

Observations on basophil degranulation as an indicator of antigen-antibody reaction

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According to Shelley and his collaborators,¹⁻⁹ the blood basophil undergoes degranulation during the antigen-antibody reaction. In vitro observations have indicated to these workers that basophil degranulation may be used as a reliable indicator of anaphylactic sensitivity which parallels in intensity the presence of specific circulating antibody in any allergic state. These authors report further that this type of observation permits the detection of specific sensitivity in a wide range of human allergic disease including such syndromes as drug hypersensitivity,^{6, 8} atopic dermatitis,³ gastrointestinal allergy,³ cold urticaria,⁹ and allergy to stinging insects.³ They have also reported that the basophil undergoes degranulation not only in vitro, but in vivo as well, as shown by observations on the blood of a patient experiencing a penicillin reaction.⁶

Various workers have suggested a number of specific functions for basophils, but there is no general agreement on the importance or even existence of many of these functions.¹⁰ While the genetic independence of mast cells and basophils is generally accepted,¹¹ it is known that the human basophil, like the tissue mast cell, contains, among other things, heparin or a heparin-like substance, histamine, and histidine decarboxylase.¹² Although the localization of histamine within the blood basophil is somewhat less conclusive than in the case of the mast cell, both cells undergo degranulation and dissolution with the liberation of histamine into the blood plasma.¹² Evidence that mast cells undergo alteration during circumstances in which histamine is released in anaphylaxis

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has suggested that the union of antigen with antibody takes place in, or very near to the mast cells.¹³ Shelley¹ advances the idea of a mastocyte-basophil system with parallel responses of the two cell lines in the immediate or anaphylactic state, and proposes that the basophil, like the mast cell, is the target cell in immediate hypersensitivity, releasing its granules of histamine-heparin complex in the allergic reaction.

METHODS AND MATERIALS

The present studies represent an attempt to confirm or clarify the observations of Shelley and his associates by studying basophil degranulation induced by adding antigen to the sera of patients or animals known to be sensitive to ragweed pollen, and to the sera of subjects with good histories of allergic reactions following the administration of penicillin. The indirect technique described by Shelley² was employed where basophils obtained from the buffy coat of rabbit blood are placed on slides prestained with neutral red, to which sera from sensitive animals or human subjects are added, and then challenged with specific antigen.

Indirect method of Shelley

Small amounts of heparinized blood, freshly drawn from albino New Zealand rabbits, are introduced into 7 cm. lengths of transparent polyethylene tubing and spun in a microhematocrit centrifuge. The buffy coat is isolated by clamping with mosquito forceps and expelled from the remaining tubing onto a glass microscope slide previously stained with neutral red, on which 0.005 ml. of the test serum has been placed. To this mixture of serum and buffy coat, 0.005 ml. of antigen solution is added. After being mixed by gentle air agitation, the preparation is covered with a coverslip edged with petroleum jelly and observed under oil immersion for the next 20 minutes. The basophil granules stain a distinct brick red within 5 minutes, and readings are begun at that time; the first 20 basophils are observed for changes in shape, stainability, and degranulation. While readings may be completed within 10 minutes, slow reactions may take 20 to 30 minutes. In a positive test, over 30 per cent of basophils show changes which are variously described as swelling, ameboid movement, visualization of nucleus, peripheral palisading of granules, degranulation, decrease in cell staining, and explosive extrusion of cell granules.⁶ Antigen controls consisting of antigen and buffy coat and serum controls consisting of test serum and buffy coat are run in parallel experiments. If antigen controls are positive, reduction in the concentration of antigen is recommended. If serum controls are positive, new specimens of serum are advised.

In the current study, the details of Shelley's indirect procedure were followed in the case of ragweed-sensitive sera, with a total of 20 cells examined within 20 minutes for each test. In the case of sera from penicillin-sensitive subjects, small test tubes, rather than polyethylene tubing, were used for centrifuging rabbit's blood. This yielded, in our hands, a greater abundance of basophils for observation, allowing rapid examination of approximately 100 basophils within 20 minutes, so that total counts were based on at least 50 cells,

and in many cases on 100 cells or more. Observations and counting were begun 5 minutes after mixing and were routinely completed by the end of 20 minutes. All tests were performed by four technicians, generally operating in teams of two, which allowed rapid handling and examination of specimens. Considerable periods of time were required for each technician to learn the techniques of handling and observing the changes in basophils. While an attempt was made to classify the changes in basophils on the basis of Shelley's description and photographs reproduced in his papers,^{1, 6} we found that the changes which lent themselves most readily to detection and agreement by the workers involved were disruption of the cellular membrane and observation of extrusion of cell granules. A test or control was considered positive if 30 per cent or more of the basophils counted showed these changes during the 20 minutes of observation.

