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Dual Effects of Formylpeptides on the Adhesion of Endotoxin-Primed Human Neutrophils

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Neutrophils, treated with sequential additions of bacterial products such as endotoxin (*E. Coli* lipopolysaccharide, LPS) and the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), undergo to metabolic activation and express membrane-anchoring proteins that promote adhesion to serum-coated culture wells. By investigating the dose–response relationships of these phenomena, we have found that: (a) resting neutrophils do not produce a significant amount of superoxide (O_2^-) and show only minimal adhesion to serum-coated plastic surfaces; (b) fully activatory doses ($> 5 \times 10^{-8}$M) of fMLP induce the release of O_2^- and a significant increase of the cell adhesion; (c) pretreatment of the cells for 1 h with LPS augments cell adhesion to serum-coated culture wells in the absence of further stimulation and primes the neutrophils to enhanced fMLP-dependent O_2^- release; (d) addition of low, substimulatory doses of fMLP (from 10^{-10}M to 5×10^{-9}M) inhibits and reverses the adhesion of LPS-treated cells, (e) high fMLP doses ($> 10^{-7}$M) are additive to LPS in promoting adhesion. Phorbol-myristate acetate ($> 10^{-9}$M) increased adhesion in both normal and LPS-treated neutrophils, but low doses of this stimulant did not inhibit adhesion. Low doses (10^{-9}M) of fMLP increased intracellular cyclic AMP in both normal and LPS-treated neutrophils, suggesting that stimulus-induced rises in cAMP may be the negative signal responsible for down-modulation of adhesion. Low (5×10^{-9}M) and high (5×10^{-7}M) fMLP doses induced the same increase of expression of CD11/CD18 integrins, indicating that the inhibition of adhesion caused by low doses is not due to quantitative down-regulation of integrins. These findings may provide an *in vitro* model of the complex biological events involved in the regulation of neutrophil adhesion.

KEY WORDS—Priming; neutrophil modulation; adhesion; endotoxin; chemotaxis; cyclic AMP.

INTRODUCTION

During inflammatory reactions human neutrophils respond to particulate and soluble stimuli by activating a number of functions such as the release of oxygen free radicals and of arachidonic acid metabolism derivatives, as well as release of granule constituents, chemotaxis, adhesion and even cytokine synthesis. The ability of neutrophils to adhere to the microvascular endothelium and to components of the extracellular matrix, and to produce oxygen-derived free radicals is regarded as an essential event in the inflammatory response and in the phagocyte-dependent host defence systems.

All these responses are subject to complex regulation by endogenous and exogenous mediators, and the functional activity of neutrophils resulting from exposure to a specific stimulatory compound may range from high responsiveness (primed state) to desensitization, adaptation or inactivation, depending on the cell environment and previous contacts with agonists or antagonists. It has been shown previously that neutrophils isolated from patients suffering from inflammation or infections,[1–5] and neutrophils treated *in vitro* with bacterial products such as endotoxin (LPS), formylated peptides or cytokines[6–10] have an increased capacity to generate reactive oxygen species, i.e. these cells are primed. Neutrophil priming is regarded as an important functional change both in the host defence against invading microorganisms[11–13] and in the potentially harmful events of the inflammatory process.[14–18]

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The primed state of neutrophils has been widely studied for its consequences on the O_2^--forming NADPH oxidase, on the expression of receptors, on arachidonic acid generation and on several pathways of signal transduction, but other responses and functions have received little attention. One of the main points that remains to be clarified is the effect of priming on the adhesion response. By using a recently developed simultaneous assay for O_2^- release and adhesion,[19] we have evaluated these functions of human neutrophils primed *in vitro* with bacterial endotoxin (*Escherichia coli* LPS) and stimulated with formylpeptides. The dose–response curves of primed and control cells have been determined. These curves highlighted an unexpected dual effect of fMLP on the adhesion of LPS-primed cells: low doses inhibited, whereas high doses stimulated the adhesion of neutrophils to serum protein-coated plastic surfaces. This phenomenon may have physiological significance in the regulation of leukocyte kinetics and chemotaxis.

