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## Leucoattractants enhance complement receptors on human phagocytic cells

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### SUMMARY

The N-formyl-methionyl peptides, F-Met-Leu-Phe, F-Met-Met-Phe and F-Met-Phe, when tested at differing concentrations, proportionally increased both *in vitro* cell locomotion and the expression of surface receptors for C3b on human peripheral blood neutrophils and monocytes. In contrast, the unformylated peptides, Met-Leu-Phe and Met-Met-Phe, had no chemotactic or complement receptor-enhancing activity at comparable concentrations. Casein and supernatants from human lymphocytes (cultured either in the presence or absence of phytohaemagglutinin), also recognized as chemotactic agents for human neutrophils and monocytes, enhanced C3b receptors on these cells in a similar dose-dependent fashion. These data, taken together with our previous findings with the eosinophil, suggest that in addition to promoting cell locomotion a further biological function of leucoattractants may be their capacity to render complement receptors more freely available thereby increasing the magnitude of adhesion of phagocytic cells to opsonized particles.

### INTRODUCTION

We recently reported that eosinophil chemotactic factors (including the ECF-A tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu), histamine and imidazoleacetic acid) markedly enhanced the expression of receptors for complement (C3b and C4) on human eosinophils (Anwar & Kay, 1977 and 1978). Therefore, it seemed important to determine whether other phagocytic cells, such as human neutrophils and monocytes, undergo similar complement receptor enhancement following incubation with substances known to promote their migration *in vitro*. If, in addition to promoting cell locomotion, leucoattractants also render complement receptors more freely available this would provide a mechanism whereby the degree of adhesion of phagocytic cells to opsonized particles was increased. The studies reported here employed synthetic bacterial-associated N-formyl-methionyl peptides, as their chemical structure is clearly defined and potent chemotactic activity well documented (Schiffman, Corcoran & Wahl, 1975; Showell *et al.*, 1976). In addition, we examined other recognized human neutrophil and monocyte leucoattractants for complement receptor enhancement. These included supernatants from cultured lymphocytes (Ward, Remold & David, 1969; Snyderman *et al.*, 1972) and casein (Keller & Sorkin, 1967).

### MATERIALS AND METHODS

F-Met-Leu-Phe was a gift from Dr Dereck Hudson (Royal Postgraduate Medical School, London). F-Met-Met-Phe, Met-Leu-Phe and Met-Met-Phe were gifts from Miles Laboratories (Stoke Poges). F-Met-Phe was obtained from Sigma Chemical Company (St Louis, Missouri, U.S.A.). Other materials were obtained as follows. Preservative-free heparin (Evans Medical, Liverpool); casein (BDH Chemicals Ltd, Poole); phytohaemagglutinin (PHA-P, Wellcome Reagents Ltd., Beckenham); dextran (Lomodex 70, Fisons Pharmaceuticals, Loughborough); Ficoll and Hypaque (Pharmacia, Uppsala,

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Sweden); ovalbumin five times crystallized (Koch Light Laboratories, Colnbrook); cellulose nitrate filters, 8 and 0.45- $\mu\text{m}$  pore size (Sartorius-Membrane Filters, Göttingen, West Germany); polycarbonate (Nucleopore) 5- $\mu\text{m}$  pore size filters (Neuroprobe, Bethesda, Maryland, U.S.A.).

*Preparation of EAC1423b (EAC).* This has been described in detail elsewhere (Anwar & Kay, 1977) and was briefly as follows. Dextrose-gelatin-Veronal buffer (DGVB<sup>2+</sup>, pH 7.4) was used for washing sheep erythrocytes (E) during sensitization and coating with various complement components and was prepared by mixing equal volumes of isotonic Veronal-buffered saline (containing 0.0015 M Ca<sup>2+</sup>, 0.0005 M Mg<sup>2+</sup> and 0.1% gelatin-Veronal buffer (GVB<sup>2+</sup>)) with 5% dextrose in water containing the same concentration of Ca<sup>2+</sup> and Mg<sup>2+</sup>. The IgM fractions of rabbit antisera to sheep red cells were prepared by Sephadex G-200 gel filtration (Shevach *et al.*, 1972). Functionally pure human complement components were added sequentially to EAM<sup>ra</sup>b to prepare C3b-coated cells. The amounts were as follows: 400 effective molecules of C1, 400 of C4, 50 of C2 and 500 (for neutrophils) or 400 (for monocytes) of C3. This amount of C4 was insufficient to give EAC14 rosettes with neutrophils, eosinophils or monocytes (Anwar & Kay, 1977).

