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Oscillatory motion in human neutrophils responding to chemotactic stimuli

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Human neutrophils treated with the secretion inhibitor 17-hydroxywortmannin were stimulated with fMLP, C5a, PAF or LTB_4, and the ensuing shape change was studied. The cells rapidly extended lamellipodia and showed regular oscillatory behaviour. The oscillations were observed in both light transmission and 90° light scattering, had the same frequency in each case, and disappeared within 30-50 seconds. Light scattering theory suggests that they reflect rhythmic changes in the shape and/or size of the chemotactically stimulated cells, possibly related to crawling or swimming movements associated with migration.

The neutrophil leukocytes constitute the main defense system of higher organisms against microbial invasion. They are activated in the blood stream by chemotactic signals arising upon infection, and migrate in large numbers into the infected tissues, where they normally ingest and kill the invaders (1). Migrating neutrophils are elongated, and indeed their change from a spherical to a polarized shape is the most conspicuous response observed following stimulation with chemoattractants (2-4). It is believed that the shape change as well as granule release, which is also induced by stimulation, are reflected by changes in light-transmission and 90° light scattering observed in stirred neutrophils suspensions (5,6). We treated human neutrophils with

Abbreviations: fMLP, N-formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; LTB_4, leukotriene B_4; HWT, 17-hydroxywortmannin; CB, cytochalasin B.

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17-hydroxywortmannin (HWT) (7), a fungal metabolite which inhibits secretion without affecting shape change, and studied their response to four different chemotactic receptor agonists. We describe here oscillations in light transmission and light scattering elicited by chemotactic stimulation and possibly related to the migratory capacity of stimulated cells.

METHODS

Human neutrophils were isolated from fresh blood or buffy coats of donor blood stored up to 20 h at 4-10°C (Swiss Red Cross Laboratory, Berne, Switzerland) by centrifugation through a Ficoll-Hypaque gradient (8), and lysis of contaminating erythrocytes in isotonic NH_4Cl (9). They were then washed three times by centrifugation in a saline medium containing 130 mM NaCl, 4.6 mM KCl, 0.05 mM $CaCl_2$, 5 mM glucose, buffered at pH 7.4 with a combination of 5.0 mM $NaHCO_3$, 1.1 mM KH_2PO_4 and 20 mM HEPES. The final suspension was adjusted to 10^7 cells per ml (95-98% neutrophils) and kept at 10°C to await measurements.

Light microscopy and scanning electron microscopy. Samples were diluted with saline medium to 2 x 10^6 cells/ml, warmed to 37°C in a cuvette used for turbidimetry and stimulated with 10^{-7} M fMLP as described below. The cells were fixed by the addition of ice-cold glutaraldehyde (final concentration 3%).

Light transmission changes. Samples of the neutrophil suspension diluted to 8 x 10^5 cells/ml in 3 ml of saline medium to ensure conformance with Beer's law were warmed to 37°C in a 1 cm square cross section plastic cuvette stirred at 13 rps in the sample compartment of a very small acceptance-angle HeNe laser turbidimeter (10). A beam stop was used to prevent strongly forward-scattered light from reaching the transmission detector, thus permitting quantitative analysis of the data by means of light scattering theory. When indicated, HWT (10^{-6} M) and cytochalasin B (5 ug/ml) were added 5 and 4 min prior to stimulation, respectively. In all cases EGTA (10^{-3} M) was added 2 min before the stimulus to prevent aggregation, which requires extracellular calcium (11). All reagents were injected in ul quantities into the stirred cell suspension.

Right-angle light scattering was measured in the laser turbidimeter (10) by means of a second photomultiplier positioned at 90°.

Materials and solutions. fMLP and PAF were obtained from Bachem AG, Bubendorf, Switzerland; cytochalasin B from SERVA Feinbiochemika GmbH, Heidelberg, FRG; C5a was kindly provided by Dr. C.A. Dahinden, Department of Clinical Immunology, University of Bern, Switzerland; LTB_4 was donated by Dr. J. Rokach, Merck Frosst Canada Inc., Pointe Claire-Dorval, Que., Canada; the wortmannin derivatives, HWT and compound 11 in Ref. 7, were obtained from Dr. T.G. Payne, Sandoz Ltd., Basel, Switzerland.

