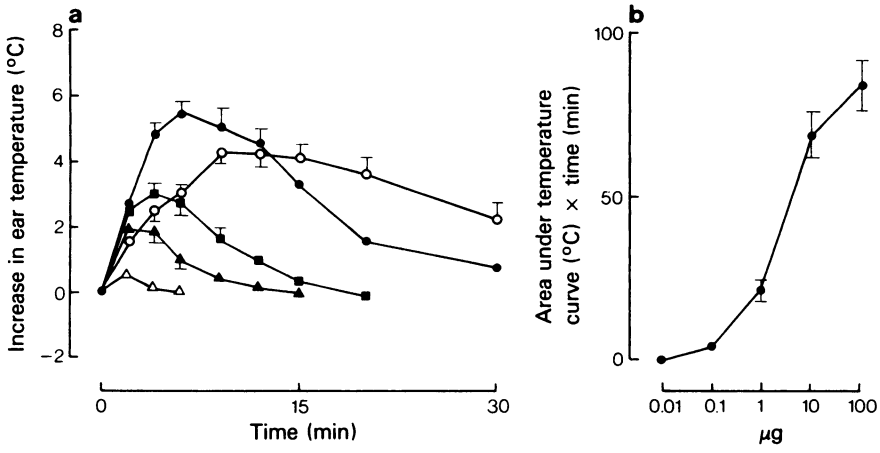

Figure 1 Effect of histamine on guinea-pig ear temperature. Histamine 0.01 μg (△), 0.1 μg (▲), 1 μg (■), 10 μg (●) and 100 μg (○) produced a dose-dependent increase in the peak value and duration of the ear temperature response (a). These results were expressed as area under the temperature curve (°C) × time (min), (b). Values are mean, $n = 6$; vertical lines, show s.e. mean

Methods

Male, albino guinea-pigs weighing 350 to 500 g were used. The animals were housed in individual cages and the environmental temperature was maintained at 22 ± 1°C.

Evaluation of inflammatory responses

The vascular changes were evaluated by techniques which permit simultaneous and quantitative measurement of vasodilatation, vascular permeability and oedema formation. The general methodology has been described previously in detail (Woodward & Owen, 1979). Briefly, mepyramine and or cimetidine were injected subcutaneously into the scruff of the neck; 0.9% w/v NaCl solution (saline) was injected into control animals. Twenty eight minutes later ^{125}I-labelled human serum albumin and ^{51}Cr-labelled guinea-pig erythrocytes were injected into the dorsal foot vein. The use of radiolabelled albumin and erythrocytes allows total tissue albumin content and blood content to be measured accurately. After a further 2 min, 2 μl of saline was injected into the right ear and 2 μl of histamine, histamine-like agonist or compound 48/80 were injected into the left ear. Vasodilatation was measured indirectly as increases in ear surface temperature and was recorded at predetermined intervals for a period of 30 min by means of an electric thermometer incorporating a surface 'touch-on' probe. Ear temperatures were also measured before subcutaneous injection into the neck and intradermal injection into the ear. At 30 min post-intradermal injection, the animals were killed. The ears were amputated, weighed, counted in a γ counter with 1 ml of venous blood as a reference and then dried to constant weight in a vacuum oven. This procedure enables the extravascular albumin content to be calculated as follows; total albumin content − blood content (≡ intravascular albumin content) = extravascular albumin content (ml/g dry wt. tissue).

Oedema formation was quantified as water content (g/g dry wt. tissue).

Saline was injected into the control ears to take account of the effect of injection trauma. It produced a small, transient increase in ear temperature. The temperature responses have been expressed as area under the temperature curve with time. This transformation of the ear temperature data is illustrated in Figure 1. The end point on the time scale was selected as the last point at which a statistically significant difference between the temperature of the treated ears and the saline-injected ears was obtained.

Active cutaneous anaphylaxis

Guinea-pigs were sensitized by injection of ovalbumin solution 1 ml × 100 mg/ml intraperitoneally and 1 ml × 10 mg/ml subcutaneously into the scruff of the neck. The animals were challenged 3 weeks later by injection of graded doses of ovalbumin into the left ear in a 20 μl volume. In controls, 20 μl of saline was injected into the contralateral ear. The dose range of ovalbumin was restricted to the highest challenge dose which produced no obvious systemic effect.