MATERIALS AND METHODS

Reagents

Hank's 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 gl^{-1} KCl, 0·06 gl^{-1} KH_2PO_4, 8 gl^{-1} NaCl, 0·09 gl^{-1} Na_2HPO_4 1 gl^{-1} D-glucose (pH 7·4); the composition of PBS was 0·2 gl^{-1} KCl, 0.2 gl^{-1} KH_2PO_4, 0·047 gl^{-1} $MgCl_2$, 8 gl^{-1} NaCl, 1·5 gl^{-1} Na_2HPO_4 (pH 7·4). fMLP, PMA, lipopolysaccharide (LPS, from *Escherichia coli,* serotype 026:B6) and 3-isobutyl-1-methyl-xantine (IBMX) were purchased from Sigma Chemical Company St. Louis, Mo; fMLP (10^{-4}M) was dissolved in dimethylsulfoxide and stored at −20°C, PMA (0·1 mg ml^{-1}) was dissolved in dimethylsulfoxide and stored at −70°C, LPS (1 mg ml^{-1}) was dissolved in PBS and stored at +4°C. Cytochrome c was purchased from Boehringer, Mannheim, Germany; purified serum bovine albumin and human albumin were from Behring Institut, Marburg, Germany; fluorescence conjugated anti-human LFA-1ß (CD18) monoclonal antibodies (mAb) and lgG1 isotype control mAb were from Becton Dickinson, San José, CA; Percoll was from Pharmacia, Uppsala; fetal bovine serum (FBS) and sterile 96-well microtitre plates with flat-bottomed wells (Linbro type) were from Flow Laboratories. FBS was inactivated by incubation at 56°C for 30 min. The microplates were precoated with FBS in order to abolish nonspecific cell activation and to provide physiological adhesion surface, as described previously[19]. Other materials and reagents were of the highest purity available. In order to avoid contamination, a possible cause of artifactual activation or priming of the cells, sterile apyrogenic solutions and disposable plasticware were used in all the experiments, which were carried out, whenever possible, under a laminar flow hood. Reagents were prepared using pyrogen-free water or 0·9 per cent NaCl solutions.

Cell Preparation

Neutrophils were obtained from the blood of healthy subjects (blood donors). Neutrophils were prepared by centrifugation over 62 per cent/73 per cent discontinuous Percoll gradients,[20,21] using either ethylene diamine tetraacetate-anticoagulated blood or leukocyte rich buffy-coats prepared by centrifugation of citrate-anticoagulated whole blood, collected in the quadruple-bag system, for 5 min at 2000 *g* at 22°C in Heraeus Cryofuge 8000. After hypotonic lysis of contaminating erythrocytes and two washings with PBS, the cells (> 95 per cent neutrophils, > 99 per cent viable as judged by the Trypan blue exclusion test) were finally suspended in HBSS, containing 0·2 per cent human serum albumin (H-A), and kept at room temperature until used. When indicated, LPS treatment was done by incubating neutrophils in suspension (3×10^6 cells ml^{-1} in H-A) for 1 h at room temperature. Ten minutes before plating for the assay of superoxide and adhesion, the cell suspensions were supplemented with $CaCl_2$ and $MgSO_4$ to a final concentration of 0·5 mM and 1 mM respectively.

Assay of Superoxide and Adhesion

The superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c.[22] The microplate assay for O_2^- release was done as described previously[19,23]. The assay medium was composed of buffer H-A supplemented with 0·5 mM $CaCl_2$ and 1 mM $MgSO_4$ (H-ACM). The FBS-coated wells were supplemented with 25 µl of 0·6 mM cytochrome c in H-ACM and with 75 µl of the cell suspensions. The assays were

done in triplicate for each experimental condition. The plate was first incubated for the desired time (15–60 min) in a humidified incubator at 37°C, then the wells were supplemented with 50 μl of H-ACM solution containing the stimulant (fMLP or PMA) at a concentration exactly three times the required final concentration. This was done because the stimulant is diluted three times in the final incubation mixture. When required, the plates were transferred rapidly 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 the standard for 1 nmole of reduced cytochrome c.[19]

For the measurement of adhesion, the plates were transferred to an automatic washer (Easy Washer 2, SLT Labs Instruments) and subjected to two washing cycles with PBS at room temperature. Adherent cells were quantified by measuring the membrane enzyme acid phosphatase and the percentage of adhesion was calculated on the basis of a standard curve obtained with known numbers of neutrophils.[19]

