MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

IMMUNOPHARMACOLOGY AND IMMUNOTOXICOLOGY
Vol. 24, No. 4, pp. 627–638, 2002

IMMUNOMODULATORY EFFECT OF GLUTOXIM ON SOME ACTIVITIES OF ISOLATED HUMAN NEUTROPHILS AND IN WHOLE BLOOD

Vincenzo Fimiani,[1,*] Antonia Cavallaro,[1] Tommaso Ainis,[1] Galina Baranovskaia,[2] Olga Ketlinskaya,[3] and Leonid Kozhemyakin[3]

[1]Department of Experimental Pathology and Microbiology, and
[2]Department of Social and Territorial Medicine, Medical School, University of Messina, 98125 Messina, Italy
[3]BAM-R&D Laboratories Ltd, 193015 Saint-Petersbourg, Russia

ABSTRACT

Activity of Glutoxim, a sulfur-containing hexapeptide with immunomodulating effect on lymphocytes, was studied on human neutrophils. Commercial available agent containing the substance, Glutoxim, was used. At the doses of 1, 3, 6 or 100 μg/mL the drug stimulated the superoxide anion and hypochlorous acid generation in resting neutrophils but inhibited them in cells stimulated with zymosan or PMA. The only inhibiting effect was observed on nitric oxide production in LPS-stimulated RAW 264.7 cells. The drug also influenced neutrophil chemotaxis showing chemoattractant activity on cells and inhibiting fMLP-induced chemotaxis. These effects on neutrophils confirm and extend range of Glutoxim immunomodulating abilities.

*Corresponding author. E-mail: vincenzo.fimiani@unime.it

DOI: 10.1081/IPH-120016039
0892-3973 (Print); 1532-2513 (Online)

INTRODUCTION

Glutoxim is an immunomodulating agent commonly used in Russia and patented in USA, Japan, Canada, China and Sweden; it is a chemically synthesized biologically active substance, a hexapeptide with stabilized disulfide bond, bis-(γ-L-glutamyl)-L-cysteinyl-bis-glycine disodium salt with a sum formula $C_{20}H_{32}O_{16}N_6S_2$ that affects immune system stimulating erythro-granulocyto- monocyto- and lymphocyto-poiesis[1] even in immunodepressive diseases,[4,5] and activating cytokine system.[1,3] Glutoxim effects on lymphocytes are well-known but barely studied on neutrophils in spite of their very important role of defense as first line cells against infectious and xenobiotic agents.

The aim of this article is to evaluate the Glutoxim effects on superoxide anion (O_2^-), hypochlorous acid (HOCl), nitric oxide (NO) generation and chemotaxis in neutrophils, inasmuch as neutrophils are involved in inflammation and defense against bacteria and tumors development.

MATERIALS AND METHODS

Reagents

Hank's balanced salt solution (HBSS), superoxide dismutase (SOD), cytochrome c, phorbol-12-myristate-13-acetate (PMA), zymosan, dextran, glutamine, Histopaque-1077, RPMI 1640, glucose, HEPES, L-arginine, fetal calf serum (FCS), bovine serum albumine, streptomycin, penicillin, ethylenediaminetetraacetic acid (EDTA), trypsin, 5,5′-dithio-bis(2-nitrobenzoic acid) (TNB), sodium borohydride, taurine, formyl-Met-Leu-Phe (fMLP), lipopolysaccharide (LPS), sulfanilamide, N-(1-naphthyl)-ethylenediaminedihydrochloride, phosphoric acid were obtained from Sigma-Aldrich srl (Milan, Italy). Glutoxim was purchased from BAM (Saint-Petersbourg, Russia), Other chemicals were of HPLC grade.

Blood

Human venous blood from health adult volunteers was collected with disposable plastic syringes and anticoagulated with 10 units of heparin per mL.

Isolation of Neutrophils

Peripheral blood was diluted 1:2 in Hank's solution, layered on Hystopaque-1077 and centrifuged at 400× g for 45 min at room temperature;

the bottom, washed twice in PBS, was resuspended in 0.6% dextran then left for 45 min to allow erythrocytes to sediment, the clear erythrocyte shore was recovered, centrifuged at 1.800 rpm for 15 min and residual red cells were removed by hypotonic lysis as described by Haslett et al.[6] Cells were found to be >98% pure and >95% viable as measured by trypan blue exclusion.

