

Human Leukocyte Histamine Release Assays with Whole Ragweed and Alternaria Antigens Using Technicon Autoanalyzer II

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A simple and commercially available automated technique for fluorometric analysis of histamine is described. It is applicable to histamine release experiments utilizing washed leukocytes, and analyzes 15 samples/h at histamine concentrations between 1 and 1000 ng/ml with a mean variance between 0.5% and 18%.

This technique is applied to a cross sectional study of leukocytes from 14 ragweed sensitive patients (7 on and 7 off immunotherapy) as well as five normals. It was also applied to 17 Alternaria-sensitive patients (4 on and 12 off immunotherapy) in addition to six normals. While antigen induced in histamine release clearly separated the sensitive group from the normals, it did not correlate with immunotherapy, and a considerable variation was seen in the sensitive patients of both the treated and untreated groups.

Key words: Alternaria; autoanalyzer; histamine release assay; ragweed

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The fluorometric analysis of histamine was first reported by Shore et al. in 1959 (6). The procedure involves the isolation of histamine by two sequential extractions, the first into alkaline butanol and then, after acidification, back into an aqueous phase. A fluorophore is then formed by complexing the histamine with *O*-phthaldialdehyde (OPT) and the fluorescence is measured in a fluorometer.

The manual fluorometric technique reported by Lichtenstein & Osler (2) is sensitive to histamine levels down to 1 ng/ml but is quite tedious, requiring one day for the analysis of 40 samples. It also has a relatively high mean variance resulting in a poor

confidence level as reported by Siraganian (7) when he introduced his automated fluorometric technique.

The first automated fluorometric technique was reported by Evans et al. in 1973 (1). They used Technicon autoanalyzer equipment to perform 30 samples/h with sensitivity down to 20 ng/ml and a mean difference of 5% in the 100 ng/ml range. This technique was not applied to human leukocyte histamine release, however, and it was Siraganian (7, 8) who improved the sensitivity of the method so that 0.1 ng/ml concentrations could be measured. These modifications facilitated histamine release experiments with relatively small

volumes of peripheral blood. His recent modifications include nitrogen-bubble injection and the use of a dialyzer. These changes improve sensitivity down to a reported 0.1 ng/ml without the dialyzer and allow for the analysis of histamine in both serum and whole blood, although at some loss of reproducibility with the dialyzer. A precision of $\pm 2\%$, without dialyzer is reported but raw data on reproducibility are not given. In our laboratory we use a modification of the methodology based on Evans' (1) technique and available from Technicon Industrial Systems. By alternating samples with blanks, maximizing sensitivity and eliminating carry-over between samples, it is possible to use this simple method and still analyze histamine from relatively small numbers of leukocytes which can be separated from 0.75 cc of peripheral blood per test.

MATERIAL AND METHODS

Equipment. All equipment was obtained from Technicon Industrial Systems (Tarrytown, New York). The basic method, with the exception of alternating blanks, is described in the Technicon Bulletin as Technicon Industrial Method no. 283-73 p/ Tentative; September, 1973. The system components are listed below.

1. Sampler IV operates at 30 samples/h and holds 40 sample cups in its tray. The ratio of sample volume to wash solution volume is 4:1 and each sample to be measured is alternated with a "blank" of 0.4 N perchloric acid in intervening sample cups.

2. Proportioning pump II assures consistent delivery of the reagents and air bubbles at a rate determined by the caliber of the tubes traversed by steel rollers.

3. Manifold No. 116-D488-01 provides the structure for the phase separators and mixing coils as outlined in the flow chart (Fig. 1). The sample containing an unknown or standard histamine concentration is injected into air bubble fractionated column of a

butanol/NaOH mixture. The histamine-containing butanol phase passes through the two sequential phase separators while the aqueous phase containing potential contaminants is discarded. After acidification, the histamine returns to the aqueous phase and the final phase separator removes the organic upper layer allowing the water soluble histamine to be complexed with OPT and be subsequently measured as a complex in the fluorometer.

4. A fluoronephelometer utilizes a 355 nm primary filter and a 455 nm secondary filter allowing for adjustment of sample and reference apertures to accommodate sample concentrations of histamine from 1 ng/ml to 1000 ng/ml.

