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STIMULATING EFFECTS OF MERCURIC- AND SILVER IONS ON THE SUPEROXIDE ANION PRODUCTION IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

GUNNAR JANSSON* and MATS HARMS-RINGDAHL

Department of Radiobiology, Stockholm University, S-106 91 Stockholm, Sweden

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In a survey of a number of heavy metal ions for effects on the oxidative metabolism (respiratory burst) of human polymorphonuclear leukocytes (neutrophils) we have found that mercury(II) and silver ions in micromolar concentration significantly increase the production of superoxide anions in cells, initiated by formyl-methionyl-leucylphenylalanine (fMLP). The stimulation of radical formation induced by a certain ion concentration varied considerably in cells isolated from different blood donors, from a moderate increase to a very large (up to 400% of control values). When the soluble stimulator phorbol myristate acetate (PMA) or the particulate stimulator Zymosan were used to initiate the cell respiratory burst, no additional stimulating effects by the metal ions on superoxide anion formation were observed. This fact might indicate that the effect of the metal ions on the fMLP-dependent initiation of cell activity is a mechanism coupled to the interaction between the chemotactic peptide and its corresponding receptor molecules on the cell surface.

By increasing the concentration of silver ions during pre-incubation of resting neutrophils, a spontaneous activation of the cells could be recorded at a concentration exceeding 5 μM. However, the silver ion concentration at which such spontaneous initiation of the respiratory burst occurred varied significantly between blood samples from different donors with a concentration range of 5 to 15 μM. This effect could not be shown for mercuric ions due to the toxicity of the metal above 5 μM. Blood samples from some donors contained neutrophils that could be activated by either mercuric- or silver ions at concentration as low at 1 μM.

The spontaneous activation of neutrophils with elevated concentrations of silver ions is kinetically similar to the PMA-induced. The onset of superoxide anion formation is preceeded by a lag period whose length varies in time with the concentration of agent applied to the cells. It is a known fact that once the neutrophils have been activated with fMLP it is not possible to reactivate the cells by a second supplementation of fMLP. However, after cessation of the fMLP-induced activation, addition of PMA or silver ions gives rise to renewed production of superoxide anions.

We propose two different mechanisms of action of silver ions on oxidative metabolism of neutrophils. At a low concentration the metal ions are thought to interact with an activating agent and a corresponding cell surface receptor molecule, while at elevated ion concentrations, we postulate an action like that of phorbol-esters on neutrophils, (i.e., an interaction between activating agent and the enzyme protein kinase C of the cells).

ABBREVIATIONS

PMNL's polymorphonuclear leukocytes
fMLPformyl methionyl-lencyl-phenylalanine
CLchemiluminescence
LUClucigenine
PBSphosphate buffered saline
HBSSHank's Balanced Salt solution
SODSuperoxide anion dismutase
NADPHReduced nicotineamidadeninedinucleotide phosphate

KEY WORDS: Polymorphonuclear leukocytes, respiraatory burst, mercuric ions, silver ions, lucigenine, chemiluminescence.

* Correspondence: Dr. G. Jansson.

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INTRODUCTION

Polymorphonuclear leukocytes (PMNL) or neutrophils are present in large numbers in circulating blood. Their task is to combat microorganisms invading the human body. This is accomplished by encompassing the immunologically "pretreated" organism in a vacuole formed by the cell membrane.[1] At the same time the neutrophil is activated by various immunological factors to initiate the production of free oxygen radicals and subsequently other oxidants. These oxygen-centered species are formed together with various hydrolytic enzymes released into the vacuole.[2] In a concerted action these agents kill and disintegrate the encompassed infectious organism. The formation of toxic oxygen-containing molecules by the immunologically activated neutrophils is not confined to the formed vacuoles but is also taking place on the entire cell membrane. These oxidants can diffuse into the extracellular environment and under adverse conditions cause damage to surrounding tissues, cells and essential macromolecules.[3] This situation is thought to exist in certain rheumatic diseases, in sepsis and in the respiratory distress syndrome.[4]

The above considerations were the rationale for the present investigation of effects of various metal ions on the oxidative metabolism of neutrophils. Information available on this subject is rather rare in current scientific documentation. Aluminium salts have been claimed to stimulate luminol-dependent chemiluminescence in activated neutrophils.[5] High concentrations of lead, zinc and cadium are reported to enhance oxidant formation in neutrophils activated with heat aggregated IgG.[6] In our study, however, these metals did not stimulate the radical formation in neutrophils activated with fMLP. In contrast, we have observed remarkable stimulating effects in neutrophils, activated with fMLP, by both silver- and mercuric ions.

