

Basophil degranulation: A new method of observation and its correlation with skin testing

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For improved study of the atopic reaction, we have devised a new method of assessing degranulation of the human basophil. The procedure involves the concentration of basophils from 20 ml. of peripheral blood, incubation of the basophil concentrate with dilutions of antigen extract, counting of nondegranulated basophils, and comparison of the maximal percent degranulation of the test suspensions with a control. A significant difference in degranulation between the control and test suspensions constitutes a positive test. In a group of 17 ragweed-sensitive patients, the mean maximal per cent degranulation was 77.4 per cent. In a group of 13 nonsensitive subjects, the mean maximal per cent degranulation was 8.8 per cent ($p < 0.01$). The procedure is reproducible, specific, and correlates with the results of skin testing.

Degranulation of the sensitized human basophil upon exposure to a specific antigen has been the basis for many types of in vitro procedures for the study of atopic disease.¹⁻⁷ Unfortunately, the reproducibility of these procedures in the hands of different investigators has been variable,⁸⁻¹⁰ apparently due to the subjectivity involved in deciding when a basophil has degranulated. For this reason, the fluorometric determination of histamine release from human leukocytes^{11, 12} has superseded basophil degranulation as the major in vitro correlate of atopic sensitivity. Though histamine may be released from the basophil without degranulation,⁵ basophil degranulation provides essentially the same information concerning the presence of the atopic state as leukocyte histamine release and is simpler to perform.

Recently, Ishizaka and associates¹³ demonstrated that the number of basophils labeled with radioactive anti-IgE decreases as histamine release increases in an in vitro system. These facts suggested that if degranulation could be observed in a more objective fashion, wider application for its use might be found, both in investigation and in diagnosis. In this paper we describe a method of observing basophil degranulation that we believe incorporates objectivity as well as specificity, reproducibility, and accuracy.

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METHODS AND MATERIALS

Preparation of antigen and control solution

All antigens are diluted in Tris buffer to which human serum albumin, calcium, and magnesium have been added and the buffer adjusted to pH 7.6 (Tris-ACM) according to Lichtenstein and Osler.¹¹

Tenfold dilutions of the antigen are prepared in Tris-ACM from 5 per cent (weight by volume) nonphenolated whole pollen extracts in buffered saline. The concentrations of antigens ranged from 1:50 to 1:50,000.

Collection of basophils

Venous blood, 20 ml., is drawn into a heparinized plastic syringe and is gently mixed. Ten-ml. aliquots are then transferred to two 15 ml. tapered, calibrated, plastic centrifuge tubes and centrifuged in an 8 inch angle Safety-Head centrifuge (Clay Adams, Inc., New York, N. Y.) at approximately 250 r.p.m. In order to attain such a low number of revolutions per minute, an extra rheostat for this type of centrifuge is required. The tubes are centrifuged for a minimum of 5 minutes or until at least 2.2 ml. of cloudy, red supernatant can be seen above the red cell layer in each tube. The supernatant is removed from both tubes with plastic capillary tubing attached to a 1.0 ml. plastic syringe and centrifuged again at 400 r.p.m. for 5 to 10 minutes or until at least 0.4 ml. of the supernatant is clear. The supernatant is then removed with capillary tubing attached to a syringe down to the 4.0 ml. mark and discarded. The remaining cells are resuspended by covering the top of the tube with Parafilm M and placing the tube on a rocking mixer for 2 minutes. This volume is then divided into five 0.5 ml. aliquots in tapered plastic 15 ml. centrifuge tubes. Collection of basophils by this method concentrates these cells approximately 1 to 2 times.

Performance of test

1. From each aliquot of 0.5 ml. of cell concentrate, 2 λ are removed with a 10 λ pipette and discarded. This is done to keep the total volume constant when 2 λ of the test antigen are added.

2. Tris-ACM buffer, 2 λ , are added to the control tube, and 2 λ of each of 4 antigen concentrations are added to the remaining tubes. The tubes are covered with Parafilm M, placed on the rocking mixer for one minute, and then incubated in a 37° C. water bath for 20 minutes.

3. After incubation, the cells are resuspended on the rocking mixer until they appear evenly dispersed at the bottom of the tubes.

4. Using separate 10 λ glass pipettes, 4 λ of the cell suspensions are withdrawn for each tube, and 2 λ (0.002 ml.) of this sample are smeared on the appropriate acid-alcohol cleaned slide using a circular motion to spread the cells over an area 7.5 mm. in diameter (previously outlined on the back of the slide with a felt-tipped pen). During this step the slide is held vertically while the pipette is held horizontally.

