

Automated histamine analysis for in vitro allergy testing

I. A method utilizing allergen-induced histamine release from whole blood

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A sensitive, automated, histamine assay system has been developed and applied for in vitro allergy testing. Nine common pollen and environmental allergens were used at three log dilutions for in vitro studies utilizing small volumes of blood (15-20 ml). The clinical evaluation was correlated with the results of the histamine release. Two different procedures were utilized. The first is the commonly used histamine release from washed leukocytes. There was excellent correlation between the clinical evaluation and the results of histamine release from washed leukocytes in 17 different individuals. The second and simpler method utilized whole heparinized blood which might better reflect the immunologic reaction which occurs in vivo. Aliquots of blood and allergen were incubated for 1 hr at 37° C and each supernatant was then analyzed for histamine release. There was excellent correlation between the two tests in 29 patients tested by both the whole blood and washed leukocyte methods. There was also good correlation between the clinical evaluation of the patients and the in vitro tests. The precision, accuracy, and sensitivity of the automated histamine assay make feasible its routine application in the clinical study of allergic patients.

Histamine release from washed leukocytes has been extensively utilized for in vitro studies of allergy. However, for several reasons the technique has had only limited clinical application: (1) histamine extraction and fluorometric assay are cumbersome and time-consuming procedures; (2) large amounts of blood are required; and (3) the assay must be performed by highly trained technicians.

Recently, a completely automated, sensitive histamine analysis system has been developed.^{1, 2} The method is capable of analyzing 30 samples per hour (sample volume of 0.4-0.6 ml) with a precision of $\pm 1-2\%$. The system can be used with a broad range of histamine concentrations and at the highest sensitivity is capable of analyzing samples which contain 0.05 to 5 ng of histamine (in 0.5 ml). When samples have a large amount of protein, the sensitivity is somewhat

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TABLE I. Allergens used for in vitro testing

Allergen	Final concentration (PNU/ml)*			
Mixed ragweed pollen Mixed grass pollen Cat dander	0.2	2	20	
Mixed tree pollen Mixed weed pollen Mixed molds Mixed feathers House dust Dog dander	2	20	200	
Anti-IgE (μ g of AbN/ml)	0.0025	0.005	0.01	0.05

*Final allergen concentration in the reaction mixture; actual concentration added to the tubes is twice this amount.

less, but samples with as little as 0.1-15 ng of histamine can still be analyzed accurately. Therefore, the use of the automated histamine assay has overcome two of the limitations for the clinical application of the manual histamine release system.

To simplify the histamine release method further the reaction is now performed with whole blood which eliminates the time-consuming procedure for the isolation of the washed leukocytes. Different allergen concentrations are added to heparinized whole blood and incubated for 60 min at 37° C. After centrifugation, the supernatants are analyzed for histamine content without further treatment. Besides saving time, the use of whole blood retains the plasma and might therefore be a better reflection of the true in vivo immunologic situation. Each test requires only 0.5 ml of blood, so that a patient can be screened against several allergens with a limited amount of blood.

This paper describes the method which was finally adopted for allergen-induced histamine release from whole blood. A panel of nine common pollen and environmental allergens were used in this study. Histamine release from whole blood correlated closely with release from washed leukocytes prepared at the same time and from the same donors. Further studies on this small sample of patients show that whole blood histamine release correlates well with the clinical evaluation of the patients. Future papers will describe more extensive clinical trials of whole blood histamine release for in vitro diagnosis of allergies.

MATERIALS AND METHODS

Solutions

A Tris buffer (pH 7.4) containing NaCl 120 mM, KCl 5 mM, and Tris 25 mM was used. Tris A was made by adding human serum albumin (HSA) obtained from A. G. Behring (Hoechst Pharmaceutical Co., Somerville, N. J.) to a final concentration of 0.03%. Tris A with Ca²⁺ and Mg²⁺ at 1×10^{-3} M each was used where indicated (Tris ACM). A stock solution of EDTA contained 0.1 M EDTA with the pH adjusted to 7.4. Purified rabbit antihuman IgE, Fc specific (710 μ g of antibody nitrogen per ml), was kindly provided by Dr. K. Ishizaka. All solutions used for the automated histamine analysis have been described previously.^{1, 2}

Allergens

The allergens used in this study were all obtained from one commercial source. The allergens were obtained in an aqueous buffered solution. Mixed ragweed pollen contained equal amounts of tall and short ragweed pollen; mixed grasses contained equal amounts of timothy, orchard, June, red top, and sweet vernal pollen; mixed tree was from ash, beech, birch, elm, hickory, maple, oak, and poplar pollens. The mixed molds consisted of equal amounts of *Alternaria*, *Horodendrum* (four varieties), *Aspergillus*, and two varieties of *Penicillium*. The mixed weeds were from lamb's-quarters, cocklebur, marsh elder, and English plantain pollens. Mixed feathers contained equal mixtures of chicken, duck, and goose feathers. Cat dander, dog dander, and house dust were also used. The mixed ragweed, grasses, trees, molds, and weeds were obtained at a concentration of 40,000 PNU/ml; the mixed feathers, cat and dog dander, and house dust were at a concentration of 20,000 PNU/ml.

