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Eosinophilia

II. CUTANEOUS EOSINOPHILIA IN GUINEA-PIGS MEDIATED BY PASSIVE ANAPHYLAXIS WITH IgG1 OR REAGIN, AND ANTIGEN–ANTIBODY COMPLEXES; ITS RELATION TO NEUTROPHILS AND TO MAST CELLS

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Summary. More eosinophils accumulate in sites of cutaneous anaphylaxis mediated by homologous IgG1 or reagin, than in skin treated with IgG2, 18 hours after intravenous challenge. The increased number of eosinophils becomes apparent at 4 hours and reaches a maximum at 12–24 hours, whereas neutrophils infiltrate skin sensitized with IgG1 within 15 minutes of challenge and are most numerous at 4–8 hours. There is a much higher ratio of eosinophils to neutrophils in skin passively sensitized with reagin and challenged after 14 or 28 days.

In anaphylactic skin, eosinophils accumulate round changed mast cells.

The numbers of eosinophils in anaphylactic skin reflect the numbers in the blood when challenged, and no increase in the number of haematogenous eosinophils occurs between the time of challenge and sampling.

The behaviour of eosinophils *in vitro* appears to differ from that *in vivo*, in that they, like neutrophils, are attracted more strongly to complexes containing IgG2 than IgG1 antibody. They are also attracted to damaged neutrophils.

It is suggested that eosinophils are not selectively attracted to sites of cutaneous anaphylaxis, but enter them with the neutrophils in the relative proportions in which they are present in the blood. They are however selectively retained in anaphylactic, or anaphylactoid tissue, while the neutrophils continue to emigrate.

INTRODUCTION

Guinea-pig eosinophils are attracted to anaphylactic skin previously sensitized with homologous IgG1 antibodies (Litt, 1967; Kay, 1970; Parish, 1970a). Eosinophilia in the blood and peritoneum is also elicited by anaphylaxis after passive sensitization with IgG1, or by injection of complexes of antigen and IgG1, and though complexes containing non-anaphylactic IgG2 attract eosinophils at the site of injection, they do not elicit a haematogenous eosinophilia (Parish, 1970a; 1972). Similarly, eosinophilia in the blood of persons sensitive to penicillin is associated with the presence of skin-sensitizing antibodies and not with IgG or IgM (Zolov and Levine, 1969).

Skin and blood have an advantage over other tissues, in that samples may be taken repeatedly without seriously affecting the number of eosinophils and other leucocytes, as may occur with peritoneal lavage which removes most of the cells.

This study examines the relation between the number of eosinophils in the blood and

the number infiltrating skin prepared with IgG1 or IgG2 and challenged with antigen, or skin injected with preformed complexes. In order to reduce any eosinophilotropism exerted by neutrophils preceding the eosinophil infiltration, guinea-pigs were passively sensitized by intravenous injection of antibody and challenged by skin prick-tests. In other tests skin was sensitized locally by homologous reagin, and the animals challenged intravenously 14 or 28 days later, when any neutrophils infiltrating the injected sites had gone.

MATERIALS AND METHODS

Antisera and complexes

Guinea-pig IgG1a, IgG1b, IgG2 anti-picryl-chloride antibodies, and IgG1a and IgG2 anti-bovine serum albumin (BSA) antibodies were those prepared for the previous study (Parish, 1972).

Guinea-pig reagin (heat-labile antibodies sensitizing homologous skin passively for at least 28 days) were prepared to β-lactoglobulin with aluminium hydroxide (Parish, 1972b) and to egg albumin with *Bordetella pertussis* (Mota and Perini, 1970).

Complexes of guinea-pig antibody with antigen at equivalence or eight times excess of equivalence, were also prepared as in the previous study (Parish, 1972).

Passive sensitization and challenge

Guinea-pigs, about 350 g weight, were passively sensitized intracutaneously with 0·1-ml volumes of serum or globulins. After varying periods, stated in the text, usually 4 hours, the animals were challenged intravenously with 0·25 mg of the appropriate antigen and 2·5 per cent Evans' blue dye in 0·5 ml. The animals were killed and skin samples taken at intervals stated in the text, usually at 18 hours.

The effect of three quantities of antibody, 0·5 μg, 5·0 μg and 50 μg globulin, on cutaneous eosinophilia was tested on nine guinea-pigs for each quantity; the animals being divided into three groups of three according to the number of circulating eosinophils (Table 1). Each animal received four injections, one each of IgG1 and IgG2 antibody to the test antigen, and to an unrelated antigen.

In order to control any effect due to regional variations in the skin, and to determine the constancy of the number of eosinophils infiltrating the test sites, further guinea-pigs with 100–250 circulating eosinophils/cu mm blood were prepared with 5·0 μg globulin. Three guinea-pigs received four injections of IgG1, one over each scapula and one over each femur. The test antigen-specific IgG2 and non-specific IgG1 and IgG2 were tested in the same manner, each injection at four sites on three guinea-pigs.

Generalized passive sensitization (Table 2) was achieved by injecting 1 mg IgG1 or IgG2 intravenously, and challenging the skin 4 hours later by a prick 3-mm deep with a medi-point needle through a drop of 1 per cent of the test antigen and an unrelated control antigen. Skin samples were taken under ether anaesthesia 18 hours after challenge. The test was repeated 24 hours after sampling by injecting each animal with the immunoglobulin not used in the first test, e.g. IgG2 into an animal previously tested with IgG1. In the first test the antigen was BSA; in the second, picryl-chloride as picryl-chloride-horse γ-globulin.