Materials

Cimetidine solutions were prepared by dissolving the base in a small quantity of 0.1 N HCl. The solution was then neutralized with NaOH and made up to the required volume.

The following substances were dissolved in saline and neutralized with NaOH as appropriate: mepyramine maleate (May and Baker), histamine acid phosphate (BDH), 2-(2-aminoethyl) pyridine dihydrochloride, dimaprit dihydrochloride, compound 48/80 (Wellcome) and egg albumin (Hopkin and Williams). ^{125}I-human serum albumin and $Na_2{}^{51}CrO_4$ were supplied by the Radiochemical Centre, Amersham.

Statistics

The results were analysed by the modified parallel line assay method described in full in the statistical appendix. The purpose of this analysis was to determine whether differences exist between the four treatment groups in response to histamine, compound 48/80 and cutaneous anaphylaxis and for this to be done, multiple comparisons between the treatment groups are necessary. Comparisons may be made between treatment groups at each individual dose of histamine, compound 48/80 and antigen by analysis of variance. However, a comparison between two treatments is more useful if it is applicable over a range of doses and this can be achieved by assay analysis. If the usual assumptions of an assay are valid, two treatments can be compared by estimation of the potency ratio which is the inverse of the ratio of the doses that produce equivalent responses in the two treatment groups.

The advantages of this method are in interpretation of results. The analysis of individual doses can show both significant and non-significant differences at points along the dose-response curves. A comparison of the effects of treatments on the entire dose-response curves would provide a better measure of the effects of drug treatment.

Since this is a multiple assay, 99.167% confidence limits were used which allows the overall level of significance to be 0.05.

Results

Effect of histamine and histamine-like agonists on the cutaneous vasculature

Histamine produced a dose-dependent increase in both the magnitude and the duration of the ear temperature response (Figure 1a). These results were expressed as area under the temperature curve with time (Figure 1b).

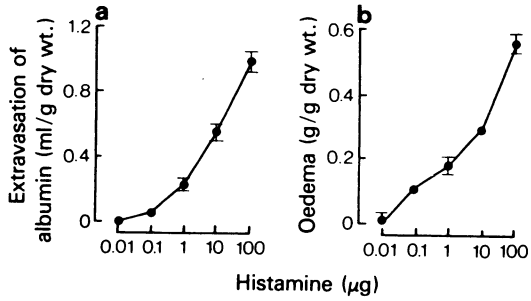

Figure 2 Effect of histamine on extravascular albumin and water content in guinea-pig ears. Histamine produced a dose-dependent increase in extravascular albumin content, (a) and water content, (b). Values are mean, $n = 6$; vertical lines show s.e. mean.

Histamine also produced a dose-dependent increase in vascular permeability (Figure 2a) and water content (Figure 2b).

Figure 3 compares the relative effectiveness of histamine, the selective H_1-receptor agonist, 2-(2-aminoethyl) pyridine, and the selective H_2-receptor agonist, dimaprit, in eliciting increases in extravascular albumin accumulation, water content and ear temperature. Histamine and 2-(2-aminoethyl) pyridine produced dose-dependent increases in extravascular albumin content, (Figure 3a), water content, (Figure 3b), and ear temperature, (Figure 3a and b), whereas dimaprit selectively increased ear temperature (Figure 3a and b).

The effect of mepyramine and cimetidine, alone and in combination, on the vascular changes produced by histamine

Mepyramine and cimetidine produced a significant parallel displacement of the ear temperature dose-response lines (Figure 4a) with potency ratios of 0.409 (0.182–0.841, 99% limits) and 0.478 (0.216–0.981, 99% limits) respectively. The combination of cimetidine and mepyramine produced a larger displacement than either antagonist alone, potency ratio 0.145 (0.055–0.311, 99% limits). The combination also produced a significantly greater displacement relative to that produced by mepyramine and cimetidine alone, potency ratios 0.354 (0.154–0.729, 99% limits) and 0.303 (0.130–0.627. 99% limits) respectively. No significant difference was obtained between mepyramine- and cimetidine-treated groups.

Mepyramine, alone and in combination with cimetidine, virtually abolished the vascular permeability, (Figure 4b) and oedema formation (Figure 4c) responses to histamine over the dose range used.

