

Eosinophilia

III. The anaphylactic release from isolated human basophils of a substance that selectively attracts eosinophils

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Summary

Isolated human basophils sensitized *in vitro* by IgE antibody, and challenged by antigen in the absence of complement, release a substance that attracts eosinophils but not neutrophils. The anaphylactic release of the eosinophilotropic substance is accompanied by morphological changes in the basophils and release of histamine and presumptive SRS-A.

The anaphylactic eosinophilotropic substance differs from that formed in fresh serum by antigen–antibody complexes. Human IgG, aggregated chemically or with antigen, induces formation of anaphylatoxin and substances attracting both eosinophils and neutrophils. IgE, similarly aggregated, does not.

Damaged basophils or neutrophils incubated in serum containing complement, confer on the serum activity attracting neutrophils and eosinophils.

The suspensions of the three types of polymorphonuclear leucocytes used in these tests were 92–97% pure, prepared by the techniques of Day.

Introduction

Several substances have recently been described that attract eosinophils *in vitro*. They either attract both neutrophils and eosinophils or are selective for eosinophils. These substances can be divided into three groups according to their properties and origin, the first being serum substances generated by incubating antigen–antibody complexes or other agents in complement-containing serum (Keller & Sorkin, 1969; Ward, 1969; Kay, 1970; Lachmann, Kay & Thompson, 1970). These substances are associated with the generation of anaphylatoxin. The several anaphylatoxins have now been shown to contain anaphylatoxin *per se* which attracts eosinophils, and a complex of anaphylatoxin and a substance designated cocytotaxin that attracts neutrophils and eosinophils (Wissler, Stecher & Sorkin, 1972a).

The second group of substances are those released from anaphylactic guinea-pig lung (Kay, Stechschulte & Austen, 1971; Parish, unpublished) and human lung (Kay & Austen, 1971) which selectively attract eosinophils.

The third group are those formed when components of neutrophils are incubated in serum containing complement, which attract neutrophils (Borel, Keller & Sorkin, 1969; Sorkin, Borel & Stecher, 1970) and eosinophils (Parish, 1972).

The results now reported are examples of each group of substances which attract eosinophils. IgG, aggregated chemically or by combination with antigen, when incubated in normal human serum generates substance(s) accompanied by anaphylatoxin activity which are chemotactic for neutrophils and eosinophils, whereas IgE aggregated by similar methods does not. Basophils isolated from human peripheral blood, sensitized *in vitro* with γ E antibody, release a substance on antigenic challenge that selectively attracts eosinophils. Basophils disrupted physically and incubated in normal serum generates activity attracting both eosinophils and neutrophils.

Materials and methods

Separation of polymorphonuclear leucocytes

Eosinophils were isolated from the leucocyte-rich supernatant fluid of heparinized blood, treated with dextran to sediment the red cells, by centrifugation on sodium metrizoate solution (Day, 1970). In some tests the sodium metrizoate was obtained as Triosil '75' from Glaxo Ltd, Greenford; in later tests as Hypaque sodium (Winthrop Laboratories Ltd, Surbiton). The Triosil was more consistent than the Hypaque. The leucocyte suspension layered onto the sodium metrizoate, density 1.148 g/ml, was centrifuged at exactly 400 g for 40 min. The eosinophils recovered from the pellet were 90–95% pure with 3–6% neutrophils. The small number of contaminating red cells were left intact lest lysing them or centrifuging again should damage the eosinophils.

Neutrophils. It was more difficult to obtain almost pure suspensions of undamaged neutrophils than of the other two types of cells. The technique usually adopted was that for the separation of eosinophils above, but with a Hypaque solution density of 1.120 g/ml. If the blood donor had very few eosinophils, the neutrophil suspension was 83–94% pure and 95% living. A good separation was not always achieved, but only samples about 90% pure were used. Elution of neutrophils with EDTA from glass beads treated or not treated with silicone, damaged them.

