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Recruitment of actin to the cytoskeletons of human monocyte-like cells activated by complement fragment C5a

Is protein kinase C involved?

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U-937 cells differentiated by exposure to dibutyryl cyclic AMP respond to complement fragment C5a with a marked increase in cytoskeletal F-actin, which can be detected by fluorescence-activated cell sorting (f.a.c.s.) analysis of their rhodamine phalloidin-stained cytoskeletons. The C5a-induced increase in F-actin content can be prevented by prior exposure of the cells to cytochalasin B and pertussis toxin. It is insensitive to removal of extra cellular Ca^{2+}, to cholera toxin or to neomycin. Phorbol myristate acetate (PMA), an activator of protein kinase C, does not induce actin polymerization in the differentiated cells. Both C5a and PMA stimulate superoxide production. The action of C5a on superoxide formation is also inhibited by neomycin, a phospholipase inhibitor. These results suggest that the cytoskeletal response to C5a requires activation of a G protein, but probably does not involve phospholipase C and protein kinase C, and is not highly dependent on the availability of Ca^{2+}. Phospholipase C and kinase C may, however, be components of the pathway leading from C5a binding to superoxide production.

INTRODUCTION

The responses of neutrophils to binding the chemotactic peptide *N*-formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) have been studied extensively. Much less is known about the responses of monocytes to chemotactic agents, probably because their low abundance makes purification difficult. Recently, Chenoweth *et al.* (1984) and Barker *et al.* (1986) have shown that a human pre-monocyte-like cell line, U-937, which does not exhibit chemotaxis, differentiates on exposure to dibutyryl cyclic AMP (dbc AMP) into monocyte-like cells which express C5a receptors on their surface. Such differentiated cells respond to complement fragment C5a by displaying a spectrum of responses, including chemotaxis, typical of monocytes (Sheth *et al.*, 1988).

One of the earliest responses of neutrophils to activation by fMet-Leu-Phe is recruitment of F-actin to their cytoskeletons from a cytoplasmic pool of G-actin (White *et al.*, 1983; Howard & Meyer, 1984). The full sequence of events linking ligand binding to actin polymerization remains obscure, although a number of features have emerged. There is evidence that a G protein sensitive to pertussis toxin is a component of the main pathway (Koo *et al.*, 1983; Okajima *et al.*, 1985; Shefcyk *et al.*, 1985), but that changes in intracellular levels of cyclic AMP, Ca^{2+} and H^+ (Sha'afi *et al.*, 1986; Sheterline *et al.*, 1986) are likely to be of major significance. Increased turnover of PtdIns(4,5)P_2 observed on ligand binding (Dougherty *et al.*, 1984; Di Virgilio *et al.*, 1985; Rossi *et al.*, 1985; Verghese *et al.*, 1986) and the ability of phorbol myristate acetate (PMA) to induce actin polymerization *in vivo* (Sheterline *et al.*, 1986) have led to suggestions that phospholipase C and protein kinase C also form part of the chain. At present, however, there is no direct evidence that phosphorylation constitutes part of the sequence of events between receptor activation and F-actin formation. Indeed discrepancies between the time courses of PMA-induced polymerization and phosphorylation (White *et al.*, 1984) and the ability of phorbol didecanoate to induce actin polymerization, although not being an activator of kinase C (White *et al.*, 1984), argue against a role for phosphorylation in promoting the early cytoskeletal changes induced by chemotactic agents.

The experiments described below were undertaken to discover whether cytoskeletal changes induced in a monocyte-like cell by C5a resemble those evoked in neutrophils by fMet-Leu-Phe. They have revealed that the U-937-derived cells are convenient for the study of molecular events underlying the early stages of chemotaxis, and they have thrown doubt on a role for phospholipase C and protein kinase C in ligand-induced actin polymerization.

MATERIALS AND METHODS

Preparation of differentiated U-937 cells

U-937 cells (0.4×10^6 cells/ml) were incubated at 37 °C for 2 days in 25 mM-Hepes + RPMI 1640 medium (Gibco) supplemented with 10% (v/v) foetal-calf serum and containing 1 mM-dbc AMP under an atmosphere of air/CO_2 (19:1) saturated with water vapour. The cells were then sedimented at 500 *g* for 5 min and resuspended in Hepes + RPMI medium and re-spun before being resuspended in the same medium, or phosphate-buffered saline (140 mM-NaCl/15 mM-KCl/8mM-Na_2HPO_4/1.4 mM-KH_2PO_4), pH 7.0, at a density of 4×10^6 cells/ml at 20 °C. After Giemsa staining, but before incubation with dbc AMP, cell nuclei were paler than the cytoplasm, whereas after differentiation they were darker.