5. A single pen recorder was used with the deflection of the peak reflecting the concentration of histamine in the sample.

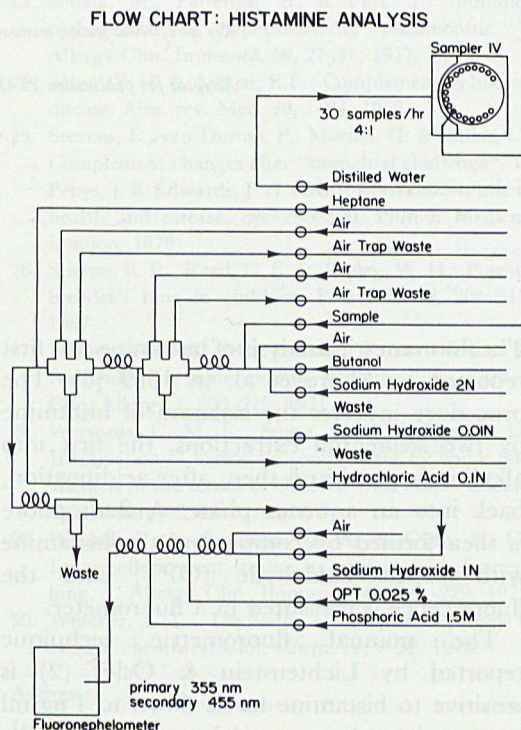


Fig. 1. (Adapted from Technicon Bulletin) demonstrates the relationship of reagents and equipment in the continuous flow system.

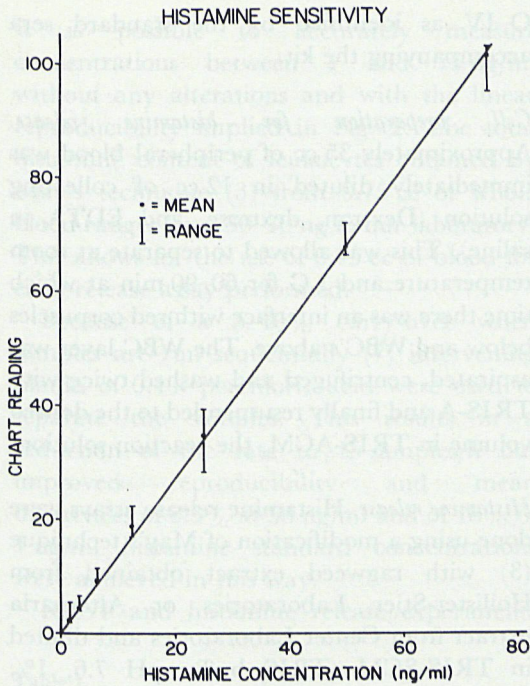


Fig. 2. The mean and range plots of eight repetitions of these histamine standards between 1.56 ng/ml and 75 ng/ml demonstrates linearity.

Reagents. All chemicals, unless otherwise stated, were ACS certified and were dissolved in deionized, glass-distilled water.

1. Butanol: suitable for fluorometric analysis, saturated by initial mixture with 0.1 N NaOH in 25% saline, separated prior to use.
2. Wash reagent: 0.1 NaOH in 25% NaCl.
3. Sodium hydroxide 2 N in 25% NaCl.
4. Heptane—technical grade.
5. Hydrochloric acid—0.1 N.
6. Sodium hydroxide—1 N.
7. *O*-Phtaldialdehyde 0.025% initially dissolved in methanol.
8. Perchloric acid 0.4 N.
9. Histamine standards were diluted in 0.4 N perchloric acid from 100,000 ng/ml stock solution (27.6 mg histamine phosphate in 100 ml of 0.4 N perchloric acid).

The flow chart (Fig. 1) summarizes the process and the relationship of the reagents.

Procedure

Before each day's use, the tubing is washed for 10 min with distilled water and Brij-35 detergent. A fresh aliquot of 200 ml OPT is mixed for each day's use and all the other reagents are started 5 min before heptane. All reagents are then run for 10 min while the fluorometer and recorder equilibrate. The first and last series of samples for each day's run are standards to establish the initial baseline and sensitivity settings as well as evaluate the possibility of drift. After the final sample has been analyzed, a 30-min wash period with distilled water and Brij-35 is essential to maintain trouble-free function.