MATERIALS AND METHODS

Formyl methionyl-leucyl-phenylalanine, superoxide dismutase, nicotin-amide adenine dinucleotide phosphate, lucigenin, luminol and ferricytochrome c were purchased from Sigma Chemical Comp., St. Louis, Mi, USA. Lymphoprep was purchased from Nycomed AB, Stockholm, Sweden. Mercuric chloride and silver nitrate were analytical grade reagents from Merck, Germany. The salts were first dissolved in distilled water and final dilutions were made in phosphate buffered saline.

Isolation of neutrophils from buffy coats was carried out as described elsewhere.[7]

Superoxide anion production of activated neutrophils was determined by measuring lucigenin-dependent chemiluminescence in a 1 ml of reaction mixture containing Hank's buffer, 3×10^6 cells, 50 μM Lucigenin and 5×10^{-7} M fMLP, at 37°C.[7] This probe is claimed to be specific for superoxide anion-dependent chemiluminescence.[8] Superoxide anions were assayed at 37°C by following ferricytochrome c reduction at 550 nm, in a reaction mixture containing HBSS, 3×10^6 cells, 100 μM ferricytochrome c with or without 100 μg SOD in a volume of 3 ml. Oxygen concentrations in cell suspensions were studied using a Yellow Spring Oxygraph. Fractionation of subcellular components was performed according to.[9]

The NADPH-oxidase activity in the $27000 \times g$ fractions of fMLP-activated neutrophils was determined according to Bromberg and Pic.[8] By adding ferricytochrome c to the reaction mixture, the electron transport to molecular oxygen could be assayed.[8] Binding of the chemotactic peptide to its receptor molecule was carried out according to Williams *et al.*[9]

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RESULTS AND DISCUSSION

Effect of Metal Ions on Superoxide Anion Production in fMLP Activated Neutrophils

When the metal ions (mercury(II), silver, lead, zinc, nickel, cadmium, copper, chromium(II)) were studied for their effect on neutrophil respiratory burst, it was found that mercuric and silver ions at a concentration of 1 μM enhanced the superoxide anion formation of cells activated with fMLP. Cupric ions showed a small enhancing effect whilst the other metal ions were either ineffective or inhibitory at the concentrations used (10^{-5} M to 10^{-8} M). Figure 1 shows a typical time dependent chemiluminescence formation in activated cells with or without pre-incubation of the cells with 1 μM silver ions, and a similar response was obtained for mercuric ions. There were however, great variations in respiratory burst activity between cell preparations from different donors in response to the two metal ions. A 50% stimulation of radical formation was considered to be a significant inter individual variation.

Since lucigenin was used, which is claimed to specifically detect superoxide anions,[10] it is clear that the metal ions stimulated superoxide anion formation. This point of view was also supported by the results obtained from reduction of ferricytochrome c and decrease in oxygen concentration upon activation of cells, showing that the