Abbreviations used: f.a.c.s., fluorescence-activated cell sorting; PMA, phorbol myristate acetate; C5a, complement fragment C5a; fMet-Leu-Phe, *N*-formylmethionyl-leucylphenylalanine; dbc AMP, dibutyryl cyclic AMP; PtdIns(4,5)P_2, phosphatidylinositol 4,5-biphosphate; Ins(1,4,5)P_3, inositol 1,4,5-triphosphate.

Preparation and staining of cytoskeletons

Incubation of cells with C5a and other agents was terminated by transfer of 0.25 ml of incubation mixture containing 10^6 cells to 0.25 ml of a lysis fixative stain solution containing 0.33 μM-rhodamine phalloidin (Molecular Probes, Eugene, OR 97402, U.S.A.), 7.4% (w/v) formaldehyde and lysophosphatidylcholine (0.2 μg/ml) in phosphate-buffered saline, pH 7.0. After 20 min at 20 °C the stained cytoskeletons were sedimented at 500 *g* for 5 min and resuspended in phosphate-buffered saline before f.a.c.s. analysis.

Preparation and properties of rabbit C5a

Highly purified rabbit C5a was a gift from Dr. P. J. Jose and Dr. T. J. Williams, MRC Clinical Research Centre, Harrow, Middx., U.K. C5a was prepared from zymosan-activated rabbit serum as described for rabbit des-Arg-C5a (Jose *et al.*, 1983), but in the presence of 1 M-6-aminohexanoic acid to prevent cleavage of the *C*-terminal arginine residue.

The binding of radioiodinated rabbit C5a to dbc AMP-treated U937 cells was indistinguishable from that of radioiodinated rabbit des-Arg-C5a reported previously by Barker *et al.* (1986). The binding was rapid, saturable and inhibited by the simultaneous addition of an excess of unlabelled C5a. Like rabbit des-Arg-C5a, radioiodinated C5a, once bound, could not be displaced from the cells, even at 0 °C in the presence of azide (conditions that prevent receptor internalization), indicating that the binding was effectively irreversible (M. D. Barker, unpublished work). Because binding is irreversible, it is not possible to determine an association constant for the binding of rabbit C5a to the cells. Chemotaxis assay performed as described by Barker *et al.* (1986) confirmed that rabbit C5a was a potent chemoattractant for dbc AMP-treated U937 cells, optimal migration occurring at a C5a concentration of approx. 10 nM (M. D. Barker, unpublished work).

Microscopy

Stained cytoskeletons mounted in Gelvatol were examined with a Leitz Diallux 22EB fluorescence microscope and photographed with an Olympus OM-2 camera on Kodak Pan-X or Konica Chrome 100 ASA 100 film.

Quantification of staining with rhodamine phalloidin

Histograms of fluorescence intensity versus channel number for cytoskeletons stained with rhodamine phalloidin were recorded with a Becton–Dickinson model 420 fluorescence-activated cell sorter linked to a Becton–Dickinson accessory computer. Stained cytoskeletons were excited at 514 nm by an argon laser within 2 h of preparation, and their fluorescence was measured through a 575 nm-band-pass filter with a band width of 26 nm. In most instances 25000 cells were analysed, and cell debris, if any, was gated out. The mean fluorescence intensity/cell was used as a measure of F-actin content (see Howard & Meyer 1984). Relative F-actin content is the ratio of mean experimental fluorescence intensity to mean control fluorescence intensity.

Loading cells with Quin 2

Cells (4.5×10^6/ml) were suspended in a Ca^{2+}-free Hanks solution containing EGTA (1 mM) and were incubated with Quin 2 acetoxymethyl ester (20 μM) for 30 min at 37 °C. Conversion of the ester into Quin 2 was monitored spectrophotometrically at 540 nm, and the concentration of free Ca^{2+} ions within the cells was calculated as described by Tsien *et al.* (1982).

Measurement of superoxide production

At 3 h before use, differentiated U-937 cells were suspended at a density of 10^7 cells/ml in ice-cold Hanks salt solution, pH 7.2, containing 1% (w/v) fatty-acid-free bovine serum albumin and lacking Phenol Red. Superoxide production was measured at 37 °C in an LKB–Wallac 1250 luminometer coupled to a Servoscribe recorder. Cells were warmed up to 37 °C in a Techne OB-3 Dri Block immediately before being transferred to the luminometer cuvette holder, which was also maintained at 37 °C. Incubation mixtures contained 6×10^6 cells and 0.5 mM-lucigenin in a final volume of 1ml.