Larger doses of histamine appeared to elicit systemic responses in control animals and consequentl

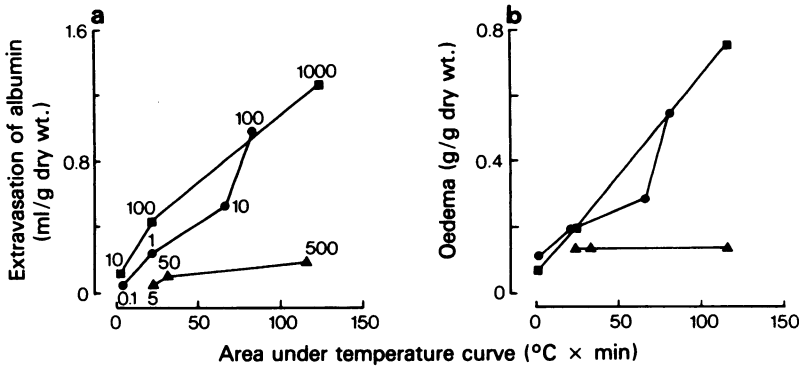

Figure 3 Comparison of the relative effects of histamine, 2-(2-aminoethyl) pyridine and dimaprit on ear temperature, vascular permeability and oedema formation. (a) Histamine (●) (0.1–100 μg) and 2-(2-aminoethyl) pyridine (■) (10–1000 μg) produced a dose-dependent increase in extravascular albumin content whereas dimaprit (▲) (5–500 μg) was almost inactive in this respect. All three agonists produced a dose-dependent increase in ear temperature. (b) Histamine (●) (0.1–100 μg) and 2-(2-aminoethyl) pyridine (■) (10–1000 μg) produced a dose-dependent increase in water content, which was not obtained with dimaprit (▲) (5–500 μg). The ear temperature responses are the same as in (a). Values are mean, $n = 6$.

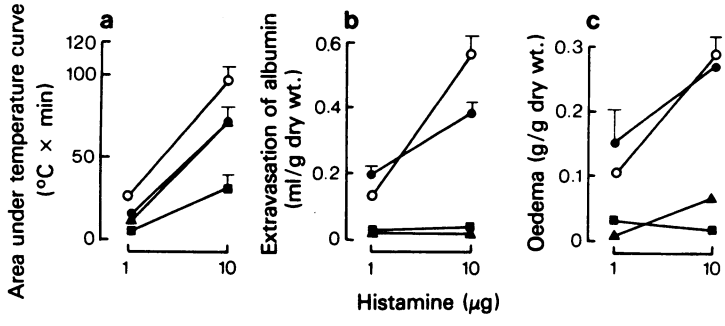

Figure 4 Effects of mepyramine and cimetidine, alone and in combination, on the vascular changes produced by histamine. (a) Ear surface temperature changes; (b) extravasation of albumin; (c) oedema formation. Dose-response curves to histamine in saline treated animals (○). Treatment with mepyramine, 5 μmol/kg (▲), significantly reduced vasodilatation, and virtually abolished extravasation of albumin. Cimetidine, 500 μmol/kg (●) significantly reduced the vasodilatation but had no significant effect on extravasation of albumin or oedema formation. The combination of mepyramine plus cimetidine (■) caused a significantly greater reduction in vasodilatation than either antagonist alone, whereas the combination of antagonists was similar to mepyramine alone on extravasation of albumin and oedema formation.

the effect of antagonists against this dose was not examined as it would have been difficult to distinguish inhibition of local responses from inhibition of systemic responses.

Effects of mepyramine and cimetidine, alone and in combination, on the vascular changes due to the release of endogenous histamine by active cutaneous anaphylaxis and compound 48/80

Mepyramine and cimetidine significantly displaced the ear temperature dose-response curve for cutaneous anaphylaxis with potency ratios of 0.037 (0.0004–0.820, 99% limits) and 0.044 (0.0005–0.960, 99% limits) respectively. The combination of antagonists produced a greater displacement relative to that produced by either antagonist alone with a potency ratio of 0.002 (0.0001–0.052, 99% limits). However, the effect of the combination was just significantly better than cimetidine but not quite significantly better than mepyramine (Figure 5a).