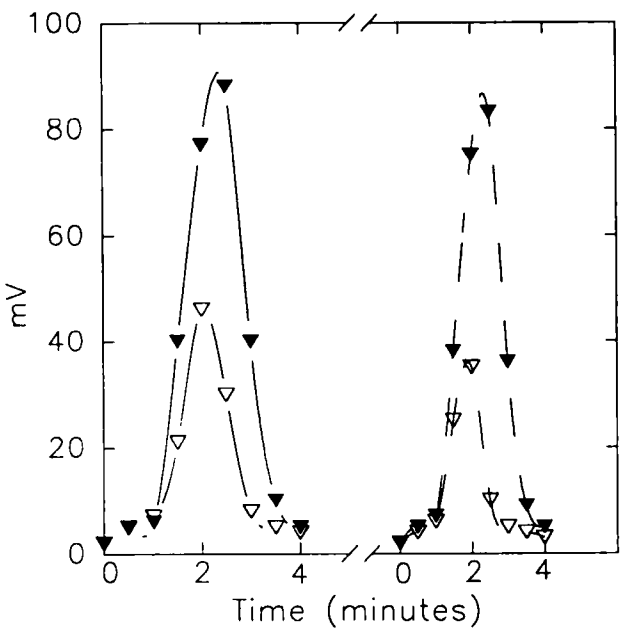

Figure 1 Stimulatory effect of Ag^{+} (1 μM, left graph filled symbols) and of Hg^{++} (1 μM, right graph filled symbols) on oxygen burst in fMLP activated human neutrophils. Graphs with open symbols represent controls without addition of metal ion. Each point in the graphs denotes the mean value of three assays. Oxygen burst was analyzed using the lucigenin assay. For details of reaction conditions see Materials and Methods.

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metal ions affect primary reactions of the respiratory burst leading to enhanced formation of superoxide anion radicals (Figures 2 and 3).

In most experiments using the chemiluminescent assay to monitor the formation of superoxide anion radicals the cells were pre-incubated with metal ions for about two minutes prior to the addition of the activating agent (fMLP). Under these experimental conditions the optimal mercuric ion concentration for enhancement of cell activity was found to be around 2 μM as is shown in Figure 4A. Similar results were obtained when superoxide anion production was monitored by ferricytochrome c reduction (Figure 4B). In contrast, the effect of silver ions on the fMLP-induced respiratory burst of neutrophils has very different kinetics. As seen in Figure 5 chemiluminescence increased steadily and was positively correlated with silver ion concentration until a plateau was reached, when millimolar concentrations were used. In contrast mercuric ions inhibited radical formation above a concentration of 5 μM. Prolonging the incubation time with silver ions (2 μM) the increase in radical formation steadily rose up to about 8 minutes (Figure 6) whereas mercury (2 μM) inhibited cell activity after about 2 minutes.

Direct Activation of Neutrophils by Metal Ions

Neutrophils from a few per cent of tested blood samples responded to silver and mercury ions at concentration of 1 to 2 μM in the absence of fMLP. The onset of

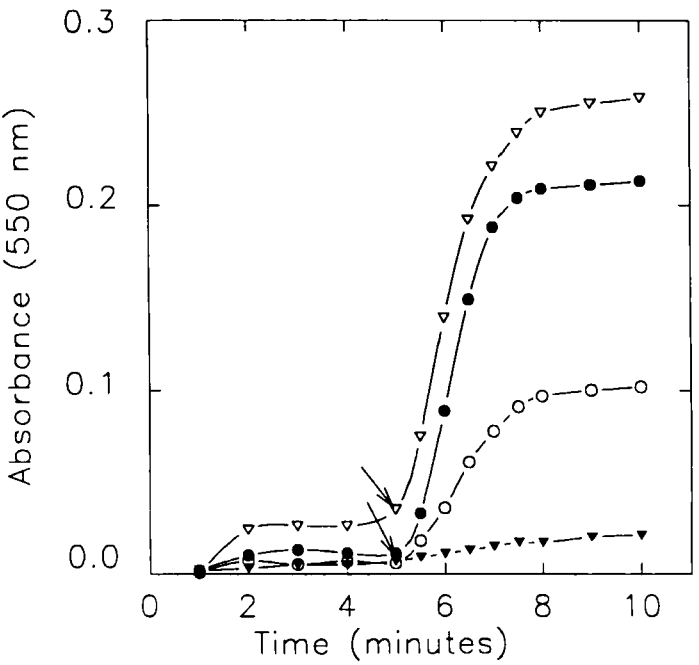

Figure 2 Determination of oxygen burst of fMLP-activated human neutrophils by reduction of ferricytochrome c. Each point in the graph denotes the mean values of three assays. ○——○ control; ●——● 2 μM Ag^+; ▽——▽ 2 μM Hg^{++}; ▼——▼ 100 μg SOD. Arrow indicates the addition of fMLP. For details of reaction conditions see Materials and Methods.

