

A new glass microfibre-based histamine analysis for allergy testing in children

Results compared with conventional leukocyte histamine release assay, skin prick test, bronchial provocation test and RAST

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A new microfibre method for allergy testing measuring histamine release from human basophil leukocytes is described. Samples of 50 µl washed blood are challenged with the suspected allergens. Released histamine is bound to microfibres and measured by a spectrofluorometrical method after removal of interfering substances by washing. The microfibre method (HR-MM) was compared to the conventional histamine release assay using the Ficoll-Hypaque gradient method (HR-FH) in 19 allergic children tested with one of three allergens. In addition, a comparison was made between the microfibre method and *in vivo* provocation tests, i.e. skin prick test (SPT), bronchial provocation test (BPT) and allergen specific serum IgE (RAST). It was found that the same individuals responded with histamine release to the same allergens in both histamine release assays, and the dose-response curves were almost identical. A positive correlation was found between the *in vivo* and *in vitro* tests. Thus it is concluded that the new method can provide reproducible, analytically precise (at the nanogram level) histamine release results in pediatric cases where: 1) a positive SPT does not correlate with case history; 2) BPT may be considered too hazardous or inconvenient; 3) confirmation of negative or inconclusive SPT or RAST is needed. In contrast to other histamine release assays it is a convenient diagnostic tool in children since only small amounts of blood are needed and at least 96 tests can be carried out in 2½ h.

Key words: allergens; allergy; basophil cell sensitivity; bronchial provocation; glass microfibres; histamine release; leukocytes; RAST; skin test.

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The *in vitro* diagnosis of type I allergy is based on estimation of total and specific IgE in serum. However, these tests have poor reproducibility, sensitivity, and specificity, especially in diagnosing less severe allergy (15).

In children with exogenous bronchial asthma conflicting results obtained by case history (CH), skin prick test (SPT) and RAST will often lead to a bronchial provocation test (BPT). This is, however time-consuming, inconvenient for the patient, and might be dangerous. Histamine release from basophil leukocytes has proven to be a valuable experimental diagnostic tool in allergy diagnosis, but its clinical

value, especially in children, is limited because the older conventional methods of histamine release are time- and blood-consuming (4, 5, 9).

Glass-microfibres have been found to bind histamine with high affinity and selectivity (7, 13). Histamine release is therefore performed in glass-microfibre-prepared microtitre plates by challenging 50 µl blood samples with different allergens. In this study the new method (HR-MM) is presented and compared with the conventional leukocyte histamine release assay (HR-FH), SPT, RAST and BPT in children.

In adults the new method has been found to fulfil the demands for a specific and sensitive *in vitro* assay for diagnosing type I allergy (7), but in children the experience is still limited and the diagnostic specificity and sensitivity of histamine release studies show great differences (6, 14, 15). Whether this principle of histamine release can altogether be applied in children is unknown and subject to debate.

MATERIAL AND METHODS

Patients

The study comprised 19 children, aged 6 to 15 years (mean 10.8) (13 boys and 6 girls) with a well-documented history of exogenous asthma verified by a family history of allergy and clinical exogenous asthma responding to bronchodilating therapy. Only children with an unequivocal CH, evaluated by detailed registration of subjective symptoms, consumption of anti-allergic drugs, and peak-flow measurements with aggravation of respiratory allergy when exposed to either *Phleum pratense*, dog dander or *Dermatophagoides pteronyssinus* were included in the study. All the children were seen in a children's allergy clinic (Rigshospitalet, Copenhagen) and were evaluated by CH, SPT, RAST histamine release by the conventional method (HR-FH) and the new microfiber-based histamine analysis (HR-MM). Finally, BPT was performed. The histamine release studies were performed prior to the *in vivo* tests and the results of the latter were unknown to the investigator of histamine release. The same allergens with the same batch No. were used throughout the investigation. The time interval between the *in vivo* and *in vitro* test was 1 or 2 days.

None of the children had ever received immunotherapy. In addition, none of the children were receiving systemic steroids at the time of the study. Bronchodilating drugs were suspended for at least 6 h and neither aminophylline nor antihistamines had been given for at least 24 h prior to the study.

