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Selective Attraction of Eosinophils and Synergism between Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A) and a Fragment Cleaved from the Fifth Component of Complement (C5a)

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Summary. ECF-A and C5a were chemotactic both for eosinophils and neutrophils. However, when eosinophils comprised approximately 10 per cent or more of a mixed leucocyte population, they were preferentially attracted by both of these agents.

Marked synergism was observed between ECF-A and C5a in their ability to attract eosinophil leucocytes.

INTRODUCTION

In previous reports, two chemotactic factors have been described which selectively attract eosinophil leucocytes from a mixed cell population. The first factor (ECF-C) required the presence of an intact complement system and was generated by incubating fresh guinea-pig serum with antigen–antibody complexes prepared from homologous IgG_1 or IgG_2 (Kay, 1970). By Sephadex G-100 chromatography, it was shown that ECF-C had an estimated molecular size of approximately 15,000 and was thought therefore to be identical to a fragment cleaved from the fifth component of complement (C5a). The second factor, an eosinophil chemotactic factor of anaphylaxis (ECF-A) was released by antigen challenge of actively sensitized lung or lung passively sensitized with IgG_1 (Kay, Stechschulte and Austen, 1971). The release of ECF-A did not require the presence of complement and, on the basis of a molecular size of between 500 to 1000, it was shown to be distinct from ECF-C.

C5a is also known to attract neutrophils (Shin, Snyderman, Friedman, Mellors and Mayer, 1968; Ward and Newman, 1969) and monocytes (Snyderman, Shin and Hausman, 1971; Hausman, Snyderman and Mergenhagen, 1972), and a purpose of the present report is to extend studies on the chemotaxis of eosinophils using C5a derived from highly purified C5 and to examine further selective chemotaxis of eosinophils by ECF-A. In addition, studies have been conducted on the relationship between ECF-A and C5a in terms of their ability to act synergistically in eosinophil chemotaxis.

MATERIALS AND METHODS

Materials were obtained as follows. Histamine acid phosphate, cytochrome C (BDH Chemicals Ltd, Poole, England); blue dextran (Pharmacia Fine Chemicals, Uppsala, Sweden); vitamin B_{12} (Glaxo Laboratories, Ltd, Greenford, England); glycogen, ovalbumin five times crystallized (Koch-Light Laboratories, Colnbrook, England); and horse serum (Wellcome Reagents Ltd, Beckenham, England).

Preparation of ECF-A

Guinea-pig anaphylactic lung diffusate was obtained from actively sensitized guinea-pig lung perfused free of blood and challenged with ovalbumin as previously described (Kay *et al.*, 1971). The cell free diffusate was extracted in 80 per cent ethanol, freed of precipitate by centrifugation, evaporated to dryness under vacuum using a rotary evaporator, reconstituted to one-tenth of the volume of the starting material in distilled water and centrifuged again at 15,000 *g* for 30 minutes. One millilitre of the concentrated lung diffusate was applied to a column of Sephadex G-25 (95 × 3·5 cm) and alternate 2-ml fractions tested for eosinophil and neutrophil chemotaxis using 0·2-ml volumes. For calibrating the column, the tubes containing the highest concentration of blue dextran and vitamin B_{12} were read visually. Histamine and slow reacting substance of anaphylaxis (SRS-A) were assayed as previously described (Kay *et al.*, 1971).

Preparation of C5a

Guinea-pig C5 was prepared by isoelectric precipitation, diethylaminoethyl (DEAE) and carboxylmethyl (CM)—cellulose chromatography and hydroxylapatite chromatography as previously described (Cook, Shin, Mayer and Laudenslayer, 1971). The C5 preparation contained approximately 400 μg of protein per ml and was treated with 4 μg of trypsin for 30 minutes at 25°. The reaction was stopped with 8 μg of soybean trypsin inhibitor (SBTI). The total volume of trypsin and SBTI added was 1/50 of the volume of C5. This mixture was used as the source of C5a. In experiments designed to show that C5a was chemotactic for both eosinophils and neutrophils, 0·3 ml of trypsinized C5 was applied to a column of Sephadex G-100 (40 × 1·0 cm) and 1-ml fractions collected.

