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The chemoattractant des-Arg^{74}-C5a regulates the expression of its own receptor on a monocyte-like cell line

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The interaction of the chemoattractant des-Arg^{74}-C5a (C5a des Arg) with its receptor on a human monocyte-like cell line, U-937, was examined. The data obtained suggest that C5a des Arg receptor expression is regulated by the extracellular concentration of C5a des Arg itself.

INTRODUCTION

The complement fragment C5a and its des-Arg^{74} derivative, C5a des Arg, are potent mediators of leucocyte chemotaxis and are thought to play an important role in the accumulation of leucocytes at sites of inflammation.

Although specific receptors for C5a have been demonstrated on murine macrophages (Chenoweth *et al.*, 1982) and human monocytes (Chenoweth & Goodman, 1983; Marder *et al.*, 1985), most work to date has concentrated on the human neutrophil C5a receptor due to the relative abundance and ease of purification of this cell type. Using a monocyte-like cell line, U-937, as a model of the monocyte response to C5a des Arg, we show here the level of C5a des Arg receptor expression appears to be regulated by the extracellular concentration of C5a des Arg itself. This may provide a mechanism by which the cell is able to maintain a chemotactic response over a wide range of attractant concentrations.

EXPERIMENTAL AND RESULTS

Materials

All tissue culture materials were from Gibco, Uxbridge, Middlesex, U.K. Bis(sulphosuccinimidyl) suberate was from Pierce (U.K.), Cambridge, U.K. Bt_2cAMP, cycloheximide, cytochalasin B and *N*-formyl-Met-Leu-Phe were from Sigma.

Purification and radioiodination of rabbit C5a des Arg

Rabbit C5a des Arg was purified and radioiodinated as described previously (Jose *et al.*, 1983).

Induction of C5a des Arg receptor expression on U-937 cells

The relatively low numbers of human monocytes obtainable from peripheral blood hinder detailed investigation of monocyte chemotaxis. Two human cell lines with monocyte properties, HL-60 and U-937, that do not normally exhibit chemotaxis can, by treatment with suitable agents, be induced to differentiate into cells that are responsive to certain chemoattractants (reviewed by Harris & Ralph, 1985). Using highly purified, radioiodinated rabbit C5a des Arg, which was chemotactic for highly purified human peripheral blood monocytes (Woof *et al.*, 1984), we examined the effect of various substances on the expression of C5a des Arg receptor by U-937 cells. Untreated U-937 cells showed no detectable binding of ^{125}I-C5a des Arg. However, Bt_2cAMP, shown previously to increase C3b receptor expression on a human lymphoblastoid cell line (Barel *et al.*, 1981) and to induce expression of receptors for bacterial peptide

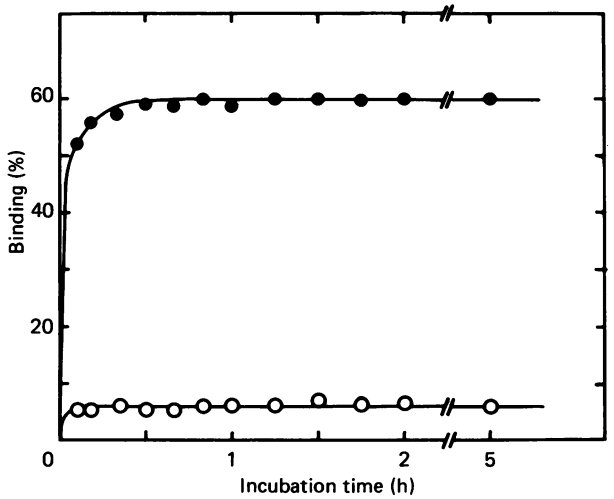

Fig. 1. Time course of ^{125}I-labelled rabbit C5a des Arg binding to Bt_2cAMP-treated U-937 cells at 0 °C

U-937 cells were treated with 1 mM-Bt_2cAMP for 2–3 days as described previously (Kay *et al.*, 1983), then washed twice in PBS/0.2% BSA/0.1% NaN_3. Two assay tubes were incubated at 0 °C in each case, one with excess (10^{-6}M) unlabelled C5a des Arg (for non-specific binding determination), and one without (for total binding). Both tubes contained ^{125}I-C5a des Arg (at 10^{-9} M) and Bt_2cAMP-treated U-937 cells at 2.5×10^7 cells/ml in PBS/0.2% BSA/0.1% NaN_3 in a total volume of 1 ml. At various time intervals 20μl aliquots were removed from each assay tube and the percentage binding of ^{125}I-C5a des Arg determined. Separation of bound and free ^{125}I-C5a des Arg was achieved by centrifuging the cells through a water-immiscible oil (Woof *et al.*, 1984). ●, Total binding; ○, non-specific binding.