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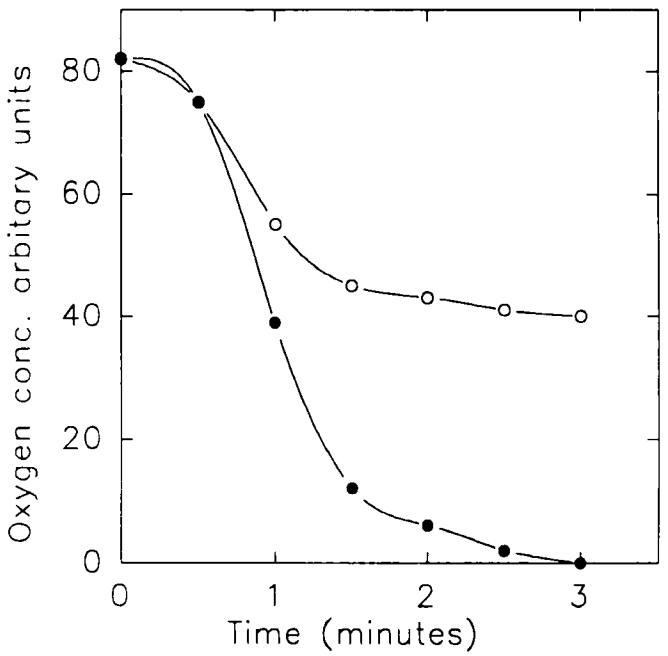

Figure 3 Determination of oxygen burst of fMLP activated human neutrophils by measuring the oxygen concentration. ○——○ control; ●——● 2 μM Ag^+. fMLP added at zero time. Each point denotes the mean value of two assays. For details of reaction conditions see Materials and Methods.

superoxide anion radical formation was immediate with kinetics resembling those found when neutrophils are activated with fMLP and with a rate of formation of radicals comparable to that found with fMLP. This observation might imply that low concentrations of these metals *in vivo* can give rise to a "forbidden" activation of neutrophils with adverse consequences to humans.

With a moderate increase in silver ion concentration (5 to 10 μM) all neutrophil preparations tested responded by production of superoxide anions in the absence of fMLP. The time response of the chemiluminescence curve so obtained, differed from those obtained using fMLP (Figure 7). The neutrophil respiratory burst was preceeded by a lag phase of different length depending both on the concentration of ion and between different donors of blood samples (Figure 8). The same effect was not observed with elevated mercuric ion concentrations because of toxic properties. Likewise with fMLP initiated superoxide anion production, there was a significant variability between blood samples with respect to silver ion concentration required to directly initiate the respiratory burst (5 μM up to 15 μM).

Mechanism(s) of Metal Ion-dependent Stimulation of the Respiratory Burst

A suspension of neutrophils was centrifuged and the supernatant medium replaced by the same amount of fresh medium. The cell suspension thus obtained showed a considerable increase in formation of superoxide anions after activation with fMLP in the chemiluminescent assay (Table 1). When centrifuged cells were resuspended in

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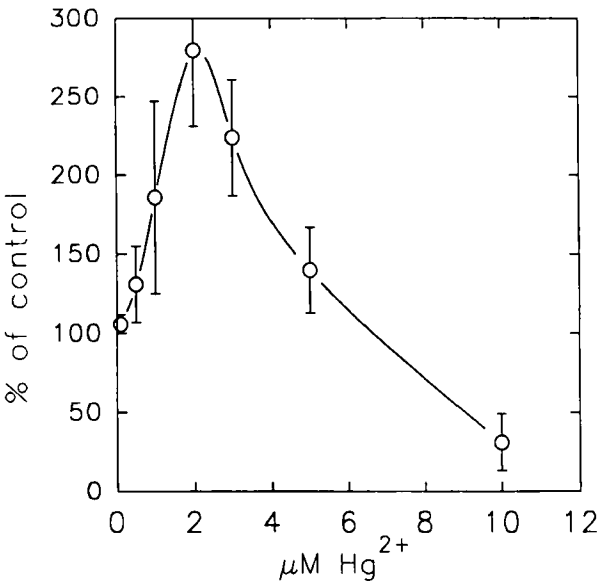

Figure 4A Dose-response curve for the stimulating effect of mercuric ions on, lucigenine assayed, oxygen burst in fMLP-activated human neutrophils. Bars represent standard error of 5 different blood samples. For details of reaction conditions see Materials and Methods.