Basophils were isolated from peripheral blood by the method of Day (1972). Blood from atopic persons weakly sensitized to allergens unrelated to those used in these tests, was taken in 15 or 30 ml volumes and mixed with sterile EDTA which had a final concentration of 0.01 M. The blood mixed 1 : 3 with a Tris buffer solution pH 7.6 was layered over a Hypaque–Ficoll solution density 1.080 g/ml and centrifuged to obtain mononuclear cells and basophils. This cell fraction was centrifuged on an 11.5% solution of Hypaque to obtain a pellet of cells which was further separated on a gradient of Hypaque and Ficoll as described by Day. Suspensions could be 92–97% pure. With the exception of one separation in which all the cells were normal, 7–16% were usually damaged by separation and subsequent passive sensitization, though not all of them took up trypan blue dye. For reasons given later, damaged cells did not affect the results. Separations in which less than 90% purity was achieved or in which more cells were damaged were not used. Usually $6-7 \times 10^5$ cells were obtained per 15 ml blood. Triosil was more consistent and less damaging than Hypaque.

Chemotaxis tests

Boyden-type chambers were made of the barrel and hollow plunger of 10 ml or 5 ml

plastic syringes (Gillette, scimitar). Millipore filters dividing the two chambers had a pore size of $3\ \mu\text{m}$ for neutrophils and $8\ \mu\text{m}$ for eosinophils. Test solutions were placed in the barrel, and cell suspensions in the hollow plunger.

The medium used as the cell diluent in all tests, and for the test solutions unless otherwise stated, was Eagle's medium containing 0.5% human albumin. The albumin had traces of α_1 globulins but no β or γ globulins. Neutrophil and eosinophil cells were used at 2×10^6 cells/ml. Equal volumes of cell suspensions and test solutions were used. In the anaphylatoxin experiments (Tables 1 and 2) 2.5 ml was placed in each chamber. In tests on basophil substances, 1 ml was used in each chamber in the 5 ml syringes, and the suspension of cells in the upper chamber was increased to 4×10^6 /ml. Chambers were incubated in air at 37°C for 3 hr. The membranes were fixed in alcohol. Those examined for neutrophils were stained by Giemsa; those for eosinophils by Peroxidase-Giemsa (Archer, 1963) or by chromatrope 2R. Cells completely penetrating to the lower surface were counted with a $\times 40$ objective and $\times 8$ eye piece. The average of ten fields was taken.

Details of tests

Table 1. The IgG was obtained from serum heated for 2 hr at 56°C by precipitation with 40% saturated ammonium sulphate and elution of the dissolved precipitate from a DEAE cellulose column with 0.005 M phosphate buffer, pH 7.8. Single radial immunodiffusion tests showed IgG only. It was aggregated with bis-diazo benzidine, and washed thrice on two days. The IgE was obtained by separating the globulins precipitated from each serum or from pools of 400 ml with 50% saturated ammonium sulphate on DEAE cellulose, taking the sample eluted with 0.025 M phosphate buffer and further separating it on Sephadex G-200, twice (Ishizaka, Ishizaka & Terry, 1967). The IgE-rich fraction was treated with insoluble rabbit anti-IgG and anti-IgA polymerized with ethyl chloroformate (Avrameus & Ternynck, 1967) to remove any traces of those immunoglobulins though it remained slightly contaminated with β_1 and α_2 globulins. The fraction was then aggregated as for IgG. The aggregated globulins, at concentrations stated, were incubated for 1 hr at 37°C and 1 hr at 4°C in fresh normal group AB serum, or the serum heated at 56°C for 1 hr, or previously dialysed against 0.01 M EDTA and incubated in its presence and then dialysed against Eagle's medium before testing for chemotaxis.

Table 2. IgG and IgE fractions of antibody-containing sera were prepared as above, but not aggregated chemically. Precipitates of IgG were prepared at twice equivalence with antigen and washed five times. Complexes of IgE antibody were formed by adding a previously determined $10\ \mu\text{g}$ IgE antibody to $25\ \mu\text{g}$ β -lactoglobulin or $50\ \mu\text{g}$ ovalbumin. Normal serum was incubated with precipitates containing $100\ \mu\text{g}$ IgG/ml or the solution containing $10\ \mu\text{g}$ IgE/ml. The serum was tested for chemotaxis after diluting 1/10 in Eagle's medium-human serum albumin.