All of the allergens were diluted into sterile diluent (NaCl 0.5%, potassium phosphate monobasic 0.036%, sodium phosphate dibasic 0.143%, without phenol) at a concentration of 2,000 PNU/ml. HSA was added to a final concentration of 0.03% and the allergens were kept refrigerated ($+4^{\circ}$ C). For histamine release experiments, the allergens were diluted in Tris ACM to twice the final concentration in the reaction mixture. The amount of phenol carried over in these dilutions (less than 0.004%) does not inhibit histamine release. The concentrations found for optimal histamine release are shown in Table I.

Clinical evaluation

Patients seen in an adult allergy clinic were evaluated by history and intradermal skin tests. The skin tests were performed with the same allergens used for the in vitro studies, but were diluted in commercial diluent. The highest concentration used for skin tests was 100 PNU/ml, and where appropriate the skin tests were initiated with lower allergen concentrations. A 0.02 ml quantity of allergen was injected at each site on the upper arm with a disposable syringe and the reactions were read at 15 min. Skin test grading was based on measurements of the greatest diameter of the wheal and erythema reaction following the usual criteria:

- Neg., the site was no different from a diluent control;
- 1+, < 5 mm of wheal, erythema less than 10 mm;
- 2+, 5-10 mm wheal with 10-20 mm of erythema;
- 3+, 10-15 mm of wheal 20-30 mm of erythema, no pseudopods;
- 4+, > 15 mm wheal with pseudopods.

The patients were also skin tested with a 1:10,000 histamine phosphate solution. Blood was obtained for histamine release studies just prior to skin testing.

Histamine release from washed leukocytes

Venous blood (18 ml) was drawn into a plastic syringe, anticoagulated with 0.01 M EDTA, and sedimented at room temperature for 1 hr with 2.5 ml of dextran (clinical dextran 6% in saline) per 10 ml of blood. The supernatant, containing leukocytes, platelets, and plasma, was transferred to another plastic tube and centrifuged at $300 \times g$ for 8 min at 4° C. The pelleted cells were resuspended in Tris A containing 4×10^{-3} M EDTA and centrifuged at $250 \times g$ for 8 min at 4° C. After a second wash in Tris A, the cells were resuspended in the desired volume of Tris ACM. The leukocyte counts were performed on the washed cell preparations; the cell counts were in the range of $0.8-4.6 \times 10^6$ /ml.

Histamine release reactions were carried out in 12 by 74 mm plastic tubes (Falcon No. 2052, Oxnard, Calif.); 0.25 ml of Tris ACM or the allergen was added to the tubes in an ice bath. Cells were added to all the tubes in a volume of 0.25 ml and the racks were transferred to a 37° C bath. In each experiment perchloric acid at a final concentration of 3% was added to some samples (completes) to obtain the total histamine content of the cells. The tubes were mixed by shaking the racks every 10 min. At the end of 60 min the reaction

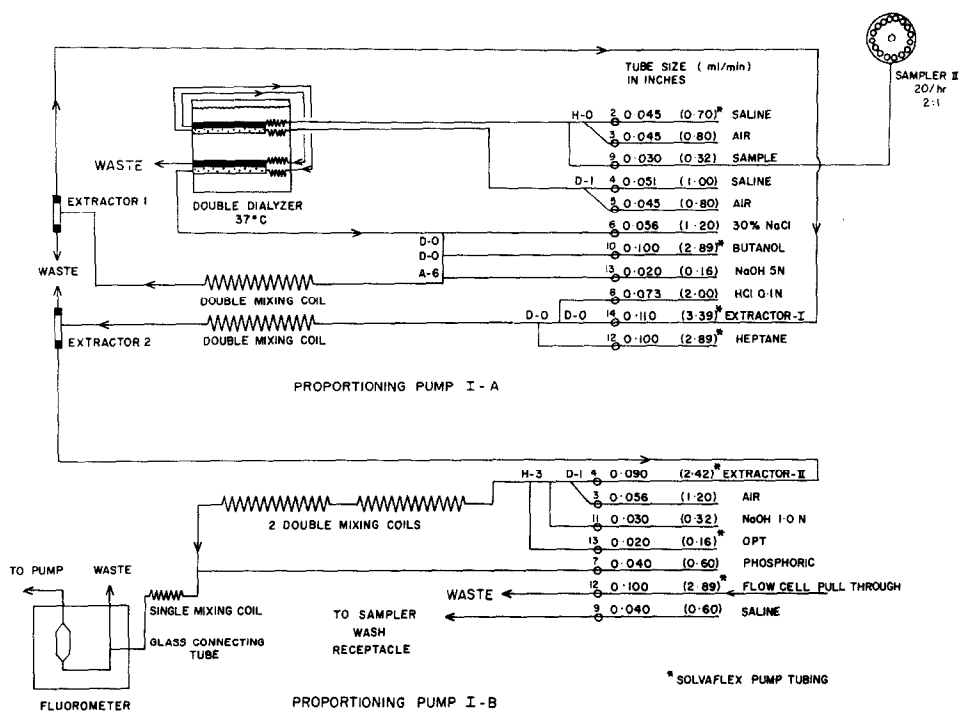


FIG. 1. Flow diagram for the automated histamine analysis in whole blood using two AutoAnalyzer pump I's. The first and second pumps have five and 10 rollers, respectively; the sample is analyzed with double dialysis. The use of N_2 instead of air at tube position 3 (on the second pump) will double the sensitivity of the system. This system has now been replaced by the use of a single larger capacity pump as described in "Methods" and by Siraganian.²