Allergens and anti-IgE

The following allergens were used: *Phleum pratense*, dog dander, and *Dermatophagoides pteronyssinus*, which were delivered by ALK, Denmark, in stock solutions of 1:10 w/v. To ensure maximum potency only fresh extracts reconstituted and diluted on the day of testing were used.

For histamine release experiments the allergens were diluted in Pipes-AMC to final concentrations in the reaction mixture of 10^{-7} , 10^{-5} , and 10^{-3} v/v.

Anti-IgE (400,000 U/ml) from Behringwerke, Germany was used in final concentrations of 4, 40, and 400 U/ml.

Skin prick test

The same person carried out the SPT on the volar surface of the patients' forearms with each extract in concentrations of 10, 3, and 1 HEP (Histamine Equivalent Prick) per ml. The wheals were marked when the reaction was at maximum and compared to a positive reference containing histamine chloride, 1 mg/ml. The contours of the wheals were marked with a felt-tipped pen and transferred to squared mm paper with a strip of tape. The size of the wheal was measured using the formula:

$$\frac{(D + d)}{2} \text{ mm}$$

where D is the largest diameter and d the perpendicular diameter.

Allergen-specific serum IgE (RAST)

RAST was determined and performed as previously described (12) by ALK Laboratory, Denmark, and the results were expressed in allergy classes 0 to 4.

Bronchial provocation test (PBT)

The technique was carried out using nebulized aqueous allergen extracts in increasing concentrations up to 1:10 w/v. Before and after

each inhalation of each concentration, forced expiratory volume in the first second (FEV_1) was recorded on an electronic spirometer (Spirotron, Dräger) and the patient was questioned about symptoms, and chest auscultation was performed before raising the concentration one decade. A drop in FEV_1 of 20% or more was defined as a positive reaction. Inhalation was started each time with phenol saline as a negative control. After BPT, PEF (peak expiratory flow) was recorded every half hour for the next 6 h to detect any late reactions. One patient had a late reaction.

Conventional leukocyte histamine release assay

Venous blood (10 ml) was drawn into a plastic syringe, anti-coagulated with 0.5 ml 0.2 M EDTA (pH 7.2), and the leukocytes were isolated by the Ficoll-Hypaque gradient method, and then washed and suspended in Tris-AMC. 100 μ l leukocyte suspensions were incubated in plastic tubes at 37°C for 40 min with the above-mentioned allergens and anti-IgE in the final concentrations as stated earlier. All concentrations were tested in duplicate. Allergens and anti-IgE were omitted for spontaneous histamine release and for determination of the total histamine content, in the latter case the samples were left at 4°C. Thereafter 3 ml ice-cold Tris-AMC was added to the samples which were then centrifuged at 2000 *g* for 10 min. The supernatant was discarded and the residual histamine was determined in the cell sediment by the spectrofluorometrical-OPT method (9). The release of histamine was expressed as a percentage of the total histamine content of the sample. In the absence of allergen or anti-IgE the spontaneous histamine release amounted to 5–10% in all experiments. It should be noted that only histamine release > 10% is considered significant, and cell sensitivity is defined as the allergen concentration causing a 20% histamine release.

Microfibre method (HR-MM)

Venous blood (3 ml) was drawn into a plastic syringe, anti-coagulated with 0.16 ml 0.2 M EDTA (pH 7.2) and used the same day. How-

ever, the blood can be stored for 24 h at 4°C before assay. The blood sample was washed 3 times at 300 *g* for 6 min at room temperature with 7 ml Pipes-AMC and resuspended to a final volume of 3 ml.

Fifty μ l samples of washed blood were placed in the glass microfibre-prepared microtitre plate. To avoid trapping of the blood cells into the microfibrils, 30 μ l of Pipes-AMC was added to the plates before the washed blood. The samples were then incubated for 60 min at 37°C with 20 μ l of allergen or anti-IgE in the final concentrations mentioned above. The net release of histamine was calculated on the basis of a standard curve, where blood samples were incubated with 0, 0.625, and 1.25 ng histamine, 2 HCl. The spontaneous release was negligible, since almost identical standard curves were obtained when histamine was added to Pipes-AMC instead of the blood.

