Shape oscillations: a fundamental response of human neutrophils stimulated by chemotactic peptides?

Markus U. Ehrengruber[a], Thomas D. Coates[b,*], David A. Deranleau[a]

[a]*Theodor Kocher Institute, University of Bern, Freiestrasse 1, CH-3012 Bern, Switzerland*
[b]*Division of Hematology/Oncology, Childrens Hospital of Los Angeles, University of Southern California, Los Angeles, CA 90027-6016, USA*

Received 16 November 1994; revised version received 3 January 1995

Abstract **Neutrophils undergo periodic cytoskeletal rearrangements that lead to cycles of shape change, ultimately resulting in cell translocation. Repeated stimulation of resting neutrophils with subsaturating chemoattractant doses induced transient sinusoidal oscillations in neutrophil filamentous actin content at the second and subsequent stimulations. Oscillation frequencies increased with increasing concentration of the first stimulus. In contrast, neutrophils pretreated with the phosphatidylinositol 3-kinase inhibitor (17-hydroxy)wortmannin displayed shape oscillations with the first stimulation, and the frequencies were independent of agonist type and dose. We demonstrate that oscillations in filamentous actin, which may be critical for neutrophil motility, can be induced in untreated cells by natural peptide chemoattractants.**

Key words: Cytoskeleton; Polymorphonuclear leukocyte; *N*-Formyl-Met-Leu-Phe; Interleukin-8; Complement fragment 5a; Cell motility

1. Introduction

Motile cells, such as neutrophil leukocytes, change their shape and 'crawl' in response to external stimuli by extruding and remodeling lamellipods, a process requiring rearrangement of cytoskeletal actin. Cycles of small scale shape changes then result in net cell translocation [1]. Neutrophil chemoattractants induce a rapid transient increase in filamentous actin (F-actin) [2–4], and a corresponding transient decrease in light scattering [5–7]. In synchronized populations of suspended cells, the optical and F-actin transients display parallel, oppositely directed sinusoidal oscillations having the same period (8–10 s) [8,9]. Scattering theory suggests that the optical oscillations result from cyclic alterations in cell body size arising from the isovolumetric extension and retraction of comparatively weakly scattering, veil-like lamellipods ('shape changes') [9,10]. Recurring lamellipod protrusion and retraction, driven by corresponding actin polymerization/depolymerization cycles [9], may be an integral feature of the mechanism responsible for the amoeboid-like crawling motions typical of cells seeking bacterial targets.

Clearly oscillating responses are rarely observed in neutrophil suspensions, but they can be routinely enabled by pretreatment with (17-hydroxy)wortmannin (HWT), an inhibitor of phosphatidylinositol-3-kinase [7,9,11]. 'Spontaneous' oscillations in 90° light scattering and F-actin content have been reported in untreated cells upon stimulation with single pulses of lipid agonists (leukotriene B_4, platelet-activating factor) but not with N-formylated peptides [8]. Flow cytometric analysis showed that all cells in the population were synchronously responding in this cyclic manner [8].

Here we show that shape-derived scattering oscillations can be reproducibly obtained by repetitive stimulation of cell populations with non-desensitizing doses of the chemoattractants *N*-formyl-Met-Leu-Phe (fMLP), interleukin-8 (IL-8) and complement fragment 5a (C5a). The oscillation frequency increases with increasing preactivating agent concentration and temperature but is independent of total F-actin content. We suspect that the cytoskeletal-related shape oscillations induced in suspension by peptide chemoattractants represent a fundamental neutrophil response critical for motile behavior.