Intracellular cAMP Concentration

LPS-treated and untreated neutrophils were suspended in H-ACM at 8×10^6 cells ml^{-1} and activated at 37°C with various doses of fMLP. Thirty seconds after the addition of fMLP (the optimal time for detecting the cAMP peak according to preliminary experiments), aliquots of 240 μl of the neutrophil suspension were withdrawn and rapidly added to tubes containing 500 μl of absolute ethanol, containing 0·8 mM IBMX, precooled to −20°C. The samples were held at −20°C for 1 h, then denaturated protein was removed by centrifuging at 10 000 *g* for 3 min and the supernatants were dried under vacuum. The lyophilates were suspended in 200 μl of 0·05 M sodium acetate buffer, pH 5·8, containing 0·02 per cent bovine serum albumin and 0·005 per cent thimerosal and the cAMP content of duplicate samples was determined by using the cAMP enzyme-immunoassay kit from Amersham Int. (Amersham, U.K.).

Immunofluorescence Flow Cytometry

Measurement of the levels of CD11/CD18 integrins in the neutrophils was done by fluorescence-activated flow cytometry. Normal and LPS-treated (90 min of preincubation with and without 500 ng ml^{-1} LPS) neutrophils (3×10^6 cells in 2 ml of H-ACM) were treated with 5×10^{-9} M fMLP or with 5×10^{-7} M fMLP for 10 min at 37°C, then 10 ml of PBS, pre-cooled to + 4°C, were added and the suspensions were immediately centrifuged for 8 min at 500 *g* at + 4°C. After a further washing with PBS, the cells were suspended in 0·3 ml of PBS and divided into two aliquots, one of which was incubated with 15 μl of fluoresceinated anti-human LFA-1ß (CD18) mAb, while the other was incubated with 15 μl of fluoresceinated control lgG1 mAb. Treatment with mAbs was done for 30 min at 4°C, then the cells were washed twice with PBS at 4°C and the fluorescence was measured with a Becton Dickinson FACScan equipped with a 488 nm argon laser. The analysis was made on 10 000 events on each sample, using software Lysys-II.

RESULTS

Figure 1a shows the dose-dependence of superoxide release by human blood neutrophils stimulated by fMLP. The optimal dose was about 10^{-7}M, doses under 10^{-8} M being totally ineffective. LPS-

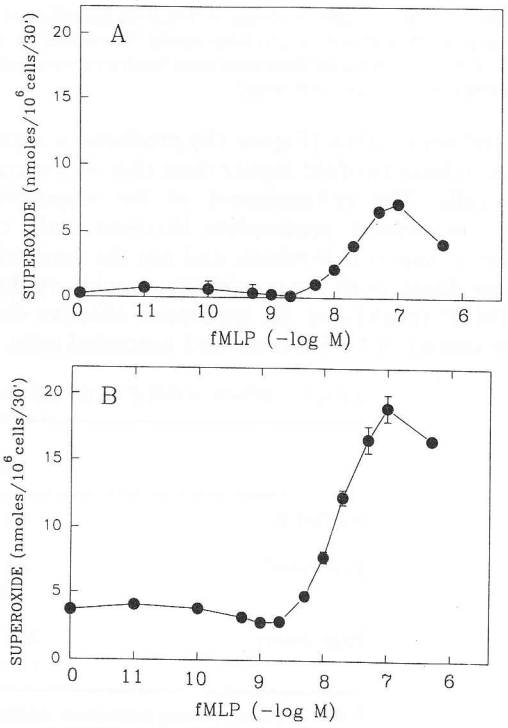

Figure 1. Effect of different doses of fMLP on superoxide release by normal (A) and LPS-treated (B) human blood neutrophils. The values are the mean ± S.D. of triplicate determinations from a representative experiment of the eight performed.