5. After the slides are air-dried under a dust cover for at least one hour, they are placed in fresh methanol overnight (or for at least 12 hours).¹⁴

6. The slides are then placed into a staining dish containing toluidine blue- AlSO_4 (0.2 per cent toluidine blue in a 2.5 per cent aqueous solution of AlSO_4 that has been filtered through Whatman No. 42 filter paper) for 2 minutes, then for a half minute in water with slow agitation, then for 30 seconds in absolute alcohol with slow agitation, and then in xylene with slow agitation until slides are cleared of alcohol. The slides are then placed in the second xylene rinse until time to coverslip and are coverslipped from a third xylene rinse.

7. Only the nondegranulated basophils are counted. All slides are counted "blind" by placing tape over the etched labeling. The eyepiece lens of the microscope is covered with tape so that a square is outlined. Using the $\times 10$ low-power objective with a $\times 10$ eyepiece and a $\times 2$ zoom attachment, a $\times 200$ magnification is obtained. As is shown in Fig. 1, each slide is counted square by square and row by row until the entire circular smear is counted.

Fig. 2 shows a typical field under $\times 200$ magnification. In the background of the slide

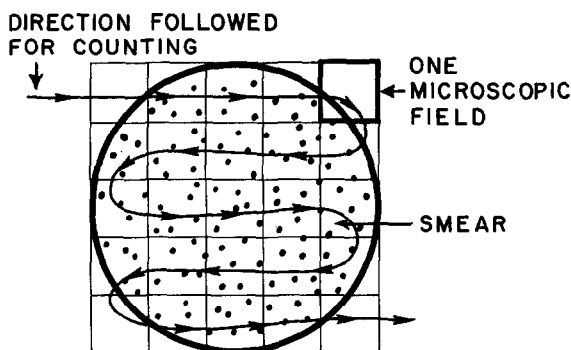


FIG. 1. Demonstration of counting method. Each slide is counted square by square and row by row under X200 magnification, using an ocular lens taped for a square field.

are the erythrocytes that stain light yellow and other leukocytes that stain light blue. The basophils appear as very dark blue, almost purple, in the field. Unless a cell is between 10 and 20 μ and is very dark blue or purple, it is not counted as a basophil. If more than 10 seconds are required to decide whether the cell is a basophil, it is not so counted.

Fig. 3 shows the basophils under $\times 1,000$ magnification. Here the tightly packed, darkly stained granules of the nondegranulated basophils may readily be seen. We performed the process of viewing the basophils first under the lower power, then under the higher power, many times until we were confident that these cells could be identified with certainty with $\times 200$ magnification. Ten minutes are required to count one slide.

Repeated counting of 4 slides, 10 times each by 3 different technicians, resulted in a mean coefficient of variation (standard deviation/mean) of 4 per cent.

RESULTS

Application of this technique to the study of atopic patients is illustrated in Fig. 4. This figure shows the results of a typical test from an untreated 12-year-old male patient with a 4 year history of ragweed hay fever and markedly positive wheal and flare skin tests. The bar labeled Tris-ACM shows the number of basophils (166) counted from the control tube after incubation with Tris-ACM buffer. Each of the other bars shows the basophil count after incubation with the concentrations of ragweed extract as listed below each bar. The difference between the basophil count of the control tube and the lowest basophil count from the antigen-containing tubes, divided by the control value times 100, is the maximal per cent basophil degranulation. The smallest number of basophils counted, which in this case was 21 (maximal degranulation of 87.3 per cent), occurred at a dilution of ragweed extract of 1:125,000 ($1/1.25 \times 10^5$). It should be noted that as the concentration of antigen decreased, the per cent degranulation increased until the optimal proportion of the antigen to antibody ($1/1.25 \times 10^5$ concentration of ragweed extract) was reached. As the concentration of antigen continued to decrease, the per cent of degranulation also decreased.