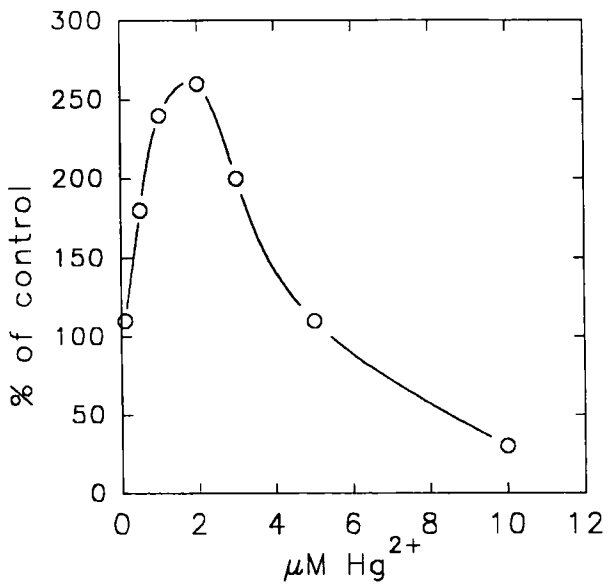

Figure 4B Dose-response curve for the stimulating effect of mercuric ions on the oxygen burst in fMLP-activated human neutrophils assayed as the SOD-inhibitible Cytochrome c reduction. Each point denotes the mean value of three determinations using the same neutrophil preparation. For details of reaction conditions see Materials and Methods.

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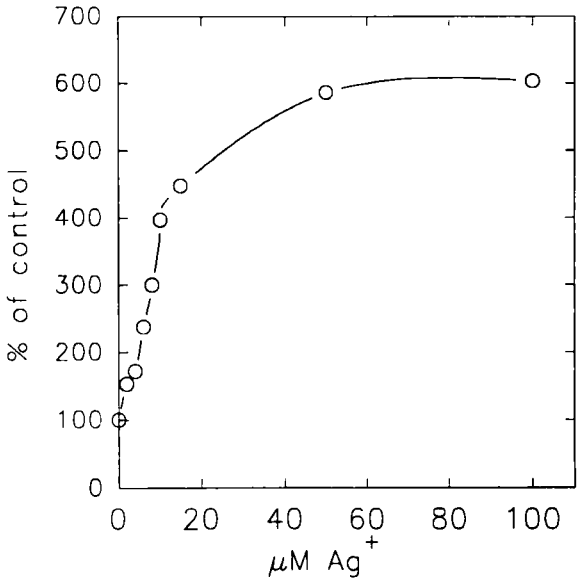

Figure 5 Dose-response curve for the stimulating effect of silver ions on oxygen burst of fMLP-activated human neutrophils. Each point in the graph denotes the mean value of three assays. Oxygen burst was analyzed as in Figure 1. For details of reaction conditions see Materials and Methods.

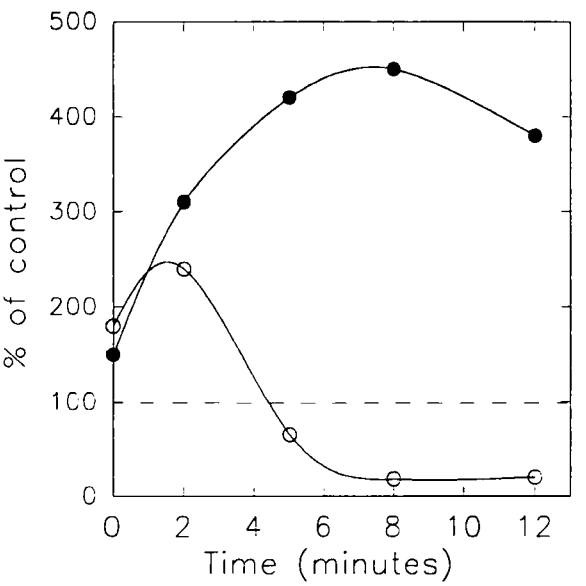

Figure 6 Influence of pre-incubation time between the addition of fMLP and metal ions (●——●——● 2 μM Ag^+; ○——○——○ 2 μM Hg^{++}), on the stimulating effect on oxygen burst in neutrophils. Each point in the graph denotes the mean value of three assays. Oxygen burst was analyzed as in Figure 1. For details of reaction conditions see Materials and Methods.