Stock solutions (10 mM) were made for fMLP and the wortmannins in DMSO, PAF in 0.9% NaCl containing 2.5 mg/ml BSA and LTB_4 in methanol. C5a was dissolved at 10 uM in 0.9% NaCl and cytochalasin B (5 mg/ml) in DMSO. These solutions were further diluted with the saline medium used to suspend the cells. The final concentration of DMSO was 0.5% or less.

RESULTS AND DISCUSSION

A typical example of shape change induced by the chemotactic peptide fMLP is illustrated by the scanning electron micrographs in Fig. 1. The main morphological alteration was the protrusion of large, thin lamellar pseudopodia, a phenomenon that occurred within a few seconds and thus coincided with the rapid increase in light transmission observed on stimulation of neutrophil suspensions (Ref. 5 and Fig. 2). The shape change was not affected by HWT, as indicated by repeated observations using light microscopy (Nomarski optics) and scanning electron microscopy (Fig. 1). On the other hand, HWT inhibited chemoattractant-induced exocytosis. In normal and cytochalasin

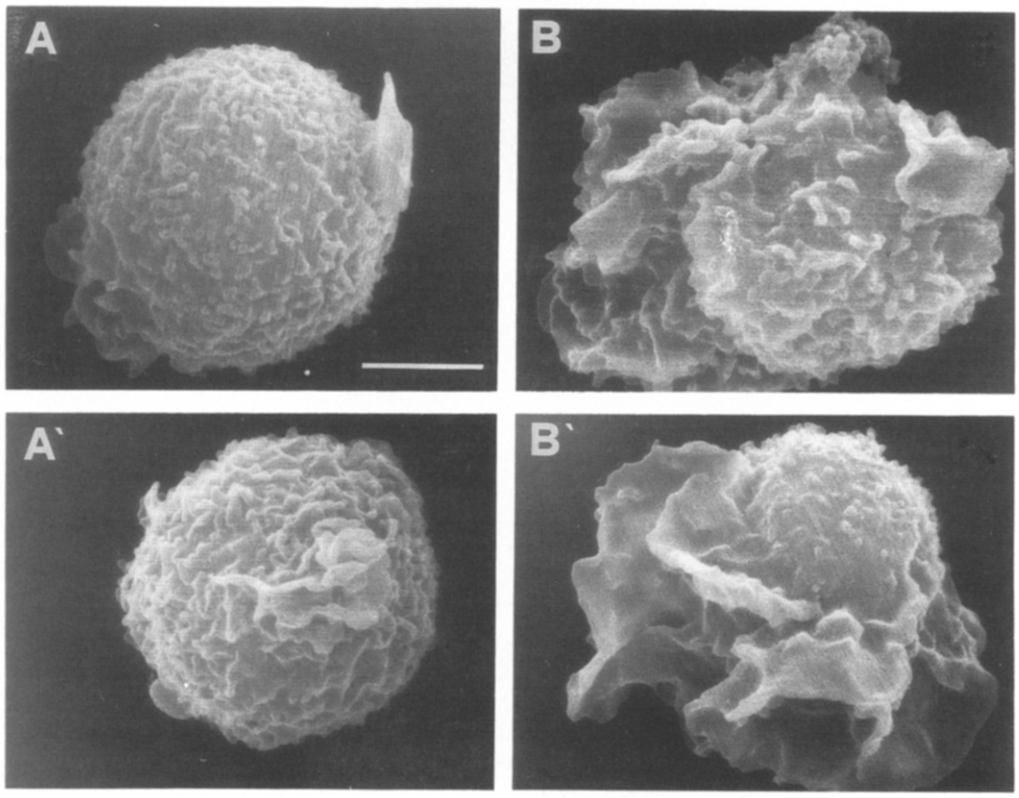

Fig. 1. Scanning electron micrographs of human neutrophils fixed with glutaraldehyde before (A, A') and 10 sec after stimulation with 10^{-7} M fMLP (B, B'). Top (A, B), no pretreatment; bottom (A', B'), pretreatment with 10^{-6} M HWT 5 min prior to stimulation. Bar 2 um.