Fig. 5 shows the results of a study of a 23-year-old nonatopic female with no wheal and flare skin reactivity to ragweed. The bar labeled Tris-ACM shows the mean number of basophils (124) counted from the control tube after incubation with Tris-ACM buffer. The other bars show the count after incubation with

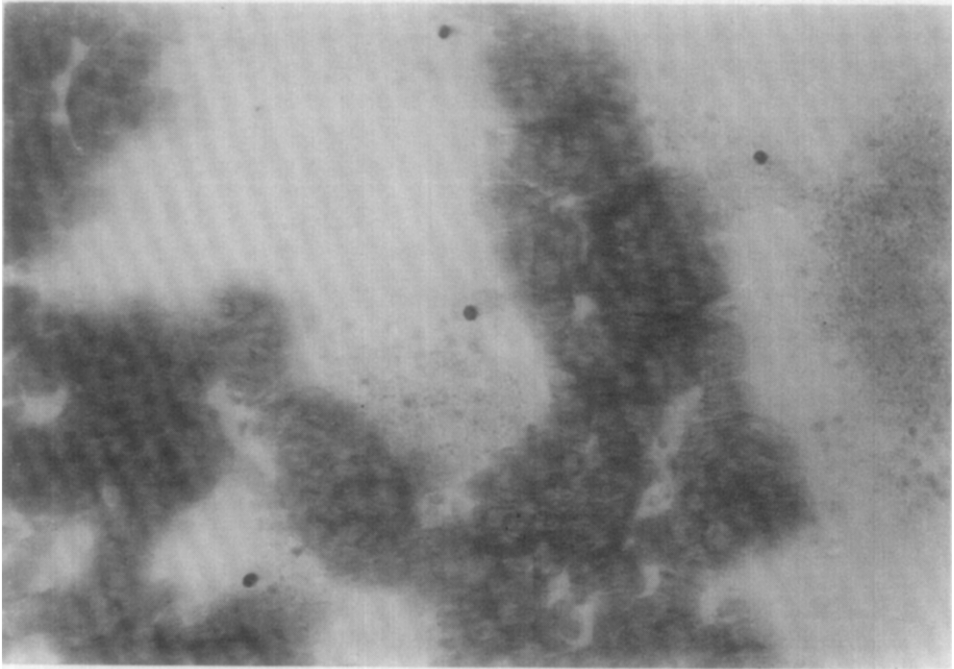


FIG. 2. A photomicrograph of a typical field under X200 magnification. The basophils appear very dark blue, almost purple, against a background of erythrocytes that stain light yellow and other leukocytes that stain light blue.

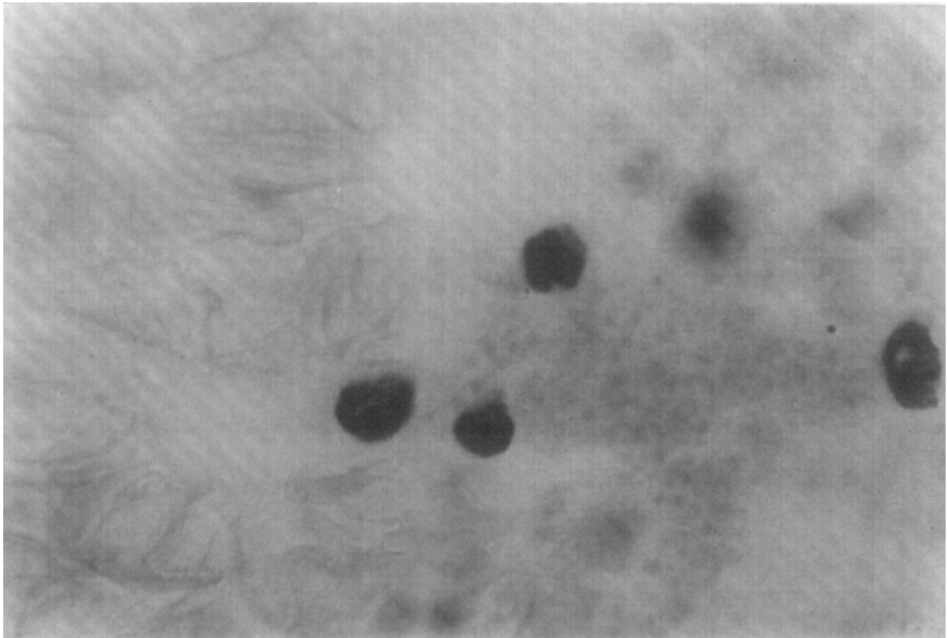


FIG. 3. A photomicrograph of nondegranulated basophils under X1,000 magnification. The tightly packed, darkly stained granules are readily seen.

