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REVIEW

SHAPE OSCILLATIONS OF HUMAN NEUTROPHIL LEUKOCYTES: CHARACTERIZATION AND RELATIONSHIP TO CELL MOTILITY

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Summary

When neutrophil leukocytes are stimulated by chemotactic factors or by substratum contact, they change their shape. Shape changes are a prerequisite for cellular migration and typically involve the extrusion of thin, veil-like lamellipods and the development of morphological polarity. Stimulation also leads to changes in the neutrophil content of filamentous actin (F-actin), which is the major cytoskeletal component. Suspensions of human neutrophils stimulated with chemoattractants exhibit sinusoidal light-scattering oscillations with a period of approximately 8 s at 37 °C. These oscillations arise from periodic fluctuations in the cell body size caused by lamellipod extension and retraction cycles. The light-scattering oscillations are paralleled by corresponding oscillations in F-actin content. This raises the interesting possibility that cyclic actin polymerization constitutes the driving force for shape oscillations of suspended neutrophils. Similar periodic shape changes are present in neutrophils crawling on a surface, suggesting that shape oscillations are important for neutrophil motion. This review summarizes our present knowledge about shape oscillations in suspended and crawling neutrophils and discusses a possible role for these oscillations in neutrophil motility.

Key words: polymorphonuclear leukocyte, locomotion, shape change, lamellipod, pseudopod, cytoskeleton, actin, turbidimetry.

Introduction

Motile cells change their shape and crawl in response to external stimuli by the extrusion and retraction of lamellipods, a process that requires rearrangement of cytoskeletal actin (Stossel, 1993). Cycles of small-scale shape changes then result in net cell translocation (Hartman *et al.* 1994). When viewed at sufficiently high temporal resolution, cyclic periodicities are found in the cellular shape changes of many crawling cell types, including neutrophil leukocytes (Hartman *et al.* 1994), *Dictyostelium discoideum* (Killich *et al.* 1993), *Physarum polycephalum* and *Amoeba proteus* (Satoh *et al.* 1985). We have been interested in the shape-change periodicities of suspended and attached human neutrophils. These phagocytic cells constitute the first-line defense system of the vertebrate organism against microbes. They migrate up chemotactic gradients to infection sources, where they kill invading microorganisms by the release of powerful oxygen radicals ('respiratory burst') and digestive enzymes. Here, we discuss our recent results supporting the hypothesis that shape oscillations are a fundamental feature of neutrophil migration.

Characterization of shape oscillations

Suspended neutrophils

Suspended resting neutrophils are spherical, with a volume of 300 μm^3 (Ting-Beall *et al.* 1993), corresponding to a diameter of 8.3 μm, and are smooth without membrane ruffling (Wilkinson and Haston, 1988; Watts *et al.* 1991). Upon stimulation with chemotactic agonists, these cells undergo a shape change which can be assessed both microscopically and turbidimetrically (Yuli and Snyderman, 1984; Wymann *et al.* 1987). The cellular protrusions formed after stimulation can be morphologically separated into lamellipods and pseudopods (Luna and Hitt, 1992; Stossel, 1994). Lamellipods (also termed 'membrane ruffles') develop early after stimulation, are thin, clear, veil-like and extruded across large areas of the cell surface, while the cell body *per se* remains spherical (Wymann *et al.* 1987; Watts *et al.* 1991). Pseudopods are produced later and are of moderate width with respect to the dimensions of the cell; they give the activated neutrophil a polarized morphology (Watts *et al.* 1991) and are similar to the pseudopod extruded

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Table 1. *Agonists provoking shape oscillations in human neutrophils*

Agonist	Origin	Reference
Peptides		
N-Formyl-Met-Leu-Phe	Bacteria	Wymann *et al.* (1987, 1989, 1990); Ehrengruber *et al.* (1995*b*)
Complement fragment 5a	Complement system	Wymann *et al.* (1987); Ehrengruber *et al.* (1995*b*)
Interleukin-8	Macrophages and endothelial cells	Ehrengruber *et al.* (1995*b*)
Neutrophil-activating peptide-2	Platelets	M. U. Ehrengruber (unpublished results)
Lipids		
Leukotriene B_4	Phagocytes	Wymann *et al.* (1987); Omann *et al.* (1989)
Platelet-activating factor	Leukocytes and tissue cells	Wymann *et al.* (1987); Omann *et al.* (1989)

Certain agonists known to induce scattering-detected shape changes, i.e. GROα/MGSA (Geiser *et al.* 1993) and complement fragment 3a (Ehrengruber *et al.* 1994*b*), were unable to provoke shape oscillations (M. U. Ehrengruber and D. A. Deranleau, unpublished results).

towards higher chemoattractant concentrations in cells crawling over a substratum (Devreotes and Zigmond, 1988). Thus, we can think of shape change as consisting of at least two superimposed events: (i) the development of a dominant pseudopod, resulting in a polar shape; and (ii) the formation of smaller, veil-like membrane ruffles superimposed on the dominant pseudopod.

