

Sensitive Glass Microfibre-Based Histamine Analysis for Allergy Testing in Washed Blood Cells

Results Compared with Conventional Leukocyte Histamine Release Assay

P. STAHL SKOV, H. MOSBECH, S. NORN and B. WEEKE

Dept. of Pharmacology, University of Copenhagen and the Rigshospital, Copenhagen, Denmark

The new microfibre method for allergy testing is based on basophil histamine release after challenge with suspected allergens in samples of 50 μ l washed blood cells. Released histamine is bound to microfibres and measured after removal of interfering substances by washing. The microfibre method was compared with the conventional leukocyte histamine release assay in 18 allergic patients tested with 10 different allergens. It was found that the same individuals responded with histamine release to the same allergens in both assays, and the number of responders was almost identical. Also the dose-response curves and the cell sensitivity were almost identical, which further substantiated identity between the results obtained by the new microfibre method and the conventional assay. A comparison between the microfibre method and *in vivo* provocation tests showed good agreement when comparing the number of positive and negative responses in these test. The new method overcomes the problems in allergy testing, where only small amounts of blood are available and many tests have to be carried out.

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

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The *in vitro* diagnosis of specific type I allergic reactions is mainly based on estimation of total and specific IgE in serum. However, these tests have poor reproducibility and sensitivity, especially in diagnosing less severe allergy (16). Histamine release from human basophil leukocytes fulfils the demands of a specific and sensitive *in vitro* assay for diagnosing type I allergic reactions (1, 10, 11). We have therefore developed a new, simple method, which only requires small amounts of blood and no isolation of cells, since histamine was released by allergen in washed blood (13). In this study, the new microfibre method was compared with the conventional leukocyte histamine release assay, and the results obtained by the new method

were found to be almost identical to those obtained by the conventional assay.

PATIENTS

Eighteen patients with a well-documented history of allergy were included in the study. Seventeen of the 18 patients suffered from asthma, 13 from conjunctivitis, 15 from rhinitis, and one from atopic dermatitis. All patients were seen in an adult allergy clinic (Rigshospital, Copenhagen) and were evaluated by case history and skin prick test, and in 16 of the patients bronchial provocation tests were performed. These *in vivo* tests were performed prior to the *in vitro* studies of histamine release

using the same allergens, and the time interval between the *in vivo* and *in vitro* tests amounted to 6 months (range 1 day to 1 year). Thirteen of the patients received immunotherapy with a mean duration of treatment of 1 year (range 3 months to 2 years).

MATERIAL AND METHODS

Allergens

The following allergens were used in this study. *Betula verucosa*, *Phleum pratense*, *Artemisia vulgaris*, horse, dog and cat dander, *Dermatophagoides pteronyssinus*, and house dust, which were delivered from ALK, Denmark in stock solutions of 1:10 w/v, and *Alternaria* and *Cladosporium herbarum* (both 10,000 BU/ml) from Pharmacia, Sweden.

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 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} v/v. Pipes-AMC, a buffered solution of pH 7.4, was used instead of Tris-AMC (3) and consisted 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, Leo, Denmark, 15 IE/ml.

Skin prick tests

The allergen concentration used for skin tests was 5 HEP (Histamine Equivalent Prick) corresponding to a positive reference of 5 mg histamine, 2 HCl/ml. Skin reactions were read after 15 min, and wheals half or more the size of the histamine reference were regarded as positive.

Bronchial provocation test

The technique was carried out as described earlier (16). A drop in forced expiratory volume in

the 1st s (FEV_1) of 20% or more was defined as a positive reaction to the allergen. The highest concentrations of allergens used were 1:10 w/v. The time interval between bronchial provocation and *in vitro* tests was in many cases at least 6 months, therefore no attempts were made to examine a correlation between histamine release and bronchial sensitivity.

Conventional leukocyte histamine release assay

Venous blood (20 ml) was drawn into a plastic syringe, anticoagulated with 1 ml 0.2 M EDTA (pH 7.2), and the leukocytes were isolated by the Ficoll-Hypaque gradient method, and thereafter washed and suspended in Pipes-AMC instead of Tris-AMC (11). 100 μl leukocyte suspensions were incubated in plastic tubes at 37°C for 40 min with the above-mentioned allergens in final concentrations of 10^{-7} , 10^{-5} , and 10^{-3} v/v, or anti-IgE in final concentrations of 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} v/v. 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 Pipes-AMC was added to the samples and they were centrifuged at 2000 g for 10 min. The supernatant was discarded and the residual histamine was determined in the cell sediment. To the cell sediment was added 400 μl NaOH/OPT mixture (2 mg OPT (o-phthaldialdehyde, Fluka) dissolved in 1 ml methanol and 9 ml 0.05 N NaOH). After 4 min 400 μl 0.175 N H_3PO_4 was added and the samples centrifuged at 3000 g for 10 min. The histamine content was determined spectrofluorometrically in a Farrand provided with a micro-flow cell and an autosampler. Samples were read at 355/455 nm (excitation/emission). 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. Therefore, it should be noted that only release > 10% is considered significant. Cell sensitivity was defined as the allergen concentration causing a 20% histamine release (10).