Observations on the sera of patients allergic to ragweed pollen

Patient's sera. Blood was drawn 2 to 12 weeks prior to the 1963 ragweed season from 32 patients who gave a good clinical history of hay fever or asthma during the preceding ragweed season and who were strongly positive by scratch test to defatted ragweed pollen extract (30,000 PNU per milliliter). No differentiation was made as to the length of time each had experienced his difficulty, nor to his treatment status. Some were receiving aqueous ragweed extract, others emulsified ragweed antigen, while still others had not received specific antigen treatment at the time blood was taken. Sera were immediately frozen and used for the test within 7 days. Blood was also obtained for testing from 20 additional allergic patients who were not clinically sensitive or skin-test reactive to ragweed pollen.

Antigens

Three ragweed antigens were utilized, and in certain instances the same serum was tested with more than one antigen:

1. *Antigen A.* Defatted aqueous extract of equal parts of short and giant ragweed in the concentration of 1,000 PNU per milliliter.

2. *Antigen B.* Short ragweed pollen extract No. 508 in the lyophilized state, supplied by the National Institutes of Health, reconstituted with 10 ml. diluent to give 480,000 standard nitrogen units per milliliter.* This solution was further diluted to give a final concentration of 48,000 standard units per milliliter.

3. *Antigen C.* Purified ragweed fraction, Pool C (Goldfarb)† reconstituted from lyophilized state to concentration of 0.37 mg. per milliliter.

RESULTS

Sixty-three tests were completed on the sera of 32 patients who were clinically sensitive to ragweed and who had evidence of reaginic antibody (Table I). Antigen or serum controls were positive or questionably positive in 29 in-

*A standard nitrogen unit is the activity represented by 0.00001 mg. N of the original standard before dialysis.

†Purified fraction, Pool C, was kindly supplied by Dr. A. R. Goldfarb, Sinai Hospital, Detroit, Mich.

Table I. *Ragweed-sensitive sera versus ragweed antigens (results in 32 patients)*

<i>Antigen</i>	<i>Total tested</i>	<i>Control positive</i>		<i>Control questionable</i>		<i>Control negative</i>	
		<i>Test negative</i>	<i>Test positive</i>	<i>Test negative</i>	<i>Test positive</i>	<i>Test negative</i>	<i>Test positive</i>
A	20	1	10	0	2	2	5
B	20	1	5	1	4	4	5
C	23	1	4	0	0	2	16

Table II. *Ragweed-sensitive sera, comparison of antigens*

<i>Patient</i>	<i>Antigen A control test</i>			<i>Antigen B control test</i>			<i>Antigen C control test</i>		
B-4	neg.	pos.	+	neg.	neg.		neg.	pos.	+
B-6				neg.	pos.	+	neg.	pos.	+
B-7	pos.	pos.		neg.	neg.		neg.	pos.	
B-15	pos.	pos.		quest.	pos.				
B-19	neg.	neg.		neg.	pos.	+	pos.	pos.	
B-22	pos.	pos.		pos.	neg.		neg.	pos.	+
B-23	quest.	pos.		neg.	pos.	+	neg.	pos.	+
B-25	pos.	pos.					neg.	pos.	+
B-38	neg.	neg.		pos.	neg.		neg.	pos.	+
B-39	neg.	pos.	+	neg.	pos.	+	neg.	pos.	+
B-40	neg.	pos.	+	quest.	pos.		neg.	pos.	+
B-41	neg.	pos.	+	pos.	pos.		neg.	pos.	+
B-42	neg.	pos.	+	pos.	pos.		neg.	neg.	
B-43				pos.	pos.		neg.	pos.	+
B-44				quest.	pos.		pos.	pos.	
B-45				pos.	pos.		neg.	pos.	+
B-46				quest.	pos.		neg.	pos.	+
B-47				pos.	pos.		neg.	neg.	
B-49				neg.	neg.		neg.	pos.	+
B-50				neg.	neg.		pos.	pos.	

Table III. *Non-ragweed-sensitive sera versus ragweed antigens (results in 20 patients)*

<i>Antigen</i>	<i>Total tested</i>	<i>Control positive</i>		<i>Control questionable</i>		<i>Control negative</i>	
		<i>Test negative</i>	<i>Test positive</i>	<i>Test negative</i>	<i>Test positive</i>	<i>Test negative</i>	<i>Test positive</i>
A	13	0	7			4	2
B	15	2	4	0	0	6	3
C	4	0	0	0	0	2	2

stances, with positive serum controls accounting for slightly more than half of this number. Positive antigen controls were most frequent with Antigen B, next with Antigen A, and least frequent with Antigen C, accounting for a greater number of negative controls in the tests made with Antigen C.