was stopped by transferring the tubes to an ice bath, followed by centrifugation at 1,600 rpm ($700 \times g$) for 20 min at 4° C. The supernatants were poured off and assayed for histamine. The histamine release in the blanks which received only cells and Tris ACM is usually less than 5% of the completes ($\bar{x} = 3.02\%$, range 0.1-7.1% $S_x = 1.36$, $N = 41$).

Histamine release from whole blood

Venous blood (18-20 ml) was drawn into a plastic syringe and transferred into a plastic tube (16 by 15) (Falcon No. 2045) containing 0.5 ml of heparin (Liquemin sodium 10, sodium heparin injectable USP, 1 ml = 1,000 units, Organon Inc., West Orange, N. J.). The blood was diluted with about 5 ml of Tris ACM and used directly for the histamine release reaction. Except where indicated, tests were performed within 4 hr after obtaining the blood.

Histamine release reactions were carried out in 12 by 75 mm plastic tubes; 0.5 ml of Tris ACM or allergen concentration was added to the tubes in an ice bath; 0.5 ml of the diluted blood was then added to all the tubes and the racks were transferred to a 37° C bath. The tubes were mixed by shaking the racks every 10 min. In each experiment perchloric acid at a final concentration of 6% was added to six or seven samples to obtain the total histamine content of the blood; these were then pooled to yield four "completes." This procedure was necessary as there was a large amount of precipitate formed and the amount of sample (supernatant) that can be poured off for histamine analysis was less than adequate for the assay. At the end of 60 min the reaction was stopped by transferring the

TABLE II. A representative protocol for in vitro allergen-induced histamine release from whole blood

Tubes			Heparinized blood	Other	Relative fluorescent intensity*	Histamine release† (%)
No.	Description	Conc. (PNU/ml)				
1	Completes		0.5 ml	0.5 ml	84	
2					85	
3				12% HClO ₄	85	
4					86	
5				↓		
6						
7	Blanks			0.5 Tris	4	
8				ACM	4	
9				↓	4.5	
10	Ragweed	0.2		0.5 ml	66	76
11		2.0		each	74	86
12		20.0‡		allergen	54	61
13	Grasses	0.2			3.5	0
14		2.0			3	0
15		20.0			29	31
16	Trees	0.2			9	6
17		2.0			52	59
18		20.0			60	69
19	Molds	2.0			5	1
20		20			5	1
21		200			6	2
22	Weeds	0.2			55	62
23		2.0			72	83
24		20			66	76
25	Dust	2	To all tubes	To all tubes	6	2
26		20			7	4
27		200			9	6
28	Cat	0.2			5	1
29		2.0			16	15
30		20			52	59
31	Dog	2			7	4
32		20			6	2
33		200			8	5
34	Feathers	0.2			4	0
35		2.0			4	0
36		20.0			4	0
		(ng AbN/ml)				
37	Anti-IgE	2.5			11	9
38		5.0			37	41
39		10			47	53
40		50			57	65
Histamine analysis standard 10 ng/ml					24	

*Peak height read from AutoAnalyzer recorder.

†Percent histamine release calculated using the formula described in the "Methods" section.

‡The highest concentration of each allergen was shown not to have fluorescent activity.

tubes to an ice bath, followed by centrifugation at 2,500 rpm (1,200 × g) for 30 min at 4° C. The supernatants were poured off and assayed for histamine.

Histamine assay

The automated histamine assay was used for the analysis of the samples.^{1, 2} Supernatants from washed leukocyte experiments were analyzed without the use of dialysis whereas whole

blood supernatants were analyzed with dialysis. Initial studies were with the automated assay system utilizing two pump I's. The system was essentially as described previously except that the manifold was modified to allow the analysis of a larger sample volume and to increase the efficiency of the dialysis system. This system allows the analysis of samples at the rate of 20/hr with volumes of 0.6 ml with dialysis (Fig. 1). During the last year the modified system with 0.32 ml uptake tube and dialysis has been used.² The sensitivity of this system is such that it allows samples with less than 25 ng/ml in plasma to be analyzed efficiently and accurately at the rate of 30 samples per hour. All histamine values are expressed for the base.

Percent histamine release was calculated by the formula:

$$\text{percent release} = 100 (e-b)/(c-b),$$

where e = fluorometric reading for experimental supernatant, b = mean of blanks, and c = mean of completes.

RESULTS

Histamine release from whole blood

The protocol for a representative experiment demonstrating histamine release from whole heparinized blood is shown in Table II. The blank tubes received Tris ACM and diluted whole blood. In this experiment there was histamine release with all three concentrations of ragweed and mixed weeds. The tree pollen and cat dander extracts induced significant histamine release ($> 10\%$) at two concentrations, and mixed grasses were positive at only the highest concentration tested. There was no histamine release with molds, dust, dog, and feather allergens. The addition of anti-IgE to whole blood also released histamine. Using the value of the histamine standard, total histamine in this experiment is calculated at 35 ng/ml.