In some experiments fresh human AB serum diluted with DGVB<sup>2+</sup> was used as a source of complement. Equal volumes of EAM<sup>ra</sup>b at a concentration of  $1 \times 10^8$  cells/ml and human AB serum were mixed and incubated for 30 min at 37°C, washed twice in DGVB<sup>2+</sup> and resuspended in the same buffer to a concentration of  $1 \times 10^8$  red blood cells/ml. The dilution of fresh serum was adjusted to give between 20 and 30% of neutrophil or monocyte rosettes and was usually between 1 in 600 and 1 in 1000.

*Neutrophils and monocytes.* Blood from healthy volunteers was drawn into plastic tubes containing 10 units of preservative-free heparin/ml. The red cells were sedimented with 70% dextran; 1 part dextran to 4 parts blood v/v for 60 min at room temperature. The leucocyte-rich plasma was then layered over Ficoll-Hypaque (specific gravity 1.078) at 4°C in 15 ml plastic conical centrifuge tubes and centrifuged at 4°C for 40 min at 400 g. The pellet, containing red blood cells and granulocytes, was resuspended in 0.82% ammonium chloride as described (Boyle, 1968), to lyse the red cells. Monocytes, usually from the same donors, were separated on Ficoll-Hypaque gradients according to the method of Böyum (1968). The separated cells were finally washed twice in medium 199, pH 7.4, and the cell count adjusted to  $2 \times 10^6$  cells/ml for neutrophils or  $1 \times 10^6$  monocytes/ml.

*Lymphocyte supernatants.* 'Stimulated lymphocyte supernatants' were prepared from suspensions of human peripheral blood mononuclear cells, separated on a discontinuous gradient of Ficoll-Hypaque as described above, and contained  $3 \times 10^6$  leucocytes/ml in medium 199. Under sterile conditions, and with added penicillin and streptomycin, 9  $\mu\text{l}$  of PHA-P (0.5  $\mu\text{g}/\text{ml}$ ) were added to 3 ml of the mononuclear cell suspension. The cells were incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After incubation the supernatants were harvested by centrifugation, divided into portions and stored at -70°C until use. The 'unstimulated lymphocytes' were prepared in an identical fashion with the exception that PHA-P was added at the termination of the 3 day incubation period.

*EAC rosettes.* Equal volumes of neutrophils ( $2 \times 10^6/\text{ml}$ ) or monocytes ( $1 \times 10^6/\text{ml}$ ) and various concentrations of the leucoattractants under study, or medium alone as control, were mixed and incubated in a shaking water bath at 37°C for 30 min. The cells were then washed twice in medium 199 and the numbers re-adjusted to their original concentration in the same medium. A portion (0.1 ml) of EAC ( $1 \times 10^8/\text{ml}$ ) was added to 0.1 ml of the leucocyte suspension, the mixtures centrifuged at 100 g for 10 min at 4°C, and the pellets incubated at 37°C for 30 min. The pellet was gently resuspended and smears prepared on clean glass slides in duplicate. These were dried quickly in air, fixed in 95% methanol and stained with May Grunwald/Giemsa. Leucocytes with three or more adherent sheep red cells were termed rosettes. In each slide 200 neutrophils or monocytes were counted and the increase in the percentage of rosetting cells following incubation with the various leucoattractants was calculated.