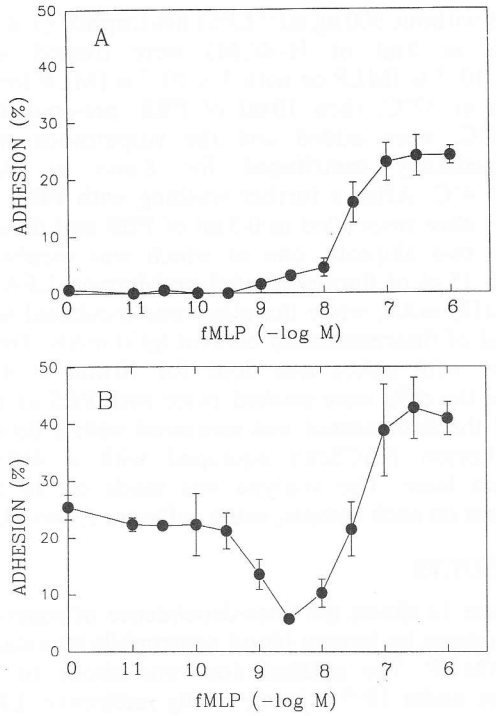

Figure 2. Effect of different doses of fMLP on the adhesion of LPS-treated (B) and normal (A) neutrophils. The values are the mean ± S.D. of triplicate determinations from a representative experiment of the eight performed.

treated neutrophils (Figure 1b) produced a superoxide release twofold higher than that in the control cells. The enhancement of the respiratory burst in primed neutrophils involved only the extent of superoxide release and not the sensitivity to low doses: both the maximum stimulatory dose of fMLP (peak) and the minimum effective dose were similar in LPS-treated and untreated cells.

The adhesion of normal and LPS-primed cells to serum-coated culture wells is shown in Figure 2. The doses of fMLP that stimulated adhesion (10^{-7} M to 10^{-6} M) were similar to, or slightly higher than the doses that stimulated superoxide formation (compare Figures 1a and 2a). However, by looking at the adhesion response of LPS-treated neutrophils (Figure 2b), we noted that a substantial proportion of these cells adhered even in the absence of fMLP and upon addition of low doses of fMLP the LPS-mediated adhesion was inhibited. A series of eight experiments showed consistently a biphasic dose–response effect in LPS-treated neutrophils, where the fMLP doses causing inhibitory effects ranged from 10^{-10}M to 5×10^{-9}M. These results, which are summarized in Table 1, indicated that : (a) untreated cells underwent only minimal adhesion to serum-coated plastic surfaces and this adhesion was slightly reduced by low doses of fMLP, (b) fully activatory doses of fMLP induced a significant increase of the cell adhesion, (c) treatment of the cells with endotoxin augmented adhesion in the absence of further stimulation, (d) low, substimulatory doses of fMLP significantly inhibited the adhesion of endotoxin-treated cells, (e) high fMLP doses were additive to endotoxin in promoting adhesion.

Low doses of fMLP not only prevented LPS-induced adhesion, but were also capable of reversing the adhesion that had already occurred. The time-course of the detachment of adherent neutrophils is reported in Figure 3. Neutrophils were treated with LPS, and left to adhere to culture wells for 40 min; then low doses (5×10^{-9} M) or high doses (5×10^{-7} M) of fMLP were added. It can be seen that low doses of fMLP caused a rapid detach-

Table 1. Effects of fMLP on the adhesion of normal and LPS-treated human neutrophils.

	Adhesion (%)	
	No LPS	+1 μg ml^{-1} LPS
No fMLP	4.5 ± 4.1	20.9 ± 10.5
Low doses*	3.1 ± 2.4 (n.s)	7.4 ± 6.1 ($p < 0.01$)
High doses†	24.8 ± 11.5 ($p < 0{\cdot}01$)	40.3 ± 16.9 ($p < 0{\cdot}01$)

* fMLP doses causing maximum inhibition of adhesion (from 3×10^{-9} M to 5×10^{-9} M according to different experiments).
† 5×10^{-7} M fMLP. The mean values ± S.D. of eight experiments are reported. In parentheses the paired *t*-Student test comparing assays with and without fMLP in each experiment is reported.