Abbreviations used: BSA, bovine serum albumin; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate; H-RPMI, RPMI 1640 + 25 mM-Hepes; ^{125}I-C5a des Arg, radioiodinated rabbit des-Arg^{74}-C5a; PBS, phosphate-buffered saline, pH 7.4.

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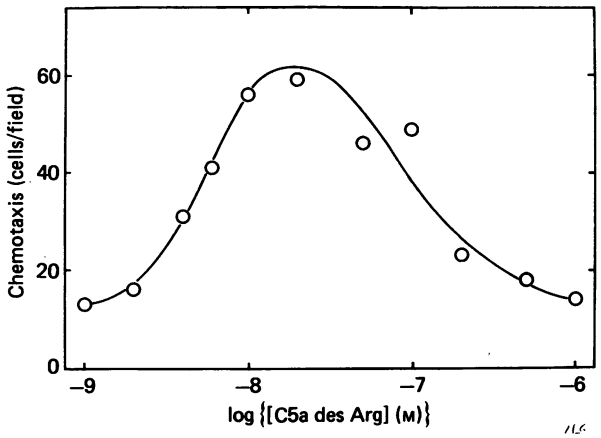

Fig. 2. Chemotaxis of Bt_2cAMP-treated U-937 cells in response to a range of concentrations of rabbit C5a des Arg

Chemotaxis was measured by a modification of the Boyden technique (Aggett *et al.*, 1979) using a nitrocellulose and polycarbonate filter (0.45 μm and 5 μm pore size respectively) sandwiched together (Campbell, 1977). Cells [maintained in H-RPMI containing 10% (v/v) foetal calf serum] were resuspended in H-RPMI/1% (w/v) BSA at 10^6 cells/ml and 0.2 ml was placed above the filters, and the C5a des Arg in H-RPMI/1% BSA was placed below. The plates were incubated for 2 h at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. The filters were fixed in propan-2-ol, stained with haematoxylin, dehydrated, cleared and mounted on a slide with Canada balsam. Cells completely traversing the upper polycarbonate filter, thus lying within the sandwich, were counted under a ×40 objective with an ocular reticle. Duplicate assays were performed at each concentration and five fields were scored/filter. Assays were also carried out (results not shown) in which different concentrations of the C5a des Arg were placed above and below the filter to ensure migration was not simply due to chemokinesis (Zigmond & Hirsch, 1973). The average number of cells migrating in the absence of a positive gradient was always less than 14 cells/field.

chemoattractant on U-937 cells (Kay *et al.*, 1983), elicited a dramatic increase in C5a des Arg receptor expression on this cell line. [This observation has been independently made by Chenoweth *et al.* (1984).] The binding of ^{125}I-C5a des Arg to the cells was specific and rapid, being virtually complete within 10 min at 0 °C (Fig. 1).

Chemotaxis

The Bt_2cAMP-treated U-937 cells exhibited positive chemotaxis to rabbit C5a des Arg over a wide range of concentrations, very high C5a des Arg concentrations being inhibitory (Fig. 2).

Affinity cross-linking

Covalent cross-linking of radioiodinated rabbit C5a des Arg to Bt_2cAMP-treated U-937 cells gave rise to a complex that ran as a diffuse band of M_r 55000–65000 on SDS/polyacrylamide gels (Fig. 3). Similar results have recently been reported (Johnson & Chenoweth, 1985; Rollins & Springer, 1985) using radioiodinated human C5a.

