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STUDIES ON EOSINOPHIL LEUCOCYTE MIGRATION

I. EOSINOPHIL AND NEUTROPHIL ACCUMULATION FOLLOWING ANTIGEN–ANTIBODY REACTIONS IN GUINEA-PIG SKIN

A. B. KAY

Department of Pathology, University of Cambridge

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SUMMARY

Eosinophil leucocytes migrated into the site of PCA reactions mediated by those fractions of 7S guinea-pig IgG containing IgG_1; neutrophils were associated with IgG_1 and IgG_2. Maximal eosinophil infiltration is seen at 12 hr and was associated with degranulation.

Intradermal histamine was not eosinophilotactic in guinea-pigs.

Preformed antigen–antibody complexes of IgG_1 and IgG_2 both promoted eosinophil and neutrophil migration in guinea-pig skin but slightly more eosinophils were seen following injections of complexes containing IgG_1. Local eosinophilia and PCA activity were mediated by a relatively heat-stable element since these effects were demonstrable even after prolonged heating of fractions containing IgG_1. Eosinophils were seen following injections of Compound 48/80 and this was accompanied by low mast cell counts; however, there was also some associated tissue destruction.

INTRODUCTION

Eosinophils are associated with certain 'allergic' tissue reactions particularly immediate-type (Type 1) hypersensitivity. Various mediators of an eosinophilia have been proposed including histamine (Kline, Cohen & Rudolph, 1932; Archer, 1963), antigen–antibody complexes (Litt, 1964) and sensitized tissue treated with antigen (Samter, Kofoed & Piper, 1953; Parish & Coombs, 1968).

Experiments *in vitro* using Boyden's millipore technique indicate that the eosinophil and neutrophil chemotaxis generated by antigen–antibody complexes require the presence of fresh serum and that the chemotactic activity is probably mediated by intermediate complement components or their fragments (Keller & Sorkin, 1969; Ward, 1969).

In the present study an initial experiment showed that following passive cutaneous anaphylaxis (PCA) reactions in the guinea-pig eosinophils and neutrophils accumulated

Correspondence: Dr A. B. Kay, Robert Brigham Hospital, Parker Hill Avenue, Boston, Massachusetts 02120, U.S.A.

in large numbers around the reaction site 24 hr following challenge with antigen. Investigations were, therefore, carried out to determine the relationship of eosinophil to neutrophil migration following PCA reactions using fractions of 7S guinea-pig immunoglobulins. In further experiments the eosinophilia was specifically studied in order to determine more precisely the mechanism of eosinophilotaxis.

MATERIALS AND METHODS

Animals

Male albino guinea-pigs weighing between 200 and 400 g were used throughout this study.

Guinea-pig antisera

Antisera to ferritin (Koch-Light) and bovine serum albumin (BSA, Armour Laboratories) were raised separately in two sets of eight guinea-pigs according to the following schedule:

Day 1. One hundred micrograms of the antigen was given in Freund's complete adjuvant to each hind footpad.

Day 14. One hundred micrograms of antigen in Freund's incomplete adjuvant was given in two subcutaneous areas in the neck.

Day 28. One hundred micrograms of antigen was introduced intradermally into two areas of the back. This process was repeated at weekly intervals until the development of a strong

