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STUDIES OF SKIN-WINDOW EXUDATE HUMAN NEUTROPHILS: Complex Patterns of Adherence to Serum-Coated Surfaces in Dependence on FMLP Doses

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Abstract—Human neutrophils were isolated both from peripheral blood (PB) and from aseptic inflammatory exudates obtained by the Senn's skin-window (SW) technique. The respiratory burst (O_2^- release) and the adherence to serum-coated wells of culture microplates was investigated using a simultaneous assay. Unstimulated PB resting neutrophils did not produce a significant amount of O_2^- and were incapable of adhering to serum-coated plastic surfaces, while unstimulated SW neutrophils showed augmented adhesion to serum-coated culture wells. SW neutrophils were primed to enhanced FMLP-dependent O_2^- release in response to *n*-formyl-methionyl-leucyl-phenylalanine (FMLP). Adhesion of SW neutrophils was significantly decreased by addition of low doses (10^{-10}–10^{-8} M) of FMLP (from 17.1% to 8.4%, $P < 0.01$, $N = 12$), while fully activating doses ($>5 \times 10^{-8}$ M) of FMLP induced a marked increase of the cell adhesion, more pronounced in SW (39.2%) than in PB cells (27.2%). Low (5×10^{-9} M) and high (5×10^{-7} M) FMLP doses induced morphological changes (polarization) and actin polymerization in the neutrophils from both sources. Biphasic dose–response curves of SW neutrophil adherence were observed using FMLP, but not using concanavalin A or phorbol myristate acetate as stimulatory agents. Therefore, the adherence of SW cells appears to be regulated in a complex fashion, nonlinearly dependent on the chemotactic peptide doses and specifically regulated according to the receptors involved.

INTRODUCTION

A number of biological activities are induced by inflammatory reactions in human neutrophils, including the ability to adhere to microvascular endothelium and to

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extracellular matrix components, to migrate towards a gradient of chemotactic substances, and to produce oxygen-derived free radicals. These and other functions are subject to a complex regulation by endogenous and exogenous mediators produced by other cell types or generated from plasma precursors. Previous investigations on animal and human models have shown that neutrophils harvested from patients with infections or from inflammatory exudates show enhanced responses to various membrane stimulants with respect to peripheral blood neutrophils (1–8). One frequently observed metabolic modification related to this phenomenon, called priming, is the enhanced production of oxygen free radicals. Neutrophil priming is the object of active investigations because it may have a role both in strengthening resistance against invading microorganisms and in predisposing the host to increased tissue damage during the inflammatory process (9–14).

One of the main points that remains to be clarified is the regulation of the cell adherence of primed neutrophils, an important phenomenon that also may have a potential pathogenic role (15–19). Increased adherence is an active response of the cells to suitable soluble or particulate agents, being due to the expression and eventually to the activation of various specific membrane-anchoring proteins. However, recent data showed that the relationship between cell stimulation by neutrophil agonists and adhesion response is neither linear nor simple. In fact, adherence to biological surfaces may either stimulate (20–22) or modulate (23, 24) other functional responses to cytokines or other soluble stimulants, according to the experimental models utilized. Moreover, our previous studies showed that adherence of neutrophils primed in vitro by endotoxin is stimulated by high doses of chemoattractants, while it is inhibited by low doses (25). Others have shown that interleukin-8 and low doses of other chemoattractants such as C5a and FMLP reduce the adhesion of normal neutrophils to cytokine-activated endothelial cells (26). Finally, we (27) and others (28) have shown that the priming phenomenon of exudate and inflammatory cells is factor-specific, depending on the stimulatory agents employed.

In this work, we have evaluated the metabolic and adhesive functions of human neutrophils primed in vivo during an experimental inflammatory reaction produced by the skin-window (SW) technique. The sensitivity of both superoxide production and adherence to the stimulation by various doses of the chemoattractant FMLP and other stimulants was determined. Comparing FMLP dose–response curves of primed (SW) and control cells gave prominence to a dual effect of FMLP on the adherence of SW neutrophils: low doses inhibited and high doses stimulated the neutrophil adherence to serum protein-coated plastic surfaces.

Since cell adherence is strictly linked to cytoskeleton structure and function, we have also explored the effects of various FMLP doses on cell morphology,

evaluated by phase-contrast light microscopy, and actin polymerization, evaluated as right angle light scattering.