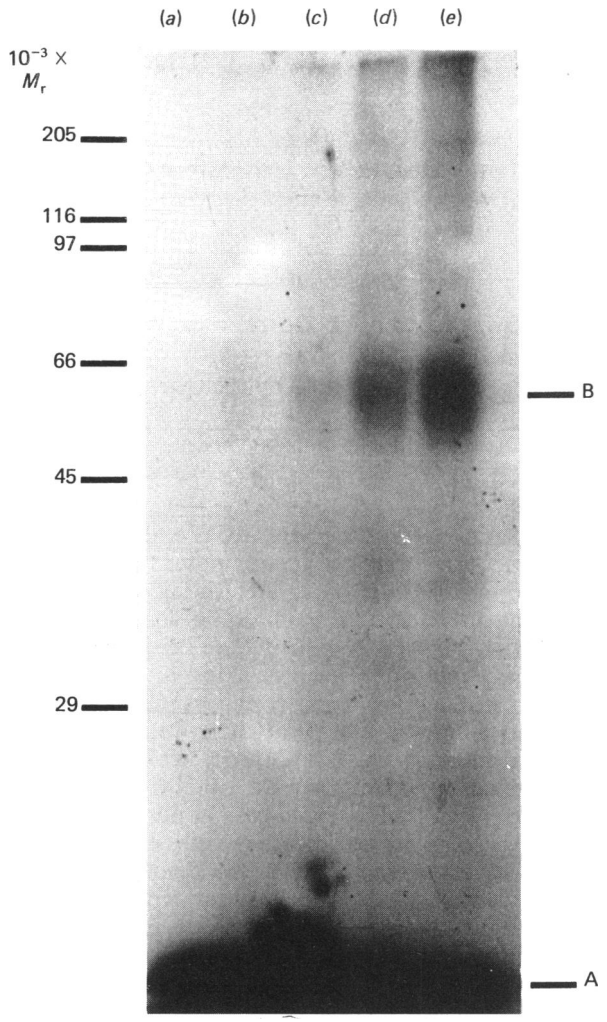

Fig. 3. Covalent cross-linking of ^{125}I-C5a des Arg to intact Bt_2cAMP-treated U-937 cells

Lanes (*a*)–(*e*) denote 0, 0.01, 0.05, 0.2 and 1 mM cross-linking agent respectively. Numbers to the left correspond to the positions of molecular weight standards ($\times 10^{-3}$). Band A is unbound ^{125}I-C5a des Arg; band B is the cross-linked complex containing ^{125}I-C5a des Arg. Washed Bt_2cAMP-treated U-937 cells (2.5×10^7 cells/ml) were resuspended in PBS/0.2% BSA/0.1% NaN_3 with 10 nM-^{125}I-C5a des Arg and incubated for 60 min at 0 °C. The cells were then washed twice and resuspended in PBS at 0 °C. Bis(sulphosuccinimidyl) suberate was freshly dissolved in PBS and added to the cells at concentrations varying from 0.01 to 1 nM. The reaction was allowed to proceed for 60 min at 0 °C with occasional mixing. The reaction was quenched with Tris-buffered saline (0.15 M-NaCl/0.025 M-Tris/HCl, pH 7.4) and the cells were washed twice with the same solution. The cells were then solubilized in SDS sample buffer [1% SDS/0.05 M-Tris/HCl, pH 6.8, containing 40% (w/v) urea] with or without 1% (v/v) 2-mercaptoethanol and heated in a boiling water bath for 2 min. The samples were then subjected to SDS/polyacrylamide gel electrophoresis (Laemmli, 1970) on a 12% gel. The gel was dried and subject to autoradiography on Fuji RX X-ray film. Depicted is an autoradiograph of a non-reducing gel. The reducing gel was identical.

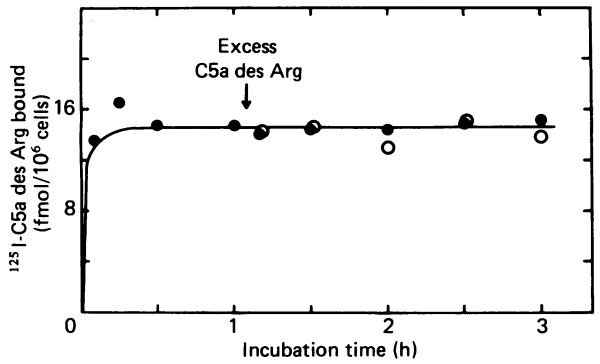

Fig. 4. Addition of excess unlabelled rabbit C5a des Arg to cells after preincubation with ^{125}I-C5a des Arg at 0 °C

Bt_2cAMP-treated U-937 cells (2.5×10^7/ml) were incubated with 10^{-9} M-^{125}I-C5a des Arg in PBS/0.2% BSA/0.1% NaN_3 at 0 °C in a total volume of 1 ml. At various time intervals aliquots were taken to determine binding. After 1 h the reaction mixture was split into two portions; to one, unlabelled C5a des Arg (10^{-6} M final concentration) was added and to the second was added an equal volume of buffer. Further aliquots were then taken from each tube for 2 h and the amount of ^{125}I-C5a des Arg bound was determined as before. ●, Cells with ^{125}I-C5a des Arg alone; ○, cells with ^{125}I-C5a des Arg and excess unlabelled C5a des Arg.

