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# Haematological effects of inhalation of *N*-formyl-methionyl-leucyl-phenylalanine in man

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## Abstract

***Background*** ***N*-Formyl-methionyl-leucyl-phenylalanine (FMLP) is a bacterial oligopeptide which stimulates neutrophil chemotaxis, degranulation and superoxide generation. Inhalation of FMLP produces bronchoconstriction in man; in the rabbit this is in part neutrophil dependent. The effects of inhalation of FMLP on peripheral blood leucocytes in normal subjects has been studied.**

***Methods*** **This was an open study in non-asthmatic subjects. Change in total peripheral white cell count were studied for 15 minutes after inhalation of 0·4 μmol FMLP in six subjects. Change in total and differential white cell count and spontaneous neutrophil chemiluminescence were then studied five and 30 minutes after inhalation of 0·4 μmol FMLP (n = 7) or diluent (n = 4). Finally, leucocytes from three subjects were labelled ex vivo with technetium-99m labelled sulphur colloid and reinfused. The effect of inhalation of FMLP or diluent on pulmonary neutrophil flux was studied by continuous gamma scanning of a pulmonary window.**

***Results*** **Leucopenia occurs rapidly after inhalation of FMLP, the nadir of the white cell count (53% of baseline) occurring at four minutes. This was followed by a rebound increase in white cell count evident at 15 minutes (154% of baseline). Five minutes after inhalation of 0·4 μmol FMLP, neutropenia (17% of baseline) and monocytopenia (40% of baseline) were seen followed again by a neutrophilia (213% of baseline at 30 minutes). The eosinophil count was significantly reduced at 30 minutes (24% of baseline). Neutrophil chemiluminescence was elevated (186% of baseline) at the time of the neutropenia. There was no influx of labelled cells to the lung during the period of neutropenia.**

***Conclusion*** **FMLP inhalation activates circulating leucocytes. In vivo production of FMLP in the airway could contribute to bronchial inflammation during bacterial infection.**


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Bacteria produce low molecular weight chemotactic factors,[1] such as *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), which are formylated oligopeptides.[2,3] FMLP activates a number of neutrophil functions, including chemotaxis, lysosomal enzyme release and oxygen free radical generation.[4] It also contracts smooth muscle. There is a close correlation between chemotactic and spasmogenic activity[5] between the different related formyl peptides. Following the finding that inhaled FMLP produces bronchoconstriction in normal subjects,[6] we suggested that it may cause bronchoconstriction during bacterial bronchial infection, particularly in patients with chronic airflow limitation.

Although FMLP contracts human bronchial smooth muscle directly in vitro,[7] its bronchoconstrictor activity in vivo may in part be neutrophil dependent as neutropenic rabbits show a reduced bronchoconstrictor response to FMLP.[8] Infusions of substances known to activate neutrophils (FMLP, platelet activating factor (PAF), C5a, granulocyte-monocyte colony stimulating factor (GM-CSF) and endotoxin) produce transient leucopenia,[9–13] as a result of reduced deformability[14] or enhanced adherence to endothelium.[15–17] This produces neutrophil margination in small vessels, particularly in the pulmonary circulation.[11,12] Activation of leucocytes by inhalation of FMLP in addition to sequestration in the lungs may explain the neutrophil dependent component of bronchoconstriction.

The aims of this study were to determine the changes in leucocyte count and neutrophil function and distribution after FMLP inhalation.

## Methods

The subjects were lifelong non-smokers aged 25–38 years with no history of asthma or recent respiratory infection. All were men, apart from one woman in study 1. Only one subject participated in more than one experiment, taking part in all three. All protocols were approved by the Institutional Ethics Committee of Concord Hospital and all subjects gave informed consent to participate.