Measurement of chemotaxis

A modification of the Millipore technique of Boyden was used as previously described (Kay, 1970). Guinea-pig eosinophils were obtained by peritoneal lavage from animals which had received multiple injections of horse serum. Neutrophils were harvested from peritoneal cavity of animals injected with glycogen 3–6 hours previously. Eosinophil and neutrophil migration were both measured using an 8·0-μ pore size, since previous studies with guinea-pig peritoneal cells had shown that this pore size gave full expression of eosinophil and neutrophil chemotaxis towards ECF-A and 'activated guinea-pig serum'. Under these conditions background counts with diluent alone were zero. Neutrophils or eosinophils were used at a concentration of 2×10^6 cells/ml. Tyrode solution containing 0·5 per cent ovalbumin was used as the diluent throughout.

RESULTS

EOSINOPHIL AND NEUTROPHIL CHEMOTACTIC ACTIVITY OF ECF-A AND C5a

In previous studies we have shown that compared with eosinophils, there was minimal

chemotaxis of neutrophils towards an anaphylactic lung diffusate (Kay *et al.*, 1971). When the concentrated anaphylactic diffusate was applied to a column of Sephadex G-25, neutrophil and eosinophil chemotactic activity eluted in the same position thus providing some evidence that both activities were a function of ECF-A (Fig. 1).

When trypsinized C5 was applied to a column of Sephadex G-100, eosinophil and neutrophil chemotactic activity eluted together (Fig. 2). The peak of activity for both cell types eluted at approximately the same position as the cytochrome C marker (mol. wt 12,270), a molecular size which closely corresponds to C5a (mol. wt approximately 15,000)

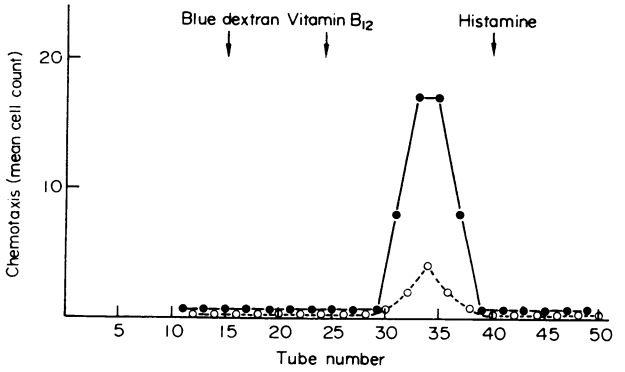

FIG. 1. Sephadex G-25 chromatography of an anaphylactic diffusate. Even numbered tubes were tested for (○) neutrophil chemotaxis and odd numbered tubes tested for (●) eosinophil chemotaxis. Although neutrophil response was minimal to ECF-A, the cell preparation gave a count of 80 towards 10 μg of trypsinized C5.

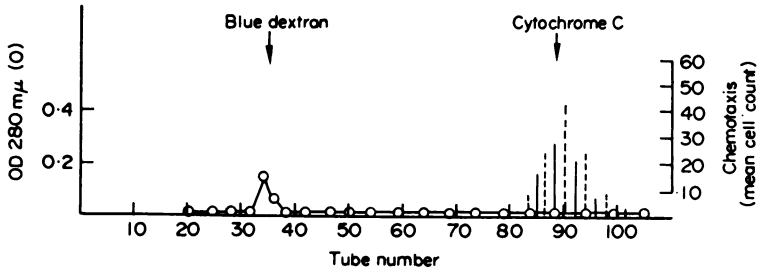

FIG. 2. Sephadex G-100 chromatography of trypsinized C5 showing the chemotactic response of fractions towards (——) eosinophils and (– – –) neutrophils.