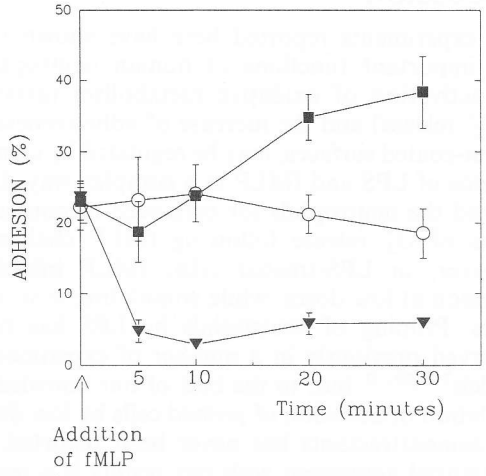

Figure 3. Effect of the addition of fMLP on the adhesion of LPS-treated neutrophils. Cells were treated with 200 ng ml^{-1} LPS for 1 h, then plated and left to adhere for 40 min in separate plates. Then either 5×10^{-9} M fMLP ▼, 5×10^{-7} M fMLP (■) or an equivalent volume of HCMGA (O) were added at zero time. The values are the mean ± S.D. of triplicate determinations from a representative experiment of the two performed.

ment of adherent cells, and that detachment was almost complete in less than 5 min. On the other hand, high doses caused the detachment of a small percentage of cells in the first 5 min, but thereafter caused a progressive increase of adherence to above the values of unstimulated LPS-treated neutrophils.

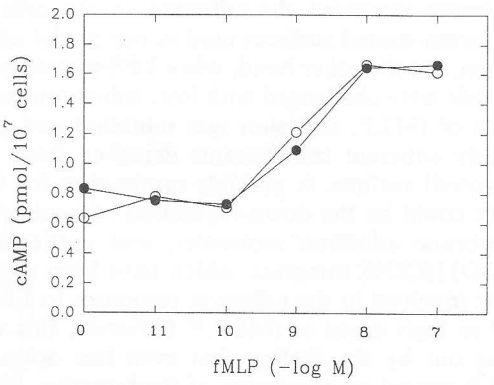

Figure 4. Dose–response of intracellular cAMP concentration in neutrophils stimulated with fMLP. Cells were pre-incubated for 90 min in suspension in the absence (○) and in the presence of (●) of 250 ng ml^{-1} LPS, then activated with the indicated doses of fMLP. cAMP was assayed on duplicate samples. The values of a typical experiment, representative of the three performed, are shown.

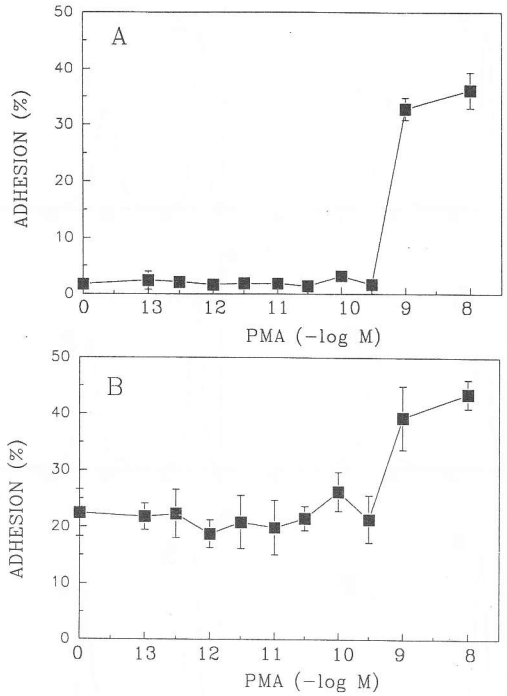

Figure 5. Effect of different doses of PMA on the adhesion of LPS-treated human neutrophils. Neutrophils were treated in suspension without (A) and with (B) 250 ng ml^{-1} LPS, then incubated for 30 min in microplates with the indicated concentrations of PMA. The values are the mean ± S.D. of triplicate determinations from a representative experiment of the four performed.

A possible biochemical mechanism underlying the observed effects of low doses of fMLP could be the rise of intracellular cAMP, a messenger to which a role as negative feedback signal has been attributed.[24–27] Therefore, the intracellular cAMP in response to fMLP was investigated in normal and LPS-treated cells. As shown in Figure 4, doses of fMLP as low as 10^{-9} M were capable of triggering a marked increase of intracellular cAMP, the maximal effect being reached with 10^{-8} M fMLP.