Table 3. Neutrophils and basophils were tested at 1.5×10^5 cells. Cells, normal or damaged, were incubated in medium or in complement containing serum, with or without the addition of $5\ \mu\text{g}$ β -lactoglobulin, for 1 hr at 37°C . They were lightly centrifuged to deposit the cells and the supernatant fluid was passed through a Millipore swinny filter $0.45\ \mu\text{m}$ pore size and made up to 1 ml for testing. Cells were also treated with serum containing γG or γE anti- β -lactoglobulin in the presence of heparin at $10\ \mu\text{g}/\text{ml}$ and 4×10^{-3} M EDTA for 2 hr at 37°C (Osler, Lichtenstein & Levy, 1968). They were then washed in Eagle's medium twice. The deposited washed cells were

challenged with a 0.1 ml of 5 μ g antigen in Eagle's medium at 37°C, and then made up to 1 ml volumes with Eagle's medium-0.5% human albumin, and the supernatant fluid tested. In other tests the cells were treated with antibody-containing sera without addition of heparin and EDTA for 2 hr. They were challenged with 5 μ g antigen in 0.1 ml for 40 min, without washing. The supernatant fluid containing the serum was tested.

Table 4. Basophils were sensitized in unheated, or heated for 2 hr at 56°C, reaginic anti- β -lactoglobulin serum and washed as above. The sensitized cells were challenged with 5 μ g antigen for 40 min at 37°C in Eagle's medium, or with monospecific rabbit anti-IgE or anti-IgG sera. The 0.1 ml volumes were made up to 1 ml for chemotaxis tests in Eagle's-human albumin medium, or for histamine and SRS-A assays in Tyrode solution.

All sensitization and washing of cells for Tables 3 and 4 was carried out in siliconed glassware.

Results

Leucocyte chemotaxis after treating normal serum with aggregated globulins

Aggregated IgG when incubated in normal human serum activates a substance that attracts human neutrophils and eosinophils (Table 1). Almost pure suspensions of neutrophils are attracted slightly more readily than eosinophils, though the difference is probably due to the pore size of the filters used for each type of cell. When neutrophils and eosinophils were mixed in equal numbers of 1×10^6 cells/ml and tested on filters with a pore size of 8 μ m, greater numbers of both cells migrated through the filter. It is possible that the neutrophils penetrating the filter slightly in advance of the eosinophils attracted more eosinophils. Complement appeared necessary for the formation of the chemotactic agent, which was not formed if the normal serum was heated before incubation with the aggregated globulin, or if dialysed against 0.01 M EDTA before incubation (Table 1).

Formation of the leucocyte-attractive substance occurred concomitantly with the formation of anaphylatoxin. The normal serum incubated with aggregated IgG induced moderate but not fatal anaphylaxis when 1 ml was injected intravenously into

Table 1. Tests for chemotactic activity in serum incubated with aggregated IgG or IgE

Ig class	Concentration (μ g N/ml)	No. of neutrophils or eosinophils/field when aggregated globulin incubated in:							
		Normal serum		Heated serum		Serum + EDTA		No serum	
		Neut.	Eos.	Neut.	Eos.	Neut.	Eos.	Neut.	Eos.
IgG	1	21	20	3	0	0	0	4	0
	10	61	18	1	2	0	0	2	1
	50	101	91	5	0	1	0	0	2
	100	121	83	6	2	0	0	4	2
IgE	1	8	6	0	0	2	1	0	1
	10	10	2	1	0	0	0	2	0
None	—	9	5	1	0				

guinea-pigs, including coughing, respiratory distress, nose-wiping and ear scratching. When injected intradermally in guinea-pigs, the substance formed by treatment with 50 μ g IgG, diluted 1/32, induced increased vascular permeability of 10×10 mm, and it contracted strips of guinea-pig ileum in a Schultz-Dale bath.

The aggregated IgE incubated in normal serum did not activate a substance chemotactic for leucocytes or generate formation of anaphylatoxin.