Microfibre method

Venous blood (5 ml) was drawn into a plastic syringe, anticoagulated with 0.25 ml 0.2 M EDTA (pH 7.2) and, if not otherwise stated, used on the same day. However, the blood can also be stored for 24 h at 4°C before assay, since this does not change the histamine release or cell sensitivity. The blood sample was washed 3 times at 300 g for 6 min at room temperature with 15 ml Pipes-AMC and resuspended in this solution to a final volume of 5 ml.

Fifty µl samples of washed blood were placed in glass microfibre-prepared microtitre plates (international patent application No. WO 83/00229). To avoid trapping of blood cells into the microfibrils 50 µl of Pipes-AMC was added to the plates before the washed blood. The samples were incubated for 60 min at 37°C with 20 µl of allergen or anti-IgE in the final concentrations mentioned above. The net release of histamine was calculated on the basis of a standard histamine 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 to the blood.

After incubation, released histamine was bound to the microfibrils and interfering substances were removed by washing the plates for 1.5 min with deionized water. Thereafter histamine was released from the fibres by addition of 20 µl HClO₄/OPT mixture (2 mg o-phthalaldehyde, Fluka, dissolved in 1 ml methanol and 1 ml 0.59% HClO₄). After 10 min at room temperature, 65 µl 0.05 N NaOH was added and the coupling reaction was terminated after 10 min by addition of 65 µl 0.59% HClO₄. 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 lowest allergen concentration releasing 0.3 ng histamine, 2 HCl.

RESULTS

Eighteen allergic patients were tested with 10 different allergens and with anti-IgE by the

conventional leukocyte histamine release assay and with the new microfibre method. Of the 180 tests performed with allergens in each method, 40% were positive, i.e. showed significant release of histamine. It appears from Table 1 that the results obtained by the microfibre method were identical to those obtained by the conventional assay. Thus, the same individuals responded with histamine release in both assays and the number of responders was also identical, with the exception of a slight difference in the response to *Betula verucosa* where six persons showed histamine release in the microfibre method, but only five of them in the conventional assay. The response to anti-IgE was also identical, since in both assays 14 of the 18 patients showed histamine release by anti-IgE (Table 1). That the two methods produced almost identical results was also demonstrated by dose-response curves where identical curves were obtained by the microfibre method and the conventional assay. Representative examples are shown in Fig. 1.

In Table 2 cell sensitivity to the 10 different allergens was determined in the 18 patients by the two assays. It is seen that a highly significant correlation was found between cell sensitivity measured by the microfibre method and

Table 1

Allergic patients showing histamine release by *in vitro* provocations with allergens or anti-IgE

	Methods	
	Conventional	Microfibre
<i>Betula verucosa</i>	5	6
<i>Phleum Pratense</i>	12	12
<i>Artemisia vulgaris</i>	9	9
Horse dander	4	4
Dog dander	7	7
Cat dander	9	9
<i>Dermatophagoides pteronyssinus</i>	8	8
House dust	12	12
<i>Alternaria</i>	2	2
<i>Cladosporum</i>	3	3
Anti-IgE	14	14

Eighteen patients were tested for histamine release by 10 different allergens and by anti-IgE. The same patients showed release in both methods and the number of persons showing histamine release is stated.

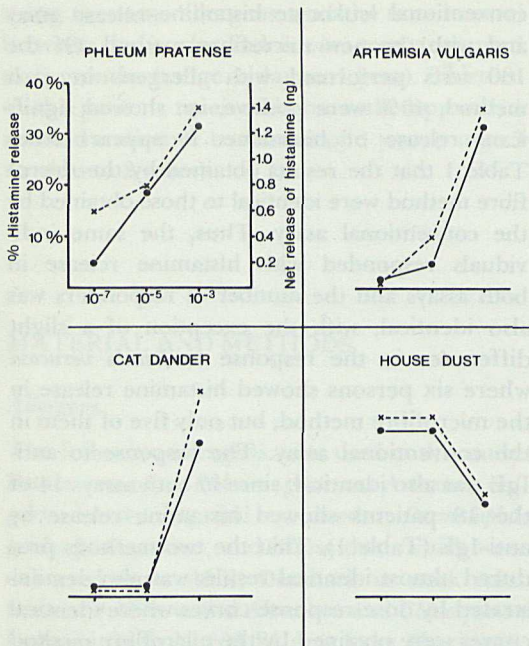


Fig. 1. Histamine release caused by different allergens in an allergic patient. Microfibre method (x---x) and conventional leukocyte histamine release assay (●—●).