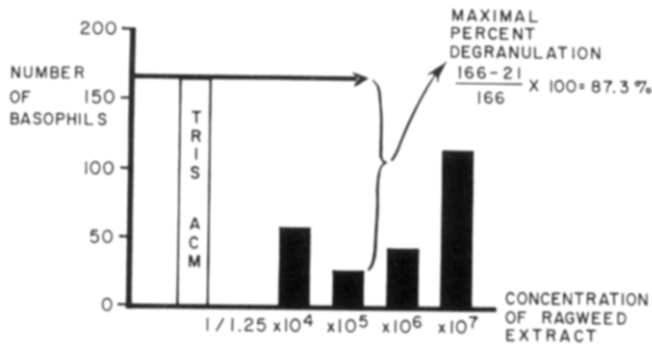


FIG. 4. A typical basophil degranulation test on a 12-year-old patient (D. L.) with a 4 year history of ragweed hay fever and strongly positive skin tests. The white bar shows the number of basophils (166) in 2λ of basophil concentrate after incubation with Tris-ACM buffer. Each of the black bars shows the number of basophils in 2λ of basophil concentrate after incubation with the concentrations of ragweed extract listed below each bar. The difference between the basophil count of the control tube and the lowest basophil count (21) divided by the control basophil count times 100 is the maximal per cent degranulation (87.3 per cent).

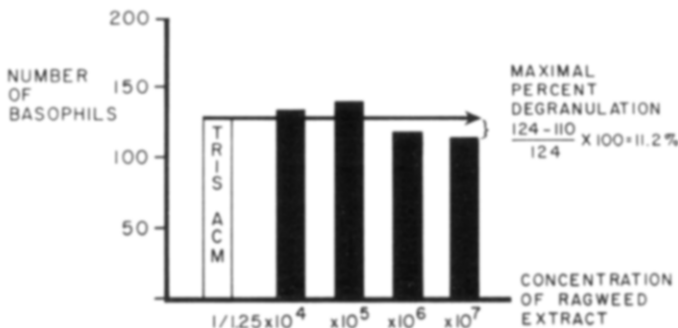


FIG. 5. A basophil degranulation test on a 23-year-old nonatopic subject (S. P.). The bars have the same designations as in Fig. 4. There is no significant decrease in the number of basophils with any concentration of ragweed extract.

the same concentrations of ragweed extract as used previously. There is no appreciable decrease in the number of basophils with any concentration of ragweed extract.

A comparison of the maximal per cent basophil degranulation from a group of 13 nonatopic subjects with the maximal per cent basophil degranulation from a group of 17 highly atopic patients is shown in Table I. The lowest basophil count obtained by one of the 4 concentrations of ragweed ($1/1.25 \times 10^4$, 10^5 , 10^6 , and 10^7) is the maximal per cent degranulation. All 13 control subjects had negative histories and negative skin tests, when tested intracutaneously to ragweed extract in concentrations from 1:125 to 1:12,500, with 2 exceptions. These 2 subjects had 1+ positive tests to ragweed at a 1:125 concentration. The mean maximal per cent degranulation for this group was 8.8 per cent. The range of

TABLE I. Comparison of results of basophil degranulation on nonatopic subjects and highly atopic patients

Nonatopic subjects	Maximal % degranulation*	Atopic patients	Maximal % degranulation*
K. S.	0.0	M. S.	35.6
C. W.	0.0	G. D.	41.2
J. Z.	0.5	R. K.	58.0
H. K.	3.2	H. P.	67.1
O. B.	6.3	M. Me.	73.9
B. M.	9.0	E. H.	75.2
S. M.	9.5	D. S.	76.7
M. M.	10.4	A. K.	76.7
L. K.	10.5	A. C.	86.9
S. P.	11.2	D. L.	87.3
A. W.	11.7	T. F.	87.9
J. F.	16.0	M. Mu.	88.1
D. J.	26.2	J. W.	88.2
		R. M.	90.4
		M. B.	92.6
		F. G.	93.8
		J. A.	96.4
Mean	8.8%		77.4%
S.E. $\pm$	2.0%	p = < 0.01	4.3%
S.D. $\pm$	7.3%		17.8%

*Maximal per cent degranulation obtained by one of the 4 concentrations of ragweed ($1/1.25 \times 10^4$, 10^5 , 10^6 , and 10^7).