ECF-A free of antigen, histamine, and SRS-A after Sephadex G-25 gel-filtration and C5a derived from highly purified C5 were then tested for their ability to attract eosinophils and neutrophils. The neutrophil suspension contained less than 1 per cent of eosinophils. The eosinophil preparation contained 52 per cent eosinophils, the remainder being only mononuclear cells. The cell counts were adjusted so that each preparation had the same number of eosinophils and neutrophils respectively. The total white cell count of the eosinophil preparation was therefore approximately twice that of the neutrophil suspension. The ability of these two cell preparations to migrate towards C5a is shown in Fig. 3. About ten times as many neutrophils migrated compared to eosinophils. With ECF-A,

however, eosinophil chemotaxis was predominant; very little neutrophil chemotaxis being noted even with the highest concentration of ECF-A (Fig. 3).

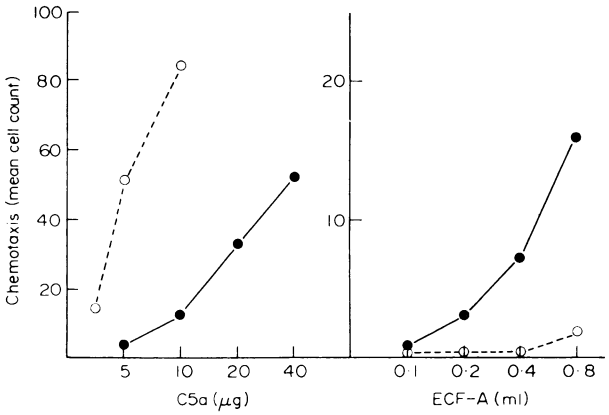

FIG. 3. The capacity of (○) neutrophils and (●) eosinophils to respond in chemotaxis to increasing doses of C5a and ECF-A. No chemotaxis was observed with untreated C5 or with the trypsin/SBTI mixture. The weight of C5a is expressed as that of the untreated C5.

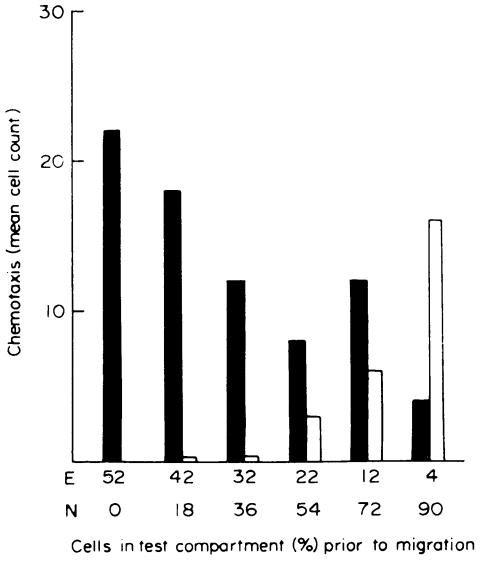

FIG. 4. The effect of alterations in the percentage of (■) eosinophils (E) and (□) neutrophils (N) in the test compartment on the chemotactic response to 0·8 ml of ECF-A.

THE EFFECT OF VARIATIONS IN PERCENTAGE OF EOSINOPHILS AND NEUTROPHILS

An eosinophil preparation containing no neutrophils was mixed in varying proportions with a suspension of neutrophils having only 4 per cent of eosinophils. When these cell preparations and their mixtures were tested against ECF-A, it was found that as the percentage of eosinophils increased this cell type was selectively attracted (Fig. 4). The

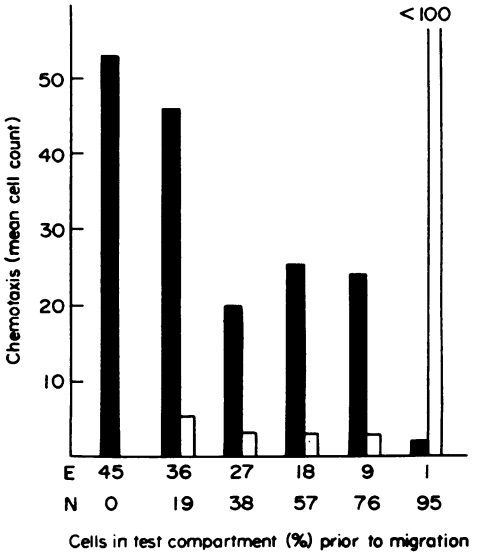

FIG. 5. The effect of alterations in the percentage of (■) eosinophils (E) and (□) neutrophils (N) in the test compartment on the chemotactic response to C5a derived from 40 μg of C5.