Table 2

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

		Microfibre method			
		No release	10 ⁻³	10 ⁻⁵	10 ⁻⁷
Conventional method	No release	109	1		
	10 ⁻³		16	3	
	10 ⁻⁵		1	23	2
	10 ⁻⁷				25

Cell sensitivity to different allergens was determined by the microfibre method and by the conventional assay and the number of determinations are given. Note that cell sensitivity was defined in different ways in the two assays, i.e. by the allergen concentration causing a net release of 0.3 ng histamine or a 20% histamine release. The correlation between cell sensitivity in these assays was highly significant (*P* < 0.01 by Student's *t*-test).

Table 3

Relationship between responses measured by the microfibre method and bronchial provocation or skin test

		Bronchial provocation		Skin test	
		Pos.	Neg.	Pos.	Neg.
Histamine release	Pos.	19	2	66	13
	Neg.	0	4	29	72

The number of tests showing positive and negative responses by challenge with allergens are given.

the conventional leukocyte histamine release assay, which further substantiates the degree of identity between the results obtained by the two methods.

To compare the microfibre method with *in vivo* provocation tests, the correlation between the responses was examined. Sixteen patients were challenged with different allergens, each patient with approx. two allergens. Table 3 indicates a complete agreement between the number of positive responses obtained in the histamine release assay and by bronchial provocation, whereas a small difference was found in the negative responses, since in six patients with negative bronchial reaction only four showed no release of histamine. When the 18 patients were challenged with the 10 allergens, positive skin reactions were obtained in 95 tests and among these 66 (69%) showed histamine release. Of 85 tests showing negative skin reaction 72 (85%) did not show histamine release.

DISCUSSION

Allergen-mediated basophil histamine release has proved to be an excellent test for diagnosing type I allergic reactions in atopic patients, since this test more closely imitates the allergic reaction occurring *in vivo* than measurements of allergen-specific IgE in serum. Thus, in allergic individuals there is good correlation between the severity of clinical symptoms, skin tests, bronchial provocations, and histamine release (4, 6, 8, 15, 16). Many attempts have therefore

been made to simplify the histamine release method (1, 7, 9, 11), or to develop new histamine analyses (2, 5, 12). We have earlier described a new microfibre method for detecting *in vitro* basophil histamine release by a sensitive and specific glass microfibre-based histamine analysis, which overcomes the methodological problems in assays available today (13). The existence of a high histamine binding to glass, which is the basis of the new assay, has later been confirmed by others, suggesting a specific process for adsorption of histamine to borosilicate-containing glass surfaces (14).

The microfibre method seems suitable for clinical use, since isolation of cells and extraction of histamine are omitted and the test only needs a small volume of blood, i.e. 50 μ l per sample. Furthermore, the blood can be stored for 24 h at 4°C before assay without changes in histamine release or cell sensitivity, which is also the case in other studies of basophil histamine release (9, 11).

The microfibre method differs from the conventional leukocyte histamine release assay not only in being a new principle but also because histamine release was determined by net release instead of by per cent release, since a total release of histamine by detergents would interfere with the histamine analysis. It was therefore essential to examine the degree of identity between results obtained by the new microfibre method and the conventional assay.

Eighteen allergic patients were therefore tested in both assays with 10 of the most common inhalant allergens used in Denmark. It was found that the same individuals responded with histamine release in both assays 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. Furthermore, the cell sensitivity to allergens, which seems to be useful for evaluating the degree of allergy (10, 16), was determined, and a highly significant correlation was found between cell sensitivity measured by the microfibre method and the conventional leukocyte histamine release assay, which further substantiates the identity of results obtained by the two methods.

As mentioned above, several authors found basophil histamine release to have a good correlation with the *in vivo* provocation tests. In the present study, the bronchial provocation test and the skin test were performed at various intervals before the histamine release assay and many of the patients also received immunotherapy. Therefore, it was only fair to compare the number of positive and negative responses obtained in the microfibre assay with those obtained by the *in vivo* provocation tests, and the comparison showed good agreement. Further examinations comparing the results obtained by the microfibre method with the clinical symptoms, skin prick test, bronchial provocation, and RAST are now in progress.

The new microfibre method overcomes the problems in allergy testing, especially in children where only small amounts of blood are available and many tests have to be performed. The method might also prove to be useful in monitoring the effect of immunotherapy and allergen standardization.

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Address:

P. Stahl Skov
Dept. of Pharmacology
University of Copenhagen
20 Juliane Mariesvej
DK-2100 Copenhagen Ø
Denmark

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