Released histamine was bound to the microfibrils and after incubation interfering substances were removed by washing the plates 1.5 min with deionized water. Histamine was released from the fibres by addition of 20 μ l $HClO_4$ /OPT-mixture (10 mg o-phthalaldehyde, Fluka, dissolved in 2.5 ml methanol and 7.5 ml 0.59% $HClO_4$). After 5 min at room temperature, 65 μ l 0.05 N NaOH was added and the coupling reaction was terminated after 10 min by addition of 65 μ l 0.59% $HClO_4$. The histamine content was determined in a Farrand spectrophotofluorometer; only net release of histamine $\geq$ 0.3 ng histamine, 2 HCl was considered a significant release, and the cell sensitivity was here defined as the allergen concentration releasing 0.3 ng histamine, 2 HCl (8).

Reagents

For histamine release experiments by the microfibre method the allergens were diluted in Pipes-AMC, a buffered solution of pH 7.4 and consisting of 10 mM piperazine-N,N (bis-ethane sulfonic acid), 140 mM sodium acetate, 5 mM potassium acetate, 0.6 mM $CaCl_2$, 1.1 mM $MgCl_2$, glucose 1 mg/ml, human serum albumin 0.3 mg/ml, and heparin 15 IE/ml, LEO, Denmark.

Tris-AMC was used for the conventional assay and consisted of a buffered solution containing 25 mM Tris at pH 7.6, 0.12 M NaCl, 5 mM KCl, 0.6 mM CaCl₂, 1.1 mM MgCl₂, 0.3 mg/ml human serum albumin, and 1 mg/ml glucose.

Score system

The results obtained with the various tests were scored in the following way: 0 = a negative test; + = a positive test. Relations between scores are seen in Table 1.

Statistics

Correlation between the two histamine release assays was calculated by Spearman's Rho.

RESULTS

Nineteen children with exogenous asthma were tested with the suspected allergen and with anti-IgE by the conventional leukocyte histamine release assay and with the new microfibre assay.

The response to anti-IgE was identical, since in both assays 18 of the children showed anti-IgE-induced histamine release (results not

Table 1		
Score system		
Score	Negative 0	Positive +
BPT	negative	≥ 20% fall in FEV ₁ with 10 ⁻⁶ v/v HEP extract
SPT	< 75% of histamine ref. with 10 HEP	≥ 75% of histamine ref. with 10 HEP
RAST	allergy class 0-1	allergy class 2-4
HR-FH	< 20% histamine release with ≤ 10 ⁻³ v/v extract	≥ 20% histamine release with ≤ 10 ⁻³ v/v extract
HR-MM	< 0.3 ng histamine, 2 HCl with ≤ 10 ⁻³ v/v extract	≥ 0.3 ng histamine, 2 HCl with ≤ 10 ⁻³ v/v extract