Leucocyte chemotaxis after treatment of serum with human antibody-antigen precipitates

The previous test was repeated using precipitates of human IgG anti- β -lactoglobulin with β -lactoglobulin, or complexes of IgE anti- β -lactoglobulin and anti-ovalbumin with the appropriate antigens. The results (Table 2) show that the precipitates of IgG and antigen when incubated in unheated normal serum generated chemotactic activity for neutrophils and eosinophils, whereas complexes of IgE antibody with antigen did not. Anaphylatoxin was also present in serum samples treated with precipitates containing IgG but not in those treated with complexes containing IgE. Doubts about the possibility that the IgE antibodies had failed to form complexes with antigen were resolved by injecting 0.1 ml samples of the IgE-antigen complexes, not incubated with the normal serum, into the skin of a monkey. This elicited immediate increased vascular permeability which is a characteristic of aggregated IgE.

Eosinophil-attracting substance released from anaphylactic basophils

Basophils sensitized with IgE reaginic serum, and washed free of the serum before testing with antigen in the Eagle's-human serum albumin medium released a substance into the medium that attracted eosinophils, but not neutrophils (Table 3). Neutrophils treated with IgE antibody, washed and challenged with antigen in medium, released no leucocyte chemotactic substance.

Basophils 'sensitized' with IgG anti- β -lactoglobulin, and challenged with antigen in medium, released no chemotactic substance, whereas neutrophils treated in the same manner released a substance weakly attracting neutrophils and eosinophils.

When basophils or neutrophils were sensitized by sera containing IgE or IgG antibody, and were challenged without washing the cells free of serum, the supernatant fluid, diluted 1/10 and placed into the test chamber, was found to contain activity

Table 2. Tests for chemotactic activity in serum incubated with complexes of IgG or IgE with antigen

Test complexes or substances incubated in:	No. of neutrophils or eosinophils/ field			
	Normal serum		Heated serum	
	Neut.	Eos.	Neut.	Eos.
IgG anti- β -lact. + β -lact.	92	86	8	2
IgG anti- β -lact. (no antigen)	14	8	0	0
β -lactoglobulin	9	7	3	1
IgE anti- β -lact. + β -lact.	11	6	2	0
IgE anti-egg + ovalbumin	8	10	0	0
IgE anti-egg	7	5	1	0
Ovalbumin	5	5	0	0

Table 3. Chemotaxis elicited by basophils and neutrophils, untreated, or treated with antibody, antigen and normal serum

State or treatment of test basophils or neutrophils	Addn β -lact.	Chamber diluent	No. of neutrophils or eosinophils attracted to treated:			
			Basophils		Neutrophils	
			Neut.	Eos.	Neut.	Eos.
Undamaged by isolation	—	Medium	2	0	0	2
Slightly damaged	+	Medium	2	0	3	0
Disrupted by dist. water	—	Serum	88	74	136	128
Treated IgG Ab, washed	+	Medium	4	2	18	11
Treated, serum with IgG Ab	+	Serum	34	26	184	132
Sensitized IgE Ab, washed	+	Medium	8	48	5	2
Treated, serum with IgE Ab	+	Serum	21	11	52	39

IgG and IgE antibodies to β -lactoglobulin as described in Materials and Methods.

moderately or strongly attractive to leucocytes. Most or all of this activity probably results from incubating in serum, cells damaged by handling or by the allergic reaction, because both basophils and neutrophils, damaged by distilled water, or in unreported tests by freezing and thawing, when incubated in serum containing complement, generate leucocyte chemotactic substances (Table 3).

Basophil release of histamine and SRS-A with the eosinophil-attracting substance

Human and monkey basophils, sensitized with reagenic anti- β -lactoglobulin, on challenge with antigen released into the medium histamine, ?SRS-A and a substance chemotactic for eosinophils, but not for neutrophils. Antigenic challenge of basophils pretreated with the reagenic serum heated at 56°C for 2 hr did not release the eosinophil-attracting substance or SRS-A, and the small amount of histamine released is believed to result from the washing and other technical procedures (Table 4). The

Table 4. Concomitant release of eosinophil-attracting substance, histamine and ?SRS-A from sensitized basophils tested with antigen or with anti-IgE

Origin of basophils	Reagenic serum treatment	Sensitized cell challenge	Post-challenge supernatant fluid activity			
			Chemotaxis		Histamine release (% total)	SRS-A release
			Neut.	Eos.		
Man	Unheated	Antigen	5	32	76	+ ?
	Heated	Antigen	3	1	12	—
	Unheated	Anti-IgE	1	18	85	+ ?
	Unheated	Anti-IgG	4	0	14	—
Monkey	Unheated	Antigen	7	22	33	nd
	Heated	Antigen	7	3	5	nd

nd = not done. Results of antigenic challenge of human and monkey basophils are a mean of two tests. Results of antiglobulin challenge are from one test.

release of these substances occurred in the absence of any complement in the Eagle's-human serum albumin medium.