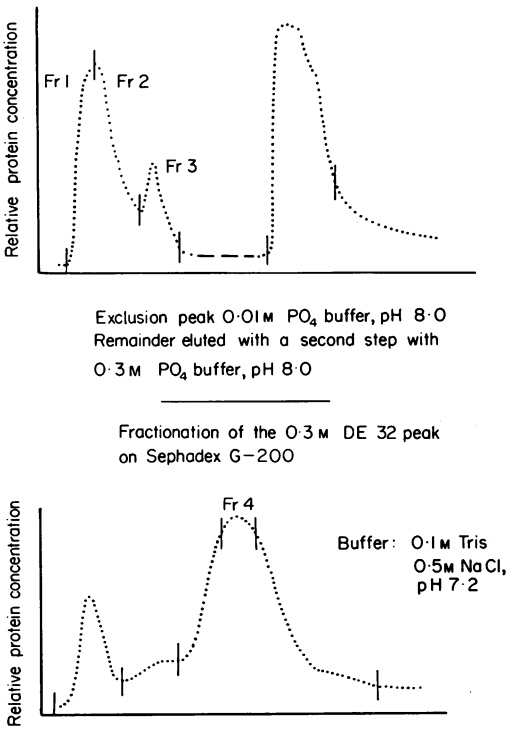

FIG. 1. Chromatographic fractionation of guinea-pig anti-ferritin. Virtually identical patterns were obtained with antiserum to BSA.

Arthus reaction. The course was usually complete within 6–8 weeks. The animals were bled 1 week after the last injection and the anti-ferritin and anti-BSA antisera were pooled separately.

Preparation of guinea-pig IgG_1 and IgG_2 immunoglobulins

The procedures followed were essentially those of Forsgren (1968). Sixty millilitres of guinea-pig anti-ferritin was precipitated with 33% saturated ammonium sulphate and the precipitate was redissolved in distilled water. The process was repeated four times and the final precipitate was redissolved in 10 ml of water and dialysed against 0·01 M phosphate buffer at pH 8·0. The sample was applied to a column of DEAE-cellulose (DE 32—Whatman) 3·5 × 26·5 cm, which had been previously equilibrated with 0·01 M phosphate buffer, pH 8. A second small peak followed the main exclusion peak (Fig. 1). The rest of the sample was eluted with a 0·3 M phosphate buffer, pH 8. The fractions were pooled as indicated in Fig. 1 and concentrated using a UM 1 Amicon-Diaflow membrane. The 0·3 M peak was then applied to a column of Sephadex G-200 (3·5 × 95 cm) in order to eliminate IgM and to further purify IgG_1. The purity of the fractions was tested using gel-diffusion and immunoelectrophoresis as described below. The guinea-pig anti-BSA sera gave virtually similar chromatographic patterns as the anti-ferritin sera (Fig. 1).

Preparation of anti-sera to guinea-pig immunoglobulins

(1) *Rabbit anti-guinea-pig 7S IgG.* The second Sephadex G-200 peak [Fraction (Fr) 4] of the guinea-pig anti-BSA was precipitated at equivalence with the antigen and the precipitate was washed eight times in 0·01 M EDTA–saline at +4°C. Half a milligram of the washed

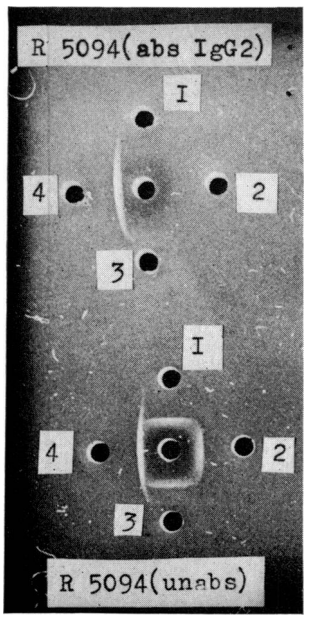

FIG. 2. Gel-diffusion studies using fractions of guinea-pig anti-ferritin. 1, 2, 3 and 4 correspond to Fr 1, 2, 3 and 4, respectively. The R5094 (abs IgG_2) gives an antisera specific for IgG_1. Similar results were obtained with R5095.

precipitate was suspended in 1 ml of saline and emulsified with 1 ml of FCA and injected intramuscularly into rabbits (R5048 and R5049). The injections were repeated 14 days later and the animals were subsequently given 0·25 mg of the precipitate intravenously in saline at weekly intervals for 2 weeks following the second injection. The animals were bled 1 week following the last injection.