2. Experimental

2.1. Materials

HWT and wortmannin were kindly provided by Dr. T.G. Payne, Sandoz Ltd., Basel, Switzerland; synthetic IL-8 was a gift from Dr. I. Clark-Lewis, Biomedical Research Center and Department of Biochemistry, University of British Columbia, Vancouver, Canada. Bovine serum albumin, fMLP, and lysophosphatidylcholine were purchased from Sigma, St. Louis, MO, USA; formaldehyde was from Merck AG, Darmstadt, Germany; EDTA was from Fluka AG, Buchs, Switzerland; and rhodamine phalloidin was from Molecular Probes Inc., Junction City, OR, USA; all other chemicals were obtained as described [12]. Concentrated stock solutions were prepared as follows: fMLP and HWT (each 10 mM) in dimethyl sulfoxide; C5a (10 μM) in 0.9% (w/v) NaCl; and IL-8 (260 μM) in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4). Stock solutions were further diluted with test buffer (130 mM NaCl, 5 mM $NaHCO_3$, 4.6 mM KCl, 5 mM glucose, 0.05 mM $CaCl_2$, 20 mM HEPES, pH 7.4), except for IL-8 and C5a, diluted with test buffer containing 0.25% (w/v) bovine serum albumin, and injected as 3 μl pulses into 3 ml of stirred cell suspension in the sample cuvette of the measuring apparatus (below). Neither stirring nor the presence of dimethyl sulfoxide in the test medium (final concentration 0.15% v/v, or less) affected any of the parameters tested.

2.2. Cell preparation

Neutrophils were isolated from buffy coats of single donors as previously described [12], suspended in test buffer, and kept at 4–8°C. Aliquots of this suspension were diluted to a final concentration $c = 8 \times 10^5$ cells/ml (determined in a Sysmex CC-130 microcell counter; TOA Medical Electronics Co., Kobe, Japan) and warmed to 37°C (or to 25°C for experiments carried out at this temperature) for measurement.

2.3. Shape change measurements

Light transmission and relative 90° scattering were simultaneously recorded in an optical multichannel analyzer [13] in ratio mode I/I_0, where $I = I_t$ is the transmitted, $I = I_s$ the 90° scattered, and I_0 the

*Corresponding author. Fax: (1) (213) 660-7128.
E-mail: tom@hemonc.usc.edu

Abbrevations: C5a, complement fragment (anaphylatoxin) 5a; F-actin, filamentous actin; fMLP, *N*-formyl-Met-Leu-Phe; HWT, 17-hydroxywortmannin; IL-8, interleukin-8.

SSDI 0014-5793(95)00048-8

incident intensity, to compensate for possible fluctuations in source intensity. We used a single frequency HeNe laser in combination with a 10× beam expander to provide a stable, highly collimated light source; an in-line optical stop prevented strongly forward-scattered light appearing outside the dimensions of the exciting beam from reaching the transmission detector (necessary conditions for quantitative extinction measurements on scattering samples [14,15]). Approximately 300,000 cells were under continuous observation at any given time. Transmission data were converted to extinction $E \equiv -\log(I_t/I_0) = \varepsilon_x c l$ (Beer's law), where ε_x is the total (decadic) scattering extinction coefficient (0.4343 × the particle scattering cross-section, a sensitve measure of cell size and/or shape [15,16]) and $l = 1$ cm is the path length. The turbidity is the extinction per unit path length $E/l = \varepsilon_x c$ (in decadic units).

Oscillations were induced in resting neutrophils as a two-step process. A first or 'oscillation-enabling' dose of either HWT or chemotactic peptide was added to the suspension; this addition did not result in the production of oscillations (in the case of chemotactic peptide, there was a single decrease and recovery in the light scattering signal). Three minutes later, a second or 'oscillation-provoking' stimulus was added. A train of oscillations ensued after the second and subsequent agonist additions. Oscillation frequencies were determined by power spectral density analysis after subtracting the mean DC component and the non-oscillating transient, as approximated by a smooth polynomial fit through the middle of the oscillating portion of the transient. A single narrow peak characteristic of a pure sinusoidal response was found in almost all instances. Data are expressed as means ± S.D.

2.4. Quantification of F-actin content

Total F-actin was determined using a variation of the method of Howard and Oresajo [4]. Neutrophils (10^6 cells/ml) were prewarmed to 37°C, allowed to react with increasing doses of fMLP for 3 min, and fixed for 15 min at 37°C with 3.7% (w/v) formaldehyde (supplemented with 2 mM EDTA to prevent cellular aggregation). The cells were then permeabilized with 67 μg lysophosphatidylcholine/ml and simultaneously stained with rhodamine phalloidin (0.33 μM) for 15 min at room temperature. Five thousand cells per sample were analyzed in a Coulter Epics Profile II flow cytometer (Coulter Corporation, Hialeah, FL, USA).

