

Development of a microassay for histamine release

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Summary

A microassay has been developed to measure histamine release from both washed human leucocytes and whole blood, in which blood samples can be diluted down to 1:20 without affecting their sensitivity to antigen. The sensitivity and reproducibility of the single isotope histamine radioenzymatic assay [REA] was compared to the conventional spectrofluorometric histamine assay. The limit of detection was 0.2 and 2.0 ng/ml, respectively, and the agreement between the two assays was good ($r=0.94$). There was also a good qualitative agreement between skin-prick test and histamine release (92%). In order to see if this assay was suitable for small volumes of blood, we measured histamine release to antigen from both diluted whole blood taken by a thumb prick (as a model of the heel prick used in obtaining paediatric blood samples), and undiluted venous blood. There was complete agreement between a positive or negative histamine release response to antigen by diluted thumb prick and undiluted venous blood samples. Leucocyte sensitivity to antigen increased slightly upon dilution, but remained within a ten-fold antigen dilution. The micro-histamine release assay described here offers an opportunity to measure basophil sensitivity to allergens where the availability of the blood is limited (in young babies for example) as well as making possible multiple allergen testing.

Introduction

The development of immediate-type hypersensitivity reactions to foods is a common occurrence in infancy [1]. In time, most atopic children lose their hypersensitivity to food but many go on to develop sensitivity to inhalant allergens [2]. The *in-vitro* assessment of sensitivity is generally based on the quantitation of total and specific IgE in serum, however, levels of antibody do not always correlate well with clinical symptoms [3,4]. In addition there is some doubt about the validity of skin tests in infants [5,6], especially if intradermal tests are used [7]; although some success has been reported in skin-prick testing of infants over 3 months old [8]. *In-vitro* histamine release is an accurate and sensitive method for diagnosing type 1 allergic reactions [9] but it is

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rarely used with paediatric blood samples because the conventional test (based upon the fluorometric histamine assay) requires too much blood (0.5 ml of blood per individual test [10]) and the most that can be easily taken for routine analysis from babies is 100–300 μ l by heel prick.

Our aim in this pilot study was to develop a micro-histamine release test that might be suitable for small volumes of blood. Blood was collected from adults by thumb prick (to simulate a heel-prick sample) and diluted in buffer to give a workable volume. Following incubation with antigen, the released histamine was assayed by the radioenzymatic assay (REA), a technique that was developed to quantitate the small concentrations of histamine that are found in plasma [11].

Subjects and methods

Antigens

Timothy grass pollen and house dust mite (*Dermatophagoides pteronyssinus*) extracts (GA 2463) were obtained as a gift from Mr B. Overell, Beechams Pharmaceuticals PLC (U.K.).

Antigens were tested in ten-fold dilutions between 0.05 ng/ml and 5 μ g/ml (final concentration) in duplicate. The antigens were diluted in Tris ACM buffer (25 mM Tris (Hydroxymethyl)amino methane, 120 mM NaCl, 5 mM CaCl_2 , 1 mM MgCl_2 , 0.03% human serum albumin) at pH 7.5. Anti-human IgE anti-serum (E chain specific) was purchased from Miles Yeda and diluted in assay buffer. Calcium ionophore A23187 was purchased from Calbiochem and diluted immediately prior to use.

Subjects

Five adult atopics (subjects A–E) were selected on the basis of a positive skin-prick test to either grass pollen or *D. pteronyssinus* and a personal history of seasonal or perennial asthma/rhinitis. Four were male and one was female. A negative control was included of one male non-atopic subject (F) who gave a negative skin-prick test response to all antigens tested.

Skin-prick tests

Subjects were tested against standard Bencard (Beechams PLC) solutions of *D. pteronyssinus*, Timothy grass pollen, cat dander and dog dander. Histamine was used as a positive control and saline as a negative control. A positive result was defined as a weal 3 mm or greater than the negative control.

Venous blood dilutions

These studies were carried out using venous blood from subjects A and B in which both washed leucocyte and whole blood preparations were tested at various dilutions as detailed below.

Washed leucocyte preparations. Fifteen millilitres venous blood were taken and washed leucocytes prepared as described by Clinton *et al.* [12]. The washed cells were resuspended in Tris ACM buffer, giving a final cell count of 5×10^6 leucocytes/ml for A and 9×10^6 leucocytes/ml for B.

The washed leucocytes were further diluted in Tris ACM to give dilutions of 1:2, 1:5, 1:10, and 1:20. Two additional dilutions of 1:50 and 1:100 were tested with subject A, but were found to give unreliable histamine release results and so were not used further with subject B.