(2) *Specific anti-guinea-pig IgG$_1$.* The ascending part of the DEAE exclusion peak of the guinea-pig anti-BSA (Fr 1) which gave a single line on gel diffusion and a single γ_2 line on immunoelectrophoresis was polymerized with ethyl chloroformate for use as an immunoabsorbent as described by Avrameas & Ternynck (1967). The rabbit anti-IgG$_1$, also containing IgG$_2$ (R5048 and R5049), was absorbed with the polymerized IgG$_2$ (Fr 1) to give an antisera specific for IgG$_1$. The results of the gel-diffusion studies are shown in Fig. 2. Fr 1 contains only IgG$_2$ and gives a line of identity with the other fractions. Fr 4, therefore, contains some IgG$_2$ in addition to the spuring IgG$_1$. The late peak of IgG$_2$ (Fr 3) shows bending of the IgG$_1$ line and therefore contains a little IgG$_1$.

Preparation of antigen–antibody complexes

Samples of anti-ferritin Fr 1 and Fr 4 (IgG$_2$ and IgG$_1$) were precipitated at equivalence with ferritin following a standard optimal proportions titration. The complexes were washed four times in 0·01 M EDTA–saline and resuspended in saline. The protein content of the final suspension was measured by the Folin method.

Passive cutaneous anaphylaxis

The recommendations of Brocklehurst (1967) were observed in all PCA reactions with the exception that 0·1-ml volumes of fractions were injected intradermally under sterile conditions. Following sensitization 1 ml of 0·25% Evans Blue containing 1 mg of ferritin was injected intravenously.

Quantitation of eosinophil and neutrophil infiltration following PCA reactions

At intervals following challenge with antigen and dye the animals were killed and the injection sites, previously marked, were immediately biopsied using a circular punch of 1 cm diameter. The biopsy included all layers of the skin including the panniculus carnosus muscle. Following fixation in formal saline, or Susa's medium in the case of mast cell experiments, sections were taken from the site and approximately 2 mm either side of the intradermal injection. The sections were wax embedded, cut and stained in the usual way using haematoxylin and chromotrope 2R. Chromotrope 2R was found to be a satisfactory stain for eosinophils as background staining was minimized. For mast cell experiments aqueous toluidine blue was used. From each section a total of fifteen random high power fields were counted in 0·3-mm strips between the junction of the epidermis and dermis and the upper limit of the panniculus carnosus using a previously calibrated graticule and the high power ($\times 40$) objective (Fig. 5). The cell count is expressed as a total of fifteen strips which represents the mean of the three sections counted, i.e. forty-five strips. Preliminary experiments showed that sections from the anterior abdominal wall were unsatisfactory due to difficulties in obtaining complete sections of the entire skin thickness. The biopsy studies reported below were prepared from multiple, well spaced sites on the back which reached laterally to the mid axillary line. Biopsies from these areas gave the most satisfactory results.

RESULTS

The association of eosinophil and neutrophil infiltration with passive cutaneous anaphylaxis

An initial experiment showed that large numbers of eosinophils and neutrophils infiltrated into the site of PCA reactions when the skin was passively sensitized with a dilution of 1:50 of the unfractionated guinea-pig antiserum to ferritin. Skin injected with saline or dye alone and the uninjected sites all showed minimal cellular infiltration.

Isolated fractions of 7S IgG showed that PCA reactions were only associated with those fractions containing IgG_1 (Fr 3 and 4), but not with Fr 1 and 2 which by gel diffusion and immunoelectrophoresis had been shown to contain IgG_2 only. Biopsies of PCA reactions using isolated fractions of IgG (Fr 1, 3 and 4) taken 24 hr after antigen challenge showed that eosinophil infiltration is only associated with those fractions containing IgG_1 and giving positive blueing reactions (Fig. 3). A substantial skin eosinophilia was noted with a