When PMA was used the dose–response curve was not biphasic. This agent is known to stimulate neutrophils through direct activation of protein kinase C. Figure 5 shows that doses from 10^{-9} to 10^{-8} M of PMA stimulated adhesion in both normal and LPS-primed neutrophils and that substimulatory doses of PMA did not affect the adhesion of LPS-treated cells (Figure 5b).

Since the fMLP - and LPS-induced adhesion of neutrophils involves the function of $ß_2$-integrin (CD11/CD18) membrane molecules,[19,28] the effect

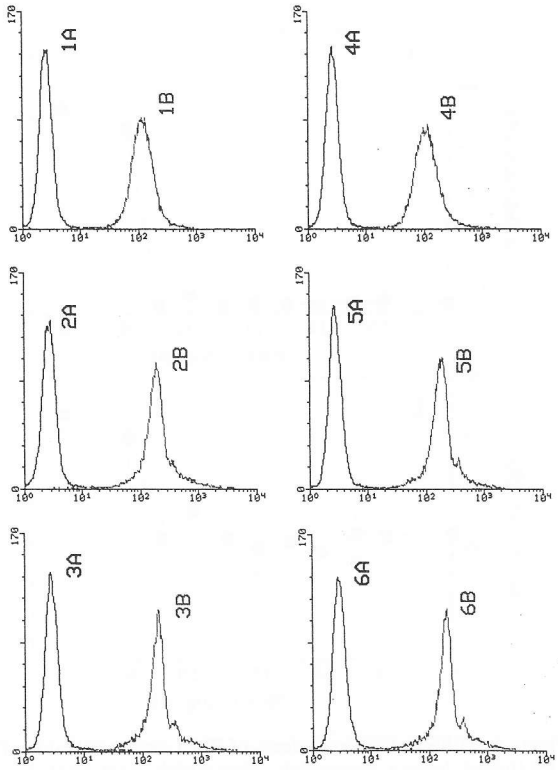

Figure 6. Expression of CD11/CD18 integrins on human neutrophils. Normal (1-3) and LPS-treated (4–6) neutrophils were activated for 10 min at 37°C with 5×10^{-9} M fMLP (2 and 5) or with 5×10^{-7} M fMLP (3 and 6). Cells in 1 and 4 did not receive fMLP. The expression of CD11/CD18 integrins was quantified by cytofluorometry. Horizontal scale: fluorescence; vertical scale: number of cells. The mean fluorescence of the peaks was: 1A, 2·6; 2A, 2·7; 3A, 2·9; 4A, 2·7; 5A, 2·8; 6A; 2·9; 1B, 116·9; 2B, 193·5; 3B, 189·7; 4B, 110·8, 5B, 180·1; 6B, 201·0.

of low and high fMLP doses on the quantitative expression of these molecules was evaluated by immunofluorescence flow cytometry (Figure 6). Both low 5×10^{-9} M) and high (5×10^{-9} M) doses of fMLP increased the expression of CD11/CD18 integrins as compared with resting cells. Moreover, it can be seen that the level of expression of integrins was not affected by treatment of the neutrophils with LPS. In the same experiment, the adhesion assay confirmed the marked inhibition of LPS-induced adhesion by 5×10^{-9} M fMLP. These results rule out the hypothesis that the inhibitory effect of low doses of fMLP was due to quantitative down–regulation of integrin-type adhesion molecules.

DISCUSSION

The experiments reported here have shown that two important functions of human neutrophils, the activation of oxidative metabolism (assayed as O_2^- release) and the increase of adhesiveness to serum-coated surfaces, may be regulated by combinations of LPS and fMLP in a complex way. LPS primed the neutrophils for enhanced responses in terms of O_2^- release following fMLP challenge; however, in LPS-treated cells, fMLP inhibited adhesion at low doses, while stimulating it at high doses. Priming of neutrophils by LPS has been observed previously in a number of experimental models[7–9,29–31] but, to the best of our knowledge, inhibition of adhesion of primed cells by low doses of chemoattractants has never been reported. In substantial agreement with our results is a recent report showing 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.[32]

LPS-dependent priming of oxidative metabolism was shown as an increase of total O_2^- release and not as a shift to the left of the dose–response curve, suggesting that, under these experimental conditions, priming is not due to enhanced sensitivity of receptors to low doses, but instead to increased responsiveness of the cells to a given dose. Similar conclusions have been drawn from an *in vivo* priming model consisting of experimentally-induced skin-window inflammatory exudates.[33]