Table 1. Comparison between radioenzymatic and spectrofluorometric histamine assay

Subject	Atopic status*	Correlation (<i>r</i>) REA vs SP†	Spontaneous HR‡	
			REA	SP
1	+	0.84	9.4	3.6
2	—	0.87	10.0	4.0
3	+	0.88	9.0	2.1
4	+	0.93	2.0	3.0
5	—	0.98	12.2	3.8
6	—	0.97	3.0	3.8
7	—	0.98	11.0	7.2
8	—	0.95	9.2	3.7
9	—	0.97	5.7	6.0
10	—	0.99	9.3	7.0
			8.1	4.4

* + = atopic, — = non-atopic.

† REA = radioenzymatic assay, SP = spectrofluorometric assay.

‡ Spontaneous histamine release values are given as mean histamine release expressed as % of total.

Aliquots of *D. pteronyssinus* in Tris ACM (50 μ l) were added to 'V-shaped' wells in Linbro Titertek microtitre plates. To each antigen dilution was added 50 μ l of each washed leucocyte dilution. The spontaneous histamine release values were determined in triplicate by incubating 50 μ l of cells with 50 μ l of Tris ACM under the same conditions as the cell and antigen incubations. The covered plates were placed in an humidified incubator at 37°C; after 1 hr the reaction was stopped by cooling the plates to 4°C followed by centrifugation at $880 \times g$ for 25 min at 4°C in a Hettich centrifuge (modified by Arnold A. Horwell Ltd). Total histamine determinations were made by boiling cells diluted 50% with 50 μ l Tris ACM for 10 min before cooling to 4°C and spinning as above. Aliquots of 50 μ l were taken from each supernatant and stored at -20°C for subsequent histamine assay.

Whole blood preparations. Venous blood was collected (10 ml from subject A, 5 ml from subject B) into heparinized Tris ACM to give 10 U heparin/ml of blood. The cell counts were 3×10^6 leucocytes/ml for subject A and 4×10^6 leucocytes/ml for subject B. The whole blood samples were further diluted to the same extent as the washed leucocyte preparations; incubations with antigen were carried out as for the washed cells. Total histamine estimations were obtained by boiling 50 μ l whole blood in 100 μ l buffer (a greater dilution of whole blood was used to facilitate removal of the supernatant after boiling) for 10 min in triplicate.

Comparison between thumb-prick and venous blood samples

This study was carried out using blood from all five subjects A–E and subject F as a negative control. A heparinized capillary tube was used to collect 200 μ l of blood taken by a thumb prick from each person and diluted 1:9 in 1.6 ml Tris ACM. In addition, 5 ml of venous blood were taken into 50 U of heparin in 200 μ l Tris ACM. The leucocyte concentration ranged from 1.5×10^6 cells/ml (mean, 3×10^6 cells/ml) in the undiluted blood samples and 2.5×10^5 cells/ml (mean, 3.7×10^5 cells/ml) in the diluted

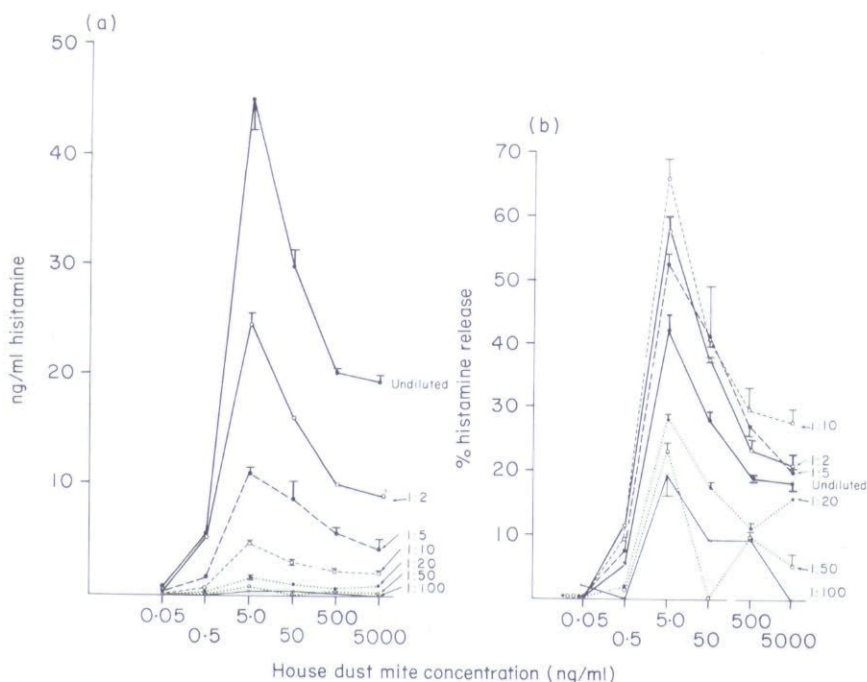


Fig. 1. Histamine release to log₁₀ dilutions of *D. pteronyssinus* from a washed leucocyte preparation from subject A; (a) actual histamine release, (b) percentage histamine release. Error bars represent duplicate values.