*Cell locomotion.* The capacity of neutrophils or mononuclear cells to migrate towards a concentration gradient of various leucoattractants has been described in detail elsewhere (Kay, Pepper & McKenzie, 1974; Campbell, 1977). For neutrophils (Böyum, 1968), cellulose nitrate filters of 8  $\mu\text{m}$  pore size were employed using an incubation time of 90 min at 37°C. The cell count was adjusted to  $2 \times 10^6/\text{ml}$  in medium 199 containing 0.5% ovalbumin at pH 7.4. Only those cells which had migrated across the entire thickness of the micropore were counted. Cells were counted under a high power ( $\times 40$ ) objective and the average number in five fields was recorded (mean cell count). Monocyte chemotaxis was measured using the double filter technique (Campbell, 1977) with the exception that the 'Boyden chamber' supplied by Neuroprobe, Inc., Bethesda, Maryland, was used instead of the Sykes-Moore type of chamber. In all experiments with neutrophils or monocytes the values obtained from the controls (medium 199 alone) were subtracted from the values obtained with the various agents being tested.

## RESULTS

The percentage increases in C3b rosettes and the numbers of neutrophils migrating through the micropore filters were directly related to the concentrations of the formyl-methionyl peptides (Fig. 1). The magnitude of these two biological events appeared to increase proportionally with increasing concentrations of the peptides. With F-Met-Leu-Phe appreciable activity was observed from  $10^{-8}$  to  $10^{-10}$  mol/l, but not at  $10^{-11}$  mol/l. With F-Met-Met-Phe neutrophil chemotaxis and complement receptor

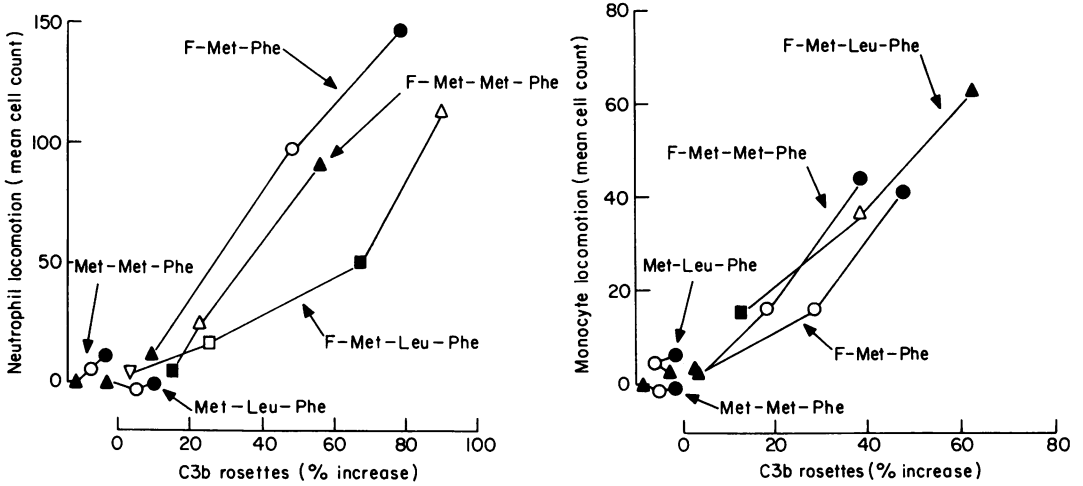


FIG. 1. The association between the percentage increase in neutrophil C3b rosettes and neutrophil locomotion by formylated and unformylated methionyl peptides. The symbols represent the concentration of the peptides in mol/l and are as follows:  $10^{-5}$  (●),  $10^{-6}$  (○),  $10^{-7}$  (▲),  $10^{-8}$  (△),  $10^{-9}$  (■),  $10^{-10}$  (□) and  $10^{-11}$  (▽). The results are the mean values of three experiments (five experiments with F-Met-Leu-Phe). Receptor enhancement and chemotaxis were performed on cells from the same volunteers (with the exception of two experiments with F-Met-Leu-Phe).

FIG. 2. The association between the percentage increase in monocyte C3b rosettes and monocyte locomotion by formylated and unformylated methionyl peptides. The symbols represent the concentrations of the peptides in mol/l and are as follows:  $10^{-5}$  (●),  $10^{-6}$  (○),  $10^{-7}$  (▲),  $10^{-8}$  (△) and  $10^{-9}$  (■). The results are the mean values of three experiments. Receptor enhancement and chemotaxis were performed on cells from the same volunteers.