Materials

All fine chemicals were obtained from Sigma Chemical Co., except pertussis toxin, which was a gift from Dr. L. Irons, Public Health Laboratory Service, Porton Down, Salisbury, Wilts., U.K., and rabbit C5a, which was also a gift (see above). Gelvatol was supplied by Cairn Chemicals, Chesham, Bucks., U.K.

RESULTS

Effect of C5a on the F-actin content of cytoskeletons

The fluoresence intensity of cytoskeletons stained with rhodamine phalloidin may be taken as a measure of their relative content of F-actin. Exposure of dbc AMP-treated U-937 cells to 10 nM-C5a for 2 min at 20 °C led to an approximate doubling in the capacity of their cytoskeletons to bind rhodamine phalloidin as revealed by the shift in the peak of the fluorescence histogram obtained by flow cytometry (Fig. 1). Pretreatment of differentiated cells for 30 s with cytochalasin B (5 μg/ml) abolished the response. The time course of the change in F-actin content after treatment with C5a is shown in Fig. 2. Maximum fluorescence occurred at about 2 min,

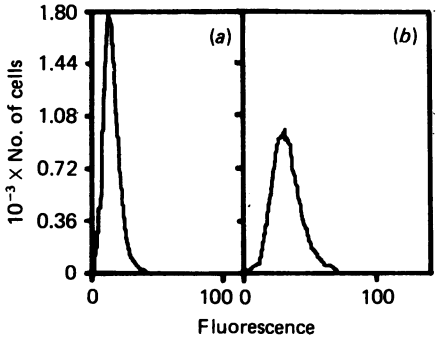

Fig. 1. Fluorescence histograms of cytoskeletons from control and C5a-treated cells

(*a*) Fluorescence histogram derived from f.a.c.s. analysis of 25000 rhodamine phalloidin-stained cytoskeletons from control, differentiated U-937 cells. (*b*) Fluorescence histogram derived from f.a.c.s. analysis of 25000 rhodamine phalloidin-stained cytoskeletons of cells treated with 10 nM-C5a for 2 min at 20 °C.

but was already well developed by 15 s. By 40 min the relative F-actin content had returned to basal levels. The dose–response curve (Fig. 3) shows that half-maximal increase in F-actin content was achieved with between 10^{-9}- and 10^{-8} M-C5a.

Fluorescence microscopy of cytoskeletons from C5a-treated cells

In the absence of C5a, a diffuse distribution of fluorescence was observed in the cytoskeletons of differentiated U937 cells. The fluorescence intensity increased within 1 min of stimulation with C5a, and by 5 min the distribution of fluorscence was patchy and the outlines of the cytoskeletons were irregular. At 20 min the cytoskeletons tended to be elongated and distinctly polarized. By 40 min they had resumed a more spherical form, but still displayed a patchy distribution of fluorescence. It should be noted that, when the cytoskeletons were most polarized, their F-actin content had fallen well below its maximal value (Fig. 2).

Ca^{2+}-dependence of the cytoskeletal response to C5a

Reducing the extracellular concentration of Ca^{2+} to 1.1×10^{-8} M by inclusion of 5 mM-EGTA in the suspending Hepes+RPMI medium (see Portzehl *et al.*, 1964) was without effect on the evoked response, although it did cause some increase in the basal level of cytoskeletal actin. Similar results were obtained when cells were suspended in a Ca^{2+}-free balanced salt solution containing 1 mM-EGTA. In a further experiment in which Quin 2-loaded cells (see the Materials and methods section) were resuspended in Ca^{2+}-free medium containing 1 mM-EGTA, C5a still evoked a response, despite

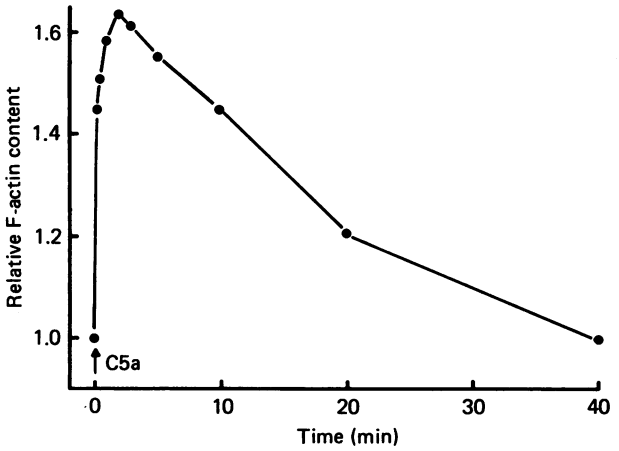

Fig. 2. Time course of the effect of C5a upon the cytoskeletal content of F-actin

Differentiated U-937 cells were incubated with 10 nM-C5a at 20 °C for various periods of time before being fixed, permeabilized, stained and subsequently analysed by f.a.c.s. as described in the Materials and methods section.