In 34 observations, controls met the necessary negative criteria. In these cases the test, combining antigen, serum, and buffy coat, was recorded as positive in 5 out of 7 experiments (70 per cent) where Antigen A was used, and in

5 out of 9 (55 per cent) where Antigen B was employed, while in 18 observations performed with Antigen C, 16 (88 per cent) were positive. For the entire group, a total of 26 out of 32 (76.5 per cent) were positive.

A comparison of tests performed on the same serum with more than one of the ragweed antigens was possible in 20 instances (Table II). In 11 cases where controls were satisfactory, 6 sera showed positive responses with 2 or 3 antigens, while dissimilar responses were recorded in 5 others.

The sera of 20 allergic patients who were not clinically sensitive or skin-test positive to ragweed pollen were subjected to 32 observations (Table III). Where controls were satisfactory, positive degranulation response was recorded in a total of 7 out of 19 tests (36 per cent). Breaking this down further, tests with Antigen A were positive in 2 out of 6 instances (33 per cent); with Antigen B, in 3 out of 9 (33 per cent) and with Antigen C, in 2 out of 4 (50 per cent).

The results indicate a high degree of basophil alteration from the direct effects of ragweed antigen, especially with Antigens A and B, as well as from the sera of both ragweed-sensitive and nonsensitive subjects. With satisfactory controls, however, the ragweed-sensitive group showed an over-all incidence of positive basophil tests of 76.5 per cent as compared to 36 per cent for sera not sensitive to ragweed. Further, the increase in positive tests obtained with purified ragweed fraction, Pool C, suggests the possibility of a higher degree of specificity for this antigen than for whole ragweed extract. This is consistent with findings of increased titers of hemagglutinating and reaginic antibody for Pool C as compared to the whole ragweed antigen.¹⁴

Observations with ragweed antigen and a homologous anti-ragweed rabbit serum

Antigen. Short ragweed extract, NIH No. 508, supplied in lyophilized form and reconstituted with physiologic saline before use.*

Antiserum. Anti-short ragweed rabbit serum, NIH pool No. 90, obtained from rabbits injected with NIH No. 508 emulsified in Freund's adjuvant.* This serum, when tested with its homologous antigen at 40,000 standard units per milliliter, produces a precipitation pattern of 14 bands. When used at a dilution of 1/32 in an Oudin test, it gives a measurable precipitation band with antigen No. 508 at 10,000 standard units per milliliter.†

RESULTS

Control observations using either saline, dilutions of antigen, or dilutions of antisera, showed a wide range in the percentage of basophil alterations induced by these substances alone (Table IV). These presumably nonspecific effects appeared more pronounced with higher concentrations of antigen or sera. In relatively few instances was it possible to obtain satisfactory negative controls against which to compare the effects of antigen plus antiserum on the

*Antigen No. 508 and anti-serum No. 90 kindly made available by Dr. R. P. Wodehouse, National Institute of Health.

†Proceedings of Conference on Purified Ragweed Fractions, National Institute of Allergy and Infectious Diseases, Washington, D. C., 1962.

Table IV. *Ragweed extract (NIH No. 508) versus rabbit antiragweed serum (NIH No. 90)*

	Percentage of degranulation in 20 minutes (50 to 100 cells counted)
Saline + buffy coat	0-14
Antigen + buffy coat	
Dilution 1,000 u/c.c.	0-3
Dilution 6,000 u/c.c.	8-40
Dilution 12,000 u/c.c.	12-38
Dilution 24,000 u/c.c.	5-33
Dilution 48,000 u/c.c.	8-29
Dilution 96,000 u/c.c.	25-64
Dilution 480,000 u/c.c.	10-55
Serum + buffy coat	
Dilution 1/16	0-24
Dilution 1/32	0-10
Dilution 1/64	0-7

Table V. *Ragweed extract (NIH No. 508) versus rabbit antiragweed serum (ARS-NIH No. 90)*

	Percentage of degranulation in 20 minutes (50 to 100 cells counted)		
	Trial No. 1	Trial No. 2	Trial No. 3
Antigen 1,000 u + buffy coat	3.5	0	—
ARS 1/32 + buffy coat	2	0	—
ARS 1/32 + antigen 1,000 U. + buffy coat	14	6.5	
Antigen 48,000 u + buffy coat	8	8	12
ARS 1/32 + buffy coat	10	8	2
ARS 1/32 + antigen 48,000 U. + buffy coat	56	36	46

rabbit's basophils. Where this was possible, dilutions of 1/32 antiserum against antigen, 1,000 standard units per milliliter, resulted in negative basophil tests, while observations utilizing 48,000 units per milliliter of antigen against the same 1/32 antiserum proved positive (Table V).