HEP = Histamine equivalent prick

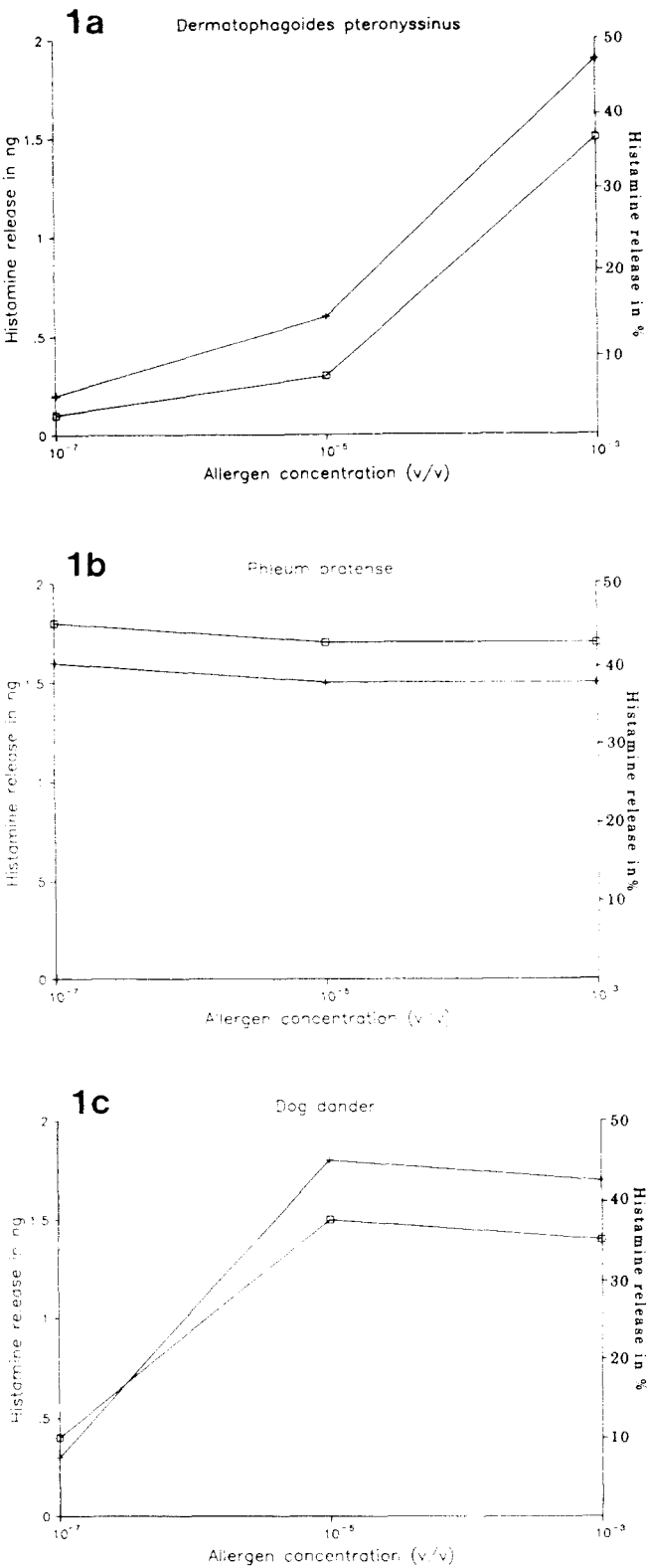


Fig 1a, 1b, 1c: Examples of histamine release caused by different allergens in three different allergic children. Microfibre method (+ — +) and conventional leukocyte histamine release assay (□ — □). Please note that histamine release is expressed differently in the two histamine release assays.

shown). The patient who failed to release histamine was also negative to the allergens.

Fig. 1 shows three representative examples of

the dose-response curves produced by the two methods (HR-FH/HR-MM). Almost identical results were obtained.

In Table 2 cell sensitivity to the suspected allergen was determined in the two histamine release assays. A significant correlation ($Rho = 0.93$, $P < 0.001$) was found, which further substantiates the degree of identity between the two methods.

To compare the new method with the other tests used in allergological diagnosis, RAST,

SPT, and BPT were included. Table 3 shows, for each patient, the relation between responses obtained by the different tests according to the suspected allergen. Positive clinical allergy (asthma) verified by BPT was found in 10 patients (53 %).

In Table 4 the agreement between the results obtained in HR-MM are correlated to the individual diagnostic tests. From the table it can be calculated that the best agreement was found between HR-FH vs HR-MM 17/19 (90 %),

Table 2

Correlation between cell sensitivity measured by the microfibre method and the conventional leukocyte histamine release assay

		HR-MM			
		No release	10^{-3}	10^{-5}	10^{-7}
HR-FH	No release	6	1		
	10^{-3}		2		
	10^{-5}		2	1	1
	10^{-7}			4	2

Cell sensitivity was defined in different ways in the two assays, i.e. by the allergen concentration (v/v) causing a net release of 0.3 ng histamine or a 20 % histamine release. The correlation between cell sensitivity in these assays was positive ($\rho = 0.93$; $P < 0.001$).