The actual recorder chart from the histamine analysis of the supernatants of a similar experiment utilizing blood from another patient is shown in Fig. 2. The tubes are identified on the chart. There was histamine release with mixed ragweed, grasses, trees, feathers, dust, cat, and dog; there was no release, however, with mixed mold allergens.

The fluorescence measured in the blank tube supernatants is the total of histamine released from the cells and fluorescent material present in the plasma (e.g., histidine). Analysis of plasma alone shows that it is the main factor in blank fluorescence (e.g., Garden³ has reported that normal plasma contains 5.5 ± 0.5 ng of histamine/ml). Therefore high blank values are not an indication of a "bad" experiment, as is usually the case with washed leukocyte preparations. Blanks averaged 10.09% of the completes and had an $S_{\bar{x}} = 3.52$ (range 3.6-18.7%, $N = 126$). The ratio of blanks to completes was greater when the absolute "complete" values were low.

Correlation of whole blood histamine release with washed leukocyte release and with clinical diagnosis

Although early experiments on allergic histamine release utilized whole blood,⁴⁻⁷ most of the recent work has used washed leukocytes.⁸⁻¹¹ The initial experiments were therefore designed to determine whether the release of histamine from whole blood was similar to release from washed leukocytes.

A 35-ml sample of blood was obtained from each of 17 patients who had

received no prior hyposensitization therapy; 18 ml were used for the whole blood histamine release and the remainder for the preparation of washed leukocytes. The experiments were run in parallel, with the same allergen concentrations used for both tests. A summary of the results is shown in Table III. From these data it is clear that when the history and skin test of an individual were positive for a given allergen the probability was very high that the in vitro tests were also positive for histamine release. For example, out of the 17 patients there were 12 who were positive by clinical evaluation for ragweed hay fever; of these, 11 released histamine by both whole blood and washed leukocytes and 1 was negative by both test systems. In contrast, all five patients who were clinically negative had negative in vitro histamine release tests. The results with the other allergens were quite similar; e.g., out of eight patients who were clinically positive for grass allergy, all eight were positive by in vitro histamine release tests. As a whole, there was close concordance between the whole blood and the washed leukocyte histamine release assays. The results are summarized at the bottom of the table (Table III). There were 55 positive clinical diagnoses; of these, 47 were positive by both histamine release methods, three were negative by both, and four were positive by whole blood but negative by washed leukocytes. The results when the patients were clinically negative were quite similar. Among the patients who were clinically positive, 51/55 were positive by the whole blood and 48/55 by washed leukocyte tests. Among the patients who were negative, the whole blood was negative in 96/98 assays and the washed leukocytes were negative in 94/98 assays. The concordance of the two in vitro tests was about 93%. The accuracy of both tests in picking the clinically positive patients is therefore above 90% in this small series, and the percentage of false positives is quite low (washed leukocytes 4%, whole blood 2%). If only the patients who released >10% histamine at two or more allergen concentrations are considered, the data show a concordance of 97.4% between the washed leukocyte and whole blood results. However, only about 72% of the clinically positive patients are then positive by histamine release.

There was good correlation between the histamine release and the skin test data (Table IV). When the intradermal test was strongly positive (>4+) the histamine release tests were all positive. When the skin tests were 3+ or 2+ the positive in vitro tests dropped to 71% and 40%, respectively.

The results of whole blood and washed leukocyte histamine release can also be compared by determining the correlation between the maximum release from washed leukocytes and from whole blood by each allergen. This correlation is shown in Fig. 3, where the maximum release from the washed leukocytes at any one of the three concentrations of each allergen is compared to the maximum histamine release from whole blood with the same allergen. There is good correlation, with a coefficient of correlation of $r = 0.95$. However, the least square regression line does not show equal release from whole blood and from washed leukocytes; the maximum release that could be obtained from whole blood was always less than the release from the washed leukocytes. This appears to be a general phenomenon, although the release at any one concentration might be less with washed leukocytes than with whole blood.