Similar results were obtained with compound 48/80. The combination produced a greater displacement of the ear temperature dose-response curve, potency ratio 0.008 (0.0002–0.076, 99% limits), than either mepyramine, potency ratio 0.047 (0.002–0.348,

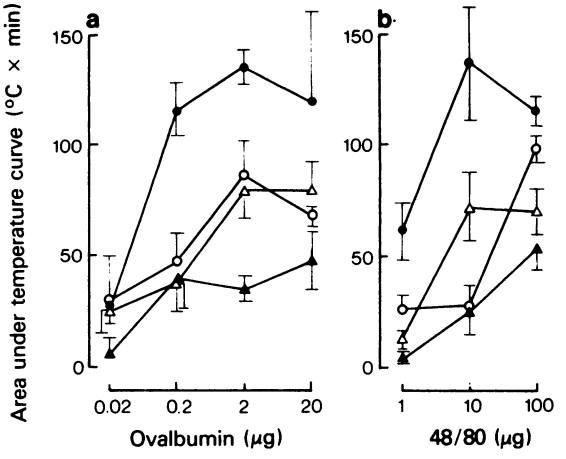

Figure 5 Effect of mepyramine and cimetidine, alone and in combination, on the increase in ear temperature (vasodilatation) produced by active cutaneous anaphylaxis (a), and by intradermal injection of 48/80 (b). Dose-response curves for animals treated with saline are indicated as (●), mepyramine, 5 μmol/kg (○), cimetidine, 500 μmol/kg (△) and mepyramine plus cimetidine (▲). Values are mean, $n = 6$; vertical lines show s.e. mean.

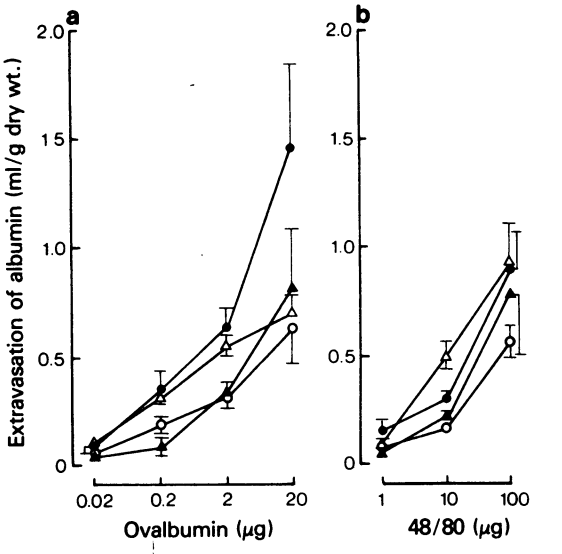

Figure 6 Effect of mepyramine and cimetidine, alone and in combination, on the extravasation of albumin produced by active cutaneous anaphylaxis (a), and 48/80 (b). Dose-response curves for animals treated with saline are indicated as (●), mepyramine 5 μmol/kg (○), cimetidine 500 μmol/kg (△) and mepyramine plus cimetidine (▲). Mean values are shown, $n = 6$; vertical lines indicate s.e. mean.

99% limits) or cimetidine, potency ratio 0.065 (0.004–0.473, 99% limits). Again the combination was just significantly superior to cimetidine but not quite superior to mepyramine. No other significant differences between treatment groups were obtained (Figure 5b).

Mepyramine alone and in combination with cimetidine significantly displaced the extravascular albumin content dose-response curve for cutaneous anaphylaxis with potency ratios of 0.125 (0.022–0.577, 99% limits) and 0.132 (0.023–0.605, 99% limits) respectively. No other significant differences were obtained (Figure 6a). No significant differences were obtained at all for compound 48/80 although mepyramine and the combination of antagonists showed a trend towards inhibition with potency ratios 0.315 (0.071–1.204, 99% limits) and 0.437 (0.102–1.671, 99% limits) respectively (Figure 6b).

Mepyramine and the combination both significantly displaced the water content dose-response curve for active cutaneous anaphylaxis with potency ratios 0.048 (0.003–0.406, 99% limits) and 0.045 (0.003–0.379, 99% limits) respectively. Cimetidine had no significant effect (Figure 7a). The parallel line assay was rejected for compound 48/80 results due to non-linearity and therefore, an analysis of variance was used. All treatments produced a highly significant reduction in the increase in water content induced by 100 μg compound 48/80 ($P < 0.001$): no other significant differences were obtained (Figure 7b).