MATERIALS AND METHODS

Reagents. The chemotactic peptide FMLP, phorbol myristate acetate (PMA) and concanavalin A (Con A) were purchased from Sigma Chemical Company, St. Louis, Missouri; cytochrome *c* from Boehringer, Mannheim, Germany; purified serum bovine albumin and human albumin from Behring Institut, Marburg, Germany; Percoll was from Pharmacia, Uppsala, Sweden. Sterile 96-well microtiter plates with flat-bottomed wells (Linbro type) were from Flow Laboratories. The microplates were precoated with fetal bovine serum (Flow Laboratories) in order to abolish non-specific cell activation and to provide physiological adherence surface, as described (29). Hanks' balanced salt solution (without calcium and magnesium) (HBSS) and Dulbecco's phosphate buffered saline (PBS) were from Gibco Ltd., Paisley, Scotland; the composition of HBSS was 0.4 g/liter KCl, 0.06 g/liter KH_2PO_4, 8 g/liter NaCl, 0.09 g/liter Na_2HPO_4, 1 g/liter D-glucose (pH 7.4); the composition of PBS was 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 0.047 g/liter $MgCl_2$, 8 g/liter NaCl, 1.15 g/liter Na_2HPO_4 (pH 7.4). Other materials and reagents were of the highest purity available.

Sterile apyrogenic solutions and disposable plasticware were used in all experiments, which were carried out, whenever possible, under a laminar flow hood.

Cell Preparation. Neutrophils were obtained from blood and from SW exudates of healthy human volunteers. Blood neutrophils were prepared from 40 ml of ethylene diamine tetraacetate-anticoagulated blood by centrifugation over discontinuous 63%/72% Percoll gradients (30). After hypotonic lysis of contaminating erythrocytes and two washings with PBS, the cells (>95% neutrophils, >99% viable as judged by trypan blue exclusion test) were finally suspended in HBSS, containing 0.2% human serum albumin (H-A), and kept at room temperature until use. A few minutes before use, 100× concentrated solutions of $CaCl_2$ and $MgSO_4$ were added to the cell suspensions at the final concentration of 0.5 mM and 1 mM, respectively.

Exudate neutrophils were isolated according to the method described by Senn and Jungi (31), with modifications (27). Briefly, an abrasion of 1 cm^2 was obtained with a rotating sterile abrasive cylinder on the volar surface of the nondominant forearm. A bell-shaped, sterile, and disposable plastic skin chamber (FAR Italia, Verona) was put on the skin abrasion and fixed with a fenestrated sticking plaster. One milliliter of autologous serum was then injected into the chamber through a hole and 24 h later the exudate was collected by aspiration. The exudate cells (>95% neutrophils, >99% viable) were then centrifuged at 1200 rpm, washed twice with phosphate-buffered saline (PBS), and finally suspended in H-A and kept at room temperature until use. Before use, the cell suspensions were supplemented with $CaCl_2$ and $MgSO_4$ as described above for blood cells.

Oxidative Metabolism and Adherence. A simultaneous assay of O_2^- production and cell adherence was performed according to previously reported procedures (25, 29), with the following modifications. The assay medium was H-A supplemented with 0.5 mM $CaCl_2$ and 1 mM $MgSO_4$ (H-ACM). The microplate wells were supplemented with 25 μl of 0.6 mM cytochrome *c* and with 25 μl of the stimulants, dissolved in H-ACM. Assays were currently done in triplicate for each experimental condition. The plate was brought to 37°C, and 50 μl of the neutrophil suspension (2 × 10^5 cells) prewarmed at 37°C, were added to each well. The plate was then incubated for the desired time (10–40 min) in a humidified thermostat at 37°C. When required, the plate was rapidly transferred into a microplate reader (Reader 400, SLT Labs Instruments) and the reduction of cytochrome *c* was measured at 550 nm using 540 nm as reference wavelength, and using 0.037 optical density units as standard for 1 nmol of reduced cytochrome *c* (29).

For adherence measurements, the acid phosphatase assay was utilized (29). Forty minutes

after cell plating, the microplate was subjected to two washing cycles with PBS at room temperature, adherent cells were lysed with 0.1% Triton X-100 and quantitated by measuring the activity of the enzyme acid phosphatase in 0.15 M acetate buffer, pH 5.3, containing 5 mM *p*-nitrophenyl phosphate. The *p*-nitrophenol produced in the reaction was measured with the microplate reader at 405 nm, and the percentage of adhesion was calculated on the basis of a standard curve obtained with known numbers of neutrophils.