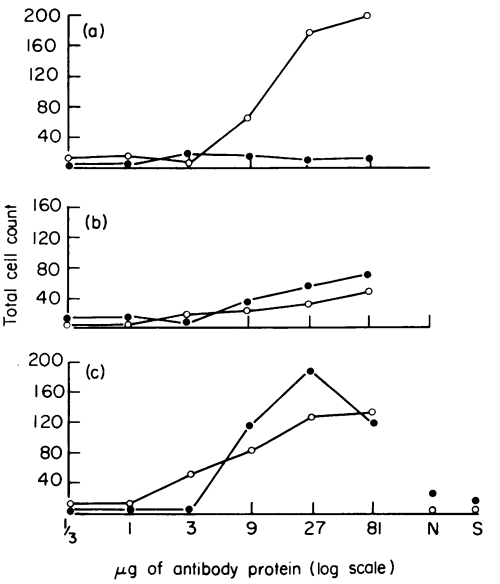

FIG. 3. Eosinophil (●) and neutrophil (○) accumulation into guinea-pig skin following passive cutaneous anaphylaxis with isolated fractions of guinea-pig IgG anti-ferritin. IgG_2 (a), IgG_2 (late peak) (b) and IgG_1 (c) are fractions 1, 3 and 4, respectively. Four hours sensitization period. Biopsies taken 24 hr following antigen challenge. N, Normal skin; S, saline.

dose as low as 9 μg of antibody protein. A few eosinophils were seen in normal skin but neutrophils were almost always entirely absent. Neutrophils appear in large numbers following treatment with IgG_1 or IgG_2 in doses as low as 9 μg of antibody protein. The results in Fig. 3 represent the counts from one animal given randomly spaced injections of the various dilutions of antibody protein. Normal skin and saline injections represent controls in the same animal implying that the migration of eosinophils and neutrophils is directional to the reactive sites. The experiment in this form was repeated once and gave essentially similar results. In further investigations a dose of 27 μg of antibody protein was chosen for fractions 1 and 4, respectively, and as seen from Figs. 4 and 7 and Table 1, the

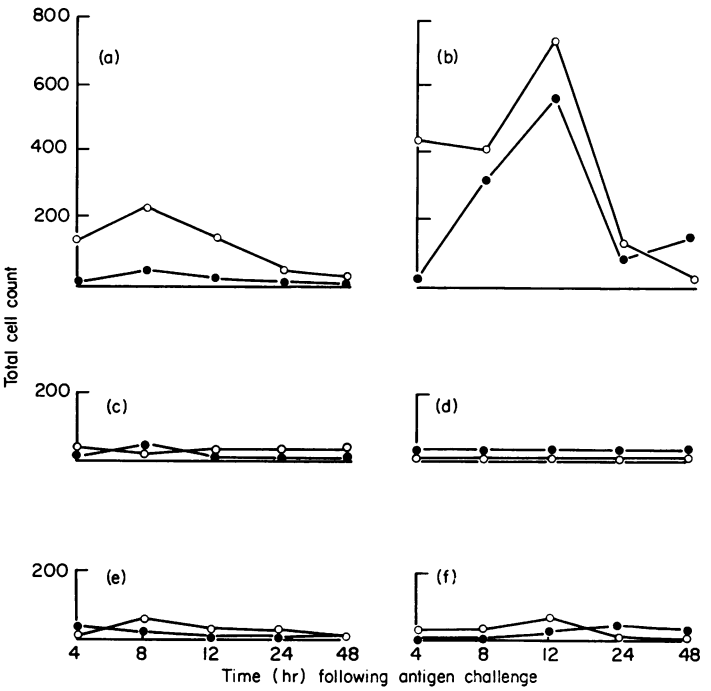

FIG. 4. The time course of eosinophil (●) and neutrophil (○) accumulation into guinea-pig skin following passive cutaneous anaphylaxis and intradermal histamine. Four hours sensitization. (a) IgG_2, (b) IgG_1, (c) saline, (d) uninjected sites, (e) 1 μg histamine, (f) 10 μg histamine.

eosinophil infiltration was associated with IgG_1 containing fractions and not with IgG_2 and so in effect the main observation of this first experiment was confirmed several times.