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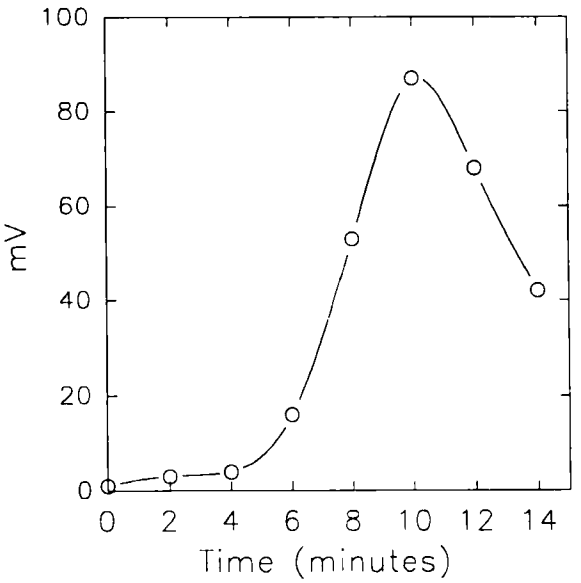

Figure 7 Time course of the respiratory burst of neutrophils induced by 8 μM Ag^+. Each point in the graph denotes the mean value of three assays. Oxygen burst was analyzed as in Figure 1. For details of reaction conditions see Materials and Methods.

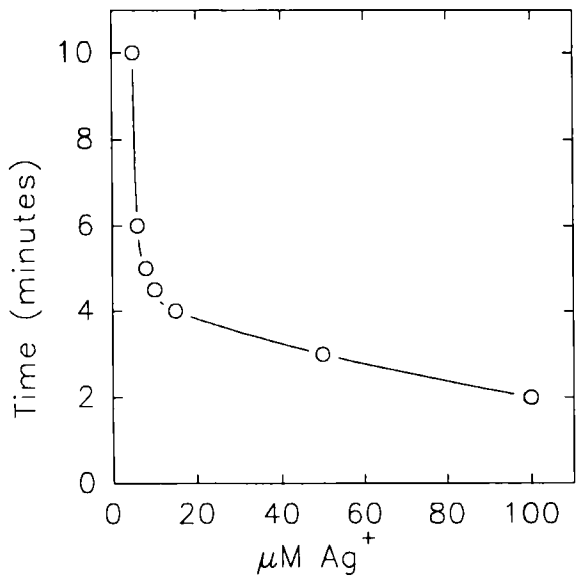

Figure 8 Effect of increasing concentrations of silver ions on the length of the lag period before onset of superoxide anion formation in neutrophils. Each point in the graph denotes the mean value of three assays. Superoxide anions were analyzed as in Figure 1. For details of reaction conditions see Materials and Methods.

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Table 1 Effects of extracellular factors on the oxygen burst in neutrophils

Treatment of cells	Peak value of chemiluminescence in mV ± S.D. n = 6
Uncentrifuged cells	24.8 ± 2.2
Uncentrifuged cells + 2 μM Ag^+	61.6 ± 3.4
Centrifuged cells suspended in the same medium	33.0 ± 1.5
Centrifuged cells suspended in the same medium + 2 μM Ag^+	65.3 ± 4.0
Centrifuged cells suspended in fresh medium	57.6 ± 4.3
Centrifuged cells suspended in fresh medium + 2 μM Ag^+	50.6 ± 8.6
Centrifuged cells suspended in a mixture of old and fresh medium	45.5 ± 3.3
Centrifuged cells suspended in a mixture of old and fresh medium + 2 μM Ag^+	78.2 ± 7.1

Oxygen burst was determined using the lucigenin assay. For details of reaction conditions see Materials and Methods.