Table 3

Relation between responses in the different tests

Pt. No.	BPT	RAST	SPT	HR-FH	HR-MM	Allergen
1	-	-	-	-	-	grass
2	+	+	+	-	+	dog dander
3	+	-	+	+	+	grass
4	+	+	+	+	+	grass
5	+	+	+	+	+	grass
6	-	-	+	-	+	dog dander
7	+	+	-	+	+	dog dander
8	-	-	-	-	-	mite
9	+	+	+	+	+	dog dander
10	-	-	-	-	-	dog dander
11	-	-	-	-	-	grass
12	+	+	+	+	+	grass
13	-	+	-	-	-	dog dander
14	-	+	-	-	-	dog dander
15	+	+	+	+	+	grass
16	-	-	+	+	+	dog dander
17	+	-	+	+	+	grass
18	+	-	+	+	+	grass
19	+ *	-	-	+	+	mite

Positive (+) and negative (-) response

*Developed a late asthma reaction

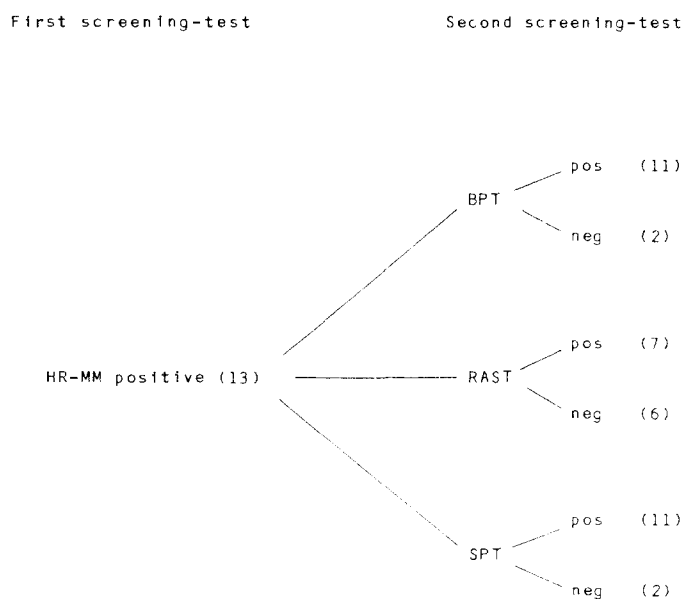


Fig. 2. Results of stepwise combination of positive micro-fibre method (first screening test) with BPT, RAST or SPT (second screening test) in relation to a final positive clinical diagnosis.

HR-MM vs SPT 17/19 (90%), and in decreasing order, HR-MM vs BPT 16/19 (84%) and HR-MM vs RAST 11/19 (58%). Complete concordance (positive or negative) between all five diagnostic procedures was only found in 8/19 (42%) of the patients.

Table 4 indicates a complete agreement between the positive responders obtained by the BPT and HR-MM, whereas a small difference

is found among the negative responders, since in eight patients with negative BPT only six showed no release of histamine. Please note that the patient who developed a late asthma reaction was included among the positive responders. Table 4 also shows that the coincidence between HR-MM and BPT/SPT (90%) is higher than between RAST and BPT/SPT (73/58%).

The screening process in allergological diagnosis is a stepwise combination of tests to enable a final diagnosis. Although our material is small, Fig. 2 shows the trend in combining HR-MM with SPT, RAST and BPT. It is seen that RAST will lead to even more conflicting results and will give no further diagnostic help to the clinician.

DISCUSSION

The first step in the diagnosis of allergy is a thorough case history. In some cases the patient's history is decisively negative or positive, but in a number of cases it is doubtful. When the patients is multi-allergic the correct diagnosis and the relevant allergens can be even more laborious and time-consuming to establish.