The cellular scattering extinction coefficient, which we determined as 65.8±6.9 for resting neutrophils at 37 °C (mean ± S.D. at 632.8 nm, N=332; M. U. Ehrengruber and D. A. Deranleau, unpublished results), is a sensitive measure of cellular size and/or shape (Latimer, 1979; Deranleau, 1987). Chemoattractants such as *N*-formyl-Met-Leu-Phe (fMLP) decrease neutrophil extinction and light scattering by inducing lamellipod formation (Yuli and Snyderman, 1984; Sklar *et al.* 1984; Wymann *et al.* 1987). In synchronized cell populations,

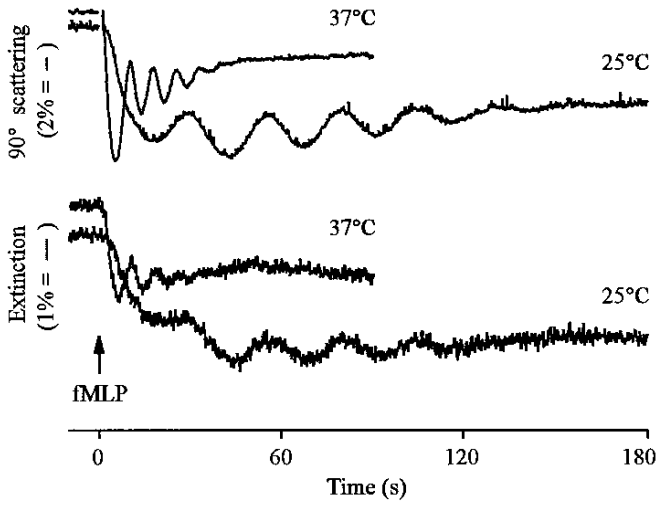

Fig. 1. Neutrophil shape oscillations at 25 and 37 °C. Relative changes in 90 ° scattering (top) and extinction (bottom) of cells pretreated with 1 μmol l^{-1} 17-hydroxywortmannin (HWT) (for 10 min) and stimulated with 10 nmol l^{-1} *N*-formyl-Met-Leu-Phe (fMLP, arrow) are shown. The extinction coefficient of resting human neutrophils at 37 °C is 65.8±6.9 (mean ± S.D. at 632.8 nm, N=332).

the agonist-induced scattering changes exhibit sinusoidal, temperature-dependent oscillations (Fig. 1) with a period of 8.4±0.8 s at 37 °C (Wymann *et al.* 1987; Omann *et al.* 1989; Ehrengruber *et al.* 1995*b*). They can be provoked by both peptide and lipid agonists (Table 1) and show the same activation energy as oscillations in neutrophil filamentous actin (F-actin) content (Omann *et al.* 1989; Ehrengruber *et al.* 1995*b*). While clearly oscillating responses are rarely observed in suspensions of resting neutrophils, they are routinely 'enabled' by pretreatment with wortmannin, 17-hydroxywortmannin (HWT; phosphatidylinositol 3-kinase inhibitors), with phorbol 12-myristate 13-acetate and by prestimulation with an agonist (Wymann *et al.* 1987, 1989; Ehrengruber *et al.* 1995*b*). We have demonstrated that the oscillation frequencies increase with increasing prestimulation of the cells (Ehrengruber *et al.* 1995*b*).

Scattering oscillations are paralleled by inverse oscillations in F-actin content (Omann *et al.* 1989; Wymann *et al.* 1990). Scattering theory suggests that the optical oscillations result from cyclic alterations in cell body size due to the extension and retraction of comparatively weakly scattering lamellipods (Deranleau, 1988; Wymann *et al.* 1990), a prediction which we recently verified (Ehrengruber *et al.* 1994*a*). As motility of cells moving on surfaces is characterized by extrusion and remodeling of lamellipods, shape oscillations of suspended neutrophils could reflect crawling motions of activated cells (Wymann *et al.* 1990). Newly formed lamellipods typically contain an F-actin network (Fechheimer and Zigmond, 1983; Zigmond, 1993) which may be more labile than that in the cell body (Cassimeris *et al.* 1990). The presence of more than one pool of F-actin has been documented operationally and biochemically (Watts and Howard, 1992, 1993, 1994), although morphological localization has not been accomplished.