The time course of eosinophil and neutrophil in filtration into the skin

In initial experiments a time of 24 hr following antigen challenge was arbitrarily chosen for measuring cellular infiltration. Using a dose of 27 μg of antibody protein pairs of animals were treated with Fr 1, Fr 4 and histamine (Fig. 4). Each pair of animals was killed

TABLE 1. Eosinophil and mast cell counts following PCA reactions and intradermal injections of Compound 48/80

	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5	
	E	M	E	M	E	M	E	M	E	M
IgG_1 (Fr 4)	909	0	557	5	258	4	157	0	350	0
IgG_2 (Fr 1)	9	46	2	51	ND	ND	ND	ND	ND	ND
Normal skin	0	39	0	75	24	171	5	99	1	89
Compound 48/80	18	57	75	51	199	2	133	27	40	103

E, Eosinophils; M, mast cells; ND, not done.
Compound 48/80, 10 μg, Experiments 1 and 2; 100 μg, Experiments 3, 4 and 5.

at 4, 8, 12, 24 and 48 hr and the injection sites biopsied as previously described. With IgG_2 (Fr 1) minimal neutrophil infiltration was seen at 8 hr but at this time very few eosinophils were noted. Treatment with IgG_1 (Fr 4) showed that substantial eosinophilia was seen at 8 hr and a considerable eosinophil count was noted at 12 hr. The eosinophil count with IgG_1 roughly paralleled the neutrophil count with the exception that neutrophils appeared earlier than eosinophils. At 48 hr eosinophils were still plentiful but few neutrophils were seen. Areas injected with saline and the uninjected sites were virtually free from eosinophils or neutrophils.

The effect of histamine

Fig. 4 shows that in addition to the study of the cellular events following PCA reactions with IgG_1 and IgG_2 the effect of intradermal histamine was also investigated. The doses of

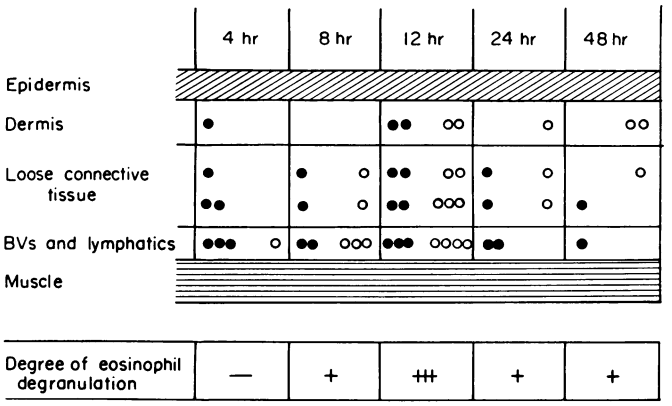

FIG. 5. A diagrammatic representation of the distribution of neutrophils (●) and eosinophils (○) following passive cutaneous anaphylaxis with IgG_1 (Fr 4).

histamine acid phosphate (British Drug Houses) was expressed as the weight of histamine base and was injected in saline at neutral pH. With 1 μg a slight neutrophilia was seen at 8 hr and similarly at 8 and 12 hr when 10 μg of histamine was injected. The number of eosinophils observed following the injection of 1 μg and 10 μg of histamine at intervals over 48 hr was no more than that seen when normal skin was biopsied. The area of blueing noted with 10 μg of histamine was slightly greater than that given by 27 μg of IgG_1.

The morphology and distribution of eosinophils and neutrophils following PCA reactions with IgG_1

At 4 hr a few eosinophils were seen in and around the small blood vessels immediately above the panniculus carnosus (Fig. 5). Considerably more were seen in this area at 8 hr, at which time some had migrated into the loose connective tissue below the dermis. At 12 hr eosinophils were seen in large numbers throughout all layers of the skin. Some were seen gathered in clusters around small vessels or randomly scattered in tissue spaces. Many of the eosinophils, especially those in large accumulations of the cell, showed degranulation with scattering of the granules for some distance around the intact nucleus. The significance

of this observation is discussed later. By 48 hr the eosinophils were confined to the dermis and upper part of the loose connective tissue whilst the remaining neutrophils were seen only in the lower layers of the skin. Neutrophils were seen in fairly large numbers throughout all layers at 4 hr although they were observed in greater numbers in the lower layers of the skin (Fig. 5). At 8 hr the number of neutrophils appeared slightly fewer than at 4 hr but the maximum number were noted at 12 hr when they were observed throughout the area of the skin counted. The neutrophils were more difficult to count, especially when present in large numbers. They often appeared in 'seams' or 'clusters' and many had pyknotic nuclei.