Besides priming the oxidative metabolism, LPS treatment increased the adhesion of the cells to the serum-coated surfaces used in our model assay system. On the other hand, when LPS-treated neutrophils were challenged with low, sub-stimulatory doses of fMLP, adhesion was inhibited and previously adherent cells became detached from the microwell surface. A possible mechanism for this effect could be the down-regulation of neutrophil membrane adhesion molecules, and particularly of CD11/CD18 integrins, which have been shown to be involved in the adhesion responses to LPS[28] and to high doses of fMLP.[19] However, this was ruled out by the finding that even low doses of fMLP caused up-regulation of the integrins. Since different doses of fMLP caused the same increase of membrane adhesion molecules but opposite effects on the cell adhesion to plasma proteins, the following alternative explanations should be considered: (a) that the primary mechanism

accounting for enhanced or diminished adhesiveness is not a quantitative difference but a qualitative alteration in integrin heterodimers, leading to changes in avidity, which have been documented in other models [34–36] or (b) that LPS-dependent adhesion requires some additional adhesion mechanism which is inhibited by low doses of fMLP. These mechanisms could be represented by L-selectins, leukocyte surface adhesion molecules which are lost from the neutrophil surface upon stimulation with cytokines and chemoattractants.[32,36,37] Further and more direct experiments are necessary before a definite conclusion regarding these points is achieved.

The intracellular signal that mediates the inhibitory effect of low doses of fMLP remains to be identified, but a conceivable candidate may be the rapid peak of cAMP that is known to occur following fMLP stimulation.[38] It has been reported by others that half-optimal O_2^- production requires the occupancy of about 30 per cent of the fMLP receptors, while, in order to stimulate intracellular calcium increase and cAMP increase, the occupancy of less than 3 per cent receptors is required.[39] Here we have shown that low doses of fMLP (from 10^{-9} to 10^{-8} M), that inhibited and reversed the LPS-induced adhesion, may also increase cAMP. On the other hand, low doses of PMA, a stimulatory agent that does not cause a rise in intracellular cAMP,[40,41] did not inhibit LPS-induced adhesion.

It has been reported by us[19] and by others[24,27,41,42] that pharmacologic elevation of intracellular cAMP can block adhesion, chemotaxis and superoxide production. The critical effect of an elevation in cAMP could be to affect the neutrophil cytoskeleton, since actin is a substrate of cAMP-dependent protein kinase[43] and microinjection of fibroblasts with the catalytic subunit of cAMP-dependent protein kinase inhibits actin polymerization.[44] Alternatively, a possible mechanism could be the alteration of the binding function of the integrins themselves, as reported in lymphocytes, where elevation of cAMP inhibits the ability of the T cell receptor to induce an increase in the avidity of the CD11a/CD18 integrin for its ligand.[45] In any case, it remains to be explained why high doses of fMLP increase adhesion even in the presence of a cAMP burst. It is conceivable that high fMLP receptor occupancy triggers a transduction pathway that by-passes the inhibitory effect of cAMP and is capable of acitivating both NADPH oxidase and adhesion molecules.

Adhesion and detachment are important for the kinetics and distribution of leukocytes in the bone marrow, in the bloodstream and in the various regions where phagocytes are recruited to accomplish their defensive or destructive functions. Sequestration of neutrophils in a variety of vascular beds, manifested as acute neutropenia, occurs shortly after the intravascular infusion of LPS and it has been suggested to result from an effect on the adhesive and mechanical properties of neutrophils.[46] Therefore, it is possible that during specific conditions where production and release of formylpeptides occurs, adherent cells in the vascular bed or on the outskirts of an inflammatory focus are exposed to low doses of chemoattractants. These low doses may induce the cells to detach and eventually to migrate towards the centre of the inflammatory focus. When the migrating (and primed) cells arrive at sites where the concentration of the agonist is high, this may activate membrane anchoring proteins and cause the cells to adhere to endothelia and eventually to connective tissue proteins. Clearly, these conclusions represent an oversimplification of the actual network comprising biochemical messengers, receptor dynamics and cell reponses that regulates the behaviour of neutrophils in the context of the inflammatory process.

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