Standardization

Standardization and data on reproducibility were accumulated by diluting the 100,000 ng/ml histamine standard to 75, 50, 25, 12.5, 6.25, 3.13, and 1.56 ng/ml. These dilutions were sampled repeatedly and in varying sequences. Fig. 3 represents data from

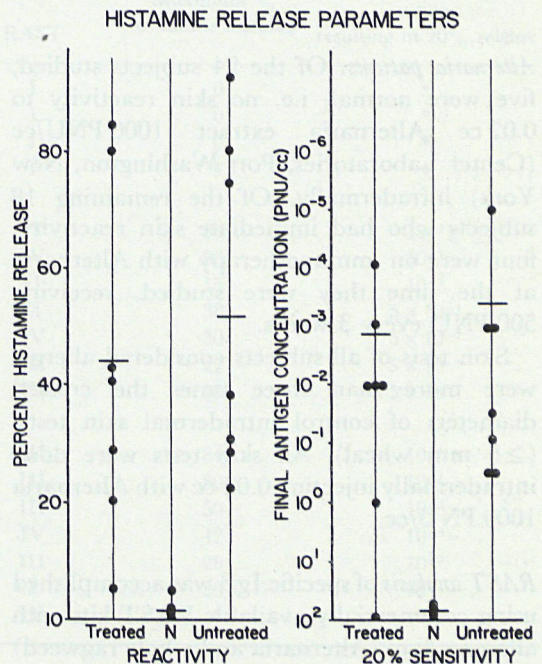


Fig. 3. Both reactivity and 20% sensitivity differentiate normals (N) from sensitive subjects but do not differentiate the treated from the untreated group.

eight repetitions of standard analysis. Mean variance was computed from at least 19 separate determinations of each dilution.

Subject selection

Ragweed subjects. Out of 19 subjects whose leukocytes were studied, 14 had skin sensitivity, i.e. $\geq$ mm wheal to intradermal injection of 0.02 cc of ragweed extract 100 PNU/cc (Hollister-Stier Laboratories, 2201 Curtis St., Downers Grove, Ill.), and had symptoms of rhinitis in a seasonal pattern during August-September. Of these 14 ragweed-sensitive individuals, seven were on an immunotherapy program and receiving 500 PNU of ragweed extract every 3 weeks. They had all been receiving the extract for more than 1 year prior to the study. The other seven were not on immunotherapy although one subject had been treated with shots more than 4 years prior to the study. The leukocyte studies were performed between February and May, well outside the ragweed season.

Alternaria patients. Of the 24 subjects studied, five were normal, i.e. no skin reactivity to 0.02 cc Alternaria extract 1000 PNU/cc (Center Laboratories, Port Washington, New York) intradermally. Of the remaining 18 subjects who had immediate skin reactivity, four were on immunotherapy with Alternaria at the time they were studied, receiving 500 PNU every 3 weeks.

Skin tests of all subjects considered allergic were more than three times the crossed diameters of control intradermal skin tests, (≥ 5 mm wheal). All skin tests were done intradermally injecting 0.02 cc with Alternaria 1000 PNU/cc.

RAST analysis of specific IgE was accomplished using commercially available RAST kits with allergen discs (Alternaria and whole ragweed) obtained from Pharmacia Diagnostics, (Division of Pharmacia, Inc., Piscataway, New Jersey). Results are expressed in RAST classes

O-IV as identified by the standard sera accompanying the kit.

Cell preparation for histamine release.

Approximately 35 cc of peripheral blood was immediately diluted in 12 cc of collecting solution (Dextran, dextrose, and EDTA in saline). This was allowed to separate at room temperature and 1 G for 60-90 min at which time there was an interface with red corpuscles below and WBC's above. The WBC layer was aspirated, centrifuged and washed twice with TRIS-A and finally resuspended to the desired volume in TRIS-ACM, the reaction solution.