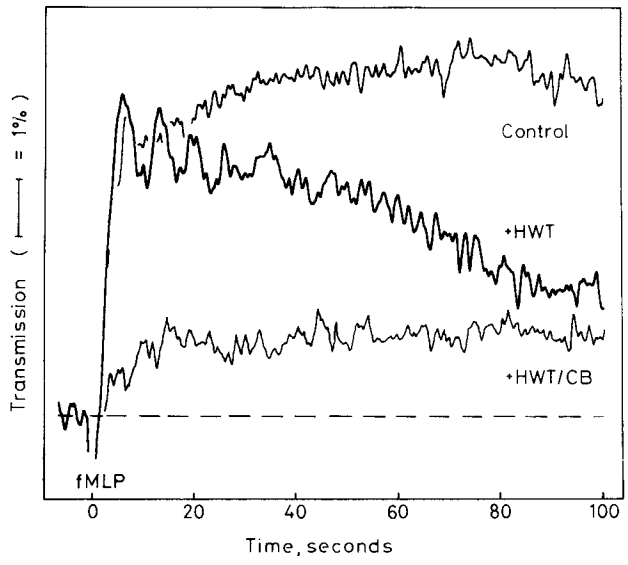

Fig. 2. Typical light transmission changes of neutrophil suspensions challenged with 10^{-7} M fMLP. Top curve (Control), no pretreatment; middle curve (+ HWT), cells pretreated with 10^{-6} M HWT 5 min prior to stimulation; bottom curve (+ HWT/CB), cells pretreated with both 10^{-6} M HWT (5 min) and 5 ug/ml cytochalasin B (4 min) prior to stimulation.

B-treated neutrophils which were stimulated with 10^{-7} M fMLP, 10^{-7} M LTB_4 or 10^{-6} M PAF, 10^{-6} M HWT reduced the release of vitamin B_{12}-binding protein (from specific granules) and B-glucuronidase (from azurophil granules) by 66 to 82%.

Stimulation of neutrophils with fMLP in the presence and absence of HWT resulted in a rapid, pronounced increase in light transmission which reached a peak within 10 sec (Fig. 2). In contrast to control samples, which showed little further change in transmission, HWT-treated samples showed a regular pattern of oscillations of progressively decreasing amplitude superimposed upon a transmission decrease. With cells which had been pretreated with cytochalasin B to prevent shape change (12), the response was markedly reduced. Aggregation, which is often invoked to explain changes in apparent light transmission following neutrophil stimulation, was absent in these experiments (see Methods) and played no role in the observed optical signals.

Except for the oscillations, our light transmission recordings appear to be qualitatively in accord with those obtained by Yuli and Snyderman (5), who attributed the optical changes to scattering by sources of subcellular dimensions. Light scattering theory, however, shows that small particles like intracellular organelles generally scatter orders of magnitude less total light than particles of cell size (13), indicating that the observed transmission changes, including the oscillations, arise from alterations in the gross morphological shape and/or size of the neutrophils. There were no significant differences in the results obtained with neutrophils from fresh or stored blood. Samples pretreated with an inactive HWT analogue, which did not inhibit secretion (10^{-6} M of compound 11, Ref. 7), showed the same turbidimetric response to fMLP as the control.

The oscillatory feature of the turbidimetric shape change progress curves was also apparent at fMLP concentrations as low as 10^{-9} M, and was not restricted to fMLP stimulation alone. Fig. 3 shows that analogous results were obtained with neutrophils stimulated with C5a, LTB_4 or PAF. In spite of differences in the later phase, the light transmission changes induced by the four agonists during the first 20-30 sec showed striking similarities. The initial rise in transmission was similar both in rate and extent, and in each case, the oscillations had a regular period of between 7 and 9 sec and were independent of stirring speed. In addition, all four agonists induced similar changes in right-angle light scattering as shown in Fig. 4 for fMLP stimulation. While the period of the oscillations was remarkably constant, the amplitude of the oscillations and the magnitude of the overall transmission or scattering change were donor-dependent. The overall transmission changes varied between 4 and 8% for different donors.