3. Results and discussion

3.1. Oscillations elicited by repeated stimulation of untreated neutrophils

In this study we examined whether shape oscillations occur in untreated neutrophils stimulated by peptide chemoattractants encountered under natural conditions. Suspensions of resting neutrophils exposed to a single pulse of IL-8 (0.003–10 nM), fMLP (0.3–100 nM), or C5a (0.001–10 nM) responded with parallel, transient decreases in extinction and 90° scattering as previously described [5–7] (Fig. 1, left panel). A second and third stimulation of the same cells by the same stimulus led to scattering changes characterized by the appearance of a damped train of prominent, regular oscillations having a period of 8–10 s (Fig. 1, middle and right panels). Because these oscillations were not evident after the first agonist addition, it seems likely that the first agonist-induced response establishes the biochemical conditions that favor oscillations in the second and third stimulation.

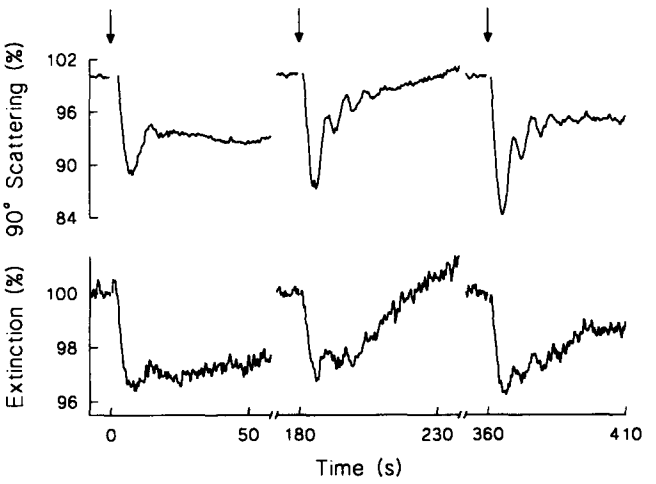

Fig. 1. Shape oscillations in untreated neutrophils revealed by repetitive stimulation. Relative changes in 90° scattering (top) and extinction (bottom) of cells stimulated at 3 min intervals (arrows); the stimulus sequence was 1 nM IL-8, 10 nM IL-8, and 10 nM fMLP. Similar results were obtained using fMLP or C5a as the first stimulus.

To determine if the oscillation frequency depends on the concentration of the preactivating agonist, we enabled oscillations with varying chemoattractant doses and held the agonist concentration of the second addition constant. The frequency of the oscillation provoked by the second agonist addition increased in a dose-dependent fashion as the concentration of the enabling stimulus was increased (Fig. 2). IL-8 generated higher oscillation frequencies, with a maximum of 0.16 Hz compared to 0.14 for fMLP. An oscillation-enabling effect of very small first-stimulus concentrations is evident from the data of Fig. 2. As little as 3 pM IL-8 (or 1 pM C5a, data not shown) led to second-stimulus oscillations of small constant frequency (0.098 ± 0.002 Hz, $n = 3$). The oscillations were strikingly similar to those observed using cells preincubated with HWT [7,9] (Fig. 3, left panel) or stimulated with lipid agonists [8]. In each case, the oscillations appear to be superimposed on an underlying average transient. This suggests that neutrophil shape change is composed of a high amplitude, low frequency component and a high frequency oscillating component. Such a behavior is predicted by the model proposed by Wymann et al. [9]. Since pretreatment with either peptide agonist or HWT permits induction of oscillations, it is likely that both enabeling treatments operate through a common pathway. As HWT [17] and C5a [18] are known to inhibit phosphatidylinositol 3-kinase, this enzyme could be involved in regulation of the oscillations.