All experiments were started between 8.30 and 10.00 am. FMLP (Sigma Chemicals, St Louis, Missouri), was dissolved at a concentration of 2·5 mmol/l in 50% dimethyl sulphoxide (DMSO)/saline. Five breaths of FMLP or DMSO solution were taken from functional residual capacity to total lung capacity, with a de Vilbiss 646 nebuliser. Automated white cell counts were performed with a Coulter Counter Model S-plus and differential counts performed on 100 cells, smears being stained with May-Grünwald-Giemsa. Total and differential white

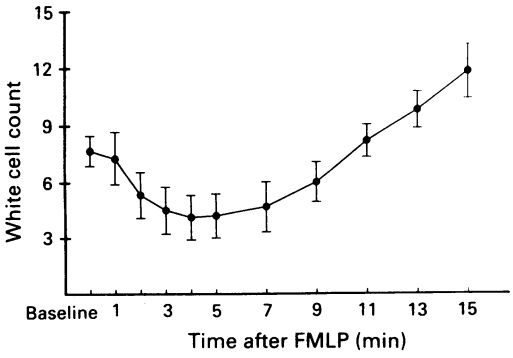


*Figure 1 Mean (SEM) change in white cell count ( × $10^9$/l) over 15 minutes after inhalation of FMLP 0·4 μmol in study 1. There was significant leucopenia between two and seven minutes after FMLP inhalation and a significant increase in white cell count by 15 minutes.*

cell counts were performed by technicians unaware of which solution had been given.

Neutrophil chemiluminescence was measured as follows: 5 ml whole blood was separated for 30 minutes on a Dextran-Hypaque gradient by a modification of Boyum's method.[18] Cuvettes containing 100 μl of the leucocyte fraction, 100 μl of phosphate buffered saline and 11 μl of luminol solution (final luminol concentration 2 × $10^{-6}$ mmol/l) at 37°C were vortexed for 15 seconds and chemiluminescence was counted for five minutes in a Hewlett-Packard luminometer. A neutrophil count was also performed on a portion of the sample. The chemiluminescence activity of the cells was then calculated according to the following formula:

Chemiluminescence (CPM) =
(measured chemiluminescence − background chemiluminescence)/cell count,

where background chemiluminescence was the cpm in the absence of cells.

For each subject the five minute and 30 minute chemiluminescence results were then expressed as a percentage of baseline.

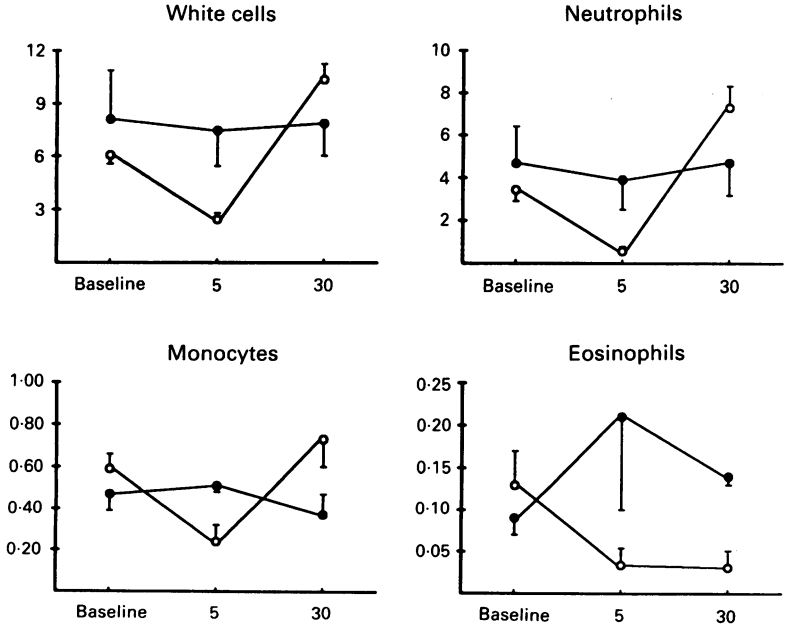


*Figure 2 Mean (SEM) change in white cell count and neutrophil, monocyte and eosinophil counts ( × $10^9$/l) in study 2 at five and 30 minutes after inhalation of FMLP 0·4 μmol (open circles) or DMSO (closed circles). After FMLP, neutropenia at five minutes was followed by neutrophilia. Monocytopenia was seen at five minutes and eosinopenia only after 15 minutes.*

PROTOCOLS

*Study 1*

Six subjects participated in this study. Venous blood was taken at baseline and at one minute intervals for five minutes and at two minute intervals for a further 10 minutes after inhalation of FMLP (0·4 μmol). A total white cell count was performed on each sample.