A comparison between washed leukocytes and whole blood histamine release

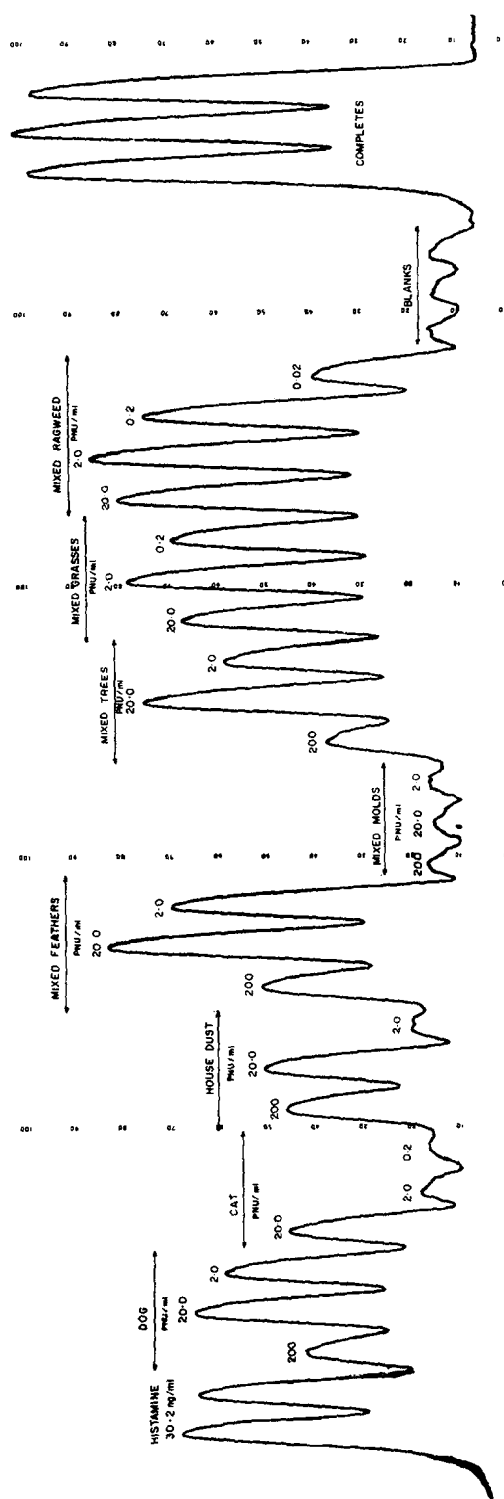


FIG. 2. Recorder chart of the whole histamine release allergy test. Recorder chart reads from right to left; "completes" refers to the total histamine content in 0.5 ml of diluted blood; "blanks" received blood and Tris ACM. This individual was highly allergic and the tests, except with the mold extract, were all positive.

TABLE III. Correlation between the clinical evaluation of patients and the results of in vitro tests with washed leukocytes and whole blood (17 untreated patients)*

Allergen	Clinical evaluation	Total No. of patients	Histamine release† (No. of patients)			
			Group I:	Group II:	Group III:	Group IV:
			Blood pos. and WBC pos.	Blood neg. and WBC neg.	Blood pos. and WBC neg.	Blood neg. and WBC pos.
Ragweed	Positive	12	11	1	0	0
	Negative	5	0	5	0	0
Grasses	Positive	8	8	0	0	0
	Negative	9	0	9	0	0
Trees	Positive	6	6	0	0	0
	Negative	11	0	11	0	0
Molds	Positive	0	0	0	0	0
	Negative	17	0	15	1	1
Feathers	Positive	4	3	0	1	0
	Negative	13	0	12	0	1
Dust	Positive	4	2	1	1	0
	Negative	13	0	10	1	2
Cat	Positive	5	4	0	1	0
	Negative	12	0	12	0	0
Dog	Positive	5	4	0	0	1
	Negative	12	0	12	0	0
Weeds	Positive	11	9	1	1	0
	Negative	6	0	6	0	0
Summary	Positive	55	47	3	4	1
	Negative	98	0	92	2	4
Totals		153	47	95	6	5
Percent of total in each group			30.7%	62%	4%	3%
			92%		7%	

*The clinical evaluation of the patients was based on the history and skin test. Positive patients were skin test-positive to the allergen with a reaction of greater than 2+ at 100 PNU/ml by intradermal test.

†A 35-ml sample of blood was obtained and used for both washed leukocytes and whole blood histamine release. The tests were performed with the same three concentrations of allergen. Histamine release greater than 10% at any one of these concentrations was considered as a positive result. Other experiments have shown that the concentration of heparin in whole blood does not affect the histamine release.

TABLE IV. Correlation of histamine release with skin test results in 17 untreated patients*

Skin test†		Histamine release‡ (No. of responses)			
		Group I:	Group II:	Group III:	Group IV:
		Blood pos. and WBC pos.	Blood neg. and WBC pos.	Blood pos. and WBC neg.	Blood neg. and WBC pos.
Response	No. of tests				
≥4+	31	31	0	0	0
3+	14	11	1	2	0
2+	10	5	2	2	1
≤1+	98	0	92	2	4

*See legend of Table III for details. Skin tests were performed as described; the highest concentration of allergens used was 100 PNU/ml.

†A ≥ 4+ reaction indicates that the test was strongly positive at lower allergen concentrations (e.g., 1 PNU giving a 3+ reaction).

‡Histamine release > 10% was considered positive.