Binding experiments

Although the binding of radiolabelled rabbit C5a des Arg was readily inhibited by the simultaneous addition of excess unlabelled C5a des Arg, subsequent experiments showed that if an excess of unlabelled C5a des Arg was added once binding was complete, little or no displacement of the radiolabelled rabbit C5a des Arg could be achieved even at 0 °C in the presence of cytochalasin B and azide, which would be expected to abolish internalization of the ligand (Fig. 4). Similar observations have been reported for the binding of radioiodinated human C5a to its receptor on neutrophils (Chenoweth & Hugli, 1978; Chenoweth & Goodman, 1983), although in this case displacement by an excess of human C5a has recently been shown to occur at 0 °C (Huey & Hugli, 1985).

Since the binding of rabbit C5a des Arg to its receptor on U-937 cells is effectively irreversible, it would be expected that, on addition of increasing amounts of C5a des Arg to a fixed number of cells, there would be a complete uptake of the ligand until saturation was achieved, whereafter there should be no further uptake. This is not the case. Fig. 5(*a*) shows, as a function of time, the effect of sequential addition of ^{125}I-C5a des Arg to the cells on the amount of ^{125}I-C5a des Arg bound. At 0.5 nM-^{125}I-C5a des Arg the ligand binds to the cells, reaching an apparently saturated level with 55% remaining unbound. However, increasing the concentration of ^{125}I-C5a des Arg to 1 nM leads to a further increase in the amount of ^{125}I-C5a des Arg bound, despite the fact there was already an excess of free ^{125}I-C5a des Arg available at the lower concentration. This process could be repeated several times, as Fig. 5(*a*) shows.

If the unbound ^{125}I-C5a des Arg was removed and added to fresh cells normal binding was still achieved, showing that the free ligand was not simply unable to bind owing to, for example, denaturation. Identical results to those shown in Fig. 5(*a*) were obtained if the particular concentration of ^{125}I-C5a des Arg was added immediately to fresh cells, rather than sequentially. Fig. 5(*b*) shows the result of Fig. 5(*a*) with the binding of ^{125}I-C5a des Arg expressed as a function of unbound ^{125}I-C5a des Arg

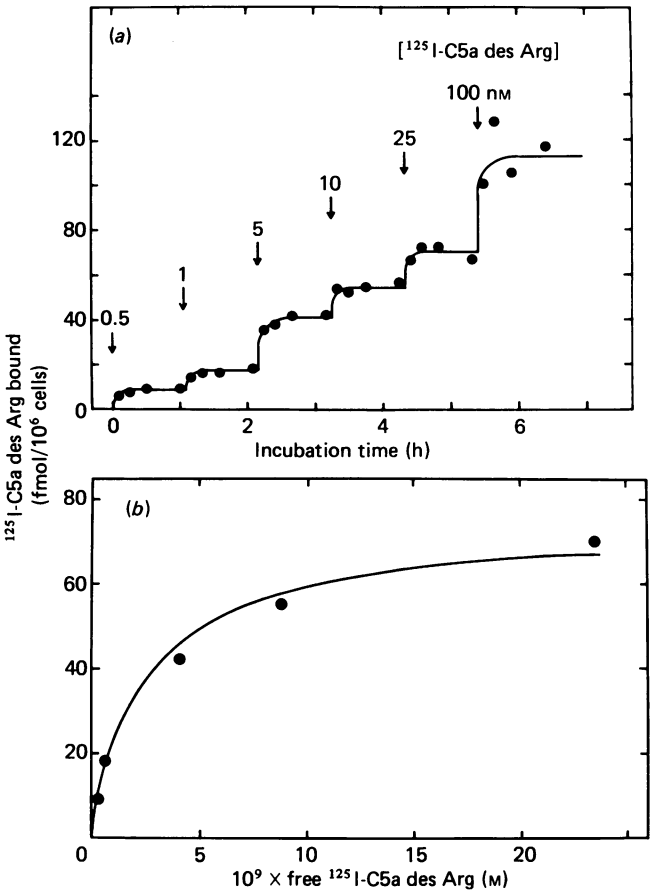

Fig. 5. Effect of adding increasing concentrations of ^{125}I-C5a des Arg to Bt_2cAMP-treated U-937 cells on the amount of ^{125}I-C5a des Arg bound at 0 °C