It should be noted that the most numerous 'loose' cells were the tissue macrophages or histiocytes. These cells were far too numerous to count but it appeared that they were more plentiful when the skin was infiltrated with eosinophils and neutrophils.

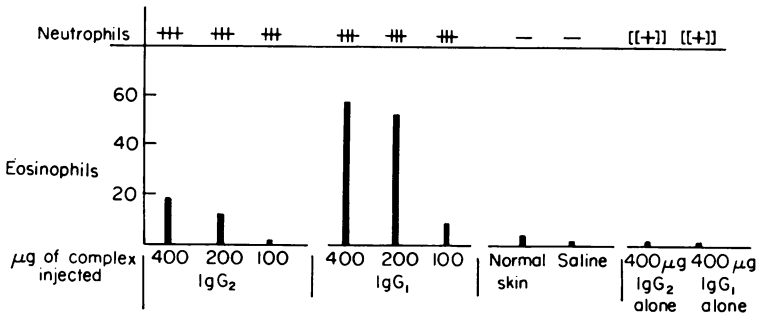

FIG. 6. Eosinophil and neutrophil accumulation into guinea-pig skin 12 hr following intradermal injections of preformed antigen–antibody complexes.

Eosinophil and neutrophil accumulation following the intradermal injection of antigen–antibody complexes

The previous experiments have shown that eosinophilia is associated with PCA reactions mediated by fractions containing IgG_1. Since histamine alone did not appear to generate this eosinophilia other possible mechanisms of eosinophilotaxis were investigated. The products of mast cell degranulation other than histamine may be chemotactic for eosinophils or alternatively IgG_1 and its antigen in a tissue bound or free form may generate such a chemotactic agent. In order to test the latter hypothesis preformed complexes of IgG_1 or IgG_2 and antigen were injected intradermally in amounts indicated in Fig. 6. Biopsies taken 12 hr later showed that with all doses of complexes the amount of neutrophil infiltration was too numerous to count, and has been scored on a plus (+) basis. Eosinophils appeared following treatment with both types of complexes although the numbers were about three times higher with IgG_1–antigen complexes than with IgG_2–antigen complexes. On a weight basis there were far fewer eosinophils with IgG_1–antigen complexes than with PCA reactions with IgG_1. Very few neutrophils were seen following injections of antibody alone, even in a dose of 400 μg. This experiment was repeated and gave a virtually similar result.

The effect of heat on the IgG_1 containing immunoglobulin fraction (Fr 4) which mediated PCA reactions and eosinophila

It is known that the ability of IgG_1 to elicit a homologous PCA reaction depends on a heat stable portion of the antibody molecule. It is conceivable, however, that the eosinophilia

associated with PCA reactions depends on a heat labile part of the molecule or that the eosinophilia is the result of interaction by an as yet unidentified heat labile immunoglobulin residing in the γ_1 region. It is shown in Fig. 7 that the ability of IgG_1 to elicit PCA reactions is partly retained even after prolonged heating and that the associated eosinophilia is proportionately reduced suggesting that the eosinophilia does not in fact depend on a heat labile moeity. Instead of a 4-hr sensitization period between intradermal antibody and antigen challenge the experiment was repeated using a 48-hr sensitization time. This was to confirm that the PCA reaction and associated eosinophilia did in fact depend on tissue bound antibody since presumably non-bound antibody would have diffused away. This experiment gave similar results to the 4 hr sensitization period experiment. Although not recorded in Fig. 7 the neutrophilia associated with IgG_2 is still apparent after a 48-hr sensitization period. There were, however, only about half as many neutrophils as when a 4-hr sensitization period was used.