Discussion

These investigations were designed to determine the presence and function of histamine H_1- and H_2-receptors in the cutaneous vasculature and to establish their quantitative contribution to inflammatory reactions produced by exogenous histamine and by the release of mast cell constituents. Previous animal studies concerning cutaneous histamine responses have been almost entirely restricted to measuring vascular permeability changes. The model used in this study permits simultaneous, quantitative measurement of vasodilatation (ear temperature), vascular permeability and oedema formation and thus, enables a more comprehensive investigation of histamine-induced cutaneous inflammation.

The presence of histamine H_1- and H_2-receptors in the cutaneous vasculature and their role in histamine-induced cutaneous inflammation has been established. Vasodilatation of resistance vessels is mediated by both H_1- and H_2-receptors, increased vascular permeability is mediated solely by H_1-receptors. These

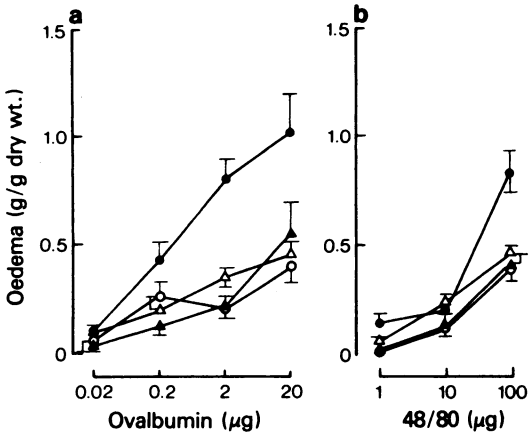

Figure 7 Effect of mepyramine and cimetidine, alone and in combination, on the oedema produced by active cutaneous anaphylaxis (a) and 48/80 (b). Dose-response curves for animals treated with saline are indicated as (●), mepyramine 5 μmol/kg (○), cimetidine 500 μmol/kg (△) and mepyramine plus cimetidine (▲). Mean values are shown, $n = 6$; vertical lines indicate s.e. mean.

conclusions are based on the following evidence. Firstly, the results obtained with selective H_1- and H_2-receptor agonists show that H_1-receptor stimulation produces both vasodilatation and increased vascular permeability whereas H_2-receptors selectively mediate vasodilatation. Secondly, both mepyramine and cimetidine reduced histamine-induced increases in ear temperature whereas mepyramine virtually abolished the vascular permeability response to histamine and cimetidine was ineffective.

Oedema formation is dependent on the permeability of the vasculature to plasma proteins, capillary hydrostatic pressure and functional capillary surface area. However, over the dose-range of agonists used in these experiments, it appears that the H_1-receptor-mediated vascular effects were predominantly responsible for oedema formation with H_2-receptor involvement being minimal.

It appears that histamine can cause vasodilatation by interacting with both H_1- and H_2-receptors to produce a common effect. Furthermore, the combination of mepyramine and cimetidine is significantly more effective than either antagonist alone in reducing increases in ear temperature elicited by histamine. The involvement of both types of receptor in mediating cutaneous vascular responses to histamine may explain the inability of potent H_1-receptor antagonists alone to abolish the flare produced by histamine injection into human skin (Bain, Hellier & Warin, 1948; Galant, Bullock, Wong & Maibach, 1973), and their partial effectiveness in the clinic in treatment of urticaria (Champion, Roberts, Carpenter & Roger, 1969; Greaves & Bentley Phillips, 1978).

Histamine injected into guinea-pig ears appeared to produce the triple response described by Lewis (1927), i.e. a small red area at the injection site succeeded by a wheal and a widespread surrounding flare. However, no attempt was made to discriminate between flare and direct vasodilatation. For this to be done reliably a method of accurately visualising the injection site and a knowledge of the cutaneous distribution of histamine is required.

Several workers, using the guinea-pig as the test species, have found H_1-receptor antagonists to be far more effective in reducing the permeability response produced by exogenous histamine than by compound 48/80 and cutaneous anaphylaxis (Miles & Miles, 1952; Alberty & Takkunen, 1957; Craig & Wilhelm, 1963). These results suggest that endogenous histamine may not play a major role in mediating inflammatory responses caused by mast cell degranulation. Therefore, the second aim of this study has been to re-examine the quantitative importance of histamine in mediating this type of inflammatory reaction with the aid of H_2- as well as H_1-receptor antagonists.