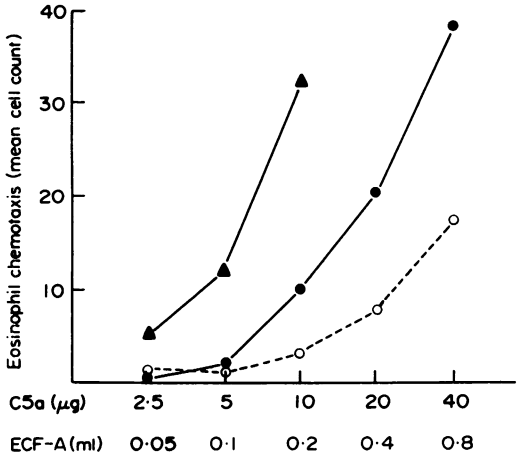

FIG. 6. Synergism between (▲) ECF-A and C5a. A dose response for (●) C5a, (○) ECF-A and a mixture of the two. The concentration of C5a is defined as in Fig. 3.

threshold for predominant eosinophil attraction was as low as 12 per cent. A similar effect was found with C5a (Fig. 5) with the threshold being 9 per cent in the experiment depicted. When there was no competition from eosinophils, neutrophils migrated toward both C5a and ECF-A.

SYNERGISM BETWEEN ECF-A AND C5a

Since ECF-A and C5a are distinct both in their molecular size and formation mechanism, it was of interest to determine the effect of combining the two agents in eosinophilotaxis. As seen in Fig. 6, the cell counts obtained after mixing were three times or greater

than that which would have been expected by summation of counts when the agents were assayed alone. This suggested that ECF-A and C5a acted synergistically in their ability to attract eosinophils.

More evidence of synergism was obtained in the experimental designs shown in Fig. 7. Chemotaxis towards ECF-A was examined in a dose-response fashion with and without the presence of a dose of C5a which gave a low chemotactic count. The counts obtained with the mixtures were far higher than would have been expected by summation (Fig. 7a). A dose-response study of C5a with a low concentration of ECF-A again revealed an increase in chemotactic counts far greater than would have been expected by addition. Since the preparation of C5a contained a trace of trypsin and SBTI, it was necessary to determine that this enzyme and inhibitor were not contributing to the synergism observed with ECF-A. Trypsin and SBTI in equivalent concentrations had no effect on the dose-response obtained with increasing dilutions of ECF-A.

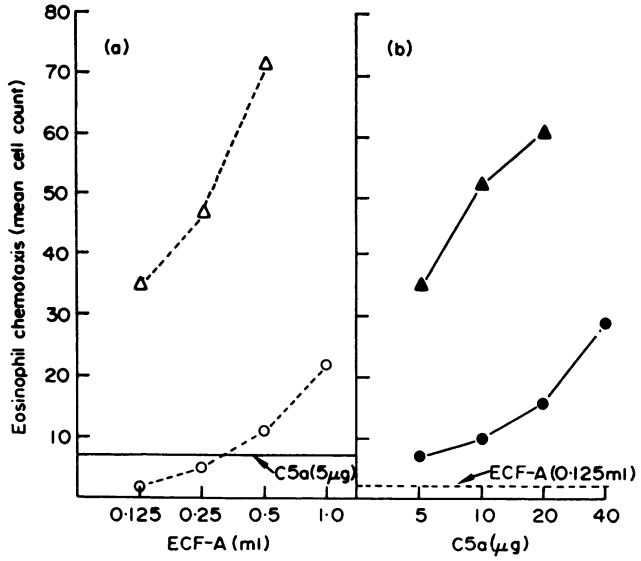

FIG. 7. Synergism between ECF-A and C5a. (a) Dose response for ECF-A (○) is compared with the same volumes of ECF-A to which 5 μg of C5a (△) giving a chemotactic count of 8 was added. (b) A dose response of C5a (●) is compared with the same volumes of C5a to which 0·125 ml of ECF-A (▲) giving a chemotactic count of 2, was added. The concentrations of C5a are defined as in Fig. 3.