*Study 2*

Eleven subjects had venous blood taken at baseline, and five and 30 minutes after inhalation of FMLP 0·4 μmol or diluent alone (n = 4). These time points were chosen to be close to the maximum fall and increase in total white cell count. Total white cell count, differential white cell count and spontaneous neutrophil chemiluminescence were determined on each sample.

*Study 3*

The effect of FMLP on phagocytic leucocytes labelled ex vivo[19] and reinfused was studied in three subjects. On each of two days, at least seven days apart, 10 ml of venous blood was taken and leucocytes labelled with technetium-99m labelled sulphur colloid (Radpharm, Canberra). After reinjection and equilibration for 45 minutes, five breaths of FMLP (0·4 μmol) or DMSO diluent were inhaled. Gamma scanning was performed continuously over the period of equilibration and for 30 minutes after FMLP or DMSO inhalation. Fields of interest were delineated for both lungs and minute counts were recorded for each. The half life of lung activity was determined for 20 minutes before and 30 minutes after FMLP or DMSO inhalation.

ANALYSIS OF RESULTS

In study 1, change in white cell count was compared with baseline values by paired Students *t* test. In study 2, change in chemiluminescence after FMLP was compared with that after DMSO by Wilcoxon's rank test. Leucocyte counts were expressed as a percentage of baseline values and the effect of FMLP and DMSO was compared by unpaired *t* test. A p value of $<0·05$ was taken as significant.

## Results

STUDY 1

There was a significant fall in white cell count within three minutes of FMLP inhalation with the nadir occurring at four minutes. At this time the mean (SE) total white cell count was 4·1(1·2) × $10^9$/l compared with the baseline value of 7·7(0·8) × $10^9$/l. There was a significant rebound increase in white cell count by 15 minutes to 11·9(1·4) × $10^9$/l (figure 1). In five of the six subjects the early fall in white cell count was greater than 40% and this was followed by a rise; the sixth subject showed a late rise without a preceding fall. Facial flushing was evident in four of the five subjects who showed transient leucopenia but not in the sixth subject.

*Half life of pulmonary activity of labelled white cells before and after FMLP 0·4 μmol/l and DMSO inhalation in study 3*

| Subject No | Sex | Age (years) | Half life of lung activity (min) | | | |
|---|---|---|---|---|---|---|
| | | | Before DMSO | After DMSO | Before FMLP | After FMLP |
| 1 | M | 38 | 140 | 163 | 73 | 233 |
| 2 | M | 32 | 60 | 108 | 132 | 578 |
| 3 | M | 28 | 90 | 92 | 302 | 308 |

DMSO—dimethyl sulphoxide; FMLP—*N*-formyl-methionyl-leucyl-pheylalanine.

STUDY 2

Neutropenia and monocytopenia were seen five minutes after FMLP inhalation. The mean (SE) neutrophil count and monocyte count were both significantly reduced, the neutrophil count to 0·57(0·21) × $10^9$/l (17% of baseline) and the monocyte count to 0·24(0·08) × $10^9$/l (40% of baseline). At this time the eosinophil count was 26% of baseline ($0·05 < p < 0·1$). At 30 minutes after FMLP, the neutrophil count was significantly elevated to 7·30(1·02) × $10^9$/l (213% of baseline) and the monocyte count had returned to 0·73(0·13) × $10^9$/l (124% of baseline). The eosinophil count was now, however, significantly reduced at 0·03(0·02) × $10^9$/l (24% of baseline) (figure 2).

All seven subjects became transiently neutropenic and five exhibited facial flushing. There was no change in leucocyte count after DMSO. Five minutes after FMLP inhalation, coincident with neutropenia, chemiluminescence was significantly increased to 186% (34%) of baseline. Thirty minutes after FMLP, chemiluminescence had returned to a normal level of 82 cpm (43% of baseline). There was no change in chemiluminescence after DMSO inhalation.

STUDY 3

Loss of activity over the lungs was biphasic. There was an initial phase of rapid activity decay with a half life of about 5 minutes. Pretreatment activity half life was calculated for the 20 minutes before FMLP or DMSO inhalation to allow five half lives of the early rapid phase to elapse for measurement of pretreatment half life. There was considerable within and between subject variability in the pretreatment half life, but no increase in lung activity during the period of neutropenia. Over the 30 minutes of the study, FMLP inhalation caused a marked increase in the half life of activity in the lung fields in two subjects and an increase in lung activity in the third (table).