Crawling neutrophils

Neutrophil migration consists of three components (Bignold, 1992): (i) the cellular driving motor; (ii) the transmission of driving forces to the cell periphery; and (iii)

the traction of the cell periphery on the substratum, where reversible adhesive interactions between lamellipods and the substratum occur. Reversible adhesive interactions formed at one stage must be reversed at a later stage, when the cell body has followed the leading lamellipod (Felder and Kam, 1994).

In the absence of a chemotactic gradient, neutrophils extend pseudopods in random directions, and the cells show undirected motility with a constant frequency of cellular turns. Chemoattractants do not alter the turning frequency of the cell, but dramatically decrease the angle between the direction of pseudopod extension and the chemotactic gradient, rapidly resulting in accurate orientation of the cell along the gradient (Zigmond *et al.* 1981; Devreotes and Zigmond, 1988; de Boisfleury-Chevance *et al.* 1989). As little as a 2 % change in concentration over 10 μm of cell length can be detected by a neutrophil. This orientation accuracy of the neutrophil is established by a mechanism that senses the change in receptor occupancy which occurs upon displacement along the cell (Devreotes and Zigmond, 1988).

Measurements made on spontaneously migrating single neutrophils show that they exhibit shape oscillations with periodicity components at 8 and 20–30 s at 37 °C. No pretreatment is required to enable oscillations and they occur in about 75 % of migrating cells (Hartman *et al.* 1994). When neutrophils migrate in a chemotactic gradient, their cell velocity oscillates (between 10 and 30 μm min^{-1}) with a period of 50–55 s. During their periodic bursts of speed, neutrophils are more flattened, possess an extended leading lamella forming contacts with the substratum and, with a slight phase lag, rapidly move their cell body over the immobile contacts (Felder and Kam, 1994). The same period was found for the characteristic time of the 'internal clock' between successive directional changes (de Boisfleury-Chevance *et al.* 1989). An elegant real-time study (Guilford *et al.* 1995) demonstrated that the force production of locomoting macrophages is periodic (period of 33–100 s for unstimulated cells, 50–200 s after addition of fMLP) and correlates with extension of the leading lamella. This suggests that mechanical oscillations are present in cells other than neutrophils. Guilford *et al.* (1995) also showed that force generation is not well correlated to cell ruffling, and concluded that ruffling and locomotion are controlled differentially and have distinct origins.

Signal transduction

The signal transduction cascade that controls shape change and motility is not clearly understood, though the number of candidate molecules seems to increase daily. Regulation of actin polymerization and cross-linking, ion channels and adhesion proteins are all involved. We attempted to investigate some possible mechanisms by using pharmacological manipulations of signal transduction pathways. While the specificity of such approaches is always in question, these studies point to directions for further detailed biochemical studies.

There are several treatments which block the neutrophil shape change and, therefore, prevent shape oscillations. We have listed various agents from our own experience or from the literature which were tested on the fMLP-induced shape change, on oscillations or on both (Table 2). For reference, we have included their effect on the respiratory burst as well. Several conclusions can be drawn from the data. First, shape change and oscillations depend on agonist–receptor binding and heterotrimeric G-proteins, as well as on the presence of an intact cytoskeleton. Second, tyrosine phosphorylation seems to be important, though no separate effect on oscillations could be seen. All agents to date which inhibit shape change also block oscillations. Third, as cell volume and shape change are regulated partially by fluid and Na^+ transport (Rosengren *et al.* 1994), Na^+ homeostasis could be involved in the oscillations. While manipulation of Na^+ transport had no effect on shape change or oscillations, replacement of all external Na^+ with choline inhibited the initial phase of the fMLP-induced shape change by approximately 50 % and almost completely blocked the oscillations. Furthermore, these manipulations had no effect on the initial fMLP-induced change in F-actin content (M. U. Ehrengruber, D. A. Deranleau and T. D. Coates, unpublished observations). This suggests that the initial shape change is partially due to actin-independent volume changes.