Histamine release. Histamine release assays were done using a modification of May's technique (3) with ragweed extract obtained from Hollister-Stier Laboratories or Alternaria extract from Center Laboratories and diluted in TRIS-SCM (TRIS buffer pH 7.6, 1% human serum albumin with 0.6 mM Ca^{++} and 1.0 mM Mg^{++}) to final concentrations expressed in PNU's of ragweed antigen. After incubation of the cells and antigen at 37° for 45 min the cells were separated by centrifugation and the supernatant analyzed. We did not use normal serum in our reaction mixtures except in blocking factor assays.

Blocking factor assay. An assessment of the influence of serum factors on the histamine release parameters in Alternaria-induced release was made by preincubating each 10 ml of antigen dilution with 10 μ l of buffer, normal serum, or serum from patients on immunotherapy with Alternaria for 1 h at 27°C before addition of leukocyte suspension. The assay was then completed as usual and comparisons made using the parallel curves obtained.

RESULTS

With proper adjustment of the fluoronephelometer it is possible to analyze histamine concentrations as low as 1 ng/ml in a 0.5 cc sample. At an intermediate setting

it is possible to accurately measure concentrations between 1 and 75 ng/ml without any alterations and with the linear reproducibility implied in Fig. 2. The total histamine content of leukocytes obtained by May's technique (3) from 0.75 cc of whole blood ranges from 30–70 ng in our laboratory. This allows for the use of 0.75 cc of blood for each release assay performed.

Because of a 5–10% carry-over when samples are run sequentially (7) intervening blanks of 0.4 N perchloric acid were used to separate the samples. This results in a reduction of the rate to 15 samples/h but improved reproducibility and mean differences of 0.5% at 50 ng/ml and of 18% of 1 ng/ml histamine standard concentrations were achieved in this way.

RAST and histamine release experiments

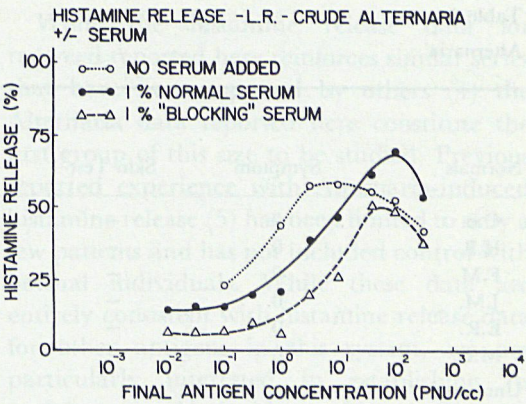


Fig. 4. Parallel histamine release curves show effect of antigen preincubation with 10% normal serum and with 10% "blocking" serum from a treated subject. Final concentrate of reaction mixture is 1%.

using washed leukocyte preparations incubated with ragweed extract produced the

Table 1
Ragweed, RAST, and histamine release data

Normals	Symptom	Skin Test	RAST	Maximum % histamine released	Conc. RW (PNU/cc) antigen resulting in 20% release
E.R.	0	—	I	0	*
R.M.	0	—	I	0	*
C.R.	0	—	I	0	*
D.R.	0	—	I	4	*
Untreated					
J.C.	R	+	IV	96	10 ⁻⁵
J.W.	R	+	IV	80	10 ⁻³
M.L.	R	+	II	75	10 ⁻³
C.F.	R	+	II	38	5 × 10 ⁻¹
T.K.	R	+	IV	30	5 × 10 ⁻²
C.C.	R	+	III	27	5 × 10 ⁻¹
K.C.	R	+	IV	20	5 × 10 ⁻¹
Treated					
L.Y.	R	+	III	84	10 ⁻¹
A.M.	A,R	+	III	80	10 ⁻¹
C.P.	R	+	III	50	10 ⁻⁴
S.H.	R	+	IV	42	10 ⁻²
A.A.	R	+	III	28	10 ⁻²
Y.Y.	R	+	I	20	10 ⁰
E.C.	R	+	IV	5	*