Table 1
Oscillation frequencies in light scattering responses of neutrophils pretreated with HWT and stimulated with a single agonist pulse

Stimulus	Oscillation frequency in Hz	
	Extinction	90° scattering
fMLP, 10 nM	0.122 ± 0.012 (45)	0.124 ± 0.009 (63)
IL-8, 25 nM	0.118 ± 0.013 (10)	0.117 ± 0.014 (13)
C5a, 10 nM	0.122 ± 0.010 (3)	0.119 ± 0.005 (4)

Power spectral density estimates. Cells were preincubated with 1 μM HWT for 10 min at 37°C. Mean values ± S.D.; parentheses enclose the number of independent experiments. Similar results were obtained using phorbol 12-myristate 13-acetate (2.5–3.5 nM) instead of HWT for pretreatment.

3.2. Oscillations in neutrophils pretreated with (17-hydroxy)wortmannin (HWT)

Oscillations of similar frequency and amplitude were elicited by single pulses of fMLP, IL-8, and C5a in cells pretreated with HWT (Table 1) or wortmannin (data not shown). Since the frequency seemed to be independent of the type of oscillation-provoking stimulus, we averaged the frequencies for all stimuli.

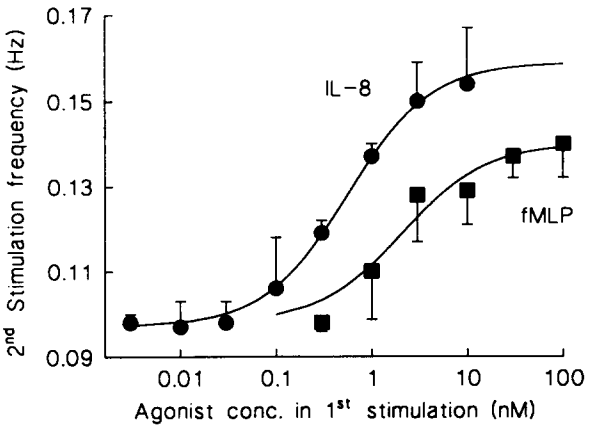

Fig. 2. Increase in 90° scattering response frequencies as a function of first-stimulus (enabling) agonist concentration. Untreated neutrophils were stimulated with variable amounts of IL-8 (circles) or fMLP (squares), followed after 3 min with a pulse of constant agonist concentration (10 nM in each case). Mean values ± S.D. of 2–19 independent neutrophil preparations are shown.

The mean frequency was 0.120 ± 0.011 Hz (n = 239; Table 2, line 1), corresponding to a mean period of 8.4 s. This frequency was independent of the agonist concentration (fMLP, 1–30 nM; and IL-8, 0.3–25 nM), which supports the concept that the oscillation frequency depends upon the oscillation-enabling event rather than the nature or concentration of the stimulus which provokes the oscillatory transient. To see if the enabling effects of peptide agonist and HWT were additive, we repeatedly provoked shape oscillations in HWT-pretreated cells (Fig. 3, middle and right panels). Each subsequent addition of peptide agonist resulted in a continued increase of the oscillation frequency (Table 2). As these cells had been pretreated with 1 μM HWT, a dose which is known to completely block phosphatidylinositol 3-kinase [17], factors other than or in addition to phosphatidylinositol 3-kinase must be involved in the regulation of the oscillations.

To explore whether the mechanism controlling oscillations in actin polymerization is similar to that which regulates oscillations in the respiratory burst [11], we determined the activation energy of the actin polymerization-coupled scattering changes. Lower frequency oscillations were found at 25°C than at 37°C and resulted in an Arrhenius activation energy of 69 (extinction) and 65 kJ/mol (90° scattering) for shape oscillations. This compares favorably with the value of 61 kJ/mol reported previously for respiratory burst oscillations [11]. Our results are consistent with other reports suggesting that regulation of the neutrophil respiratory burst is linked to regulation of the cytoskeleton [19]; however, the exact mechanism of the oscillations at the molecular level remains to be determined.