The relation between eosinophilia and time (Fig. 1) was tested on six guinea-pigs with 5 μg antibody at seven sites, and six with 50 μg antibody at six sites. One skin sample

was taken under ether anaesthesia at 2, 4, 8, 12, 24 and 48 hours after challenge. The tests at 8, 12 and 24 hours were repeated to show that the ether anaesthesia had not changed the eosinophilia in the animals from which samples were taken at intervals, as follows. Two animals were tested with 5 μg and two with 50 μg antibody at each time interval. Each animal was sensitized over the left scapula and the right femur, so that the response at two sites could be compared. The twelve animals sensitized at six or seven sites were challenged at 9 a.m. and twelve animals sensitized at two sites were challenged at 9 p.m.

In further tests, skin samples were examined for the relative numbers of neutrophils and eosinophils after sensitization and before challenge, and at intervals from 5 minutes to 2 hours after challenge.

Preparation and examination of skin samples

Thin pieces of full depth thickness skin were fixed in 10 per cent formol saline, embedded in wax and cut at 5 μm thickness. Three sections of each sample were stained with haematoxylin and chromatrope 2R, and one with Giemsa.

Further stains were used on sections from the samples taken at intervals after challenge (Fig. 1). Serial sections were stained selectively for eosinophils by Archer's (1963) modification of the peroxidase stain of Dominici, and haematoxylin with chromatrope 2R. Other sections were stained by May-Grunwald Giemsa and by Ehrlich's haematoxylin and eosin.

Sections were examined with a ×40 objective and ×8 eyepiece. Eosinophils in sixty-six fields were counted on each section in the following order, the connective tissue between the deep dermis and panniculus carnosus, the mid-dermis, and the upper dermis (ninty-nine fields were counted, but as the thirty-three fields of the epidermis so rarely contained eosinophils, these results are excluded). The results given are the average of sixty-six fields after counting at least three sections.

Mast cells were examined in skin fixed in Suza and stained by toluidine blue or Giemsa. Mast cells and eosinophils were also examined in the subcutaneous loose connective tissue, which was gently spread on coverslips, treated with 80 per cent and absolute alcohol, and cleared in xylol (Parish, 1964). These spreads were stained by Archer's peroxidase technique.

Antigen–antibody complexes attracting eosinophils in vitro

In tests, to be described in detail later, complexes of IgG1a and IgG2 antibody with antigen were tested for their ability to attract eosinophils in Boyden-type (1962) chambers of non-wettable plastic divided by Millipore membranes 8 μm pore size and 11 mm diameter.

Eosinophils were obtained from the blood of guinea-pigs primed by once weekly peritoneal injections of 1 ml diphtheria toxoid, and separated by the method of Day (1970) using a Triosil '75' (Glaxo Ltd) gradient, to give a suspension 97 per cent pure with <1 per cent taking up trypan blue. The upper chamber was filled with 2·5 ml of $1{\cdot}25 \times 10^5$ eosinophils/ml in Eagle's medium containing 10 per cent fresh guinea-pig serum. The immunoglobulins, at 50 μg/ml, with antigen at equivalence, were added to the Eagle's medium with 10 per cent fresh complement-containing serum, and 2·5 ml placed in the lower test chamber. Chambers were incubated at 37° for 3 hours, when the membranes were fixed, stained and examined by counting 100 fields with a ×40 objective.

RESULTS

ANAPHYLACTIC CUTANEOUS EOSINOPHILIA AFTER LOCAL PASSIVE SENSITIZATION

In skin passively sensitized for 4 hours and examined 18 hours after intravenous antigenic challenge, more eosinophils entered sites sensitized with specific IgG1 than entered sites injected with specific IgG2 or with IgG1 and IgG2 with different antigenic specificity (Table 1). The number of eosinophils entering the specific IgG1 sites is influenced by the number in the peripheral blood of the recipient at time of challenge and the amount of antibody injected. Eosinophils were selectively attracted to sites containing IgG1 when 0·5 or 5 μg antibody was tested on recipients having a maximum of 250 eosinophils/cu mm blood. Increasing the amount of antibody to 50 μg, or testing recipients with many haematogenous eosinophils, resulted in a slight to moderate increase of eosinophils at sites containing specific IgG2 compared with sites containing antibodies not specific for the test antigen (Table 1).

TABLE 1

NUMBER OF EOSINOPHILS IN SKIN INJECTED WITH IgG1a ANTI-PICRYL-CHLORIDE (CONTROL) AND WITH IgG1a ANTI-BSA (TEST), 18 HOURS AFTER I.V. CHALLENGE WITH BSA. EACH CONCENTRATION OF ANTIBODY WAS TESTED ON NINE GUINEA-PIGS IN THREE GROUPS OF THREE ACCORDING TO THE NUMBER OF EOSINOPHILS IN THE PERIPHERAL BLOOD

Antibody (μg)	Antibody class	Control or test	No. of eosinophils in skin sites Recipients eos./cu mm blood in range of		
			0–25	100–250	1125–2000
0·5	IgG1	Control	3	6	31
	IgG2	Control	1	6	29
	IgG1	Test	6	18	83
	IgG2	Test	1	5	44
5	IgG1	Control	3	13	54
	IgG2	Control	2	15	48
	IgG1	Test	16	82	652
	IgG2	Test	6	19	233
50	IgG1	Control	4	22	66
	IgG2	Control	4	19	71
	IgG1	Test	38	101	788
	IgG2	Test	10	64	594

Guinea-pigs passively sensitized for 4 hours before challenge.