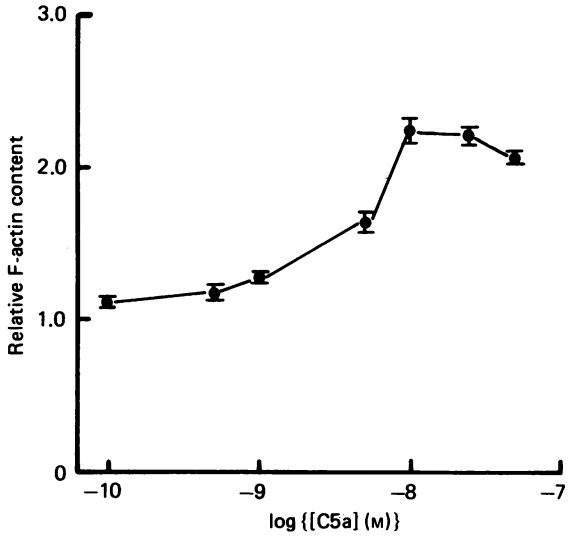

Fig. 3. Dose–response curve for the effect of C5a on the F-actin content of cytoskeletons

Differentiated U-937 cells were incubated with various concentrations of C5a for 2 min at 20 °C before being fixed, permeabilized, stained and subsequently analysed by f.a.c.s. as described in the Materials and methods section. The vertical bars indicate the S.D. ($n = 4$).

Table 1. Ca^{2+}-dependence of the cytoskeletal response to C5a

Differentiated U937 cells suspended in Hepes+RPMI 1640 medium with or without added EGTA (5 mM) were stimulated for 2 min with C5a (10 nM) at 20 °C. In the presence of EGTA the calculated concentration of extracellular Ca^{2+} was 1.1×10^{-8} M. Another sample of the same batch of cells was loaded with Quin 2 as described in the Materials and methods section and resuspended in a Ca^{2+}-free medium (148 mM-NaCl/4.9 mM-KCl/9.0 mM-D-sorbitol/2.6 mM-K_2HPO_4/1 mM-KH_2PO_4/10.1 mM-Hepes, adjusted to pH 7.3), containing 1 mM-EGTA. The calculated intracellular concentration of free Ca^{2+} was 35.5 nM. The Ca^{2+}-depleted cells were incubated for 2 min at 20 °C with 10 nM-C5a before their cytoskeletal content of F-actin was determined.

	Relative F-actin content [mean ± S.D. (*n*)]					
	Cells suspended in Hepes+RPMI 1640 medium				Cells suspended in Ca^{2+}-free balanced-salt medium	
C5a...	−	+	−	+	−	+
EGTA...	−	−	5 mM	5 mM	1 mM	1 mM
Quin-2...	−	−	−	−	+	+
	1.0 ± 0.02 (2)	2.41 ± 0.44 (2)	1.39 ± 0.09 (2)	2.71 ± 0.65 (2)	1.30 ± 0.17 (2)	2.09 ± 0.41 (2)

the intracellular concentration of free Ca^{2+} being only 35.5 nM (Table 1).

Effects of pertussis toxin and cholera toxin on the response to C5a

Preincubation of cells with pertussis toxin (1 μg/ml) for 5 h at 37 °C caused a marked reduction in the amount of actin recruited to the cytoskeletons of cells treated with C5a (10 nM) for 2 min at 20 °C. C5a alone increased the relative F-actin content from 1.0 ± 0.02 (4) to 1.93 ± 0.03 (4), whereas after pretreatment with pertussis toxin it increased the value to only 1.24 ± 0.01 (4). In contrast, preincubation with cholera toxin (1 μg/ml) for 2 h at 37 °C had no significant effect on the response to C5a. C5a alone increased the relative F-actin content from 1.0 ± 0.01 (4) to 1.67 ± 0.03 (4), whereas after pretreatment with cholera toxin it still increased the value to 1.60 ± 0.02 (4).