Table 2
Increase in light scattering oscillation frequency of repeatedly stimulated HWT-pretreated neutrophils

Stimulus	Oscillation frequency in Hz and significance P for:			
	Extinction	P	90° scattering	P
1st	0.119 ± 0.012 (91)	–	0.121 ± 0.010 (148)	–
2nd	0.130 ± 0.013 (98)	<0.001	0.134 ± 0.012 (122)	<0.001
3rd	0.142 ± 0.016 (15)	0.0016	0.145 ± 0.013 (22)	<0.001

Power spectral density estimates. Combined results using fMLP, IL-8 and C5a (each 1–30 nM). The cells were preincubated for 10 min with 1 μM HWT at 37°C and stimulated with an agonist at 3 min intervals. Mean values ± S.D.; parentheses enclose the number of independent experiments. P is the probability that the oscillation frequency is the same as that obtained in the previous stimulation (Student's t-test).

Since the oscillations have such a clearly sinusoidal waveform, it seems likely that these oscillations represent a dynamic equilibrium between a small number of molecular species. Candidate species involved in this equilibrium could be globular and F-actin, or the labile and stabile pools of F-actin, thought to drive lamellipod protrusion [20,21]. Proteins which modulate actin polymerization and cross-linking offer many possibilities for frequency and amplitude regulation of this dynamic equilibrium.

3.3. The role of F-actin

Because the oscillations reflect periodic transitions between the pools of globular and filamentous actin [9], the size of the total F-actin pool at the time the oscillation-provoking agonist is added could determine the frequency. We measured the F-actin 3 min after addition of the first peptide agonist, i.e. at a time just before the second stimulation. Although increasing fMLP concentrations led to increasing total F-actin content 3 min after stimulation, total F-actin was not linearly correlated with the oscillation frequency. Furthermore, IL-8 produced no changes in total F-actin sampled at the same time point. Thus, total F-actin at the time the second stimulus is added does not appear to influence the frequency of the resulting oscillations.

The mechanical oscillations due to actin polymerization and detected by light scattering are likely to represent a process that is critical to amoeboid movement. During their random walk on surfaces, neutrophils move in approximately straight paths separated, at certain time intervals, by discrete changes in direction. At 37°C, a mean transit time of slightly under a minute was determined as the characteristic time of the 'internal clock' between successive directional changes [22]. This time corresponds nicely to the duration of the train of shape oscillations we see in suspension. Interestingly, our recent studies on migrating neutrophils clearly detect mechanical oscillations with

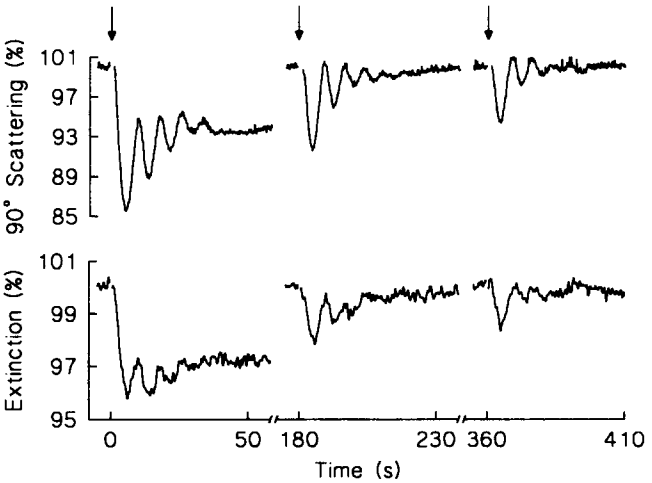

Fig. 3. Shape oscillations induced by repeated stimulation of HWT-pretreated neutrophils. Relative changes in 90° scattering (top) and extinction (bottom) of cells pretreated with 1 μM HWT (10 min) and stimulated at 3 min intervals with 10 nM fMLP (arrows). Similar results were obtained using IL-8 or C5a as the first stimulus.

a period of 8–10 s [23], corresponding closely to those detected in suspension. Taken as a whole, these data suggest that the peptide-induced oscillations seen in cell suspensions are likely to be relevant to periodic shape alterations detected in crawling neutrophils. Furthermore, our finding that the oscillation-enabling event determines the frequency characteristics of subsequent oscillations could provide a plausible explanation for the neutrophil memory which results in highly correlated [23], persistent [24] motion.