ANAPHYLACTIC CUTANEOUS EOSINOPHILIA AFTER GENERALIZED PASSIVE SENSITIZATION

Local injection of antibody damages the tissue by trauma and by excessive deposition of globulin, attracting inflammatory cells and influencing the eosinophil response reported in Table 1. In order to examine eosinophilia in skin subjected to the minimum of trauma, recipients were sensitized by intravenous injection of IgG1a or IgG2, challenged by antigenic prick test 4 hours later, and the sites examined after 18 hours.

The results (Table 2) show that IgG1a sensitizes skin to the anaphylactic attraction of eosinophils, and, in contrast to the results obtained by local injection (Table 1), circulating IgG2 has no eosinophilotropic activity in undamaged skin, even in recipients containing many eosinophils in their blood.

Each animal was tested with IgG1a and with IgG2, though the antibodies had different specificities (Table 2). It made no difference which antibody was tested first;

TABLE 2

NUMBER OF EOSINOPHILS AT 18 HOURS BSA PRICK TEST CHALLENGE SITES IN GUINEA-PIGS RECEIVING IgG1a OR IgG2 ANTI-BSA BY INTRAVENOUS INJECTION. IN A REPEAT TEST 24 HOURS LATER, EACH ANIMAL RECEIVED IgG1a OR IgG2 ANTI-PICRYL-CHLORIDE AND WAS CHALLENGED WITH PICRYL-CHLORIDE GUINEA-PIG ALBUMIN

First test					Second test		
Blood eos. of recipients	No. of recipients	Class Ig.	Test antigen BSA	Control antigen EA	Class Ig.	Test antigen Pic. Cl.	Control antigen β-lact.
100–250	3	IgG1a	52	8	IgG2	6	5
	4	IgG2	2	4	IgG1a	88	4
1125–2000	3	IgG1a	166	22	IgG2	19	16
	4	IgG2	18	19	IgG1a	236	16

Guinea-pigs passively sensitized for 4 hours before challenge.
EA: egg albumin; β-lact: β-lactoglobulin; Pic Cl: picryl-chloride guinea-pig albumin.

eosinophils only entered sites of prick tests with the antigen to which the animal had been passively sensitized by IgG1. Increased vascular permeability at these sites was detected by exudation of Evans' blue dye injected intravenously just before the antigenic prick test.

There were fewer neutrophils relative to the number of eosinophils in prick test sites of animals sensitized by intravenous IgG1, than in animals sensitized by intracutaneous IgG1.

ANAPHYLACTIC CUTANEOUS EOSINOPHILIA AFTER LOCAL PASSIVE SENSITIZATION WITH REAGIN

Eosinophils infiltrated anaphylactic skin treated with unheated guinea-pig reaginic sera 14 or 28 days previously. No increase in the number of eosinophils occurred in skin treated with the same reaginic sera previously heated at 56° for 3 hours, when compared

TABLE 3

NUMBER OF EOSINOPHILS AND NEUTROPHILS INFILTRATING SKIN PASSIVELY SENSITIZED FOR 14 OR 28 DAYS WITH UNHEATED OR HEATED REAGINIC SERA, EXAMINED 18 HOURS AFTER INTRAVENOUS CHALLENGE

Test antigen	Pas. sens. challenge interval	Lesion diameter[a] and number of eosinophils and neutrophils[b] Unheated			Heated 56°, 3 hours		
		Les. diam.	Eos.	Neut.	Les. diam.	Eos.	Neut.
β-lact.	14 days	14 mm	38	20	0	2	9
Unchallenged	14 days	0	3	0	0	1	2
β-lact.	28 days	6 mm	12	11	0	0	2
Unchallenged	28 days	0	1	0	0	2	1
Egg albumin	14 days	17 mm	46	18	0	4	5
Unchallenged	14 days	0	4	0	0	2	0
Egg albumin	28 days	5 mm	9	2	0	4	3
Unchallenged	28 days	0	1	1	0	0	2

Three guinea-pigs examined at each time interval. Each was injected twice with 0·1 ml unheated or heated antiserum to one antigen. Results expressed as the mean of each group of three guinea-pigs.
[a] Diameter (mm) of Evans' blue on outer skin surface at 30 minutes after challenge.
[b] Number of eosinophils and neutrophils in skin samples 18 hours after challenge.

with skin of other animals treated with the same sera but not challenged with antigen (Table 3). The eosinophilia was related to the severity of the anaphylactic response, because more eosinophils infiltrated skin passively sensitized for 14, than for 28 days when the response was weak as judged by the faint colour and small diameter of the lesion. The antiserum to egg albumin, prepared with *Bordetella pertussis* as adjuvant, prepared skin for a slightly greater response and eosinophilia than the reaginic anti-β-lactoglobulin prepared with aluminium hydroxide adjuvant.

There were very few neutrophils in these samples of anaphylactic skin, compared with the neutrophilia occurring after passive sensitization with IgG1 (Fig. 1).