* 20% release not achieved.
A: Asthma
R: Rhinitis
0: No disease

Table 2
Alternaria

Normals	Symptom	Skin Test	RAST	Maximum % histamine released	Conc. AL (PNU/cc) antigen resulting in 20% release
C.B.	0	—	0	0	*
H.B.	0	—	0	0	*
F.M.	0	—	0	0	*
J.M.	0	—	0	0	*
E.R.	0	—	0	0	*
M.C.	0	—	0	0	*
Untreated					
J.H.	A,R	+	III	93	10%
T.T.	A	+	III	80	5×10^{-2}
L.R.	R	+	II	79	5×10^{-2}
C.B.	A,R	+	II	63	10^{-1}
R.C.	R	+	III	57	10^{-1}
D.C.	A	+	*	52	10^{-2}
M.C.	A,R	+	III	49	10^0
R.R.	A,R	+	III	45	5×10^0
P.E.	A,R	+	III	40	10^{-1}
T.S.	A,R	+	**	36	5×10^{-2}
J.H.	A	+	III	0	*
M.L.	A,R	+	III	0	*
H.W.	A,R	+	I	0	*
Treated					
L.M.	R	+	II	75	10^{-2}
R.R.	A	+	III	63	5×10^{-3}
J.R.	A	+	III	59	10^0
D.D.	R	+	0	24	10^2

* 20% release not achieved.
** Not done.
A: Asthma.
R: Rhinitis.
0: No disease.

data as summarized in Table 1. The cellular responses of the ragweed insensitive (normal) subjects were negative as expected. The ragweed sensitive subjects, by contrast, had widely varying positive responses as has been previously reported (4).
The untreated group could not be differentiated from the group that was receiving immunotherapy on the basis of RAST, cellular reactivity, or sensitivity as measured by antigen concentration resulting in 20% histamine release (Fig. 3). The 20%

sensitivity was selected rather than the 50% sensitivity because it separates sensitive and non-sensitive subjects in this group most accurately while the 50% release point was not achieved by eight of the 14 clinically sensitive subjects assayed. Good group correlation is evident for clinical sensitivity, RAST, and positive histamine release assay.
The results of RAST and histamine release assays for the Alternaria subjects are summarized in Table 2. Once again, both RAST and histamine release correlated well

with clinical sensitivity but did not differentiate the treated and untreated groups nor degree of allergy.

While blocking activity was not measured in all the subjects in this study, Fig. 4 represents the results of one such combination of assays. The addition of serum preincubation of antigen results in a reduction of allergenic potency and a shift of the curve so that at a concentration of antigen such as 10 PNU/cc washed leukocytes release 60%, those with normal serum 45%, and those with treated patient serum 30%. Because of the depression resulting from normal serum preincubation the specificity of this blocking activity of *Alternaria*-induced histamine release is open to question and is under continued investigation. These results led us to eliminate serum from our routine histamine release assays using *Alternaria*.

DISCUSSION

The purpose of this report is two-fold. It demonstrates the usefulness of a simple, commercially available auto analysis system in a clinical research setting and it extends the reported histamine release experience with *Alternaria* antigen.

The simplicity, speed, and accuracy of this technique in the range of histamine concentrations between 1 and 75 ng/ml makes it useful in clinical studies where a large number of histamine release assays are to be done using a minimal volume of fresh peripheral leukocytes. A set-up time of 45 min and 30 min shut-down time as well as virtually automatic operation for 50-min intervals allow the operator to continue involvement in repetitive cell incubations or other activities. It eliminates the expense and complication of the dialyzer and nitrogen as described by Siraganian (8). While his technique is faster and 10 times more sensitive than the present techniques and applicable to analysis of histamine in serum and whole blood, this simplified technique is obviated by the high protein concentrations in serum.

While the histamine release data for ragweed reported here reinforces similar series that have been reported by others (4) the *Alternaria* data reported here constitute the first group of this size to be studied. Previous reported experience with *Alternaria*-induced histamine release (5) has been limited to only a few patients and has not included control with normal individuals. While these data are entirely consistent with histamine release data for other antigens in this system, we are particularly interested in establishing its usefulness in *Alternaria*-sensitive patients in preparation for application of histamine release as a measure of antigenic potency in *Alternaria* antigen purification experiments.

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