Therefore, the need for a fast, reliable procedure for the measurement of histamine

Table 4

Combination of results of histamine release by conventional method (HR-FH), bronchial provocation (BPT), skin prick test (SPT), and allergen specific-serum IgE (RAST) with the results of histamine release by the new microfibre method

		BPT		RAST		SPT		HR-FH	
		pos	neg	pos	neg	pos	neg	pos	neg
HR-MM	pos	11*	2	7	6	11	2	11	1
	neg	0	6	2	4	0	6	1	6
Coincidence %		90		58		90		90	
RAST	pos	7	2			6	3		
	neg	3	7			5	5		
Coincidence %		73				58			

*The response includes a late asthmatic reaction.

release from human basophil leukocytes is increasingly apparent as more correlations become established between the release of histamine from whole blood *in vitro* and allergic reactions in atopic diseases (6, 7, 14, 15).

We examined prospectively, in a controlled and blinded fashion, the concordance of histamine release by the new microfibre method and the conventional histamine release assay, BPT, RAST, and SPT in 19 allergic children.

The new method is a simplified histamine release assay which can be used for children with atopic allergic diseases. The specificity and sensitivity of the method and the methodological problems have been described in detail earlier (7, 8). The fact that isolation of cells and extraction of histamine are omitted makes the method suitable for clinical screening and diagnosis in children. The test only needs a small volume of blood, i.e. 50 μ l per sample – 96 samples can be incubated and analyzed in 2½ h and only requires 5.0 ml blood (since this investigation, we have found that it is possible to dilute the blood samples 1:1 without losing any sensitivity). Furthermore, the blood can be stored for 24 h at 4°C before being assayed.

The microfibre assay differs from conventional methods not only in being a new principle, but also because histamine release is determined by net release in nanograms instead of in per cent. It was therefore essential to examine the degree of identity between results obtained by the two methods. Nineteen children were tested in each histamine release assay (HR-MM/HR-FH) and it was found that the same individuals responded with histamine release and that the number of responders was almost identical. A close degree of identity between results obtained by the two methods was also demonstrated by the identical dose-response curves. In addition, the cell sensitivity to allergens, which appears useful for evaluating the degree of allergy (10), was determined, and a highly significant correlation ($Rho = 0.93$, $P < 0.001$) was found.

There are conflicting views regarding basophil histamine release, *in vivo* tests and the clinical manifestation of allergy. In previous adult studies histamine release has shown to be

of considerable diagnostic help, but this was not confirmed in a recent study in children by Stafanger et al. (11).

In this study a high coincidence was noted between histamine release from whole blood and BPT/SPT (90 %), which was higher than the coincidence between RAST and BPT/SPT (73/58 %). This indicates that the new method is superior to the RAST method, and the high coincidence between HR-MM and BPT/SPT demonstrates the value of the new method.

Several studies assume that a positive provocation test (most often bronchial) is an expression of the “true” diagnosis (1, 2). However, the response to a test antigen, especially in children with hyperreactive airways and with allergen extracts of varying purification standards and antigen-dosages, is not sufficiently physiological and can be questioned.

In our study three patients, of whom one had a late reaction, were positive by the HR-FH/HR-MM tests and did not react on BPT. This could be related to a potential allergy to the allergen in question, which at that moment was clinically unimportant.

We consider the value of histamine release from human basophil leukocyte requires further clarification in large clinical, controlled studies, where one differentiates between the demonstration of IgE reactivity (diagnostic tests) and the specific diagnosis (clinically manifest bronchial allergy). Until now antigen-mediated histamine release has been used extensively in *in vitro* studies of allergy, but has been given only limited clinical application. This is mainly due to the large amount of blood required, the slow kinetics of histamine release, and the difficulty of the histamine determination.

As the microfibre method becomes commercially available, these problems in allergy testing in children, where only small amounts of blood are available and many tests have to be performed, might be overcome. The method can be of value in children with skin symptoms, where SPT cannot be performed, in large scale screening, in monitoring the effect of immunotherapy, and in allergen standardization, and probably also in follow-up studies in atopically predisposed infants. The analysis is precise, re-

producible, and amenable to routine application.

In pediatric cases it is a safe *in vitro* adjunct to the traditional *in vivo* tests and an alternative in cases where a positive SPT does not correlate with CH, or BPT may be considered too hazardous or inconvenient, and when confirmation of negative or inconclusive SPT is needed.

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