TABLE II. Results of degranulation repeated 6 times on 3 atopic patients and one non-atopic subject

Trial number	Maximal % degranulation			
	Atopic patients			Nonatopic subject
	F. G.	M. B.	R. M.	B. W.
1	93.8	92.7	90.6	9.0
2	71.2	87.8	74.9	8.1
3	85.4	89.4	86.1	0.0
4	84.1	94.7	94.2	6.2
5	82.9	91.2	91.0	6.7
6	78.8	90.0	75.0	5.7
Mean	82.7	91.0	85.3	5.9
S.D. $\pm$	7.5	2.4	8.4	3.2

maximal per cent degranulation varied from 0.0 per cent to 26.2 per cent. The 17 patients in the highly atopic group were all untreated, had definite histories of ragweed-induced symptoms, and had 2 to 3+ skin tests at dilutions of 1:12,500 to 1:12,500,000 of ragweed extract. The mean maximal per cent degranulation for these patients was 77.4 per cent. The differences between the degree of degranulation of the atopic group and the nonatopic subjects, as shown in Table I, is highly significant ($p < 0.01$).

In order to establish reproducibility of the procedure, degranulation was performed 6 times on each of 3 atopic patients and 6 times on one nonatopic subject, as shown in Table II. The mean maximal per cent degranulation for 6

TABLE III. Comparison of maximal per cent basophil degranulation from ragweed and walnut extracts in patients clinically sensitive to ragweed only

Patient	Maximal degranulation		Intracutaneous test to walnut	
	Ragweed	Walnut	Skin reaction	Dilution
A. C.	86.9	0.0	3+	1:125,000
E. H.	75.2	33.5	3+	1:125,000
A. K.	76.7	0.0	3+	1:125,000
M. Mu.	88.1	13.0	3+	1:12,500
T. F.	87.9	8.7	2+	1:1,250
R. M.	90.4	2.0	+	1:1,250
M. B.	92.6	0.0	+	1:1,250
M. S.	35.6	11.2	0	1:1,250
G. D.	41.2	9.5	0	1:1,250
D. L.	87.3	0.0	0	1:1,250
J. We.	88.2	0.0	0	1:1,250
M. Me.	73.9	0.0	0	1:1,250

trials each on Patients F. G., M. B. and R. M. were 82.7 per cent (S.D. $\pm$ 7.5), 91.0 per cent (S.D. $\pm$ 2.4), and 85.3 per cent (S.D. $\pm$ 8.4), respectively. The mean maximal per cent degranulation for 6 trials on Subject B. W. was 5.9 per cent (S.D. $\pm$ 3.2).

Specificity of this procedure was demonstrated by studying 12 of the ragweed-sensitive patients with an extract of walnuts to which they had no clinical sensitivity. The maximal per cent degranulation from ragweed and walnut extracts in these patients is compared in Table III. Seven of these patients also had positive skin tests to walnuts. Though none of these patients exhibited significant degranulation from walnut extract, as compared to the ragweed extract, 4 out of the 7 patients with positive skin tests to walnuts did degranulate. Though one patient, E. H., showed 33.5 per cent degranulation, this value is still not significant when compared to the 75.2 per cent degranulation occurring from ragweed in the same patient.

In a procedure of this type it is important to know what per cent degranulation is necessary in order to have a significant result. Table IV shows the results of 19 degranulation tests on 4 atopic and 2 nonatopic subjects using only Tris-ACM buffer and dividing each specimen into 6 or 12 aliquots. A basophil count was then performed on 2 λ samples from each aliquot. The variation of the control (Tris-ACM incubated) basophil counts was therefore determined on 180 aliquots. The mean standard deviation of the 19 degranulation studies was 14.1 per cent. Considering variation beyond 2 standard deviations to be significant and including evaluation of the available data, we feel that there must be a maximal per cent degranulation of at least 35.0 per cent before statistically significant degranulation has taken place.

The number of patients in this initial study is too small for a firm conclusion, but there is some correlation between the positivity of the skin test and the dilution of ragweed extract resulting in maximal degranulation. Table V shows the correlation between the dilutions of ragweed extract producing a 2 or 3+ intracutaneous test and the dilution of ragweed extract that resulted in maximal

TABLE IV. Studies on the variability of the control basophil counts

Subject	Number of determinations	Mean basophil count	Standard deviation	Coefficient of variation $\left(\frac{S.D.}{\bar{X}} \cdot 100 \right)$ %
<i>Nonatopic</i>				
B. W.	6	120	14.9	12.4
B. W.	6	184	36.8	20.0
B. W.	6	176	31.7	18.0
B. W.	12	123	13.8	11.2
J. Z.	6	305	31.2	10.2
J. Z.	12	237	23.0	9.7
J. Z.	12	410	52.0	12.6
<i>Atopic</i>				
M. S.	6	143	21.0	14.6
M. S.	6	166	13.8	8.3
M. S.	12	118	21.3	18.0
R. M.	6	109	10.1	9.2
R. M.	12	153	14.8	9.6
R. M.	12	187	28.0	14.9
S. H.	12	76	22.2	29.1
S. H.	12	39	5.9	15.3
S. H.	12	34	6.9	20.3
T. S.	12	187	13.9	7.4
T. S.	12	199	27.4	13.7
T. S.	12	247	33.0	13.3
Mean				14.1