Treatment with PMA

Treating dbc AMP-differentiated U-937 cells with PMA for 2 min over a range of concentrations from 0.01 to 1.0 μg/ml and at 1.0 μg/ml for up to 10 min failed to cause any significant change in the F-actin content of cytoskeletons, although C5a alone more than doubled the amount. Pretreatment of cells with PMA (1 μg/ml) for 1 min had no effect on subsequent stimulation with C5a (10 nM for 2 min). The response to C5a was unaffected by the presence of dimethyl sulphoxide, which was used as the vehicle for PMA.

Effects of neomycin on the cytoskeletal response to C5a

Incubation of differentiated cells with neomycin (10–100 μM) for 1 h at 20 °C before stimulation was without effect upon the increase in cytoskeletal actin induced by exposure to 10 nM-C5a for 2 min.

Superoxide production in response to C5a and PMA

Both C5a (10 nM) and PMA (1 μg/ml) caused increases in superoxide production by cells incubated at 37 °C after 3 h at 40 °C. The response to C5a rose rapidly and declined almost to baseline levels by 5 min, whereas the response to PMA rose more slowly, achieved a lower maximum, but was much more persistent. Superoxide-induced fluorescence of lucigenin was quenched by treatment with superoxide dismutase. The effect of C5a was stongly inhibited by 100 μM-neomycin.

DISCUSSION

The rapid increase in cytoskeletal F-actin induced by brief exposure of differentiated U-937 cells to C5a resembles that evoked in neutrophils by the chemotactic peptide fMet-Leu-Phe. It can be inhibited by prior treatment with cytochalasin B and pertussis toxin, is relatively independent of Ca^{2+} and is insensitive to cholera toxin. On the other hand, PMA, which has been shown to cause a marked recruitment of F-actin to the cytoskeleton of several cell types, is without such effects on these human monocyte-like cells. This ineffectiveness of a much-used activator of protein kinase C suggests that phosphorylations carried out by the kinase do not stand on the main pathway of receptor-mediated actin polymerization in U-937-derived cells. Such a possibility is consistent with the finding that, although both PMA and phorbol didecanoate induce rapid polymerization of F-actin in neutrophils, only the former causes increased protein phosphorylation. Moreover the increases in actin polymerization caused by both agents are unaffected by concentrations of trifluoroperazine which inhibit kinase C, phosphorylation and degranulation (White *et al.*, 1984). Sklar *et al.* (1985) have shown that the rapid initial phase of actin polymerization in neutrophils occurs at a much lower level of receptor occupancy than some other responses, such as the rise in intracellular Ca^{2+} concentration, superoxide production and elastase secretion. This observation may suggest a branching of effector pathways at a point soon after ligand binding.

It has been proposed (Verghese *et al.*, 1986) that binding of fMet-Leu-Phe to its receptor activates a pertussis-sensitive G protein, which stimulates phospholipase C to hydrolyse PtdIns(4,5)P_2 to diacylglycerol and inositol 1,4,5,-trisphosphate (Ins(1,4,5)P_3). The former is then thought to activate kinase C and thereby initiate actin polymerization. In support of that view, Vergehese *et al.* (1986) have shown that fMet-Leu-Phe causes a rapid, transient and pertussis-sensitive increase in Ins(1,4,5)P_3 production in human monocytes. A similar response to the chemotactic peptide has been found in human neutrophils (Di Virgilio *et al.*, 1985).

It is possible that changes in Ins(1,4,5)P_3 concentration caused by G-protein activation of phospholipase C could modify the behaviour of proteins which regulate actin polymerization. Janmey & Stossel (1987) found that PtdIns(4,5)P_2 has complex effects on the actin-filament severing and nucleating properties of gelsolin, and Lassing & Lindberg (1985) have described interactions between PtdIns(4,5)P_2 and profilin. On the other hand, actin polymerization might be controlled more directly by interactions between regulatory proteins and activated G proteins. The failure of neomycin, a known phospholipase inhibitor (Bittner *et al.* 1986), to prevent C5a-induced actin polymerization in the present experiments tends to favour the latter possibility. The ability of neomycin to inhibit C5a-evoked superoxide production also suggests that cytoskeletal recruitment and superoxide-production pathways diverge at the level of G-protein activation. It is clear that, whatever the precise mechanism by which it is achieved, treatment with the chemotactic peptide causes a rapid increase in the ability of the membranes of polymorphonuclear leucocytes to act as nucleating centres for actin polymerization (Carson *et al.*, 1986).

The experiments described above indicate that further study of differentiated U-937 cells may shed light on the steps involved in coupling a chemotactic stimulus to actin polymerization. They suggest that there may be a rather direct interaction between an activated G-protein and membrane-bound elements of the cytoskeleton which does not involve activation of either phospholipase C or kinase C.

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