RAW 264.7 Cells Culture

Cells were maintained as an adherent culture in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 U/mL penicillin G and 50 μg/mL streptomycin. After detachment with trypsin-EDTA and two washing, cells were suspended in culture medium at the concentration of 10^6/mL estimated by trypan blue exclusion, and plated into flat-bottomed 24-well plates at 1 mL/well.

Superoxide Anion Release Assay

O_2^- release was estimated using the superoxide dismutase-inhibitable reduction of cytochrome c assay described by Bellavite et al.[7] Briefly in plastic test-tubes were added 0.4 mL of Krebs-Ringer-Phosphate buffer (KRP) and 1.5 mg of cytochrome c, in the half of the tubes 50 μg of SOD and in one third 0.5 mg of zymosan or 0.5 μg of PMA; then 0.1 mL of blood or 10^6 neutrophils treated and untreated with Glutoxim. The final volume of the reaction mixture was 0.5 mL and reaction was allowed to proceed for 30 min at 37°C under continuous shaking. The incubation was stopped by the addition of 2 mL of ice-cold KRP and the tubes were centrifuged at 1.800 rpm for 5 min, the absorbance of the cell-free supernatants measured at 550 nm was determined by dividing the average difference in absorbance in samples tested with and without SOD by extinction coefficient for the reduced cytochrome c (0.0245) and multiplied for 2.5 (dilution factor). The assays were carried out in duplicate. The results were expressed as nmoles of $O_2^-/10^6$ granulocytes, on the basis of the total and differential counts of white blood cells.

Hypochlorous Acid Generation Assay

Generation of HOCl was determined by assay for taurine-chloramine.[8] TNB used as oxidant substrate was generated by treating 1.0 mM 5,5′-dithio-bis(2-nitrobenzoic acid) with 2.0 mM sodium borohydride for 6 h at 37°C. TNB (100 μL) was added to supernatant (900 μL) derived from treated and untreated neutrophils (2×10^6/mL HBSS) incubated in the presence of 15 mM

taurine and stimulated with 0.5 mg/mL opsonized zymosan for 1 h at 37°C under continuous shaking. The ΔOD at 412 nm was determined and amount of hypochlorous acid generated was calculated from a standard curve using aliquots of HBSS-15 mM taurine buffer to which known amounts of sodium hypochlorite had been added. The assays were carried out in duplicate. The results were expressed as nmoles of taurine-chloramine/10^6 cells.

Nitrite Production Assay

Nitrites were measured by the Griess reaction as described by Miles et al.[9] Briefly culture media of RAW 264.7 cells were removed and centrifuges at 1.800 rpm for 10 min, 500 μL of supernatants were mixed with 500 μL of Griess reagent (1% sulfanilamide; 0.1% N-1-napthyl-ethylenediamine dihydrochloride; 2.5% H_3PO_4) and incubated at room temperature for 10 min.

Chemotaxis Assay

Neutrophils migration was measured with the Boyden chamber technique, as described by Boyden[10] and modified by Zigmond and Hirsh[11] using plastic disposable chambers (FAR Division Diagnostic, Verona, Italy) in which two compartments were separated by a cellulose nitrate filter with a pore size of 3 μm. Treated and untreated neutrophils were suspended in HBSS supplemented with 20 mM Hepes and 1% bovine serum albumin at concentration of 2×10^6 cells/mL, aliquots (200 μL) of this suspensions were added to the upper chamber compartment and 10 nM of fMLP in the lower compartment was used as chemoattractant. After 60 min incubation at 37°C the filters were removed, fixed, rinsed, stained with Harris's hematoxylin and mounted on slides. Neutrophil migration within the filter was determined under light microscopy by the leading front method, in which the distance is measured from the top of the filter to the farthest plane still containing two cells with at ×40 objective.[11] The assays were carried out in duplicate and the migration extent was determined at ten different randomly chosen filter sites. In some chambers the chemoattractant was replaced by drug to observe influence on cell migration. Results were expressed as distance in μm traveled by the cells into the filter.