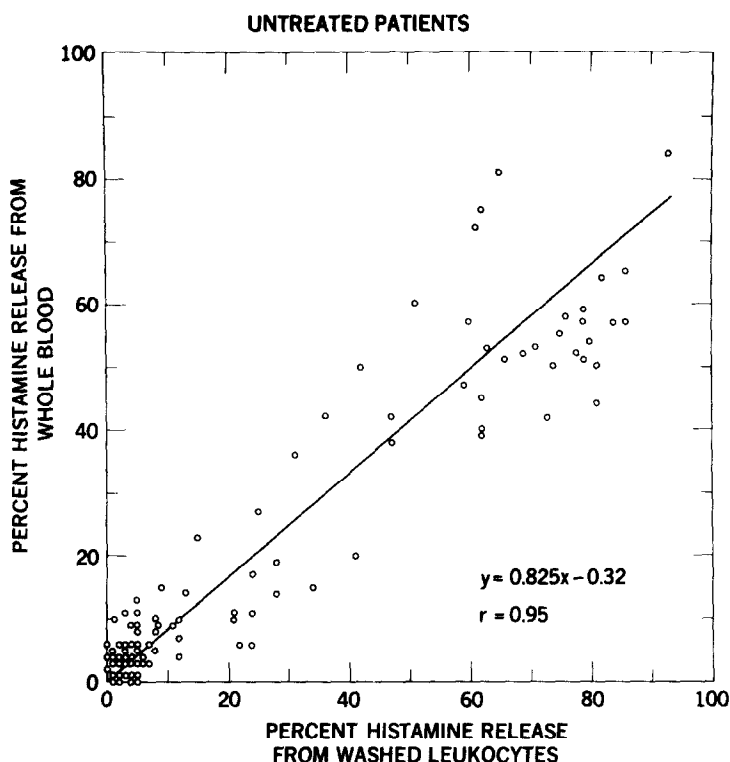


FIG. 3. Correlation between allergen-induced histamine release from washed leukocytes and whole blood from 17 different donors who had received no prior hyposensitization therapy. Washed leukocytes and whole blood were tested with the nine allergens at the indicated three concentrations. The maximum histamine release with each allergen from washed leukocytes and whole blood was used in these calculations. The linear regression line was calculated by the least-squares method; the linear correlation coefficient was $r = 0.95$.

was also performed on samples from 12 individuals who had been on hyposensitization therapy for over 2 yr and had reached maintenance doses. Blood was obtained 2 to 3 wk after their last injection. The results are shown in Table V. In 93% of the tests the washed leukocytes and the whole blood test results were comparable. The maximum histamine release from whole blood and washed leukocytes with the different allergens showed a very good correlation of $r = 0.93$ (Fig. 4). However, one difference from the untreated patients was the concentration at which allergens induced histamine release in washed leukocytes as compared to whole blood. In the untreated patients, the maximum histamine release by both assay methods was with the same allergen concentration 81% of the time. In contrast, patients on hyposensitization therapy had maximum histamine release by both methods with the same allergen concentration in 60% of the tests. If only the tests with the allergen used for a patient's hyposensitization are considered, then the allergen concentration required to give maximal histamine release with whole blood was higher in 70% of the tests.

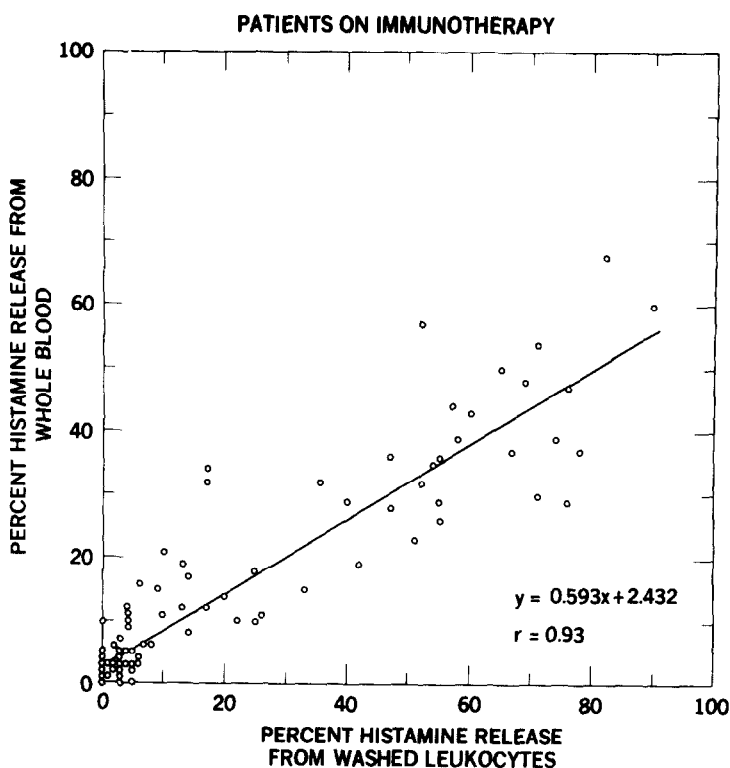


FIG. 4. Correlation between allergen-induced histamine release from washed leukocytes and whole blood in 12 patients on immunotherapy. The maximum histamine release with each allergen was used in these calculations (see Fig. 3 for details).