Right-Angle Light Scatter Assay. Measurements were performed at 37°C in an F2000 Hitachi spectrofluorometer with excitation and emission monochromators at 340 nm, as described by Omann et al. (32). The cells were in 2 ml of a stirred suspension at 2×10^6 cells/ml in HBSS containing 0.5 mM $CaCl_2$ and 1 mM $MgSO_4$.

RESULTS

Figure 1A shows the dose dependence of superoxide release by human neutrophils stimulated by FMLP. The optimal dose was about 10^{-7} M, and the threshold dose was about 3×10^{-9} M, in both cell populations. SW neutrophils exhibited a superoxide release two- to threefold higher than PB cells. The dose dependence of the neutrophil adhesion to serum-coated culture wells is shown in Figure 1B. The doses of FMLP that stimulated adherence (optimal dose, 10^{-6} M; threshold dose, 3×10^{-8} M) were slightly higher than the doses that were able of stimulating the superoxide formation (see Figure 1A). The adhesion of resting PB cells was very low, while a substantial proportion of SW cells adhered even in the absence of FMLP. The dose–response curve of SW neutrophils showed a biphasic pattern: upon addition of low doses of FMLP the adhesion was inhibited, whereas higher agonist doses caused adherence response higher than that of PB cells.

Table 1 reports the results obtained from a series of 12 experiments. We noted that the extent of functional responses and of priming of neutrophils, both in terms of superoxide production and of adhesion, varied over a wide range in different experiments carried out using cells from different individuals. However, comparing paired data of each experiment, the inhibition of SW neutrophil adherence by low FMLP doses was highly significant and reproducible.

The dual effects of FMLP on cell adhesion were also documented by phase-contrast microscopy of the bottom of culture wells. Microscopic examination was not suitable for precise quantitative evaluations, but allowed inspection of the cell morphological changes associated with FMLP treatment. As can be seen from Figure 2, resting neutrophils (Figure 2A) appeared spherical, with a smooth membrane, and with little emission of pseudopodia, while on addition of either low (Figure 2B) and high (Figure 2C) FMLP doses the cells changed to an elongated shape, with several pseudopodia and a rough, irregular membrane. The typical cell morphology corresponded to chemotactic factor-induced neutrophil polarization (33, 34). A few activated cells show a broad ruffled edge

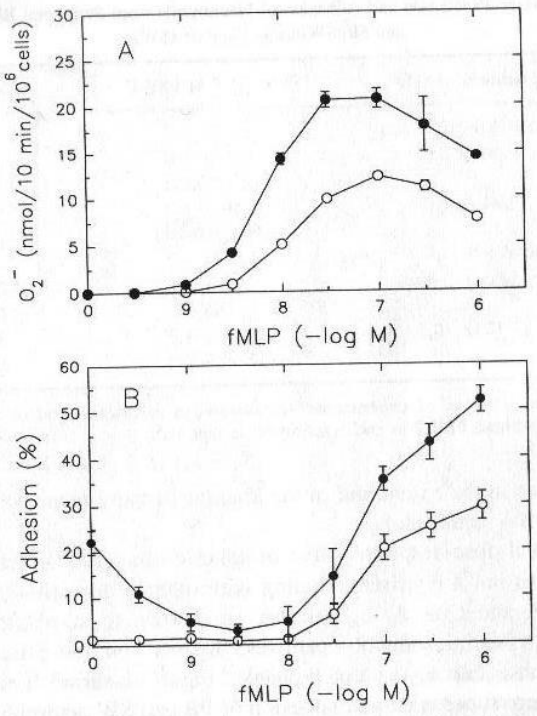

Fig. 1. Effect of different doses of FMLP on superoxide release (A) and adhesion (B) of human neutrophils. Neutrophils were isolated both from peripheral blood (○) and skin-window inflammatory exudate (●), plated and incubated in the presence of FMLP at the indicated concentrations. Superoxide release and adhesion were measured as described in Materials and Methods. Values are mean ± *SD* of triplicate determinations from a representative experiment of 12 performed.

and a narrow tail, typical of migrating neutrophils. Panels D–F of Figure 2 are representative of the residual cell population that was present on the bottom of the microwells after aspiration of the supernatant and washing. Washing caused the removal of a high percentage of unstimulated cells (Figure 2D) and of almost all the cells that had been stimulated by low FMLP doses (Figure 2E). Only neutrophils treated with high FMLP doses (Figure 2F) showed a substantial adhesion response, strong enough to resist washing stress.