Treatment of the sensitized basophils with anti-IgE also released the eosinophil-attracting substance, histamine and γ -SRS-A whereas treatment with anti IgG did not.

Similarities between results of tests on human basophils and rat mast cells

The results obtained from tests on human basophils are similar to our previous results on rat mast cells (to be published). Isolated intact rat mast cells, about 96% pure, obtained from the peritoneal cavity, were sensitized in a serum fraction containing rat IgE-like antibody, or in serum containing IgE-like and some IgG₂ antibody, or in normal serum. When these cells were washed free of the unfixed serum proteins, and then challenged with antigen in Eagle's medium or Tyrode salt solution containing bovine serum albumin, those cells sensitized by IgE-like antibody released into the medium a substance, which on testing in Boyden chambers, attracted rat eosinophils but not neutrophils.

When the medium with eosinophil-attracting activity was injected into rats intraperitoneally it elicited an eosinophilia of the blood and peritoneal fluid. However, the increase in number of the eosinophils was about the same as that resulting from passively sensitizing the rats with the appropriate antibodies and challenging *in vivo*. The increase in number of eosinophils elicited by mast cell-derived substances, or passive anaphylaxis *in vivo*, was always much less than the number of eosinophils found after active sensitization and challenge *in vivo*, even though at the time of challenge the actively sensitized rats had low levels of circulating antibody.

It is concluded that in the rat the anaphylactic mast cell-derived substance is probably responsible for most of the eosinophilia of passively sensitized rats, but other mediators, e.g. from lymphocytes, probably contribute to the eosinophilia of active anaphylaxis.

Mast cells damaged physically or allergically, incubated in even small amounts of serum, release into the serum substance(s) attracting both neutrophils and eosinophils.

Discussion

The findings reported above illustrate three types of reaction resulting in the release, activation or formation of substances which attract eosinophils *in vitro*. The first is the generation of a substance attracting both neutrophils and eosinophils when aggregated globulins are incubated in serum containing complement. The second, which is of much greater significance in anaphylaxis, is the release of a substance attracting eosinophils, not neutrophils, when almost pure suspensions of basophils sensitized with IgE reagent are challenged with antigen in the absence of serum or complement. The third is the formation or the release of a substance attracting neutrophils and eosinophils, when damaged basophils or neutrophils are incubated in serum.

These tests could not have been carried out without the techniques of Day (1970, 1972) for the preparation of almost pure samples of each type of leucocyte. Unfortunately in our hands 7-16% basophils tend to be damaged during separation and passive sensitization, though in one test separation was achieved without damage and the results of the chemotaxis tests with these cells were identical with those of the slightly damaged cells. Any chemotactic substance formed when slightly damaged cells are incubated in unheated reaginic sera is removed with the unfixed serum proteins when the cells are washed prior to antigenic challenge in Eagle's medium. This also

applies to the removal of histamine and SRS-A released spontaneously before antigenic challenge.

The release of the eosinophil attracting substance from sensitized basophils on antigenic challenge appears to result from anaphylaxis. The substance is released from basophils sensitized passively by IgE antibody, and not by heated IgE, or by IgG antibody to the same antigen. The substance is released on antigenic challenge in the absence of complement, and the release is associated with degranulation or severe vacuolation of the basophils and is accompanied by release of histamine and ?SRS-A. Moreover anti-IgE, which reacts with the IgE bound to basophils releasing their histamine (Ishizaka, Tomioka & Ishizaka, 1970) also releases the eosinophil-attracting substance.