RELATION BETWEEN EOSINOPHILIA AND TIME

Skin samples taken at intervals after challenge showed that eosinophil infiltration started between the 4th to 8th hours, reaching the greatest number at 12 hours. Most of the eosinophils persisted until 24 hours, but thereafter the number was greatly reduced

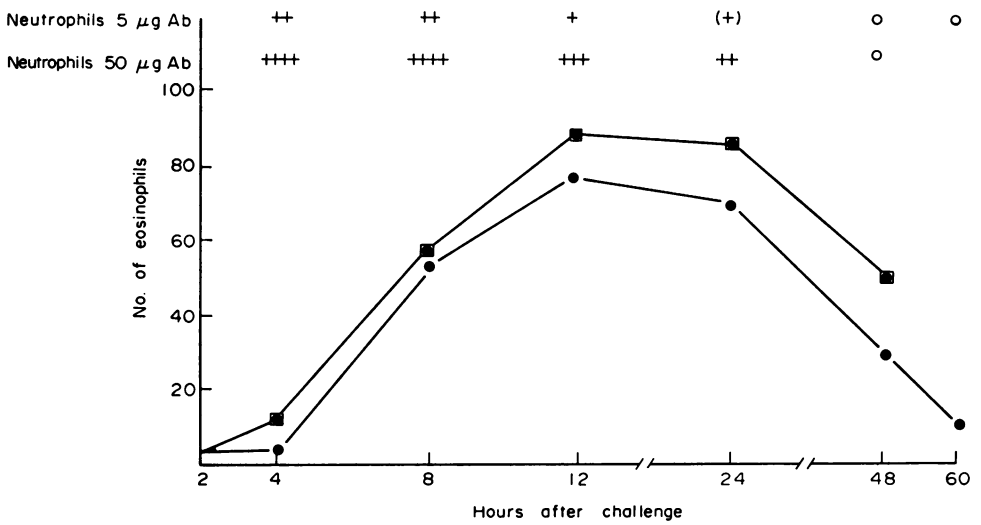

FIG. 1. Relation between the number of cutaneous eosinophils and neutrophils and time after challenge. Mean No. of eosinophils/sixty-six fields of sites sensitized with ●, 5 μg Ab and ■, 50 μg Ab. Assessment of neutrophilia per field, 0<6; + 6–20; + + 20–50; + + + 50–200; + + + + very many.

(Fig. 1). Increasing the concentration of passive sensitizing antibody from 5 μg to 50 μg made little difference to the rate of eosinophil infiltration and only a slight difference to the number, though it greatly influenced the number of neutrophils (Fig. 1).

The numbers of eosinophils in skin samples removed at intervals under anaesthesia from recipients sensitized at six or seven sites were very similar to those from recipients sensitized at two sites and killed at time of sampling, showing that anaesthesia did not influence the eosinophilia in recipients examined several times. Moreover, there was no significant difference between recipients challenged at 9 a.m. and at 9 p.m., so cyclical fluctuations in blood eosinophilia did not change the number entering the skin sites. The skin sample from the scapula of each animal tended to have 10 per cent more eosinophils than the skin from the thigh.

CUTANEOUS EOSINOPHILIA WITHOUT HAEMATOGENOUS EOSINOPHILIA

In order to see whether the infiltration of eosinophils at sites of cutaneous local anaphylaxis was accompanied by any increase in the number of eosinophils in the blood, four guinea-pigs passively sensitized intracutaneously with IgG2 and IgG1a for 4 hours were challenged intravenously, and examined for haematogenous eosinophilia every 2 hours for 18 hours when they were killed. Sections prepared from skin sites injected with IgG1a had an average of eighty eosinophils/sixty-six fields, and sites injected with IgG2 had an average of fourteen eosinophils. These results are similar to those reported in Table 1. Only one of the four test guinea-pigs had an increase in the number of eosinophils in the blood beyond the slight diurnal changes also observed in the four control animals not injected with antibody, yet in this animal the average of seventy-four eosinophils in the skin sites injected with IgG1a was less than the average for the group.

In a similar test in which four recipients were sensitized with reagin and were challenged on the 14th day, the average number of eosinophils at the IgG2 sites was six, and at the reagin sites was sixty-two. During the 18 hours post-challenge period the number of eosinophils in the blood of two of the test animals rose from 6 to 25 and from 10 to 51 cu mm, but the number of eosinophils at the reagin sites was intermediate between those of the other two animals with decreased numbers from 14 to 6 and 9 to 5 cu mm. Moreover, the increase in the number of blood eosinophils in the two animals could not be attributed to cutaneous anaphylaxis, because two of the four control unsensitized animals also had increased numbers of blood eosinophils, one from eight to twenty-one, the other from eight to thirty-three.

The increase in number of eosinophils at a site of local anaphylaxis appears to result from attraction of eosinophils already in the blood, and not result from a stimulus to their release from the bone marrow.

TABLE 4

NUMBER OF EOSINOPHILS INFILTRATING SKIN 18 HOURS AFTER INJECTION OF COMPLEXES OF ANTIGEN WITH IgG2 OR IgG1a ANTI-BSA INTO RECIPIENTS WITH FEW OR MODERATE NUMBERS OF EOSINOPHILS IN THE BLOOD

Complexes of:		No. of Eos. in skin of recipients with blood eos. of	
50 μg Ig-class	Antigen conc.	0–25	100–250
IgG1a	Equiv.	8	31
IgG1a	× 8 excess	6	44
IgG2	Equiv.	0	10
IgG2	× 8 excess	0	12
IgG1a	Nil	0	5
IgG2	Nil	2	4
Nil	× 8 excess	0	2
Nil	Nil	1	3

EOSINOPHILIA ELICITED BY COMPLEXES OF ANTIGEN WITH ANAPHYLACTIC ANTIBODY

Complexes of IgG1a with antigen at equivalence, or in eight times antigen excess of equivalence, elicited similar numbers of eosinophils 18 hours after intradermal injection;

the number of eosinophils infiltrating the skin sites was influenced by the number in the blood of the recipients (Table 4). Fewer eosinophils infiltrated skin injected with complexes containing 50 μg antibody (Table 4) than after passive cutaneous anaphylaxis with the same amount of the same antibody (Table 1). Complexes containing IgG2 attracted fewer eosinophils than complexes containing IgG1a in recipients having 100–250 eosinophils/cu mm blood.