These morphological changes occur at FMLP doses that correspond to those causing significant changes of light scattering of neutrophil suspensions, a parameter that has been shown to be strictly associated with actin polymerization (32, 35). In fact, Figure 3 shows that doses of FMLP as low as 10^{-9} M triggered a rapid actin polymerization response, that was maximal at 10^{-7} M. No signif-

Table 1. Superoxide Production and Adhesion of Neutrophils from Peripheral Blood (PB) and Skin-Window Exudate (SW)[a]

	Unstimulated cells	3×10^{-9} M FMLP	3×10^{-7} M FMLP
Superoxide (nmol/10 min/10^6 cells)			
PB	0.56 ± 0.6	1.6 ± 1.0	11.5 ± 3.0
		($P < 0.01$)	($P < 0.0001$)
SW	0.84 ± 1.0	5.46 ± 2.0	23.9 ± 5.3
		($P < 0.0001$)	($P < 0.0001$)
Adhesion (% of total cells)			
PB	5.06 ± 3.4	3.7 ± 1.6	27.25 ± 8.1
		(NS)	($P < 0.001$)
SW	17.12 ± 10.3	8.43 ± 4.9	39.22 ± 14.7
		($P < 0.01$)	($P < 0.001$)

[a] Values are mean ± *SD* of 12 experiments. In parentheses the paired Student *t* test comparing assays with and without FMLP in each experiment is reported.

icant differences in the extent and in the kinetics of these responses were found in the PB and SW neutrophils.

The unusual dose–response curve of adhesion that was found with FMLP (Figure 1B) was not a consistent finding with other tested stimulants. In fact, by using PMA and Con A, agents that are known to stimulate neutrophils through receptors and transduction pathways distinct from those used by FMLP, the dose–response curve was not biphasic. Figure 4 shows a representative experiment where superoxide and adhesion of PB and SW neutrophils was measured as a function of Con A concentration. Activation of superoxide release (Figure 4A) required Con A doses much higher than those necessary in order to stimulate adhesion, evidence in agreement with our previous findings (29). Unstimulated SW neutrophils showed a basal adhesion that was enhanced with respect to unstimulated PB neutrophils, as expected (Figure 4B). Addition of increasing doses of Con A caused a progressive increase of adhesion without any inhibitory effect, the curves of both PB and SW neutrophils showing a roughly sigmoidal shape. Using PMA as stimulatory agent (0.01–100 ng/ml), the curves of both adhesion and superoxide release were sigmoidal; low doses of this agent (0.01–0.2 ng/ml) did not cause either stimulation or inhibition of adhesion (data not shown).

DISCUSSION

Neutrophils present in inflammatory exudates are functionally modified with respect to "virgin" cells, which may be isolated from peripheral blood. In this report, we have used a well-established ex vivo human experimental