TABLE 1. Human neutrophil and monocyte complement receptor enhancement by lymphocyte supernatants and casein. N = neutrophils, M = monocytes. The results are the mean  $\pm$  s.e. of three experiments. The lymphocyte supernatants and casein were tested for neutrophil and monocyte chemotactic activity and gave results comparable to that described in our previous study (Kay & McVie, 1977)

		C3b Rosettes (% increase)			
		Dilution			
		1:4	1:8	1:16	1:32
Lymphocyte supernatant (PHA-stimulated)	N	64 $\pm$ 4	49 $\pm$ 4	34 $\pm$ 2	16 $\pm$ 8
	M	—	84 $\pm$ 5	52 $\pm$ 7	-2 $\pm$ 3
Lymphocyte supernatant (unstimulated)	N	69 $\pm$ 3	33 $\pm$ 5	25 $\pm$ 5	1 $\pm$ 5
	M	—	39 $\pm$ 7	11 $\pm$ 7	-2 $\pm$ 3
		Concentration (mg/ml)			
		3.0	2.0	1.5	1.0
Casein	N	60 $\pm$ 11	64 $\pm$ 6	34 $\pm$ 3	23 $\pm$ 5
	M	97 $\pm$ 12	76 $\pm$ 17	—	39 $\pm$ 4

enhancement were observed at  $10^{-7}$  and  $10^{-8}$  mol/l and with F-Met-Phe at  $10^{-5}$  and  $10^{-6}$ . The unformylated peptides, Met-Leu-Phe and Met-Met-Phe, gave virtually no responses either with neutrophil C3b-receptor enhancement or chemotaxis.

Similar results were obtained with human blood monocytes (Fig. 2) although the concentrations of the formyl-methionyl peptides required to demonstrate chemotaxis and complement receptor enhancement were higher than those required for the neutrophil. With F-Met-Leu-Phe activity was observed at  $10^{-7}$  and  $10^{-8}$  mol/l with little enhancement and cell locomotion at  $10^{-9}$  mol/l. With F-Met-Met-Phe and F-Met-Phe a clear effect on cell locomotion and rosette expression was demonstrable at  $10^{-5}$  and  $10^{-6}$  mol/l, but not at  $10^{-7}$  mol/l. The unformylated peptides, Met-Leu-Phe and Met-Met-Phe, gave very low values in monocyte complement receptor enhancement and monocyte chemotaxis.

Casein, previously shown to be a potent chemoattractant for neutrophils and monocytes (Keller & Sorkin, 1967), also enhanced C3b receptors on these cell types (Table 1). Similar results were obtained with cultured lymphocyte supernatants. A greater amount of neutrophil and monocyte chemotactic activity was found in cultures prepared in the presence of PHA, although the 'unstimulated' lymphocyte supernatants also promoted the migration of human leucocytes, as was previously shown (Snyderman *et al.*, 1972). Similarly, complement receptor enhancement was greater with 'stimulated' as compared to 'unstimulated' supernatants for both neutrophils and monocytes (Table 1).

The time-course of complement receptor enhancement on neutrophils and monocytes was similar, maximal effect being observed between 30 and 60 min. Irrespective of whether the indicator red cells were EAM with whole serum as a source of complement, or EAM and purified C1, C4, C2 and C3, the percentage increase in rosettes either as a function of time or the concentration of chemoattractant, was virtually identical for both neutrophils and monocytes.

## DISCUSSION

In this report we have shown that formyl-methionyl peptides, in addition to promoting cell migration *in vitro*, also increased the expression of C3b receptors on neutrophils (Fig. 1) and monocytes (Fig. 2). We have therefore, confirmed previous reports on the leucoattractant properties of these compounds (Schiffman *et al.*, 1975; Showell *et al.*, 1976) and shown that in addition they have a previously undescribed biological activity—complement receptor enhancement. Both activities, i.e. leucoattraction and complement receptor enhancement, occurred in parallel since increasing concentrations of the peptides promoted concomitant increases in both the degree of receptor enhancement and the numbers of neutrophils or monocytes migrating through the micropore filters. In contrast, the unformylated peptides, Met-Leu-Phe and Met-Met-Phe, were inactive in both biological systems suggesting that the specificity for formylation applies to both complement receptor enhancement and locomotion.