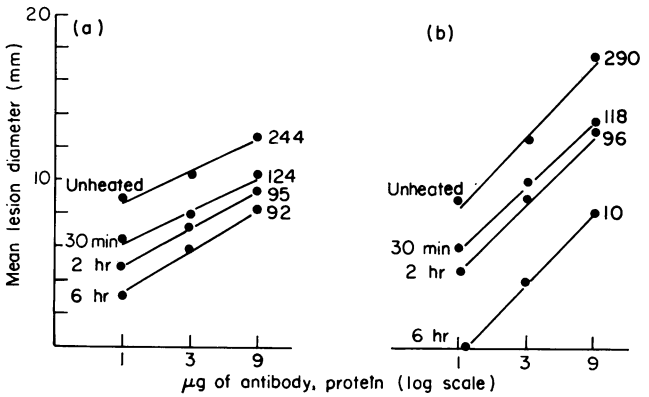

FIG. 7. The effect of heat on PCA reactions and subsequent eosinophilia mediated by IgG_1 (Fr 4). (a) Four hours sensitization, (b) 48 hr sensitization. Figures at end of lines indicate eosinophil counts.

Eosinophil accumulation following injections of Compound 48/80

Since histamine did not appear to be chemotactic for eosinophils and preformed antigen–antibody complexes generated comparatively less eosinophil migration than PCA reactions with IgG_1 the possibility was investigated that a mast cell component other than histamine released eosinophilotactic agents.

Experiments were designed to compare the eosinophil migration following PCA reactions with IgG_1 and injections of Compound 48/80. Preliminary studies showed that it was not possible to get comparative areas of blueing with Compound 48/80 and PCA reactions due to the flat dose–response curve for 48/80. Doses of 10 μg and 100 μg of 48/80 were chosen. In order to show that the effect of 48/80 was in part due to histamine release following mast cell degranulation it was demonstrated that 25 mg of mepyramine maleate inhibited the blueing reaction of 48/80 up to doses of 75 μg. Above this dose the area of blueing was only partially inhibited. Mast cell counts were performed by staining alternate sections with aqueous toluidine blue. It will be seen in Table 1 that with 10 μg of 48/80 eosinophil migration was small (Table 1, Experiments 1 and 2). The fact that there was little alteration in the mast cell count is in agreement with previous observations (Mota & Vugman, 1956a, b) on

the peculiarity of guinea-pigs to treatment with 48/80. In two of the experiments using higher doses of 48/80 eosinophil migration was seen and this was accompanied by low mast cell counts. There was, however, considerable distortion and destruction of the tissue. In one experiment (Experiment 5, Table 1) a small eosinophilia was noted even with 100 μg of 48/80. These experiments do suggest, however, that treatment leading to low mast cell counts, presumably by degranulation, is followed by eosinophil infiltration.

DISCUSSION

Eosinophil infiltration has been observed following PCA reactions mediated by fractions containing IgG_1. This agrees with the work of Parish (1969).

Recent reports have suggested that several classes or subclasses of immunoglobulins may exist in the electrophoretically fast γ_1 region and that PCA reactions may represent an activity in an immunoglobulin as yet unidentified by standard immunoelectrophoretic techniques (Pondman & Van Es, 1969; Van Es & Pondman, personal communication, 1969). In the present study, however, the comparison is made between IgG_1 and the electrophoretically slow IgG_2, the latter being unassociated with PCA activity or eosinophil migration.

Since the eosinophilia is not associated with IgG_2 it would seem unlikely that all antigen–antibody reactions prepare the tissue for a local eosinophilia as implied by Litt (1964). It is improbable that all the IgG_2 would have diffused away during the 4 hr sensitization period since it has been shown that homologous IgG_2 is absorbed onto guinea-pig tissue (Brocklehurst & Colquhoun, 1965) and that IgG_2 contains those antibodies cytophilic for macrophages (Jonas *et al.*, 1965). The neutrophilia associated with IgG_2 could be a result of bacterial contamination or the liberation of complement chemotactic factors (Ward, Cochrane & Müller-Eberhard, 1965). The neutrophil migration observed with IgG_1 (Fr 4) may be the result of contamination with IgG_2 or alternatively substances attracting eosinophils are also chemotactic for neutrophils. The eosinophil degranulation noted when the cells are gathered in large numbers could be artifactual resulting from the fixation process. The eosinophil membrane is, however, fairly resistant and can survive osmotic shock and acetone treatment. The fact that other cells surrounding degranulated eosinophils usually appeared normal indicates that the observation may be significant. Morphological changes accompanying eosinophil chemotaxis *in vitro* have also been noted (Kay, unpublished observations).