Mepyramine and cimetidine reduced the increase in ear temperature produced by compound 48/80 and active cutaneous anaphylaxis, the combination of antagonists producing larger shifts in the dose-response curves than either antagonist alone. Moreover, the combination produced a substantial quantitative reduction in ear temperature indicating that histamine plays the major role in mediating the vasodilatation response.

Although mepyramine virtually abolished the permeability response to histamine, it was less effective in reducing the permeability response to compound 48/80 and active cutaneous anaphylaxis. There is no satisfactory explanation for this phenomenon. Certainly other vasoactive substances are released when mast cell degranulation is evoked (Piper, 1976; Soter & Austen, 1976) but their implication in mast cell-mediated skin reactions is not yet proven.

Histamine-induced increases in water content were also greatly reduced by mepyramine and the combination but again mepyramine was less effective in inhibiting water accumulation due to the release of vasoactive mast cell constituents. However, there seemed to be a general trend notably for cimetidine but also the other treatments to exert a greater influence on oedema formation than would be expected from examination of their effects on vascular permeability.

This may be explained as follows. Extravascular water accumulation is a function of vascular permeability to plasma proteins, capillary hydrostatic pressure and functional capillary surface area. In this

study, a reduction in vasodilatation and consequently, blood flow (measured as ear temperature) appears to have reduced the increase in water content due to compound 48/80 and cutaneous anaphylaxis but not to histamine. The reason for this difference is unclear but may be related to the fact that the peak increases in ear temperature to histamine, over the dose range used, are comparatively transient whereas the release of mast cell constituents produces a prolonged maximum response with a slower decline. Therefore, the vasodilatation evoked by compound 48/80 and cutaneous anaphylaxis is likely to play a more significant role in oedema formation than the transient vasodilatation produced by histamine which may augment the oedema response to such a small extent that the method used is insufficiently sensitive to detect it. The relationship between cutaneous blood flow and temperature is exponential and is roughly linear only up to approximately 32°C. (Honda, Carlson & Judy, 1963). Compound 48/80 and cutaneous anaphylaxis produced well maintained ear temperature changes greater than 32°C. Therefore, indirectly measuring blood flow as surface temperature provides an underestimate of blood flow changes above 32°C and consequently, an underestimate of the effect of drugs which reduce ear temperature.

The reduction in vascular permeability obtained with cimetidine against the highest challenge dose of antigen was unexpected as the study with selective histamine H_1- and H_2-receptor antagonists and agonists provides no evidence of H_2-receptor involvement in histamine-induced increases in vascular permeability. We are unaware of properties of cimetidine independent of H_2-receptor blockade and cimetidine has no detectable activity as an H_1-receptor antagonist on histamine-induced contraction of the guinea-pig ileum *in vitro* (Brimblecombe *et al.*, 1975) nor can any H_1-receptor blocking activity be detected in a variety of *in vivo* preparations (Owen, unpublished observation). This phenomenon, therefore, is not readily explained.

In summary, the vascular permeability response to histamine is mediated solely by H_1-receptors whereas vasodilatation is mediated by H_1- and H_2-receptors. The results indicate that a combination of both types of histamine receptor antagonist is required to obtain maximum inhibition of histamine-induced inflammation. It is suggested that the combination may be of greater therapeutic value than either antagonist alone in the treatment of inflammatory skin disorders where histamine has been implicated.

Statistical appendix

In this analysis we wish to compare the dose-response relationships of a particular variable in m different preparations. The variables looked at, all showed a lack of homogeneity of variance when Bartlett's test was applied (Bartlett, 1937). In order to stabilize the variance, a square root transformation was used.

The method used is that of parallel line assay analysis with an unsymmetric design which is described by Finney (1963). However, unlike the conventional multiple assay considered by Finney, in this assay comparisons are made between each pair of preparations. Since the number of possible pair-wise comparisons is

$$^{m}C_2 = \frac{m!}{(m-2)!2!}$$

this necessitates a careful choice of significance level for each comparison. The modification to the LSD test suggested by Fisher (1935) was adopted. This involves reducing the significance level for each comparison from

$$\alpha \quad \text{to} \quad \frac{\alpha}{(^{m}C_2)}$$

The overall significance level required was 0.05 and, taking $m = 4$, this gives a level of

$$\frac{0.05}{\left(\frac{4!}{2!2!}\right)} = 0.00833$$

for each comparison.