Acknowledgements: We thank the Swiss Red Cross Central Laboratory for providing buffy coats, A. Blaser, R. Stuber and R. Müller for neutrophil isolation, Prof. H.U. Keller for the use of the flow cytometer, and Dr. M. Torres for comments on the manuscript. This work was supported by the Swiss National Science Foundation (Grant 31-36467.92 to D.A.D.) and the National Institutes of Health, USA (AI23547 to T.D.C.).

References

[1] Stossel, T.P. (1993) Science 260, 1086–1094.
[2] Fechheimer, M. and Zigmond, S.H. (1983) Cell Motil. 3, 349–361.
[3] Sklar, L.A., Omann, G.M. and Painter, R.G. (1985) J. Cell Biol. 101, 1161–1166.
[4] Howard, T.H. and Oresajo, C.O. (1985) Cell Motil. 5, 545–557.
[5] Yuli, I. and Snyderman, R. (1984) J. Clin. Invest. 73, 1408–1417.
[6] Sklar, L.A., Oades, Z.G. and Finney, D.A. (1984) J. Immunol. 133, 1483–1487.
[7] Wymann, M.P., Kernen, P., Deranleau, D.A., Dewald, B., von Tscharner, V. and Baggiolini, M. (1987) Biochem. Biophys. Res. Commun. 147, 361–368.
[8] Omann, G.M., Porasik, M.M. and Sklar, L.A. (1989) J. Biol. Chem. 264, 16355–16358.
[9] Wymann, M.P., Kernen, P., Bengtsson, T., Andersson, T., Baggiolini, M. and Deranleau, D.A. (1990) J. Biol. Chem. 265, 619–622.
[10] Deranleau, D.A. (1988) Experientia. 44, 679–684.
[11] Wymann, M.P., Kernen, P., Deranleau, D.A., Baggiolini, M. (1989) J. Biol. Chem. 264, 15829–15834.
[12] Wymann, M.P., von Tscharner, V., Deranleau, D.A. and Baggiolini, M. (1987) J. Biol. Chem. 262, 12048–12053.
[13] Deranleau, D.A. and Stüssi, E. (1987) Rev. Sci. Instr. 58, 1840–1842.
[14] Latimer, P. (1975) J. Theor. Biol. 51, 1–12.
[15] Deranleau, D.A. (1987) Trends Biochem. Sci. 12, 439–442.
[16] Moser, B., Schumacher, C., von Tscharner, V., Clark-Lewis, I. and Baggiolini, M. (1991) J. Biol. Chem. 266, 10666–10671.
[17] Arcaro, A. and Wymann, M.P. (1993) Biochem. J. 296, 297–301.
[18] Dobos, G.J., Norgauer, J., Eberle, M., Schollmeyer, P.J. and Traynor-Kaplan, A.E. (1992) J. Immunol. 149, 609–614.
[19] Woodman, R.C., Ruedi, J.M., Jesaitis, A.J., Okamura, N., Quinn, M.T., Smith, R.M., Curnutte, J.T. and Babior, B.M. (1991) J. Clin. Invest. 87, 1345–1351.
[20] Cassimeris, L., McNeill, H. and Zigmond, S.H. (1990) J. Cell Biol. 110, 1067–1075.
[21] Watts, R.G. and Howard, T.H. (1993) Blood 81, 2750–2757.
[22] de Boisfleury-Chevance, A., Rapp, B. and Gruler, H. (1989) Blood Cells. 15, 315–333.
[23] Hartman, R.S., Lau, K., Chou, W. and Coates, T.D. (1994) Biophys. J. 67, 2535–2545.
[24] Zigmond, S.H., Levitsky, H.I. and Kreel, B.J. (1981) J. Cell Biol. 89, 585–592.