The only treatment that selectively affects oscillations without blocking the shape change *per se* was internal ATP depletion (Table 2). Addition of fMLP to ATP-depleted neutrophils induced a non-oscillating shape change, which did not return to baseline (up to 30 min), whereas agonist-induced transients reversed to resting levels after approximately 3 min in control cells. This persistent shape change was reversed by addition of receptor antagonist. Subsequent addition of another agonist (interleukin-8), acting through a different receptor, stimulated a second sustained shape change (M. U. Ehrengruber and D. A. Deranleau, unpublished results). These data imply that ATP is not required for actin polymerization but is required for remodeling actin. Furthermore, the presence of an agonist is required to maintain a shape change. Our conclusion that ATP is not required for actin polymerization agrees with the finding that inhibition of tyrosine phosphatases alone, which do not depend on ATP, elicits neutrophil shape change, whereas shape change is prevented by tyrosine kinase inhibitors (Bennet *et al.* 1993). In addition, we found that inhibition of tyrosine phosphatases prevents subsequent fMLP-induced shape changes and oscillations (Table 2). Thus, one can speculate as to a possible role for tyrosine kinase/phosphatase balance in the control of neutrophil shape.

Parallel sinusoidal oscillations in cellular shape and respiratory burst activity occur in stimulated neutrophils, suggesting the existence of a common regulator in their signal transduction pathways (Wymann *et al.* 1989). However, the fact that internal Ca^{2+} depletion, protein kinase C inhibition and HWT pretreatment block the respiratory burst but not shape change suggests that the respective signal transduction pathways diverge at an early stage (Baggiolini and Kernen, 1992).

Table 2. *Effect of various treatments on fMLP-induced neutrophil respiratory burst, shape change and oscillations*

Treatment	Site of action	Block of respiratory burst	Block of shape		Reference[1]
			Change	Oscillations	
N-t-Boc-Met-Leu-Phe	Receptor antagonist	Yes	Yes	Yes	Wymann *et al.* (1989)
Bordetella pertussis toxin	Heterotrimeric G-proteins (G_i type)	Yes	Yes	Yes	Wymann *et al.* (1989)
Cytochalasin B (10 μmol l^{-1})	F-actin	No[2]	Yes	Yes	
Chlorpromazine (100 μmol l^{-1})	Microtubules, calmodulin		Yes	Yes	
Staurosporine	Ca^{2+}-dependent protein kinases	Yes	No	No	Wymann *et al.* (1989)
Internal Ca^{2+} depletion	Ca^{2+}-dependent protein kinases	Yes	No	No	Wymann *et al.* (1989)
Erbstatin, methyl-2,5-dihydroxy-cinnamate (20–35 μmol l^{-1})	Tyrosine kinases	Yes*	Yes†	Yes	Naccache *et al.* (1990)*; Bennet *et al.* (1993)†
Genistein (100 μmol l^{-1})	Tyrosine kinases	Yes*	Yes	Yes	Azuma *et al.* (1993)*
Calyculin A (20 mmol l^{-1})[3]	Serine/threonine phosphatases (type 1 and 2A)	No*	No	No	Djerdjouri *et al.* (1995)*
Sodium orthovanadate (1 mmol l^{-1})	Tyrosine phosphatases	No*	Yes	Yes	Mayer and Spitzer (1994)*; Lloyds and Hallet (1994)*
Manoalide (0.2 μmol l^{-1})[4]	Phospholipases A_2	Yes*	No	No	Mayer and Spitzer (1994)*
Exoenzyme C3	Rho proteins		No	No[5]	Ehrengruber *et al.* (1995*a*)
17-Hydroxywortmannin	Phosphatidylinositol 3-kinases	Yes	No	No	Wymann *et al.* (1989)
Monensin (10 μmol l^{-1})	Na^+/H^+ ionophore		No[6]	No	
Amiloride (1 mmol l^{-1}), dimethyl-amiloride (100–500 μmol l^{-1})[7]	Na^+/H^+ antiporter		No	No	
Ouabain (1 mmol l^{-1})	Na^+/K^+ antiporter		No	No	
pH 4.9 (90 min)			No	No	
Removal of external Na^+			Yes[8]	Yes	
2-Deoxy-D-glucose (5 mmol l^{-1})	ATP depletion		No[9]	Yes	

[1]M. U. Ehrengruber and D. A. Deranleau, unpublished results if not otherwise indicated.
[2]Enhancement.
[3]Concentration high enough to induce shape change by itself (Kreienbuhl *et al.* 1992).
[4]Concentration high enough to inhibit fMLP-induced degranulation (Barnette *et al.* 1994).
[5]Enhancement.
[6]Inhibition at higher concentrations (50–100 μmol l^{-1}).
[7]Concentration high enough to block fMLP-dependent volume changes and chemotaxis (Rosengren *et al.* 1994).
[8]Partial inhibition.
[9]Shape change sustains at elevated level, reverses upon antagonist addition, and can be provoked again with a different agonist.