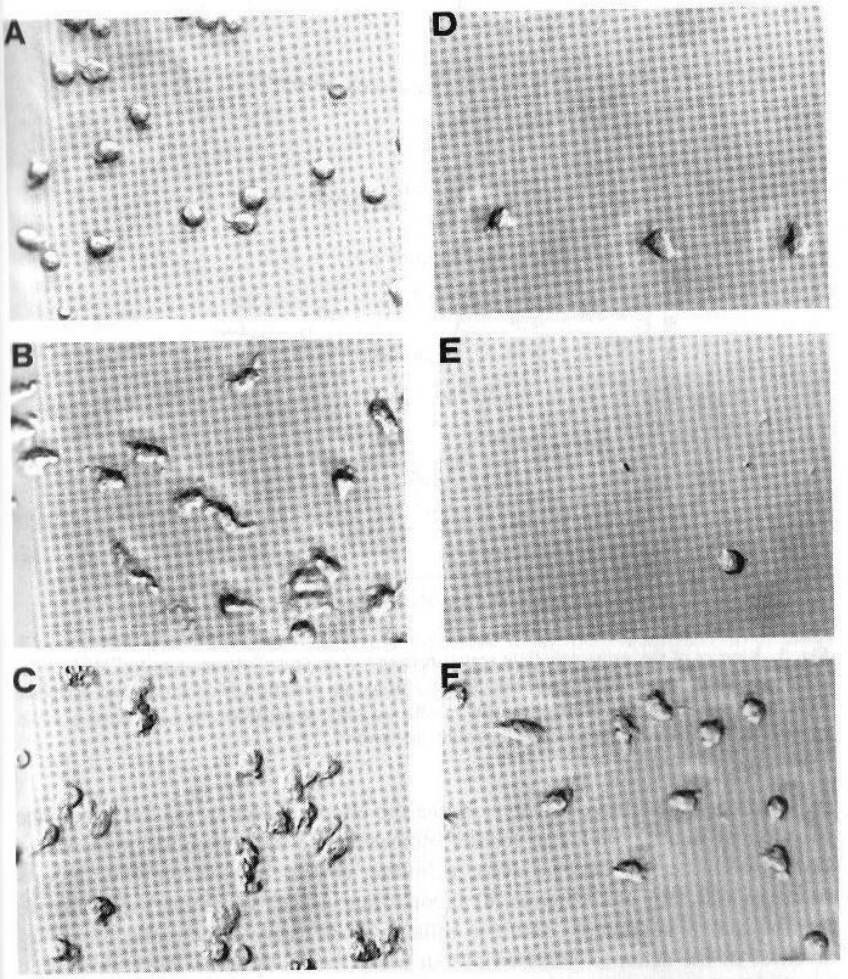

Fig. 2. Microscopic examination of settled (A–C) and adherent (D–F) neutrophils in the presence of different FMLP doses. SW neutrophils were incubated in serum-coated microplate wells for 40 min in the absence of FMLP (A, D), in the presence of 3×10^{-9} M FMLP (B, E), and in the presence of 3×10^{-7} M FMLP (C, F). The bottom of the wells was then examined with phase-contrast microscopy. A–C are the cells before washing, representing the cell population that settled to the bottom due to gravity; D–F are the cells after washing out nonadherent cells and supplementing the wells with 0.2 ml of PBS.

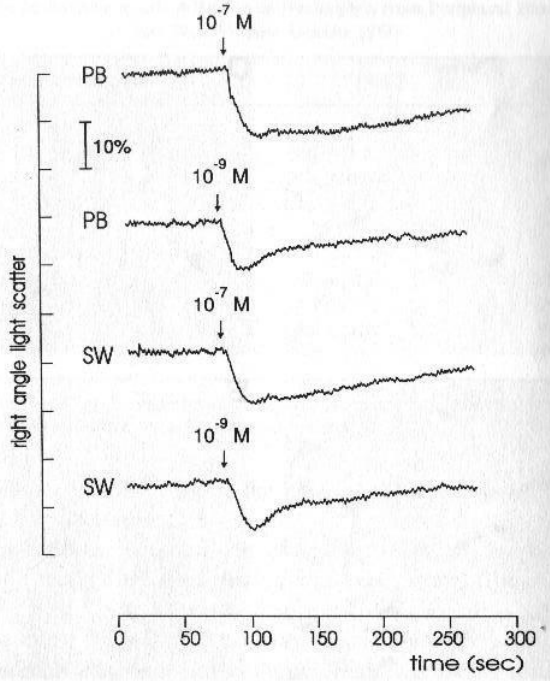

Fig. 3. Kinetics of the right-angle light scatter response of neutrophil suspensions to FMLP. Measurements performed at 37°C under continuous stirring, as described in Materials and Methods. Right-angle light scatter was observed for 60–80 sec prior stimulation, then 20 μl of a 100× concentrated FMLP solution was added as stimulus (arrow).

inflammatory model in order to examine the effect of priming on the adherence function of neutrophils. The results indicated that the metabolic response to FMLP is primed in exudate cells, a finding consistent with previous reports from our (27, 36) and other (1–8) laboratories. Besides priming the oxidative metabolism, accumulation into the inflammatory exudate increased the adhesiveness of the cells, as assessed by our assay model system. The chemotactic peptide FMLP behaves as an agonist of the adhesion response, but the dose-response for adhesion was shifted to the right as compared to the dose response for O_2^- release. On the other hand, when exudate neutrophils were challenged with low, substimulatory doses of FMLP, adherence was inhibited. A similar paradoxical effect of FMLP was recently noted by us on an in vitro priming model, using lipopolysaccharide-treated neutrophils (25) and tumor necrosis factor-α-treated neutrophils (P. Bellavite, unpublished), suggesting that it represents a typical behavior of primed cells exposed to varying FMLP concentrations.