The nature and properties of the anaphylactic basophil substance attracting eosinophils will be studied further. It appears, however, similar to, or identical with, the eosinophil chemotactic factor (ECF-A) described by Kay & Austen (1971). This substance is released from human lung, passively sensitized by IgE antibody and challenged *in vitro*, with a concomitant release of histamine and SRS-A. The substance was also released by treating the sensitized lung with anti-IgE instead of antigen. The results of tests on human lung are so very similar to those of the tests on basophils, that it is highly probable that the mast cells of the human lung are the source of the eosinophil attracting substance, just as both mast cells and basophils are the major if not the only source of anaphylactic histamine.

This conclusion is strengthened by the results of tests on rat mast cells, briefly reported above. Rat peritoneal mast cells, sensitized by the homologous IgE-like antibody and challenged with antigen in the absence of complement, release a substance that selectively attracts eosinophils. Furthermore, when sufficient of this substance is injected into normal rats it elicits an eosinophilia similar in number to that occurring on challenge of passively sensitized rats, but less than that in challenged actively sensitized rats. Therefore the eosinophilia of anaphylactic passively sensitized rats may be elicited mainly by the substance released from the mast cells and basophils.

These findings also provide an explanation for the attraction of eosinophils to changed mast cells in anaphylactic tissues. Eosinophils accumulate round anaphylactically damaged mast cells in rats and guinea-pigs, adhering to the mast cell membrane, and shedding eosinophilic granules with consequent vacuolation (Parish, 1970, 1972). In the anaphylactic subcutaneous tissues of guinea-pigs, eosinophil infiltration occurs with the mast cell changes, and in the first 30 min eosinophils are strongly attracted to the changed mast cells and cluster round them, but by 4 hr, though there are many eosinophils in the tissue, there is no obvious attraction to mast cells (Parish, 1972). On the evidence obtained from tests on human basophils, these events may be interpreted as the release of an eosinophil-attracting substance from the anaphylactic mast cells which first attracts eosinophils directly to them. When the substance diffuses away, the eosinophils continue to infiltrate the tissue but are no longer attracted to the mast cells.

Though molecular weight determinations have not been made of the basophil-released and the anaphylatoxin-associated substance generated in fresh serum, these two substances differ greatly in their origin and activity. Release of the basophil substance follows sensitization by IgE, does not require complement and selectively attracts eosinophils. The serum chemotactic substance is formed after incubation with IgG, aggregated chemically or with antigen, and requires complement. It attracts both

neutrophils and eosinophils. IgE, aggregated in a similar manner, failed to generate a chemotactic agent when used in concentrations at which the chemically aggregated IgG was active (Table 1). Aggregated IgE does not fix complement.

The recent careful, critical studies by Wissler and Sorkin (Wissler, 1972; Wissler *et al.*, 1972a, b) show that the biological activities attributed to anaphylatoxin are induced by at least two substances. One induces the changes of classical anaphylatoxin, that is, shock in guinea-pigs and contraction of isolated guinea-pig ileum. It also attracts eosinophils *in vitro*. The other substance, cocytoxin, attracts eosinophils. The two substances together attract neutrophils and eosinophils though the activity varies with their molar ratio when combined.

We have not succeeded in separating two such substances from human sera, but like Kay (1970) we separated substances attracting neutrophils from those attracting eosinophils in guinea-pig sera previously incubated with precipitates. We found that anaphylatoxin was associated with eosinophil attracting activity but not with neutrophil attracting activity. However, on acrylamide gel separation more than one band was present, and it was not known if anaphylatoxin and eosinophil attraction was the property of one substance.

Basophils behave like neutrophils in that the damaged cells incubated in serum confer on the serum the ability to attract neutrophils and eosinophils. Fractions of neutrophils incubated in serum become chemotactic for neutrophils (Borel *et al.*, 1969; Sorkin *et al.*, 1970) as damaged neutrophils in serum similarly attract eosinophils (Parish, 1972). It is still to be determined whether the basophil-serum substance is the basophil anaphylactic substance together with an anaphylatoxin-associated substance, anaphylatoxin alone, or yet another chemotactic substance.

Acknowledgments

I thank the Asthma Research Council for a grant for the study of anaphylactic antibodies. I also thank Mr R. P. Day and Professor J. Pepys of the Institute of Diseases of the Chest, Brompton, London, for advice on cell separation techniques and prepublication data.

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