Biophysical description of the oscillations

The light-scattering changes of resting neutrophils stimulated once with an agonist (Yuli and Snyderman, 1984; Sklar *et al.* 1984; Ehrengruber *et al.* 1995*b*) follow the kinetics of a critically damped oscillator. Enabling of oscillations could therefore consist of removing a damping effect (Fig. 2). From a biophysical viewpoint, the oscillations appear to be damped and superimposed on a lower-frequency transient change in shape which lasts 40–60 s (Wymann *et al.* 1990). Both the high- and low-frequency shape changes have been closely linked to polymerization and depolymerization of actin (Yuli and Snyderman, 1984; Omann *et al.* 1989; Wymann *et al.* 1990; Watts *et al.* 1991; Coates *et al.* 1992). Therefore, the biochemical reactions which account for the low-frequency transient, the oscillations and the damping probably target proteins involved in controlling the structure of the cytoskeleton. Water and Na^+ transport may also be important (Rosengren *et al.* 1994).

The nature of the damping component in the neutrophil signal transduction has yet to be resolved. The fact that fMLP-induced light-scattering changes appear to be critically damped in neutrophils that have been both ATP-depleted and HWT-pretreated (M. U. Ehrengruber and D. A. Deranleau, unpublished results) indicates that internal ATP is involved in the damping of the oscillations. Furthermore, because oscillations are enabled or enhanced by certain treatments which inhibit phosphatidylinositol 3-kinase activity, i.e. pretreatment with HWT or wortmannin (Wymann *et al.* 1987; Ehrengruber *et al.* 1995*b*), prestimulation with an agonist (Ehrengruber *et al.* 1995*b*), and Rho inhibition (Ehrengruber *et al.* 1995*a*), this enzyme activity may be involved in the regulation of the oscillations. However, as the oscillation frequency can be modulated even under complete phosphatidylinositol 3-kinase inhibition (Ehrengruber *et al.* 1995*a*,*b*), factors other than or in addition to this enzyme must be involved in the regulation. While there are several candidate pathways for control of the oscillations, the major mechanisms remain unknown.

Correlation with neutrophil migration

Recurring lamellipod protrusion and retraction, driven by corresponding actin polymerization–depolymerization cycles (Wymann *et al.* 1990), are an integral feature of the mechanism responsible for the amoeboid-like crawling motions typical of neutrophils seeking bacterial targets (Hartman *et al.* 1994; Felder and Kam, 1994). During their walk, neutrophils move in approximately straight paths separated, at certain time intervals, by discrete changes in direction. At 37 °C, a mean transit time of 50–55 s is the characteristic time between successive directional changes (de Boisfleury-Chevance *et al.* 1989). This time corresponds nicely to the 40–60 s decay time (envelope) of shape oscillations we see in suspension (Fig. 1; Wymann *et al.* 1987, 1990; Omann *et al.* 1989; Ehrengruber *et al.* 1995*b*). Interestingly, our studies on individual migrating neutrophils clearly detect shape oscillations with a period of 8–10 s, which may be identical with the lamellipod formation-dependent light-scattering oscillations of suspended neutrophils (Hartman *et al.* 1994). We hypothesize that these high-frequency shape oscillations reflect the fundamental cyclic driving force for neutrophil migration. If such a motor were directly translated into movement, then the neutrophil displacement magnitudes or turn angles would be cyclic (Hartman *et al.* 1994). Such periodic neutrophil motion has been demonstrated recently (Hartman *et al.* 1994; Felder and Kam, 1994). Our current understanding of the involvement of shape oscillations in neutrophil migration is summarized in Fig. 2.