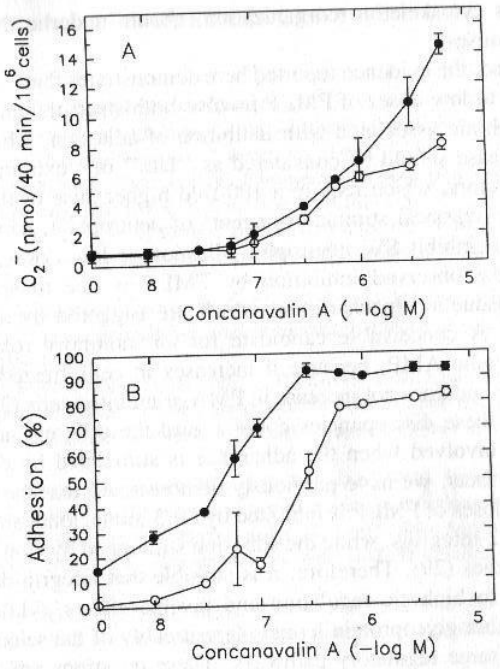

Fig. 4. Effect of different doses of Con A on superoxide release (A) and adhesion (B) of human neutrophils. Assays on neutrophils from peripheral blood (○) and skin-window inflammatory exudate (●), were performed as described in Materials and Methods. Values are mean ± *SD* of triplicate determinations from a representative experiment of four performed.

A recent report showed that interleukin-8 and low doses of other chemoattractants such as C5a and FMLP reduce the adhesion of normal neutrophils to cytokine-activated endothelial cells (26). In that system, low doses of chemoattractants caused major morphological changes, and the authors suggested that the inhibitory effects could be due to rearrangement of the neutrophil cytoskeleton, leading to loss of focal points of contact between the neutrophils and the endothelial cells. This possible explanation could be the case in our experimental system also, where marked shape changes and actin polymerization were observed in neutrophils treated with low, adhesion-inhibiting, FMLP doses. However, here we have shown that membrane ruffling, cell polarization, and actin polymerization are present also in cells stimulated with high doses of FMLP, capable of stimulating marked adhesion in both PB and SW neutrophils. Therefore, more specific molecular changes of the membrane adherence prop-

erties, besides cytoskeleton reorganization, should underlie the observed dual adhesion responses.

In any case, the evidence reported here demonstrates that "early" responses of neutrophils to low doses of FMLP involve both structural and morphological changes, which are associated with inhibition of adhesion, while adhesion and superoxide release should be considered as "late" or "extreme" responses to chemotactic factors, which require a 100-fold higher dose of this stimulant.

Two other classical stimulatory agents of neutrophils, namely Con A and PMA, failed to inhibit SW neutrophil adhesion at low doses. This fact may indicate that the observed inhibition by FMLP is due to specific receptor-dependent transduction mechanisms, which are triggered by some agents but not by others. A conceivable candidate for an inhibitory role on neutrophil adherence is cyclic AMP, because it increases in cells treated with very low doses of FMLP and does not increase in PMA-stimulated cells (25, 37). Another explanation of these discrepancies could regard the different adhesion mechanisms that are involved when the adherence is stimulated by different agents. As a matter of facts, we have previously demonstrated that the adhesion stimulated by high doses of FMLP is inhibited by 60.3 monoclonal antibodies, which bind to CD18 β2-integrins, while the adhesion stimulated by Con A is unaffected by these antibodies (29). Therefore, it is possible that integrin-dependent adhesion is subject to biphasic regulation and inverse effects, while the adhesion dependent on other glycoprotein ligands (presumably of the selectin type) is not affected by the same regulatory pathways. These questions are open to further investigations, based on more direct molecular approaches.

From a pathophysiological standpoint, the dual effects of FMLP on adhesion of SW cells may reflect the actual complexity of the regulation of neutrophil kinetics and functions. Adhesion and detachment are important for the distribution of leukocytes in the bone marrow, in the bloodstream, and eventually in the inflammatory sites. Low chemotactic factor doses may induce the weakly adherent cells to detach and then to migrate towards the center of the inflammatory focus, where a higher concentration of the agonist may stimulate them to adhere to endothelia and other connective tissue structures. Moreover, considering the phenomenon on a cell scale, locomotion of neutrophils on a surface depends on a fine balance between attachment and detachment. During the directional migration through a chemotactic gradient, neutrophils take on a bipolar shape; adhesion occurs in the ruffled edge of the cell oriented towards the higher concentration of chemotactic agent, whereas at the cell's tail, where the concentration of the chemotactic agent is lower, the membrane undergoes detachment from the adhesion surface. Our observation of a dual effect of different FMLP doses is consistent with these interpretations.

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