(*a*) Time course. The method was as described in the legend to Fig.1 with 2×10^7 cells/ml and an initial concentration of 5×10^{-10} M-^{125}I-C5a des Arg. The arrows denote addition of ^{125}I-C5a des Arg to give the final concentrations indicated. Non-specific binding was deducted from the total binding to give the specific amount bound shown. The percentage of ^{125}I-C5a des Arg bound ranged from 45% at 0.5 nM-^{125}I-C5a des Arg to about 2% at 100 nM-^{125}I-C5a des Arg. At concentrations of ^{125}I-C5a des Arg above about 25 nM the very low percentage binding relative to the non-specific binding leads to large errors in the data points; consequently it is not possible to determine precisely at what concentraion of ^{125}I-C5a des Arg complete saturation of receptors occurs. (*b*) The results of (*a*) are plotted as a function of free ^{125}I-C5a des Arg. The line represents the theoretical equilibrium binding curve for a $K_{ass.}$ of 0.5 nM^{-1} with a total number of 40000 receptors/cell, omitting, for the reasons outlined above, binding data at concentrations of ^{125}I-C5a des Arg above 25 nM. Adopting the model proposed in the text, the $K_{ass.}$ refers to the binding constant for the reversible site and the number of receptors/cell refers to the sum of reversible and irreversible sites. The model requires that a small number of reversible sites modulate a proportionally larger number of essentially irreversible sites.

concentration. The increase in binding with increase in C5a des Arg concentration was unaffected by cycloheximide and so is not due to protein synthesis *de novo* of receptor. Furthermore, binding was measured at 0 °C and inhibitors of respiration (azide and fluoride) and of microfilament rearrangement (cytochalasin B) were all unable to prevent the increase. Addition of the peptide chemoattractant *N*-formyl-Met-Leu-Phe had no effect on ^{125}I-C5a des Arg binding.

Discussion

Taken together the above results seem to indicate that the cell, sensing the extracellular concentration of C5a des Arg, is able to induce the expression of a related number of high-affinity receptors.

Such a mechanism would appear to be compatible with monocyte chemotaxis to C5a. It has been suggested that the rapid binding and internalization of C5a by neutrophils plays an important role in clearing this potent and potentially lethal anaphylatoxin from the circulation (Chenoweth & Goodman, 1983). This is a role in which monocytes may also be envisaged to play a part. However if all the receptors were initially available for binding of C5a, the very slow rate of dissociation would rapidly lead to their saturation, even at low concentrations of attractant. Unless these receptors could be rapidly recycled and/or resynthesized, the cell would thus be rendered chemotactically insensitive. In the case of neutrophils at least, the re-expression of the C5a receptor after down-regulation appears to be a relatively slow process, taking several hours (Chenoweth & Goodman, 1983). If however, as these results suggest, the cells were able to restrict the numbers of receptors expressed at any one concentration of C5a des Arg, and only allow expression of an ever increasing number as the cell moved up the concentration gradient, it would allow the cells to respond over a much greater range of attractant concentrations without saturation of all the receptors. Neutrophils have been shown to contain an intracellular pool of receptors for the peptide chemoattractant *N*-formyl-Met-Leu-Phe which have been postulated to play a similar role in the chemotactic response to this attractant (Gallin *et al.*, 1983; Fletcher & Gallin, 1983).

The mechanism proposed requires that the cells are able to detect concentrations of C5a des Arg at which the high-affinity binding sites should be saturated, which would seem to implicate the existence of further binding sites of lower affinity. For this to be the case the high-affinity binding sites must greatly outnumber the lower affinity sites, which would otherwise be detectable as such in the displacement studies.

One possibility is that there are a relatively small number of low-affinity binding sites on the cell surface, effectively monitoring the extracellular C5a des Arg concentration. Binding of C5a des Arg to these sites results in the localized expression of a proportional amount of a relatively large number of high-affinity binding sites. Binding of C5a des Arg to these high-affinity sites is then responsible for maintaining chemotaxis.

Such a mechanism would serve to amplify the chemotactic signal at low concentrations of C5a des Arg whilst enabling the cells to still respond at much higher concentrations.

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