TABLE V. Correlation between whole blood and washed leukocyte histamine release in 12 treated patients*

Allergen		Histamine release (No. of patients)			
		Group I: Blood pos. and WBC pos.	Group II: Blood neg. and WBC neg.	Group III: Blood pos. and WBC neg.	Group IV: Blood neg. and WBC pos.
Ragweed	(10)†	8	4	0	0
Grasses	(9)	7	5	0	0
Trees	(3)	3	8	1	0
Molds	(4)	3	9	0	0
Feathers	(0)	5	6	1	0
Dust	(8)	4	6	2	0
Cat	(0)	2	10	0	0
Dog	(0)	4	7	1	0
Weeds	(1)	4	6	1	1
Totals		40	61	6	1
Percent of total tests		93.5%			

*Blood from patients who had received hyposensitization therapy for over 2 yr was used in the experiments. A 35-ml sample of blood was obtained and used for both washed leukocytes and whole blood histamine release studies. The tests were performed with the same three concentrations of each allergen as shown in Table I. Histamine release greater than 10% at any one of these allergen concentrations was considered positive.

†The number of individuals who were receiving this allergen for hyposensitization. These patients were not skin-tested.

TABLE VI. Effect of blood storage on the in vitro allergy test*

Condition under which blood is stored	Donor	Time blood stored (hr)	Allergen (% histamine release)					
			Ragweed (PNU/ml)			Grasses (PNU/ml)		
			0.25	2.5	25	2.5	25	250
Refrigeration (4°C)	D. P.	0	14	39	15	0	0	3
		2	18	45	20	0	1	3
		6	18	48	23	0	2	4
		22	10	42	25	0	0	3
	T. B.	0	34	44	33	37	34	21
		2	36	41	30	36	33	21
		6	45	50	38	47	41	33
		22	31	38	31	38	38	31
Room temperature (22°C)	F. D.	0	34	47	20	3	37	39
		2	32	47	20	5	37	36
		6	29	47	21	5	36	41
		24	25	43	14	0	29	32
	R. S.	0	23	38	24	1	1	4
		2	24	42	26	0	2	0
		6	27	43	29	0	0	0
		24	13	39	24	0	0	2

*Each donor was bled 20 ml into a sterile plastic tube containing 0.5 ml of heparin (500 U). The blood was either refrigerated or left at room temperature. At indicated times 5 ml of blood were removed, diluted with 3.5 ml of buffer, and used in the experiments. Reaction mixture contained 0.5 ml of diluted blood and 0.5 ml of the allergen or buffer for the blanks. There was no appreciable change in the blanks from 0 to 24 hr. Total histamines in the reaction mixtures were 18-32 ng/ml.

TABLE VII. Effect of prior medication on in vitro histamine release*

Challenge with	% histamine release from			
	Whole blood		Washed leukocytes	
	No medication†	After medication‡	No medication	After medication
Dog dander (PNU/ml)				
2	16	1	61	0
20	39	1	45	0
200	15	1	9	1
Anti-IgE (μg AbN/ml)				
0.0025	1	1	13	3
0.005	4	1	14	3
0.010	6	1	17	4
0.050	20	1	22	3

*Histamine release tests performed on the blood of the same patient one week apart.

†Patient had received no medications for 12 hr.

‡Patient had taken a tablet containing ephedrine, theophylline, and hydroxyzine 2 hr prior to donating blood.

Effect of storage of blood on the in vitro allergy test

The results of histamine release tests using stored (instead of fresh) whole blood are shown in Table VI. Venous blood (20 ml) was obtained from several donors, then heparinized and allowed to stand either at room temperature or in the refrigerator. At indicated intervals, aliquots of the blood were removed, diluted with buffer, and used for whole blood histamine release with several allergens (Table VI). Even blood stored for 24 hr did not yield "blanks" different from blood which was tested immediately ("blanks" =

spontaneous release in tubes which received only buffer). Similarly, control tubes which received allergens to which an individual was not allergic did not show any histamine release. There was no decrease in allergen-induced histamine release in blood stored for up to 24 hr.

Effect of medication

Early in the course of these experiments it was noticed that medications interfered with *in vitro* histamine release (Table VII). One patient, on medication, had negative results for both the whole blood and washed leukocyte assays when screened against dog dander and anti-IgE. When the patient had received no medication for 12 hr she was strongly positive. As a result of these findings all *in vitro* tests were performed after patients had abstained from taking any medication for at least 24 hr.

Histamine content of blood

The total histamine content of blood was calculated after correcting for the dilutions of the blood. In a group of 83 donors (most of whom were from an allergy clinic) the values were $\bar{x} = 83.18$ ng/ml, $S_{\bar{x}} = 29.54$, and range 39-209. These values were not corrected for the fluorescence readings obtained from plasma (serum).

DISCUSSION

Allergic reactions depend on a number of factors, including the presence of specific IgE, the concentration of blocking antibodies, and the ability of basophils or mast cells to release mediators. The amount of histamine released when allergen is added to whole blood *in vitro* is due to the interaction of all these factors. This test more closely simulates the allergic reaction occurring *in vivo* than measurements of the concentration of allergen-specific IgE. Biologically cell-bound IgE is a more significant measure of the allergic state than is allergen-specific serum IgE. The use of whole blood also eliminates the cumbersome leukocyte isolation technique and therefore simplifies the assay system.