There were many more neutrophils and mononuclear cells after injection of complexes than after the PCA reaction, and mild Arthus-type reactions with thrombosis and venular degeneration occurred at sites of complexes containing each antibody.

RELATION BETWEEN EOSINOPHILIA AND NEUTROPHILIA

Eosinophil infiltration occurs in skin about 4 hours after anaphylactic challenge and is greatest at 12 to 24 hours (Fig. 1). There is no, or only sparse eosinophilia at sites of

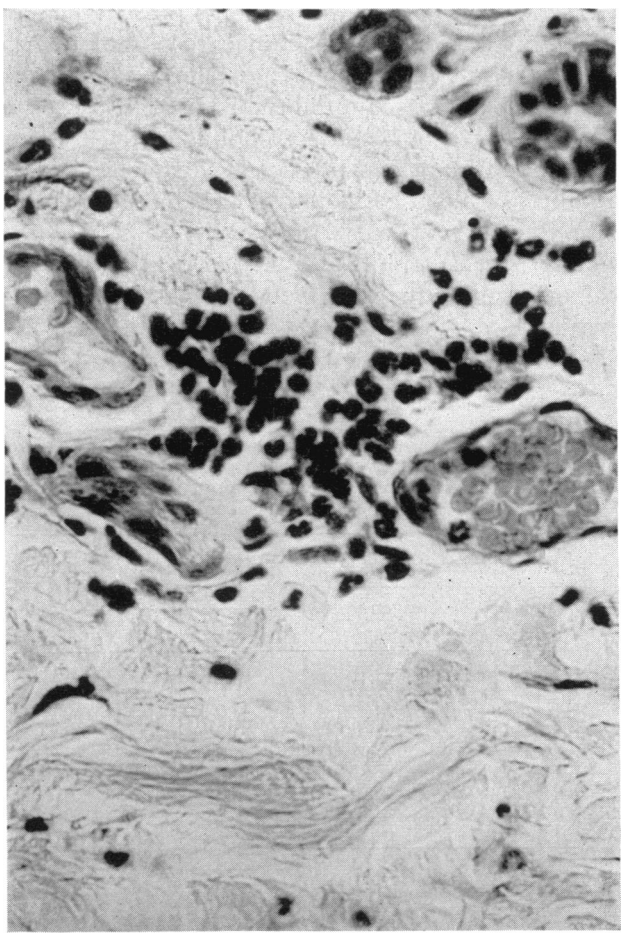

FIG. 2. PCA mediated with 5 μg IgG1, 4 hours after challenge with BSA. Eosinophils (densely staining) in dermal perivenular connective tissue. (Dominici, ×420.) (Figs 2 to 4 were selected from recipients with 640 and 720 eosinophils/cu mm blood. The tissue eosinophils are therefore abnormally numerous.)

IgG1 or IgG2 without anaphylaxis (Table 1). Neutrophil infiltration, however, starts within an hour of cutaneous injection of antibody and increases until the 4th hour, when intravenous challenge is usually made. The supervening anaphylactic changes are adherence of more leucocytes to the endothelium, venular endothelial degeneration, and oedema, followed by leucocyte emigration. Thus the anaphylactic emigration of neutrophils and mononuclear cells is super-imposed upon the leucocyte emigration induced by the injection of the serum, and accounts for the large number of neutrophils in lesions before the start of the eosinophil infiltration (Fig. 1).

The number of neutrophils per field 8 hours after challenge, at sites sensitized with 5 μg antibody was 20 to 50, and with 50 μg they were too numerous to count, whereas the number of eosinophils was fifty-three and fifty-seven/sixty-six fields respectively. Thus the ten-fold increase in the concentration of the antibody greatly influenced the number of neutrophils but not that of the eosinophils.

FIG. 3. PCA, as Fig. 2, 8 hours after challenge. Neutrophils infiltrate the intervascular connective tissue while most of the eosinophils surround venules, especially in the deep dermis. (Giemsa, × 80.)