Experimental Protocols

Whole blood was subdivided in aliquot parts of 2 mL and preincubated with 1, 3, 6 of 100 μg/mL of Glutoxim for 1 h at 37°C under continuous shaking. Isolated neutrophils were suspended in opportunely supplemented HBSS Ca^{2+} and Mg^{2+} free without phenol red at concentration of

10^6 cells/mL for superoxide assay, 2×10^6 cells/mL for hypochlorous and chemotaxis assays, then incubated as whole blood. RAW 264.7 cells were allowed to adhere a night at 37°C in a humidified 95% air -5% CO_2 atmosphere, then treated with different concentrations (1, 3, 6 or 100 μg/mL) of the drug for 1, 6 or 24 h before stimulation with LPS (1 μg/mL); at the experiment end, cell viability was estimated by trypan blue exclusion assay and found >90% both in control and in treated cells. Controls were incubated without drug in the same conditions.

Statistical Analysis

The data were analyzed by the unpaired Student's test, significance was considered attained when $p \geq 0.05$.

RESULTS

Production of O_2^-

Figure 1 shows the effect of different concentrations (1, 3, 6 or 100 μg/mL) of Glutoxim on unstimulated neutrophils in whole blood; we

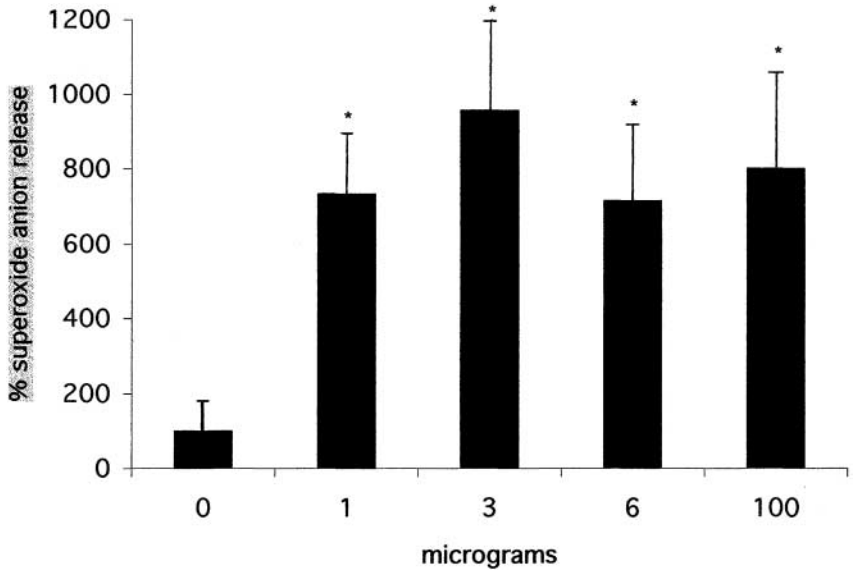

Figure 1. Effect of Glutoxim on O_2^- production by human neutrophils in whole blood. The blood samples were incubated with 1. 3, 6 or 100 μg/mL of the drug for 1 h. Values are the means ± standard deviation for three or more separate experiments. $*P \leq 0.001$ significantly different from control.