DISCUSSION

Chromatographic separation of ECF-A and C5a (Figs 1 and 2) demonstrated that both eosinophil and neutrophil chemotactic activity eluted at the same bed volume characteristic for the size of these molecules (mol. wt approximately 1000 and 15,000 respectively).

When ECF-A and C5a were examined in a dose-response fashion against a suspension of cells which contained greater than 95 per cent of neutrophils this cell type migrated towards C5a whereas neutrophils migrated only in small numbers to ECF-A (Fig. 3). When eosinophils were examined in the same manner using a suspension of cells containing 52 per cent eosinophils and 48 per cent mononuclear cells, migration was demonstrated in a dose-response fashion towards both factors (Fig. 3). Under these conditions

the neutrophil was attracted in larger numbers, however, attraction of eosinophils to C5a may have been impaired, in part, by the presence of the mononuclear cells.

The highest doses of C5a and ECF-A shown in Fig. 3 were then examined for their effect on various mixtures of eosinophils and neutrophils. When eosinophils comprised only about 10 per cent of the target-cell population they were selectively attracted by either ECF-A (Fig. 4) or C5a (Fig. 5). The preferential attraction of eosinophils by ECF-A and C5a was particularly striking when eosinophils and neutrophils were present in the cell compartment in approximately equal numbers. In both situations there was marked eosinophil migration whereas neutrophil chemotaxis was virtually absent. The experimental design illustrated in these studies involved dilution of either eosinophils or neutrophils by the other cell types. Dilution is not considered to account for the selective eosinophil migration since previous studies (Kay, unpublished observations), have shown that each cell type would respond maximally at the lowest concentration used in these studies. It should be noted that the summation of eosinophil and neutrophil responses is less for mixtures than for the original suspensions in which one or the other of the cell types predominates.

A broad specificity of chemotactic factors can be demonstrated when conditions for migration are optimal and when other cells which may be preferentially attracted are absent. Thus, in unusual cases of basophilia in association with chronic leukaemia, basophils migrated in small numbers towards C5a and an anaphylactic diffusate containing ECF-A (Kay and Austen, 1972). In this situation, the conditions for basophil chemotaxis were optimal since there was little competition from other cell types. In addition, mononuclear cell chemotaxis has been demonstrated with C5a when this cell type predominated in the cell chamber (Snyderman *et al.*, 1971; Hausman *et al.*, 1972). The present studies confirm the report of Shin *et al.* (1968) and Ward and Newman (1969), who described neutrophil chemotaxis to C5a. In addition, conditions have been demonstrated under which eosinophil chemotaxis can predominate.

Synergism between ECF-A and C5a was shown in three sets of experiments. When C5a and ECF-A, which when tested alone gave low cell counts, were mixed together, the resultant chemotactic counts were three times or more than would have been expected by summation alone (Fig. 6). When a low dose of C5a was added to increasing doses of ECF-A, the resultant increase in chemotaxis was also three times or more than would have been expected by addition of the counts (Fig. 7a). Virtually identical results were obtained when ECF-A was added to increasing doses of C5a (Fig. 7b). It is possible that eosinophils have more than one receptor for chemotaxis and that, if different types of receptors are stimulated at a low threshold, this produces an increased chemotactic response. These observations on synergism may be of significance in parasitic infestations, many of which are associated with a pronounced eosinophilia. Homocytotropic antibody and complement-fixing antibody occur together in a variety of parasitic diseases, situations in which ECF-A and C5a might act together.

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