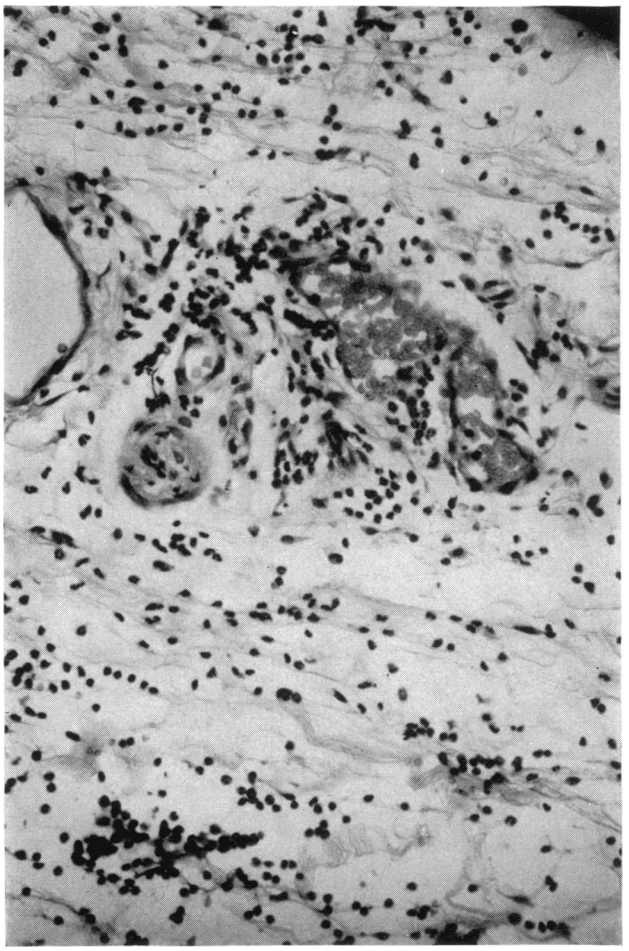

FIG. 4. PCA, as Fig. 2, 18 hours after challenge. Eosinophils diffusely infiltrate the intervascular tissue. Compare with Fig. 2. (Dominici × 180.)

TABLE 5

SUMMARY OF THE NUMBER OF EOSINOPHILS ATTRACTED TO ANTIGEN–ANTIBODY COMPLEXES IN BOYDEN-TYPE CHAMBERS

Test substance in medium with complement	Eosinophils 100 fields
Complexes with IgG2 and IgG1a in whole serum	244
Complexes with IgG2	298
Complexes with IgG1a	68
Complexes with IgG1b	51
Antigen with 10 per cent normal serum	8
10 per cent normal serum	10
Crushed guinea-pig neutrophils	271

Complexes were formed of guinea-pig anti-picryl-chloride antibodies with picryl-chloride guinea-pig albumin.

Four hours after challenge, eosinophils are partly obscured by the neutrophils which also surround venules in the subdermal connective tissue, and, less intensely, in the mid-dermis. They are best seen in sections stained with chromatrope 2R or Dominici (Fig. 2). At 8 hours neutrophils emigrate further from the vessels than the eosinophils thereby increasing the proportion of perivascular eosinophils (Fig. 3). At 12 to 24 hours the eosinophils infiltrate the intervascular tissue (Fig. 4) while some of the neutrophils already there degenerate, being identified only by the nuclear debris. It is possible that the degenerate neutrophils participate in attracting the emigrating eosinophils, because crushed neutrophils strongly attract eosinophils *in vitro* (Table 5). Changes also occur in the eosinophils; they are stained less readily by the Peroxidase technique than they were at 4 to 8 hours, and some shed intact granules which either lie free or adhere to the surface of macrophages.

In guinea-pigs passively sensitized for 24 hours before challenge, few of the neutrophils elicited by injection of IgG1a remain at the site. This avoids confusion with the leucocytosis occurring in anaphylaxis. In sections of these lesions taken at intervals after challenge, leucocytes adhere to the venular endothelium within 5 minutes of challenge, and neutrophils with a few monocytes emigrate within 1 hour. Eosinophilia starts at 4 hours when the venules are surrounded by the mononuclear cells and some neutrophils, while other neutrophils infiltrate the intervascular connective tissue.

At sites of sensitization with reaginic antibody for 28 days (Table 3) eosinophils occur around and between the blood vessels at 18 hours, with fewer neutrophils than are present 18 hours after challenge of animals sensitized with IgG1a for 4 or for 24 hours.

RELATION BETWEEN EOSINOPHILS AND MAST CELLS

Injection of serum into the skin causes mast cells at the site to release a little diffuse material staining blue with toluidine blue or Giemsa, or shed occasional intact granules. The proportion of changed to normal cells in the dermis is about 1:12 and in the subcutaneous connective tissue 1:18. On intravenous anaphylactic challenge there is no consistent change at the site of passive sensitization with IgG1a or b for 30 minutes to 1 hour. After this interval one in three mast cells in the dermis releases homogeneous metachromatic material, and by 18 hours mast cell changes in the dermis may be so complete as to be detected more by their absence than by identifying changed cells. The eosinophils infiltrating the mid-dermis at 4–8 hours do not appear to be attracted to the mast cells, though as they emigrate they pass close to the perivascular mast cells.

In the subcutaneous tissue spreads, changes occur 30 minutes after anaphylactic challenge. Nearly all the mast cells become changed, particularly when challenge follows 24 hours passive sensitization. They enlarge, become round instead of elongated, with homogeneous cytoplasm containing a few intact granules staining pale purple or pink instead of dark blue. Some of the homogeneous material leaks from the cell.

Eosinophil infiltration in the subcutaneous tissue starts much earlier than in the dermis, at about 30 minutes after challenge, just when the mast cell changes occur. The first eosinophils to infiltrate the tissue are attracted to the mast cells, so that one to three eosinophils surround them, lying near to or over the mast cell and occasionally appear to enter it, though this is difficult to distinguish from overlying (Fig. 5). A few eosinophils shed some of their granules and have small vacuoles in their cytoplasm. By 4 hours after challenge, though some eosinophils remain close to mast cells, the majority are more diffusely dispersed in the tissue.

EOSINOPHIL CHEMOTAXIS *in vitro*

Preliminary tests with 97 per cent pure suspensions of guinea-pig eosinophils in Boyden-type chambers show that they are much more strongly attracted to complexes containing IgG2 antibody in the presence of 10 per cent fresh complement-containing serum, than to an equal concentration of complexes containing IgG1a or b (Table 5). This is the reverse of the results obtained with these antisera in tissues, where IgG1 was eosinophilotropic and IgG2 was not, unless the skin was damaged.