It is apparent from this group of observations that considerable nonspecific alteration of basophil occurs either spontaneously or as a result of handling, and to an even greater degree from the effects of serum and antigen. In the small number of tests with satisfactory negative controls, the occurrence of a greater degree of degranulation with higher concentrations of antigen suggests a specific effect, although the possible additive nonspecific effects of serum and antigen cannot be discounted.

Observations on patients with history of previous penicillin reaction

Patients' sera. Blood was obtained from 40 atopic subjects who gave satisfactory clinical histories of previous reaction to penicillin. These reactions had occurred 1 month to 11 years prior to the date of examination. All were pa-

Table VI. *Sera from patients with history of penicillin reaction**

	Total of patients	Degranulation test	
		Positive	Negative
All controls negative	36	13 (36%)	23 (64%)
All controls positive	1		
Serum control positive	3		
Antigen control positive	0		
Total	40		

*Antigen: penicillin 1/100.

Table VII. *Comparison of basophil degranulation test (BD) with penicilloyl-polylysine skin test (PPL)*

	No. of patients	
Both tests positive	4	(17.4%)
Both tests negative	8	(34.7%)
PPL-positive BD-negative	7	(30.5%)
PPL-negative BD-positive	4	(17.4%)
	Total	25
PPL-positive—47.8%		
BD-positive—34.7%		

tients who had other clinical allergies and who had been urged to avoid further use of penicillin on the basis of their clinical histories. Eight patients had experienced what could be interpreted as immediate-type anaphylactic reactions, while the remainder had such adverse effects as serum sickness, immediate or delayed urticaria, generalized dermatitis, or severe local swelling. Many potential subjects were eliminated from consideration because of clinical histories which were too vague or inconsistent with what is generally considered to be an allergic reaction.

Antigens: (1) Potassium penicillin G* was prepared in physiologic saline, 1/100 (W/V), immediately prior to use in the basophil degranulation procedure.

(2) Penicilloyl-polylysine (Cilligent†) was used in direct skin testing of 24 subjects. The initial test dilution was 1×10^{-5} M penicilloyl-polylysine. If reactions were positive, one-tenth of this amount was used. When reactions were negative to the initial strength, concentrations of 6×10^{-5} M and 12×10^{-5} M were tested.

Results (Table VI). Antigen controls with penicillin 1/100 were considered satisfactory in all except one instance, while serum controls induced a positive reaction in 4 patients. In 36 observations, where antigen and serum controls were negative, the basophil reaction was recorded as positive in 13 cases (36 per cent) and negative in 23 (65 per cent).

*The Upjohn Co.

†Sigma Chemical Co.

In 24 patients where the penicilloyl-polylysine skin test could be compared with the basophil degranulation reaction, both tests were positive in 4 (16.6 per cent), and both were negative in 9 (38.3 per cent). Positive penicilloyl reactions occurred in 44.0 per cent of this small group as compared with 37.5 per cent positive basophil reactions. (Table VII).

In 7 patients with histories of immediate anaphylactic reaction, both tests were positive in one case and negative in 4 others, while in the remaining two cases, the results were dissimilar.

It must be recognized that the penicillin-sensitive subjects had experienced reactions to penicillin more than two years before these tests were performed. Since it is known from a purely clinical point of view that penicillin sensitivity is often variable and evanescent, this time element must be taken into consideration. Shelley has indicated that positive basophil tests are generally not seen within the first two weeks following a penicillin reaction, and are less apt to occur more than two years after the incident has happened.⁶ While this point could perhaps be resolved by rechallenging the subjects with injections of penicillin, the possible hazards make this unjustifiable. The incidence of positive penicilloyl-polylysine skin tests in this group is lower than that reported by Parker and co-workers,¹⁵ but it is more in line with a larger study by Rytel and colleagues¹⁶ of Naval recruits with clinical histories of penicillin sensitivity. This latter series was challenged with penicillin regardless of skin test results, with a resulting systemic reaction rate almost ninefold greater in patients with a strongly positive skin test than in those with a negative skin test.¹⁵

COMMENT

The use of ragweed-sensitive sera would seem a reasonable starting point in the study of an *in vitro* diagnostic test, since the clinical syndrome and immunologic reactions of pollinosis are definite enough to satisfy the criteria of antigen-antibody reaction from almost every aspect. In the case of drug hypersensitivity, considerably less is known about the occurrence or persistence of the clinically sensitive state, and only a little is known about its immunologic status. Recent studies of penicillin sensitivity have begun to contribute more to our knowledge of this, and the diagnostic possibilities of antigenic penicilloyl-protein complexes¹⁴ and the significance of hemagglutinating antibodies to penicillin and penicillin allergy^{17, 18} are being explored. If the basophil degranulation phenomenon could be added to the diagnostic approach as a reliable *in vitro* test, this could indeed be an important contribution to the detection of clinically important syndromes of hypersensitivity, especially those revolving about the use of drugs.