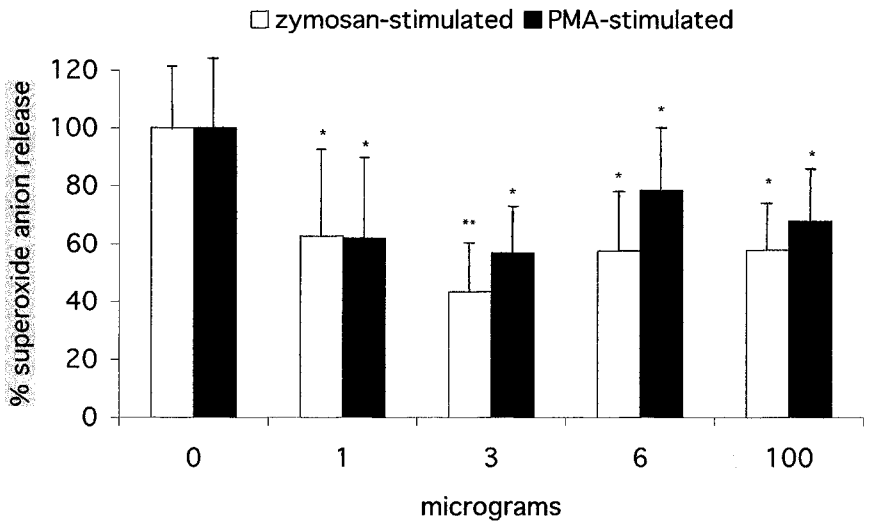

Figure 2. Effect of Glutoxim on O_2^- production by stimulated human neutrophils in whole blood. The blood samples were preincubated with 1, 3, 6 or 100 μg/mL of the drug for 1 h followed with zymosan or PMA stimulation for 30 min. Values are the means ± standard deviation for three or more separate experiments. *$P \leq 0.05$; **$P \leq 0.01$ significantly different from control.

observed an highly significant stimulating effect that was not dose-dependent and 5–9 times greater than control. Figure 2 shows the drug effect on neutrophils in whole blood after stimulation with zymosan or PMA; we observed an inhibiting effect comprised between 37.25% and 56.6% after zymosan stimulation, and between 21.6% and 43.1% after PMA stimulation that was not dose-dependent in both cases. The more efficacious dose was always 3 μg/mL.

Production of HOCl

Figure 3 shows the effect of different Glutoxim concentrations (1, 3, 6 or 100 μg/mL) on isolated unstimulated human neutrophils; we observed a significant dose-dependent stimulating effect, excepted for 1 μg/mL. Figure 4 shows the drug effect on isolated human neutrophils stimulated with zymosan or PMA; we observed an inhibiting dose-dependent effect of the drug comprised between 26.3% and 67.3% after zymosan stimulation, and between 19.4% and 50% after PMA stimulation.

Production of NO

We could not study the drug effect on isolated neutrophils, since their viability is limited to 8 h including the time for isolation, and as observed in

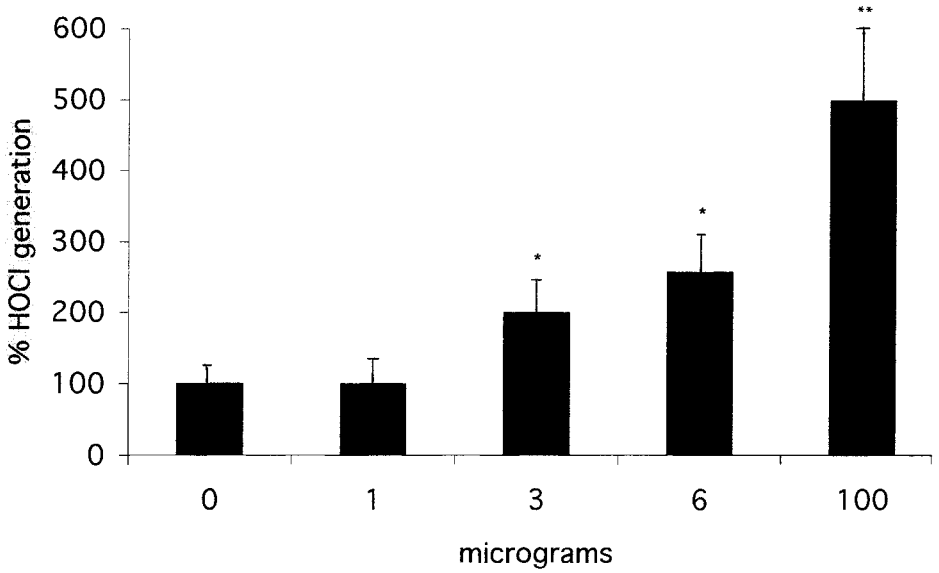

Figure 3. Effect of Glutoxim on HOCl production by isolated human neutrophils. The cells were incubated with 1, 3, 6 or 100 μg/mL of the drug for 1 h. Values are the means ± standard deviation for three or more separate experiments. *$P \leq 0.01$; **$P \leq 0.001$ significantly different from control.