the same supernatant, no change in radical formation as compared to the uncentrifuged control cells could be observed. After suspending the centrifuged cells in a mixture of old and fresh medium an intermediate value of superoxide anion formation was recorded. The decrease in chemiluminescence response in the latter case could be counteracted by the addition of silver or mercuric ions. Further, if centrifuged cells were resuspended in fresh medium and also supplied with metal ions, only a small increase in radical formation was shown. The data from these experiments (Table 1) seem to indicate the presence of a extracellular substance regulating the magnitude of the respiratory burst of neutrophils activated with fMLP. The effects of the metal ions on the cell activity may be explained by the ability of the ions to impede the action of this substance. The substance is removed by centrifugation of the cell suspension but released again by the cells during further incubation (Figure 9). If the substance is physiologically real or an artefact released from damaged cells is at the moment not known. Further work is needed to clarify this issue.

NADPH-oxidase is the primary enzyme system in the chain of events leading to the formation of superoxide anions and subsequently to other oxygen-centered molecules after initiation of the respiratory burst in neutrophils. An explanation of the metal ion effect could be that the ions influence this enzyme system. The enzyme oxidizes it substrate, NADPH, and the released electrons are transported via flavins and cytochromes to the ultimate electron acceptor, molecular oxygen, to form superoxide anions. There was however no stimulating effect of any of the metal ions on the enzyme activity in the 27000 × g fraction from sonicated cells (data not shown). Nor could any inhibitory action of supernatants from neutrophils be seen. The NADPH-oxidase activity was assayed by spectrophotometric determination of the NADPH concentration. By including ferricytochrome c in the reaction mixture it was also possible to study effects of the metal ions on the transport of released electrons to molecular oxygen. The formed superoxide anions will reduce the ferricytochrome c which may be assayed by a increase in absorbancy at 550 nm. No stimulating effects of silver- or mercuric ions on the ferricytochrome c reduction could be found (data not shown).

As the initiation of the respiratory burst in neutrophils by fMLP is mediated by a receptor (F_c) on the cell membrane, it seemed plausible to suppose that the assumed respiratory burst regulating factor in some way interacted with the F_c receptor thereby impeding the effect of the chemotactic peptide. The effect of mercuric- or silver ions

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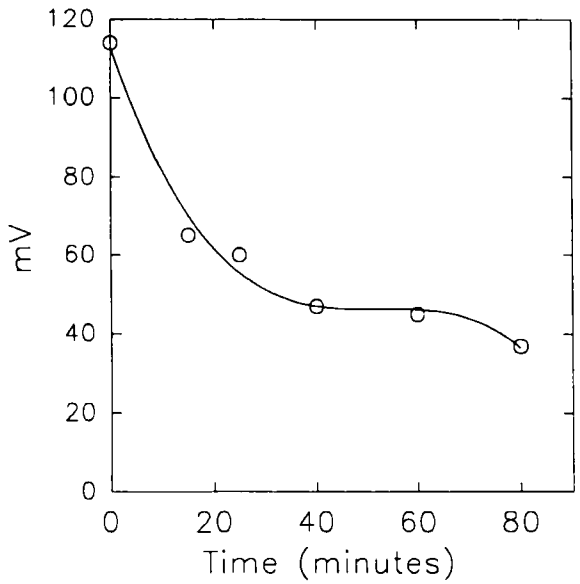

Figure 9 Oxygen burst in fMLP-activated human neutrophils as a function of time between addition of new cell medium, to the time for the assay of oxygen burst activity. Each point in the graph denotes the mean value of three assays. Oxygen burst analyzed as in Figure 1. For details of reaction conditions see Materials and Methods.

on the respiratory burst may depend on the ability of these ions to bind to the regulatory factor and so enhance the binding of the chemotactic peptide to the receptor, with subsequent stimulation of cell activity. In order to study such a mechanism for the metal on effect, receptor binding experiments with tritiated fMLP were performed. No difference in fMLP-binding capacity in control cells compared to cells treated with metal ions, at concentration stimulating the respiratory burst, could be observed (data not shown).