## Discussion

In this study inhalation of FMLP activated circulating neutrophils and produced transient neutropenia and monocytopenia and a more prolonged eosinopenia. Neutropenia was followed by neutrophilia which lasted for more than four hours. During the phase of neutrophil leucocytosis, neutrophils were retained within the lungs.

Neutrophils were activated at the time of neutropenia, as measured by increased chemiluminescence activity in unstimulated cells, but the activation was transient. The separation of red blood cells from the leucocyte fraction avoided centrifugation with its inevitable neutrophil activation. However, with this method, separation of red cells took about 25 minutes and counting therefore occurred about 30 minutes after the FMLP stimulus. Because of this delay between FMLP stimulus and counting, and because activated neutrophils tend to be marginated in the lungs or elsewhere, the observed increase in chemiluminescence may have substantially underestimated the total degree of neutrophil activation in the peripheral blood and could not measure activation occurring in the tissues.

The mechanisms responsible for FMLP induced monocytopenia are probably similar to those for neutrophils, as exposure to chemotactic factors in vivo produces monocyte adherence and extravascular migration in a similar fashion to that seen with neutrophils.[20] Interestingly, eosinopenia was also found 30 minutes after FMLP inhalation. Eosinophils respond in vitro to FMLP with directional chemotaxis,[21] superoxide generation[22] and leukotriene production.[23] Eosinopenia is seen in rabbits after FMLP infusion and, in contrast to the transient neutropenia, the eosinopenia is prolonged, lasting up to six hours.[24] This may indicate that the cycle of eosinophil adhesion and release after stimulation in vivo is slower than that of neutrophils, or that eosinophils are more likely to migrate into tissues after adhesion.

The initial step in the migration of leucocytes to sites of inflammation is adherence to endothelium. Neutrophil activation by a range of agonists, including FMLP,[14] results in enhanced adhesiveness; the parallel of this in vivo is the transient neutropenia which is seen after infusion of FMLP,[10] endotoxin[12] and GM-CSF.[13] Margination is influenced by the activation state of both leucocyte and endothelium.[9] Generally, when the activating stimulus is given via the venous circulation, margination is seen in the pulmonary circulation. However, after intravascular complement activation by cobra venom factor, margination is seen throughout the microcirculation,[25] suggesting that neutrophils may marginate passively in the first capillary bed they encounter subsequent to activation.

After reinfusion of labelled leucocytes, the decline of lung activity was biphasic with variation of slope of the second phase within and between subjects. Sequestration of labelled cells in the lungs after FMLP inhalation should have produced an increase in pulmonary activity during the period of neutropenia. No subject showed a major change in lung activity during the phase of neutropenia so we could not confirm sequestration in the pulmonary circulation. After the neutropenic phase the rate of decay of activity was reduced in two subjects and the loss of activity reversed in the third subject. Retention or accumulation of labelled leucocytes within the lungs could be a consequence of enhanced pulmonary endothelial adhesiveness[15] or reduced neutrophil

deformability[14] (both would slow neutrophil passage through the pulmonary circulation) or emigration of cells into the interstitium.

The flushing response was consistently associated with neutropenia, although neutropenia did occur without flushing in one subject. When incremental doses of FMLP are administered by inhalation, flushing is seen after the first dose or not at all.[6 12] Bronchoconstriction may occur with this dose, a higher dose or not at all, implying that the mechanisms producing neutropenia and flushing may be unrelated to those which produce decrements in airway function.

The effects of inhaled FMLP and inhaled platelet activating factor show some similarities and some differences.[27] Both produce flushing, activate neutrophils and produce transient neutropenia followed by rebound neutrophilia. Platelet activating factor inhalation had no effect on circulating eosinophil count whereas FMLP produced significant eosinopenia, an interesting finding since, of the two agents, platelet activating factor is generally regarded as more eosinophil specific. Bronchoalveolar lavage neutrophils are elevated whereas four hours after inhalation of platelet activating factor neutrophils are retained in the lung after FMLP over a shorter period.

Thus FMLP inhalation activates circulating neutrophils and produces transient neutropenia, monocytopenia and more prolonged eosinopenia. Over a longer period neutrophil retention is seen in the lung. These findings may be relevant to the accumulation of leucocytes within the lung during bacterial bronchial infection.

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