Eosinophils were also strongly attracted to crushed neutrophils in 10 per cent normal serum, indicating that the prior emigration of neutrophils into damaged tissue may also participate in the later attraction of eosinophils.

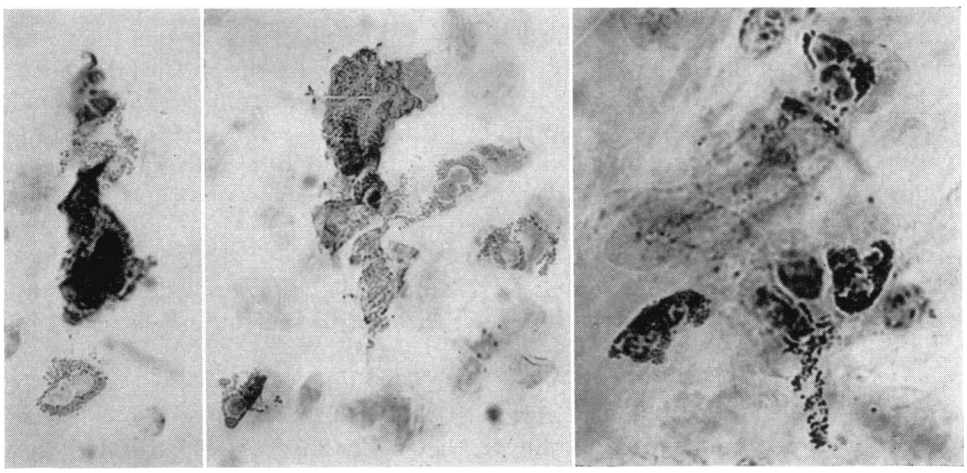

FIG. 5. Three fields of subcutaneous tissue at PCA sites. Two at 20 minutes and one at 30 minutes after intravenous challenge. At 20 minutes the mast cells are enlarged and homogeneous blue or metachromatic material hides the granules and begins to leave the cell. At 30 minutes nearly all the mast cell substance is released. Eosinophils adhere to the mast cells, usually shedding granules and becoming vacuolated. Occasional vacuoles contain metachromatic ? mast cell substance. (Peroxidase—Giemsa. × 1800.)

DISCUSSION

Three findings in this study are adduced. Eosinophils are attracted to anaphylactic skin which may contain a selective chemotropic substance. Eosinophilia in anaphylactic skin probably results from an accumulation of eosinophils already in the blood, without any additional release of cells from the bone marrow. Eosinophils, whatever special functions they have evolved, are still phagocytes and resemble neutrophils in their response to antigen–antibody complexes and some other substances.

Eosinophilia in anaphylactic skin

Eosinophils are attracted to anaphylactic skin passively sensitized with IgG1 or with reagin. No such attraction occurs in skin treated with IgG2, or IgG1 with a different antigenic specificity, unless relatively large amounts of these globulins are injected, or the recipients have many circulating eosinophils (Table 1). Even then, fewer eosinophils infiltrate sites containing these globulins than sites of anaphylaxis. The failure of IgG2 to mediate cutaneous eosinophilia if trauma caused by the local injection is avoided, is

shown by the results of antigenic prick tests in animals passively sensitized intravenously (Table 2). The eosinophilotropism of anaphylactic tissue is more evident in skin treated with reagin, for the eosinophilia is not accompanied by the numerous neutrophils occurring in tests with IgG1.

As treatment of skin with IgG2, or injection of complexes containing IgG2 does not attract eosinophils, though the skin is infiltrated by many neutrophils, it is very probable that an eosinophilotropic substance accumulates or is released from anaphylactic tissue. It is tempting to suggest that this substance is associated with mast cells, which in guinea-pig anaphylaxis usually release homogeneous cytoplasmic material (Mota and Vugman, 1956), or become enlarged and vacuolated, and occasionally shed intact granules (Parish, 1964). A relation between mast cells and eosinophils has long been considered. Riley (1956) observed large numbers of eosinophils in tissues rich in mast cells after their histamine had been released chemically. Eosinophils ingest granules released from rat mast cells (Welsh and Greer, 1959; Mann, 1969) and apparently ingest substances from guinea-pig anaphylactic mast cells (Parish, 1970a). It was also found (Parish, 1970a) that after anaphylaxis, eosinophils accumulate in guinea-pig tissues containing many mast cells and sometimes clustered round changed mast cells in the mesentery in a manner similar to that occurring in the subcutaneous tissue, as described above (Fig. 5).

The mast cell origin of the eosinophilotropic substance is not confirmed by all the evidence. Rat mast cells disrupted mechanically or anaphylactically, when injected into normal rats elicit a moderate eosinophilia, which is less than that occurring in anaphylactic rat tissues (Jeffery and Parish, unpublished). Guinea-pig mesenteries actively or passively sensitized, show no mast cell changes when incubated with antigen *in vitro*, though changes occur after *in vivo* challenge (Parish, unpublished). It is conceivable that mast cell changes may sometimes occur as a result of eosinophil or other leucocyte infiltration, rather than be the cause of the infiltration.

Therefore, though a relation certainly exists between anaphylactic mast cells and eosinophils, there is no definite evidence that the eosinophilotropic agent is a mast cell substance.

Local without haematogenous eosinophilia

The eosinophilia at local anaphylactic skin sites probably results from the accumulation of eosinophils randomly infiltrating the site from those already in the blood at time of challenge.