In a study of penicillin-sensitive sera, Shelley and his associates were able to quantitate the basophil reaction by diluting either antigen or serum,⁶ reporting that corticosteroids inhibited the degranulation phenomenon,⁶ while antihistamines failed to prevent it. In rabbits sensitized to egg albumin, these workers found that high concentrations of antigen induced the *in vitro* basophil response.¹ The same animals, when challenged with intravenous

antigen, suffered from anaphylactic shock associated with complete loss of basophils from the circulating blood immediately following the reaction, with their gradual reappearance after a period of days. For some time afterward loss of *in vitro* basophils was noted and attributed to a "desensitized state."¹

In the present study, because of frequent antigen- and serum-induced alteration in basophils, it was not possible to quantitate the basophil response in a manner desirable for establishing the specificity of the reaction. While some of the results in the ragweed studies may point to a specificity directed toward certain fractions of the ragweed antigen (Pool C), a total correlation with clinical sensitivity and presence of reaginic antibody was not found. It is possible, of course, that a closer relation may be found with blocking or hemagglutinating antibody, which also does not parallel clinical sensitivity or levels of reaginic antibody.

The experiences encountered in the present studies indicate that there exist many technical and theoretical problems which require solution before the specificity of the basophil degranulation phenomenon can be confidently related to antigen-antibody reaction. The technical aspects of the indirect technique require considerable practice and ability on the part of the observer. Aside from the recognition of the cells involved, opinions as to the state of alteration of the basophils frequently vary from one observer to another and from one preparation to another. The exact reproducibility of the reaction is seldom possible. Furthermore, there are problems involved in obtaining satisfactory controls due to high concentrations of antigen or nonspecific serum factors. It is unfortunate that one cannot at this point determine a specific antigen-antibody-induced change in basophil cell morphology, rather than rely on a quantitative determination of altered cells which presumably result from an antigen-antibody reaction. It has been pointed out by Mota¹⁹ in a discussion of a paper presented on this subject⁷ that degranulated basophils can be seen in normal blood subjected to a certain degree of manipulation before fixation and staining. Another factor requiring clarification is the selection of rabbit basophils as the indicator in Shelley's technique. Rabbit blood was suggested by Shelley² for this technique because basophil leukocytes abound (4 to 12 per cent) in contrast to their scarcity in human blood. At the same time mast cells, common in human tissues, are relatively scarce in the rabbit, and it has been claimed by workers in this field that in the case of the rabbit the histamine released by antigen-antibody reaction originates from platelets.¹³ If the basophil is not the prime source of histamine released in the rabbit's blood, it is possible that this animal's cells may not be the best choice for an indirect indicator upon which to base studies of basophil degranulation.

SUMMARY

These observations involve the study of basophil degranulation induced by the addition of antigen to the sera of patients or animals known to be sensitive to ragweed pollen and in subjects with good histories of allergic reactions to penicillin. An indirect technique described by Shelley was used, wherein basophils obtained from buffy coat of rabbit blood are used as indicator cells.

Although nonspecific alteration of basophils was frequent from antigen or serum alone, the incidence of positive basophil degranulation from human ragweed-sensitive sera was 76 per cent, compared to 36 per cent in sera from non-sensitive controls. A greater percentage of positive reactions was noted with purified ragweed fraction, pool C, over whole ragweed antigen. Limited observations with an antiragweed rabbit serum and its homologous antigen showed positive responses only with a high concentration of antigen.

In 36 patients with histories of previous penicillin reaction, the basophil response was positive in 36 per cent. In a group of 24 patients, in 44 per cent of whom penicilloyl-polylysine skin tests were positive, the incidence of positive basophil degranulation was 38 per cent.

In the present study, nonspecific alterations in basophils interfered with efforts to quantitate the basophil response. While the results with ragweed-sensitive sera suggest a possible specificity directed toward a fraction of the ragweed antigen, total correlation with clinical sensitivity and reaginic antibody was not found. Many technical and theoretical problems require solution before the specificity of the basophil degranulation phenomenon can be confidently related to antigen-antibody reaction.

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