The results presented above seem to indicate that silver ions act on the respiratory burst of neutrophils by two different mechanisms. In the first, which also applies to mercuric ions, the metal ions at a concentration about 1 μM augment the respiratory burst initiated by the chemotactic peptide fMLP. At somewhat higher concentration of silver ions (around 5 μM) the cells are activated without participation of chemotactic peptide. Due to toxicity this second mechanism of cell activation is not shown by mercuric ions at elevated concentrations. In neutrophils activated with fMLP there is a rapidly induced respiratory burst as shown in Figure 1. A maximum in chemiluminescent yield is reached within 2 minutes and after 3 to 4 minutes cell activity has ceased. A second addition of fMLP does not induce a new response. If however, silver ions in a concentration of 5 to 10 μM are added after cessation of fMLP-induced activity, the cells again start to produce radicals after a short lag period (Figure 10). This second initiation of superoxide anions is evidently not mediated by the F_c receptor. A similar effect by phorbol myristate acetate on radical formation in fMLP activated cells has been observed (data not shown).

The experimental data presented in this report show that mercuric- and silver ions

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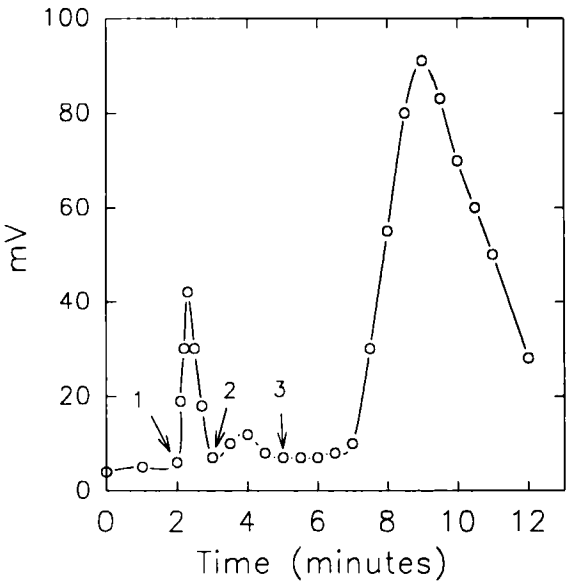

Figure 10 Oxygen burst in fMLP-activated human neutrophils, re-activated with 5.10^{-7} M fMLP (arrow 2) and with 5 μM Ag^+ (arrow 3). First addition of fMLP at arrow 1. Each point in the graph denotes the mean value of two assays. Oxygen burst analyzed as in Figure 1. For details of reaction conditions see Materials and Methods.

in low concentrations significantly enhance the formation of free oxygen radicals in human neutrophils activated with the chemotactic peptide fMLP. In addition silver ions alone, in somewhat higher concentraton, induce a respirtory burst with kinetics very similar to those induced by phorbol myristate acetate. This fact may indicate that silver ions in this case initiate respiratory burst via an effect on protein kinase C, the postulated mechanism by which phorbolymyristate acetate acts on neutrophils.[11] Due to the toxic properties of mercuric ions, this kind of neutrophil activation was not observed.

It has been suggested that the enhancing effect of aluminium ions on the luminescence of luminal caused by activated neutrophils may be due to increased oxidative capacity of O_2^- caused by a complex formation between Al (III) and superoxide anions.[13] However, from the experiments suggesting the presence of an extracellular factor with which the metal ions interact (Table 1), we have drawn the conclusion that the mechanism of action of mercuric- or silver ions on fMLP-dependent initiation of the respiratory burst of neutrophils is due to the ability of the ions to impede the effect of a extracellular factor involved in the regulation of the respiratory burst. Binding experiments with tritiated fMLP do not indicate effects of the ions on the interaction between the chemotactic peptide and the corresponding receptor on the cell membrane. Nor do the ions seem to have any influence on the capacity of partially purified NADPH-oxidase to oxidize its substrate, NADPH. This enzyme is the first to be actuated during the respiratory burst producing a flow of electrons to molecular oxygen via flavins and cytochromes. Neither is the transport of the released electrons affected by mercuric- or silver ions. It might be speculated

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that the ions facilitate the assembly of the membrane-bound NADPH-oxidase with vital co-factors known to be present in the cytoplasm of the cells.[12] Such assembly of enzyme and co-factors has been shown necessary for the respirtory burst upon activation with chemotactic peptide.

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Accepted by Professor B. Halliwell

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