No increase was seen in the number of circulating eosinophils in the 18 hours between challenge and sampling the skin; the number of eosinophils infiltrating anaphylactic skin sites of animals treated with the same amount of antibody was related to the number in the blood when challenged. The rate at which eosinophils infiltrated anaphylactic sites is almost linear up to 12 hours when the chemotropic activity is dissipated (Fig. 1) which is also consistent with local attraction.

Thus, any eosinophilotropic substance in skin tests exerts only local activity, in contrast to peritoneal or intravenous challenge which elicits a generalized eosinophilia (Parish, 1970a, 1972). Similarly Eidinger, Raff and Rose (1962) and Fowler and Lowell (1966) found that accumulation of eosinophils at skin test sites of actively or passively sensitized persons was not accompanied by increased numbers in the blood, and Samter (1970) observed pulmonary eosinophilia in the absence of haematogenous eosinophilia in guinea-pigs.

Similarities in behaviour of eosinophils and neutrophils

In all the skin samples, except those treated with reagin, neutrophils infiltrated the tissue more quickly and in greater numbers than eosinophils. This even occurred in skin injected with IgG1, when challenge was delayed for 24 hours so that neutrophils attracted by the globulin and trauma had gone before the anaphylactic response. In suitably stained skin sections, occasional eosinophils are to be found among the many neutrophils adhering to the venular endothelium and emigrating at 5, 15 and 30 minutes after challenge. There are a few more at 2 and 4 hours though it is often difficult to detect them. Eosinophilia is apparent at 8 to 12 hours when many neutrophils leave the immediate perivascular tissue. This accounts for the finding of 'few eosinophils' in guinea-pig anaphylactic skin examined 2 hours after challenge by Lieberman and Ovary (1968).

It may be misleading to state that eosinophils are selectively attracted to anaphylactic tissue. When there are many eosinophils in the blood, they are found with the neutrophils in skin injected with complexes containing non-anaphylactic IgG2 (Table 4). In this instance, skin damaged by injection appears to function like the membrane in a Boyden chemotaxis chamber, in which eosinophils are strongly attracted to complexes containing IgG2.

It may be more accurate to consider that eosinophils are retained in anaphylactic tissues rather than selectively attracted to them. Eosinophils and neutrophils probably infiltrate anaphylactic skin in the relative proportions in which they are present in the blood. Some substances in the anaphylactic tissue retains the eosinophils which continue to accumulate while the neutrophils migrate away. If there is no anaphylactic substance the eosinophils migrate with the neutrophils. This phenomenon was seen in guinea-pig mesentery treated with histamine and examined continuously by phase contrast illumination; the neutrophils emigrated more quickly than the eosinophils from the vessel wall (Parish, 1970a).

In cutaneous anaphylaxis mediated by reagin, the eosinophils are more numerous than the neutrophils (Table 3) in contrast to the very high proportion of neutrophils in anaphylactic skin prepared with IgG1 (Fig. 1). This may be due in part to the 14-day interval between sensitization with reagin and challenge, during which the skin heals and excess serum proteins are removed, not occurring in the 4 or 24-hour interval between sensitization with IgG1 and challenge. It is also probable that reagin being an extremely potent anaphylactic sensitizing antibody may generate more of the hypothetical substance retaining eosinophils on challenge. Eosinophils are the predominant cells in reagin-mediated responses in the skin of man as well as in guinea-pigs. Cellular exudates sampled on glass slides applied to antigen challenged skin of allergic persons sometimes had 60 per cent eosinophils (Eidinger *et al.*, 1962; Eidinger, Wilkinson and Rose, 1964; Fowler and Lowell, 1966), and transfer of cutaneous sensitivity to normal persons by the heat-labile antibody, elicited on challenge more eosinophils than the control sites, or in one person an infiltration greater than found in some actively sensitized persons (Eidinger *et al.*, 1962). Guinea-pigs also vary in susceptibility to anaphylactic eosinophilia (Parish, 1972).

The tests in the Boyden chambers are to be described separately. However complexes containing IgG2 attracted eosinophils strongly, whereas those containing IgG1 did not. Guinea-pig neutrophils were also attracted strongly to complexes containing γ2 anti-DNP but not to those containing γ1 (Keller, Nussenzweig and Sorkin, 1968). The leucocyte chemotropic activity of IgG2 complexes may be due to the greater ability of this antibody

to fix complement than IgG1 (Bloch, Kourilsky, Ovary and Benacerraf, 1963) because one substance known to attract eosinophils and neutrophils *in vitro* is the trimolecular complement complex C ($\overline{567}$) generated in complement containing serum by antigen–antibody complexes (Ward, 1969; Lachmann, Kay and Thompson, 1970).

In these *in vitro* tests eosinophils are attracted to the same complexes that attract neutrophils, though in the *in vivo* tests, anaphylaxis mediated by IgG1 elicits eosinophilia.

It is concluded therefore, that eosinophils are attracted to inflammation by some forces that attract neutrophils, and the cells infiltrate the tissue in numbers relative to their numbers in the blood. If the inflammatory change is anaphylactic, or possibly anaphylactoid, a substance is formed or released that retains the eosinophils, which accumulate at the site, while the neutrophils are dispersed. Haematogenous eosinophilia, which follows selective release of eosinophils from the bone marrow, as occurred in the previous experiments (Parish, 1972) may result from an excess of the anaphylactic substance, or another substance, in the blood, or as a result of an overstimulation to replace the eosinophils that infiltrated the tissues.

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