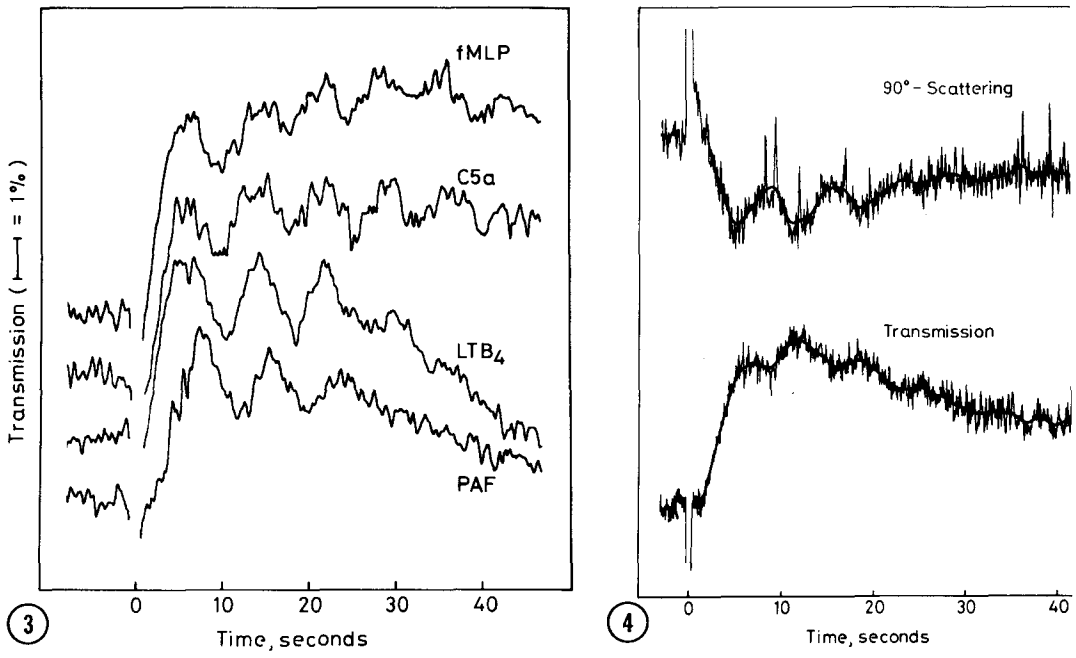

Fig. 3. Comparison of the responses induced by fMLP (10^{-7} M), C5a (10^{-8} M), PAF (10^{-7} M) and LTB_4 (10^{-7} M) in neutrophils pretreated for 5 min with 10^{-6} M HWT. Each curve has been offset from its neighbour by 1.5 scale units; zero time is the point of stimulus addition.

Fig. 4. Changes in 90° light scattering (top) and light transmission (bottom) induced by 10^{-7} M fMLP in a suspension of neutrophils pretreated with 10^{-6} M HWT. The heavy smooth curves shown superimposed on the raw data were obtained by removing high frequency noise by means of a quasi-Gaussian filter algorithm (18). Note that in Figs. 2 and 3, one or two cycles of filtering have been used to emphasize the oscillatory nature of the response.

Stimulus-induced changes in 90° scattering more or less paralleling changes in apparent light transmission have been reported by both Yuli and Snyderman (5) and Sklar et al. (6), and ascribed to a combination of shape change and secretion. It should be noted, however, that in contrast to total scattering (as measured by light transmission along a collimated beam as in the present experiments), it may be difficult to verify the sources of right-angle scattering by existing theory (14,15). We are presently investigating the origins of the shape-related alterations in transmission by means of Rayleigh-Debye and anomalous diffraction scattering theory, as well as the

possibility of using kinetic models to simulate the turbidimetric effects in a fashion similar to that previously applied to blood platelets (16,17).

It has been shown by Zigmond et al. (3) that the well known polarization of chemotactically-stimulated neutrophils reflects locomotory behaviour rather than orientation in a chemotactic gradient. The uniformity of the responses elicited by agonists acting on different receptors suggests that regular shape oscillations may represent an intrinsic property of chemotactically stimulated neutrophils, and could be an expression of Zigmond's "behavioural polarity" that cannot be easily assessed with the microscope. Activated neutrophils move into the tissues, and the oscillations revealed by our experiments may be a reflection of the crawling or swimming behaviour of the migrating cell.

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