thumb-prick samples. Aliquots of each subjects' diluted and undiluted whole blood were added to *D. pteronyssinus* and Timothy grass pollen at the same concentrations as before and incubated as above. Total and spontaneous release determinations were also carried out as above. A positive result was defined as a maximum release of histamine above a threshold value of 10% of total histamine (after subtraction of non-specific histamine release) for both diluted and undiluted blood.

Radioenzymatic histamine assay

The reagents and methods used are exactly as described previously [12].

Comparison between spectrofluorometric and radioenzymatic histamine assays

Ten subjects (not including subjects A–F), three atopic and seven non-atopic were assayed in parallel using a washed leucocyte challenge preparation with house dust mite or grass pollen, depending on the sensitivity of the individual patient, at a concentration of 100, 10, 1, and 0.1 ng/ml. Anti-human IgE anti-serum was used at a dilution of 1:100, 1:500, 1:2500, 1:12500 and 1:62500, and calcium ionophore A23187 at 0.1, 1 and 10 µg/ml. Spontaneous release was measured in buffer alone. Total cell histamine values were obtained from a lysed cell aliquot by the addition of an equal volume of 12% perchloric acid. Venous blood (20 ml) was separated by dextran sedimentation [9], washed and resuspended in Tris ACM at either 6×10^6 leucocytes/ml for the radioenzymatic assay or 3×10^6 leucocytes/ml for the spectrofluorometric assay. The concentration of cells used in the spectrofluorometric assay was lower than in the radioenzymatic assay but is compensated in part by the larger volume used.

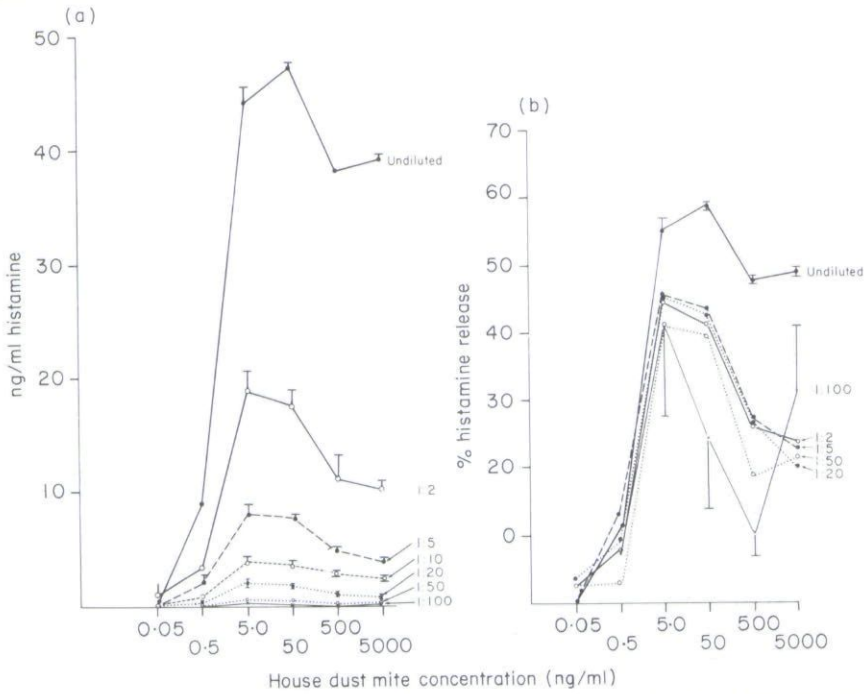


Fig. 2. Histamine release to log₁₀ dilutions of *D. pteronyssinus* from a whole blood preparation from subject A; (a) actual histamine release, (b) percentage histamine release. Error bars represent duplicate values. Because of lack of space in b, error bars are only drawn for the undiluted and 1:100 dilution blood samples.

Fifty-microlitre aliquots of 6×10^6 or $300 \mu\text{l}$ of 3×10^6 cells/ml were incubated with equal volumes of challenge material for 40 min at 37°C . Aliquots of the supernatants were then assayed by the radioenzymatic assay or spectrofluorometrically.