Various reports have concluded that histamine has eosinophilotactic properties. The evidence has recently been reviewed (Archer, 1968a). Other studies have been unable to support this view (Cohen & Sapp, 1963; Felarca & Lowell, 1968) and it could be that the situation in horses is exceptional (Archer, 1963). The argument that large local concentrations of histamine are required for eosinophilotaxis is invalid (Archer, 1968b) since in the present study the area of blueing with 10 μg of histamine was greater than that observed following PCA reactions with 27 μg of IgG_1. Both reactions were inhibited by mepyramine maleate but only IgG_1 PCA reactions were associated with an eosinophilia. The failure to produce eosinophil chemotaxis with histamine using the *in vitro* millipore technique (Keller & Sorkin, 1968; Kay, 1969) provides more convincing evidence that histamine is not eosinophilotactic. One explanation of the eosinophilia associated with PCA reactions involving

IgG_1 is that the IgG_1–antigen complex activates a serum or tissue factor, possibly complement, in a different manner to the IgG_2–antigen complexes although earlier reports suggested that IgG_1 was incapable of completing the complement haemolytic sequence (Bloch *et al.*, 1963). This has been recently challenged with the report that preformed IgG_1–antigen complexes can consume certain complement intermediates (Osler *et al.*, 1969). If IgG_1 and its antigen generate eosinophilotactic products intradermal injection of the complex should promote a substantial eosinophila. Both IgG_1 and IgG_2 produce eosinophil migration when injected with antigen as the preformed complex but more eosinophils were noted with IgG_1. However, the numbers on a weight basis were far less than when IgG_1 was introduced as for PCA reactions. Although this makes it unlikely that IgG_1 and its antigen react uniquely with complement to produce eosinophilotactic products it could be that this reaction is only possible when configurational changes have taken place in the antibody molecule as a result of tissue sensitization. Injection of the preformed complex may be an unsatisfactory method of obtaining adequate tissue fixation since the success of this procedure (using human IgE) requires a critical antigen–antibody ratio (Ishizaka & Ishizaka, 1968).

The slight eosinophilia noted with high doses of the IgG_2–antigen complex could be a result of IgG_1 contamination which was not detected by gel-diffusion. However, it has been observed that in *in vitro* studies using the millipore technique of Boyden chemotactic substances for eosinophils and neutrophils have been observed when fresh serum is incubated with IgG_1 and IgG_2 antigen complexes (Kay, unpublished observations). The substantial neutrophilia observed following the injection of preformed complexes may have prevented the recognition of the eosinophilotactic effects.

Although Compound 48/80 is said to be one of the least toxic of the agents affecting mast cells in rats, dogs, cats and man its action in the guinea-pig is thought to be atypical in that it had little effect on mast cell morphology and its toxic action was not prevented by antihistamines (Feinberg & Sternberger, 1955; Mota & Vugman, 1956a, b). It was concluded by Miles & Miles (1952), however, that 48/80 injected intradermally liberates local histamine and that the effect could be blocked by an antihistaminic agent. The present study confirms the findings of Miles and reports an association of a local eosinophilia especially with large doses of 48/80 injected intradermally. These experiments give some support to the view that a mast cell product may be eosinophilotactic but the superadded toxic effects of Compound 48/80 limits the interpretations of these *in vivo* studies. The eosinophilia following PCA reactions, antigen–antibody complexes and Compound 48/80 could represent the effect of several different eosinophilotactic agents generated by distinct mechanisms.

Experiments are in progress using the Boyden millipore technique as a method of quantitating eosinophil chemotaxis *in vitro*. The difficulties of *in vivo* studies have been largely overcome since individual components free from other tissue or serum factors can now be tested directly.

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