TABLE V. Correlation between skin testing and dilution of ragweed extract resulting in maximal degranulation in 17 patients

1:1.25 ×	Dilutions of ragweed extract producing a 2-3+ intracutaneous test			
	10 ⁴	10 ⁵	10 ⁶	10 ⁷
Dilution of	10 ⁵	10 ⁵	10 ⁵	10 ⁴
ragweed	—	—	10 ⁵	10 ⁵
extract	—	—	10 ⁶	10 ⁶
resulting in	—	—	10 ⁶	10 ⁶
maximal	—	—	10 ⁶	10 ⁶
degranulation	—	—	—	10 ⁶
1.25 ×	—	—	—	10 ⁷
	—	—	—	10 ⁷
	—	—	—	10 ⁷
	—	—	—	10 ⁷

degranulation; 8 of the 17 patients showed maximal degranulation with more concentrated solutions of ragweed than that required to produce a 2 to 3+ intracutaneous test. Another 8 patients degranulated maximally at the same concentration of ragweed producing a 2 to 3+ intracutaneous test, and one patient showed maximal degranulation at a lesser concentration of ragweed. The considerable variation within each skin testing group may be due to the imprecision of skin testing rather than the vagaries of basophil degranulation. The results imply that degranulation is slightly less sensitive than the skin test.

DISCUSSION

The test described consists basically of the concentration of human basophils followed by incubation with a control solution and dilutions of antigens similar to that used in skin testing. After incubation, exact aliquots of the basophil-antigen suspension are smeared on microscope slides, stained with toluidine blue, and all the nondegranulated basophils on each slide are counted. Though toluidine blue stains heparin and not histamine, both heparin and histamine¹⁶ are complexed within the basophilic granule and are released together upon antigenic challenge.⁵ Once complete degranulation has occurred, the cell can no longer be recognized as a typical basophil. The number of basophils determined in the control suspension is compared with the number of basophils from the antigen-containing test suspensions. If the number of basophils from the test suspensions is significantly less than the number from the control suspension, one may consider the test to be positive.

Though the release of histamine is the hallmark of the IgE-mediated immunologic reaction, Ishizaka and associates¹³ have shown that the combination of anti- γ E with basophils results in both histamine release and degranulation under identical circumstances. Our studies with several dilutions of ragweed extract have shown proportional changes in degranulation that are sufficiently similar to the results of these and other investigators¹¹⁻¹³ to lead us to believe that we are observing the same degranulation that takes place in histamine release systems. The significance of basophil degranulation therefore appears to be the same as histamine release. Further confirmation of this observation will be dependent on the establishment of a similar dose response curve having the same Ca^{++} , Mg^{++} , pH, and temperature requirements as histamine release.

The correlation between the dilution of antigen resulting in maximal degranulation and a 2 to 3+ skin test appears to be acceptable within the limits of clinical variation. A similar general correlation between skin-sensitizing antibody titers, as determined by passive transfer and histamine release from leukocytes of allergic donors, was described by Lichtenstein and associates.¹⁷ These investigators concluded, however, that the correlation of the patients' symptom scores was better with histamine release than with passive transfer. They ascribed their observation as due to the less precise end point inherent in skin testing. In contrast, Pruzansky and Patterson¹⁸ indicated that patients with strongly positive direct prick tests released a large proportion of histamine from their leukocytes but that histamine release was probably not quite as sensitive as the skin test. Our data comparing basophil degranulation with the results of intracutaneous tests tend to agree with the conclusions of the latter group of investigators.

We believe that this method of observing basophil degranulation significantly decreases the subjectivity present in other methods. The present test has been shown to be reproducible and specific. During the developmental stages of this procedure, different quantities of blood and antigen were used with the same results as the described method; however, our present format is the most convenient for our laboratory. Though we have not attempted the simultaneous performance of the test with multiple antigens, the potential application of the

method in this direction holds promise in the field of clinical diagnosis. We do not envision, however, that this procedure could replace the skin test but rather that it might be complementary. The major contributions of this particular in vitro procedure are the use of human basophils and the relative simplicity of performance.

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