Results

Comparison of spectrofluorometric and radioenzymatic histamine assays

Table 1 shows the results of ten parallel challenges with ionophore, antigen or anti-IgE. There was a close correlation between the assays when percentage histamine release was compared (sixteen data points per subject, mean $r=0.93$, range 0.84–0.99). The mean spontaneous release in the spectrofluorometric assay was 4.41% compared with 8.13% in the radioenzymatic assay. Although this difference ($P < 0.001$) is significant, both tests have acceptably low levels of spontaneous release. There was no difference in either correlation coefficient or mean spontaneous release in the atopic as compared with non-atopic groups.

Whole blood and washed leucocyte dilutions

The levels of histamine release from both the washed leucocyte and whole blood preparations of subject A in response to stimulation with *D. pteronyssinus* are shown in Figs 1 and 2. Similar findings were observed for subject B. The accuracy of each dilution was confirmed by measuring both the total histamine and the numbers of leucocytes. Total histamine levels were on average 0.95 times the expected value for each dilution whilst the total leucocytes numbers were 1.05 times the expected value for

Table 2. Comparison between skin-prick tests and histamine release tests

Subject	Skin-prick test*	Histamine release†	Histamine release (dil. WB)‡
<i>Antigen = D. pteronyssinus</i>			
A	+\$	+	+
B	+	+	+
C	+	—	—
D	—	—	—
E	+	+	+
F	—	—	—
<i>Antigen = Timothy grass pollen</i>			
A	+	+	+
B	—	—	—
C	+	+	+
D	+	+	+
E	+	+	+
F	—	—	—

* A positive skin-prick test result = > 3 mm weal.

† Histamine release = % histamine release from undiluted venous whole blood. A positive histamine release = > 10% histamine release after subtraction of the spontaneous release.

‡ Histamine release (dil. WB) = % histamine release from a diluted thumb-prick sample of whole blood.

§ + = positive result, — = negative.

each dilution. In both cases histamine release exhibits a clear dose-response to different concentrations of antigen, even at a blood dilution of 1:100, although at these high dilutions the error increases.

Cell sensitivity was unchanged at dilutions as high as 1:20 where the antigen concentrations giving 30% histamine release (HR₃₀) for whole blood ranged from 1.4–2.0 ng/ml (mean = 1.8, number of blood dilutions = 7), whilst the average HR₂₀ for washed leucocytes was 1.3 ng/ml (range = 0.79–2.5 ng/ml, *n* = 5). The 1:50 and 1:100 dilutions of washed leucocytes were generally less sensitive.

Comparison between thumb-prick and venous blood samples

Five of the six subjects chosen for this study were atopic as judged by a positive skin-prick test to one or more of the common inhalant antigens; only subject F, the negative control, gave a negative skin response to all allergens tested. There was good qualitative agreement between skin-prick test and histamine release, with a concordance of 92% in obtaining a positive or negative result for the two allergens tested (Table 2), which was statistically significant by the chi-squared test (*P* < 0.001, *n* = 12).

There was complete agreement between histamine release from undiluted venous whole blood and diluted thumb-prick whole blood when assessed as a positive or negative result.

Less than 8% of total cell histamine was released in response to both antigens in the control subject. Subjects B and D, who were only allergic to one of the allergens, also acted as their own negative controls by releasing less than 10% histamine to the other allergen tested. The spontaneous histamine release values ranged from 4–20% (mean = 12%) in the six subjects. The apparent blood dilutions were on average 1.9

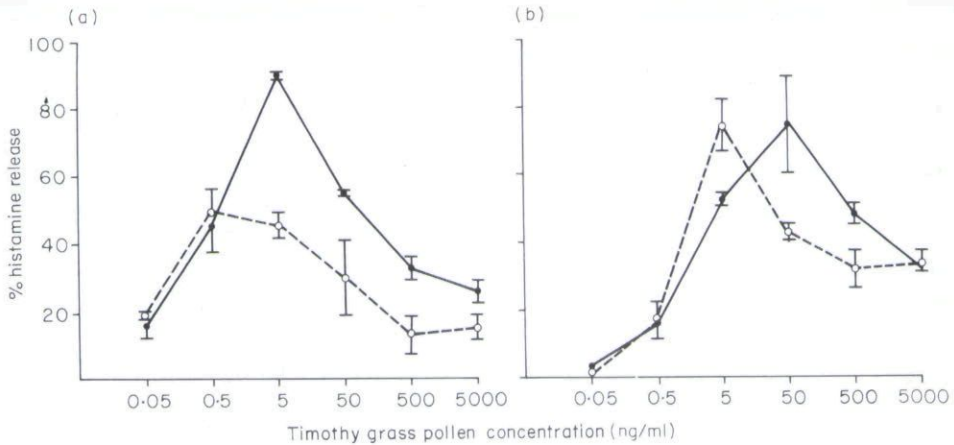


Fig. 3. Histamine release to Timothy grass pollen by (a) subject C and (b) subject D. (●—●) Undiluted venous whole blood, (○—○) diluted thumb-prick whole blood.