In ragweed-allergic individuals, there is good correlation between the severity of clinical symptoms and *in vitro* histamine release.^{9, 12-16} In these patients there is excellent correlation of histamine release with the skin tests, symptom score, and the level of serum IgE specific for antigen E.¹⁴ Both the antigen concentration at which histamine is released and the percentage of histamine release are good correlates of the clinical severity of the disease. Patients who have high concentrations of ragweed-specific IgE release histamine with low concentrations of antigen E ($r = 0.74$).¹⁴ The correlation between histamine release and a patient's symptoms is better than the correlation of the symptoms with the P.K. titers.¹⁷ There is good correlation in ragweed- and grass-allergic individuals between the concentration of antigen required to release 50% histamine and the skin sensitivity as determined by intradermal tests.^{14, 15} The data therefore indicate that histamine release can be used as a quantitative measure of the clinical sensitivity of a patient. The

present experiments demonstrate that whole blood histamine release is as reliable as the washed leukocyte assay and suggest that the system is also applicable to other allergens.

There are a number of advantages of an *in vitro* allergy testing system. Of utmost importance is the absolute safety for the patient. Performing an allergy test in a test tube instead of in the skin completely avoids the possibility of an anaphylactic reaction. Obviously, the *in vitro* tests would be more useful with allergens that induce more anaphylactic reactions. Second, there is no danger of sensitizing a patient or boosting a previous immune response, as might occur during repeated skin tests. This possibility depends on the concentration of the allergen and the frequency of skin tests. Third, an *in vitro* test would be useful in the rare patients who cannot be skin tested because of dermatologic conditions. Fourth, the data from histamine release studies are quantitative, i.e., antigen concentration required to release 50% histamine. The fifth advantage of the *in vitro* test is that it permits the use of antigens which have not been purified or which cannot be used clinically. One such example was the use of the whole blood histamine release in a patient with seminal plasma allergy.¹⁸ In this case, the method was extensively used for immunologic studies on the mechanisms of the reaction and as a tool in the purification of the allergen. A sixth advantage of the *in vitro* test is its use as a measure of the amount of blocking antibody that develops in patients during hyposensitization therapy.^{19, 20} The use of histamine release as a clinical investigative tool has been amply demonstrated during the last 10 yr. The automated histamine analysis system makes it far simpler to apply this technique to routine use.

The histamine release results compare favorably with published reports of the use of the RAST technique in allergy diagnosis.²¹⁻²³ Several reports have shown that the RAST has an overall accuracy of about 70% when correlated with a patient's clinical history. The present results showed that the histamine release was positive in over 90% of the patients who were allergic by clinical evaluation. However, this is a small sample group and no generalizations or extrapolations should be made. Therefore, a further investigation utilizing over 80 patients is under way. The RAST assay has the advantage of utilizing serum. This is convenient for the patient as serum can be stored indefinitely and also can be shipped to a central location for processing. However, histamine release from whole blood is probably a more biologically sensitive index of a patient's sensitivity and might indicate the interrelationships existing in the patient between the different antibodies and cell-control mechanisms of histamine release.

Histamine-binding proteins have been postulated to be present in plasma.²⁴ However, their presence would not affect the analysis of histamine in whole blood as performed with the automated assay system. The binding of histamine to protein requires calcium²⁵; in the automated analysis excess EDTA is added in the first step which would remove the cations and free any protein-bound histamine.

The presence of the enzyme histaminase in plasma would not significantly

affect the results of whole blood histamine release. The amount of histaminase in plasma is very small (1.1 ± 0.6 pmol of histamine deaminated/ml of plasma/hr²⁶) and would only break down 0.122 ng of histamine/hr/ml of serum. As the total histamine content in our reaction mixture is 25-30 ng/ml it is clear that the effect of the histaminase enzyme would be insignificant. However, there are marked increases in serum histaminase levels during pregnancy, and these might conceivably interfere with the whole blood histamine release assay.

A major problem in any allergy investigation is the lack of standardized and purified allergens.^{27, 28} The present experiments were undertaken to correlate the in vitro histamine release from whole blood with the clinical evaluation of patients. Therefore, the same allergens were used for the histamine release studies as were in routine use in the clinic. The use of purified and standardized allergens would improve the histamine release data because the false-positive results appear to be due to nonspecific toxicity of the crude extracts. The dilutions of allergen finally used for in vitro histamine release are shown in Table I; at higher concentrations some of the extracts caused nonspecific histamine release from leukocytes of all individuals tested. Obviously, these allergen concentrations might not be applicable for preparations from a different lot or from a different manufacturer.

The usefulness of any in vitro diagnostic test depends on a number of factors, including the accuracy and reliability of the system, its convenience for patients and physicians, its capacity to handle a large number of samples, and the total cost. In this small group of patients the test was very accurate; in the strongly positive patients histamine release was positive 91% of the time. Furthermore, the storing of blood for 24 hr had no effect on the allergen-induced histamine release from whole blood. This is a clear-cut advantage for both patient and allergist. The total cost to the patient is difficult to determine but does not appear to be prohibitive.

In summary, the two in vitro histamine release methods using whole blood or washed leukocytes are useful techniques for diagnosis and study of allergy. They are also useful complements to the clinical evaluation of patients, and could function as in vitro alternates under certain conditions. An integral part of the test is the automated histamine assay system: a technique which is very sensitive, reproducible, and automated. The sensitivity of the system allows in vitro quantitative studies with nine allergens to be performed with only 20 ml of blood.

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