Enhancement of complement receptors by 'chemotactic factors' was first described using human eosinophils (Anwar & Kay, 1977, 1978). In these reports we showed that receptors for C3b and C4 (but not C3d or IgG (Fc)) were 'enhanced' in the sense that previously recognized eosinophilotactic agents such as the ECF-A tetrapeptides, histamine or imidazoleacetic acid produced an increase in the percentage of eosinophils forming rosettes with the appropriate indicator red cells. As described elsewhere (Anwar & Kay, 1978) possible explanations of the mechanisms of complement receptor enhancement include 'membrane unfolding', 'receptor externalization' and 'subunit association'. The generation of 'new' receptors during the incubation with chemotactic factors was considered unlikely since optimal enhancement of eosinophil complement receptors was found to be between 30 and 60 min. A similar time course for complement receptor enhancement on neutrophils and monocytes has also been observed with neutrophils and monocytes (Glass, E.J., Salter, D.McG. & Kay, A. B., unpublished observations).

The term 'leucoattraction' and 'cell locomotion' has been used only in the sense that the various agents which we tested produced migration of neutrophils and monocytes towards a gradient across a micropore filter. Although we have drawn the general conclusion that agents which promote cell locomotion also enhance complement receptors on the leucocytes they chemoattract, we cannot say whether this effect is related to their chemotactic and/or chemokinetic principles. Showell *et al.* (1976), using the 'chequer-

board' titration method previously described (Zigmond & Hirsch, 1973) to distinguish true chemotaxis from chemokinesis, were able to show that F-methionyl peptides caused directional migration as well as increasing the rate of cell movement. In order to establish whether the chemotactic and/or chemokinetic property is necessary for complement receptor enhancement on human leucocytes further studies will be required using substances which increase random migration, but are not chemotactic and *vice versa*. For instance, Wilkinson *et al.* (1977) reported that mouse lymph node lymphocytes responded in chemokinesis, but not chemotaxis, to human and bovine serum albumins.

The relative activities of the formylated peptides used in this study were, for neutrophils, F-Met-Leu-Phe > F-Met-Met-Phe > F-Met-Phe. Similar results, in terms of relative potencies were observed in the study by Showell *et al.* (1975) in which chemotaxis and lysosomal enzyme release from rabbit neutrophils were tested. With monocytes about one hundred-fold more F-Met-Leu-Phe, F-Met-Met-Phe or F-Met-Phe was required to give comparable biological effects to that of the neutrophil (Figs 1 and 2). Furthermore in the human monocyte studies (Fig. 2), F-Met-Phe had a similar potency to F-Met-Met-Phe in both cell locomotion and rosette enhancement. This is in contrast to the report of Showell *et al.* (1976) in which F-Met-Met-Phe was found to be 1900 times more active than F-Met-Phe in promoting chemotaxis of rabbit neutrophils.

We have not yet had the opportunity to examine fully the effects of chemotactic agents on cell surface markers other than those for complement. However, in a previous report we showed that eosinophilotactic agents had no apparent effect on IgG (Fc) receptors (Anwar & Kay, 1977 and 1978) and recent preliminary data indicate that on neutrophils and monocytes these receptors are also unaffected by leucoattractants (Salter, D.McG. & Glass, E.J., unpublished observations). Also, we have yet to ascertain whether, like eosinophils, complement receptor enhancement on neutrophils and monocytes applies only to C3b and C4 and not C3d.

It is tempting to speculate that complement receptor enhancement may be an important biological phenomenon in terms of the events which surround (i) the directional migration of cells into sites of inflammation and (ii) the subsequent adhesion of leucocytes to particles opsonized by complement, i.e. that leucocytes are primed for the adhesion process prior to their actual attachment. The N-formyl-methionyl peptides and the other leucoattractants tested (Table 1) enhanced C3b receptors on those cells in which they induced locomotion. Therefore, the present work, taken together with our previous findings with the eosinophil (Anwar & Kay, 1977 and 1978), supports the general hypothesis that leucoattractants enhance complement receptors on human phagocytic cells.

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