These data suggest that the fundamental neutrophil motor may be under tonic inhibition. Addition of agonist removes the inhibition and enables biochemical oscillations which lead to the detected cycles in shape change (Ehrengruber *et al.* 1995*b*). The exponential decay of oscillations seen in suspension after agonist addition (Wymann *et al.* 1987, 1990; Omann *et al.* 1989; Ehrengruber *et al.* 1995*b*) and the bursts of persistent (straight-line) motion which die out after 30–60 s on surfaces (Hartman *et al.* 1994; Felder and Kam, 1994) may be due to the same processes. The relative contributions of the high-frequency and low-frequency shape-change components on directed movement are not known. In fact, we do not know for certain that the oscillations seen in suspension are due to the same process that occurs on a surface. Furthermore, motility observed on a two-dimensional surface may not be the same as in a three-dimensional tissue. It is clear that lamellipod formation and polar shape change occur perfectly well in suspension without the need for a surface or a stimulus gradient (Yuli and Snyderman, 1984; Watts *et al.* 1991; Coates *et al.* 1992). Thus, both aspects of neutrophil shape change are intrinsic properties of the cell. Could it be that surface contact is the natural biochemical event that enables oscillations? Similarly, encounter with a chemoattractant gradient could act to enable mechanical oscillations, as is the case for repetitive stimulation with peptide agonists in suspension (Ehrengruber *et al.* 1995*b*). Certainly, the specific adhesive interactions of the neutrophil with a surface serve to modulate biochemical events within the cell. Carefully regulated adhesive interaction with the substratum could convert the shape changes generated within the cell into directed movement. This model, which portrays neutrophil migration as repeating cycles of small- and large-scale shape change, opens the door to many predictions about net neutrophil displacement that could be derived from

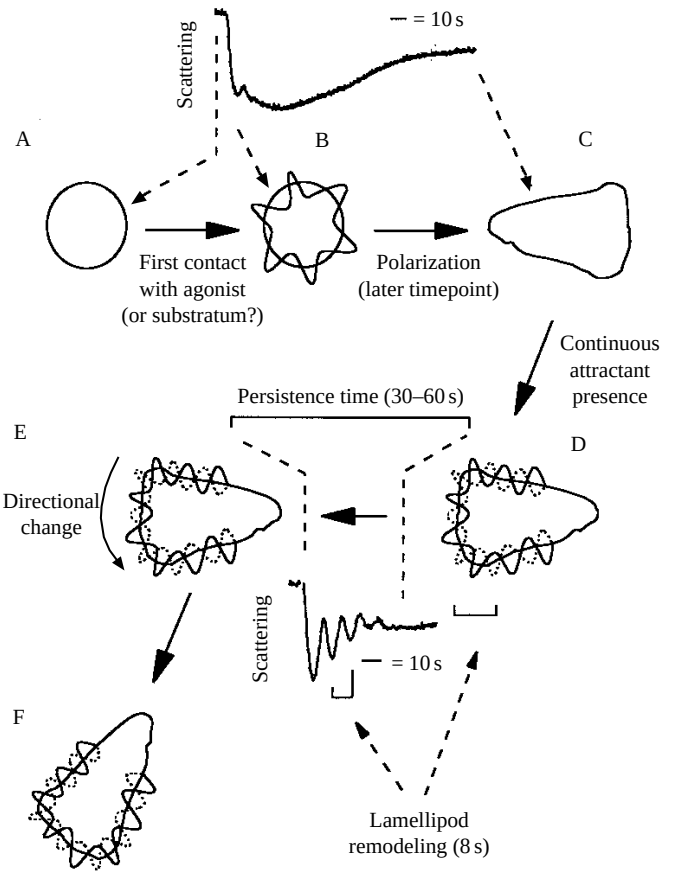

Fig. 2. Hypothetical role of shape oscillations in neutrophil migration. Two real-time tracings of 90 ° scattering changes stimulated with 10 nmol l^{-1} fMLP (at 37 °C) are included. Resting neutrophils are spherical (A) and develop lamellipods (‘membrane ruffles’) within seconds after their first encounter with a chemoattractant or possibly a substratum (B). The neutrophil shape polarizes through the formation of a dominant pseudopod (C) as total F-actin content and light-scattering return towards baseline levels (Wymann *et al.* 1987; Watts *et al.* 1991; Coates *et al.* 1992). In suspension, the chemoattractant or pharmacological agents enable 8 s period oscillations, which are then provoked when the cell encounters the triggering stimulus (D). Exactly what provides the natural enabling event is unclear, but surface contact would be a reasonable first candidate. A chemoattractant gradient might be able of enabling and provoking oscillations in a manner analogous to repeated stimuli (Ehrengruber *et al.* 1995*b*). The cell then moves forward from D to E with the persistence time of 30–60 s, similar to the length of time that it takes the train of oscillations to die out in suspended neutrophils. As one cycle of the ‘internal clock’ (de Boisfleury-Chevance *et al.* 1989) is completed, the neutrophil can then change its direction of movement (E) before another train of oscillations propels it on another straight-line segment (from E to F) of its path. Repetitions of this process lead the neutrophil to its final destination.

experimental measurements of the amplitude and directions of the oscillations in shape.

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