As indicated below an overall analysis of variance was carried out and tests of significance performed on the regression and on the departures from parallelism and linearity. Each test was made at the 0.01 level. If the assay was valid, estimates of potency with 99.167% confidence limits were made.

Formation of the analysis of variance table

Nomenclature:

Y_{ijk} represents the transformed response in unit k in preparation j on dose level i.

X_i is the logarithm to the base ten of the dose at dose level i

m is the number of preparations.

p is the number of dose levels.

N_{ij} is the number of units at dose level i in preparation j.

N_j is the total number of units in preparation j.

N is the total number of units.

We then form the sums of squares as follows:–

The total response sum of squares is:

$$T = \sum_{i}^{p} \sum_{j}^{m} \sum_{k}^{N_{ij}} Y_{ijk}^2 - \frac{(\sum \sum \sum Y_{ijk})^2}{N}$$

The between preparations sum of squares is

$$P = \sum_j \frac{(\sum\sum Y_{ijk})^2}{N_j} - \frac{(\sum\sum\sum Y_{ijk})^2}{N}$$

The sum of squares between doses within preparations is

$$D = \sum_i \sum_j \frac{\left(\sum_k Y_{ijk}\right)^2}{N_{ij}} - \sum_j \frac{\left(\sum_i \sum_k Y_{ijk}\right)^2}{N_j}$$

The last sum of squares is partitioned into components due to regression, departures from parallelism and deviations from linearity.

We write:

$$\sum_i^p N_{ij} X_i^2 - \frac{\left(\sum_i N_{ij} X_i\right)^2}{N_j} = (Sxx)_j$$

$$\sum_i^p \sum_k^{N_{ij}} X_i Y_{ijk} - \frac{\left(\sum_i N_{ij} X_i\right)\left(\sum_i \sum_k Y_{ijk}\right)}{N_j} = (Sxy)_j$$

Then the regression sum of squares is,

$$A = \frac{\sum_j [(Sxy)_j]^2}{\sum_j (Sxx)_j}$$

The parallelism sum of squares is,

$$B = \sum_j \frac{[(Sxy)_j]^2}{(Sxx)_j} - A$$

The linearity sum of squares is given by subtraction from the between doses within preparations sum of squares.

The residual sum of squares also follows by subtraction from the total sum of squares.

We may now form the analysis of variance table.

The common slope, b, is estimated by:

$$b = \frac{\sum_j (Sxy)_j}{\sum_j (Sxx)_j}$$

The logarithm of the potency ratio between preparations j and j' is estimated by,

$$M_{jj'} = \bar{x}_j - \bar{x}_{j'} - \frac{(\bar{y}_j - \bar{y}_{j'})}{b}$$

Where $\bar{x}_j$ and $\bar{y}_j$ represent the mean values of Log_{10} (dose) and transformed response respectively for preparation j.

$$g = \frac{t^2 S^2}{[b^2 \sum (Sxx)_j]}$$

Where S^2 is the mean residual sum of squares and t is

Table 1 Analysis of variance for multiple assay

Source of variation	*Sum of squares*	*Degree of freedom*	*Mean sum of squares*
Regression	A	1	A
Parallelism	B	$m - 1$	$\frac{B}{(m-1)}$
Linearity	D − A − B	$m(p-2)$	$\frac{(D-A-B)}{m(p-2)}$
Between doses: within preparations	D	$m(p-1)$	$\frac{D}{m(p-1)}$
Between preparations	P	$m - 1$	$\frac{P}{(m-1)}$
Residual	T − D − P	$N - mp$	$\frac{(T-D-P)}{(N-mp)}$
Total	T	$N - 1$	

the critical value of the t statistic on N-*mp* degrees of freedom for a two-sided test at the 0.0833 level of significance.

The 99.167% confidence limits for the logarithm of the potency ratio are then calculated as:

$$\frac{M_{jj'} \pm \frac{ts}{b}\sqrt{\left\{(1-g)\left(\frac{1}{N_j}+\frac{1}{N_{j'}}\right)+\frac{(M_{jj'}-\bar{x}_j+\bar{x}_{j'})^2}{\sum_j (Sxx)_j}\right\}}}{1-g}$$

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