Table 3. Comparison of leucocyte sensitivity to inhalant antigens in undiluted venous whole blood and diluted thumb-prick samples

Subject	Antigen	Threshold antigen concentration in ng/ml for			
		50% HR		15% HR	
		Undil. WB	Dil. WB*	Undil. WB	Dil. WB
A	<i>D. pteronyssinus</i>	1.8	0.25	0.22	0.05
	TGP†	a	a	63	112
B	<i>D. pteronyssinus</i>	0.011	0.011	b	b
	TGP	—	—	—	—
C	<i>D. pteronyssinus</i>	—	—	—	—
	TGP	0.5	0.63	0.05	0.036
D	<i>D. pteronyssinus</i>	—	—	—	—
	TGP	4.5	1.9	0.45	0.4
E	<i>D. pteronyssinus</i>	0.011	0.016	b	b
	TGP	39.8	a	8.9	5.9
F	<i>D. pteronyssinus</i>	—	—	—	—
	TGP	—	—	—	—

* WB=whole blood preparation.

† TGP=Timothy grass pollen.

a=maximum histamine release < 50%; b=minimum histamine release > 15%.

—: negative histamine release, i.e. maximum histamine release = < 10% total cell histamine.

times greater than their expected value when assessed by total cell histamine and 1.2 times their expected value by leucocyte number. These levels of total histamine are higher than expected in the diluted blood samples; this may explain an unexpected finding where the levels of maximum histamine release to antigen (as a percentage of total cell histamine) in diluted blood were approximately 50% of the histamine release

maxima in undiluted blood in subjects B, C and E (4/7 positive results). Figure 3a illustrates this finding in one subject. Figure 3b illustrates another common finding where the concentration of antigen required for maximum histamine release appears to decrease in diluted blood samples. Nevertheless, the sensitivity of leucocytes to antigen was found to be similar in diluted and undiluted blood (Table 3), despite differences in maximum histamine release.

Discussion

Allergen-mediated basophil histamine release is a useful test for helping to diagnose type 1 hypersensitivity reactions *in vitro*. However, its usefulness could be extended if the sensitivity of the test were improved, allowing a greater number of tests per ml of blood. Various other attempts have been made to minaturize this test. In 1985 Stahl Skov *et al.* [13] reported that as little as 50 μ l of washed blood could be challenged per tube, using microtitre plates containing glass microfibres to capture the released histamine. By using diluted whole blood, our test goes further than this as only 100 μ l of blood is required to test the reaction of leucocytes to six dilutions of antigen in duplicate, giving a clear dose-response curve in allergic subjects. Additional advantages include the speed of the test since very little manipulation of blood is required (although measurement of the released histamine by REA does take an extra day) and no special equipment is needed that cannot be obtained easily from normal suppliers.

Despite the good qualitative agreement between undiluted venous whole blood and diluted thumb-prick whole blood in their histamine release response to antigen, the quantitative agreement is not so close, the maximum of histamine differing in the two types of blood sample. One reason for these differences may lie in the fact that not only the effects of dilution but also the differences in blood collection and handling were being compared. Another reason for the lower maximum histamine release in diluted compared with undiluted blood samples is that the spontaneous histamine release values are at the lower detection limit of the assay (0.1–0.2 ng/ml). As a result this value becomes proportionately greater with dilution of the blood. This was seen most clearly when blood or washed leucocytes were diluted to 1:50 or 1:100. It was for this reason that a dilution of 1:9 was used for the thumb-prick samples.

Maximum histamine release occurred at a lower antigen concentration in diluted whole blood (both thumb-prick and venous) compared to undiluted venous blood (although this was not the case in diluted and undiluted washed leucocyte preparations). When the threshold antigen concentrations that gave 15% and 50% histamine release from diluted thumb-prick and undiluted venous blood were compared, they were within 1 log dilution of each other (Table 3), indicating that the sensitivities of the diluted and undiluted leucocytes were similar.

In summary, as little as 100 μ l of blood diluted 1:10 can be used to demonstrate a clear, quantifiable dose-relationship between histamine release and antigen concentration in allergic subjects. This test compares well with the skin-prick test and is of value in situations where either the supply of blood is limited or a large number of tests are required.

Acknowledgment

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