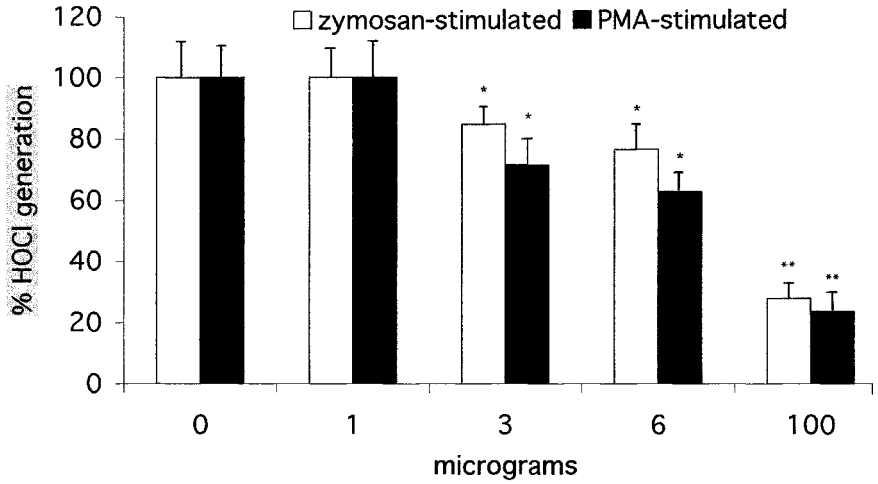

Figure 4. Effect of Glutoxim on HOCl production by stimulated human neutrophils. The cells were preincubated with 1, 3, 6 or 100 μg/mL of the drug for 1 h followed with zymosan or PMA stimulation for 30 min. Values are the means ± standard deviation for three or more separate experiments. *$P \leq 0.05$; **$P \leq 0.001$ significantly different from control.

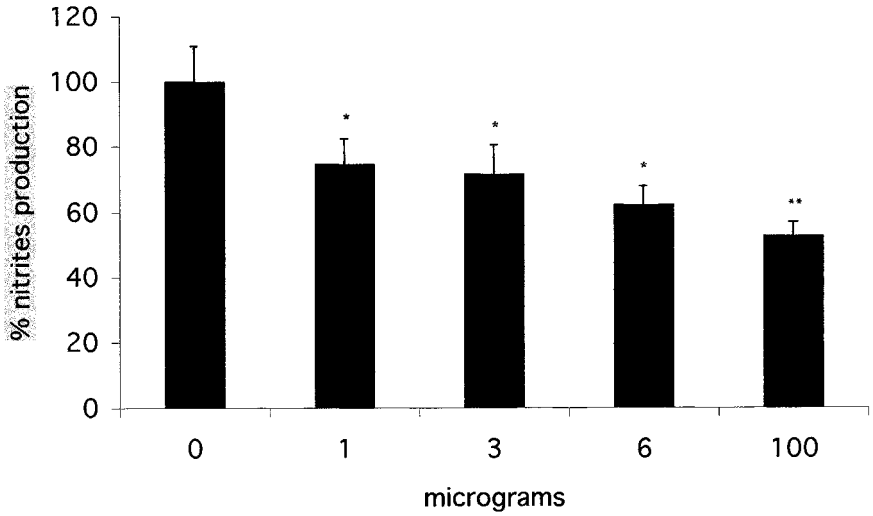

Figure 5. Effect of Glutoxim on nitrites production by RAW 264.7 cells. The cells were preincubated with 1, 3, 6 or 100 μg/mL of the drug for 24 h followed with LPS stimulation for 24 h. Values are the means ± standard deviation for three or more separate experiments. $*P \leq 0.05$; $**P \leq 0.01$ significantly different from control.

RAW 264.7 cells cultivated in vitro, it was necessary to treat them for 24 h with the studied agent to influence the NO production (data not shown). Figure 5 shows the effect of different Glutoxim concentrations (1, 3, 6, or 100 μg/mL) on in vitro cultivated RAW 264.7 cells treated for 24 h with the drug following with stimulation with 1 μg/mL of LPS; we observed an inhibiting dose-dependent effect from 25% to 47.2%. No activity of the drug was found in unstimulated cells. The cell viability, at the study end was found to be >80% both in controls and in tested cells. Chemotaxis: Fig. 6A shows the effect of different Glutoxim concentrations (1 or 6 μg/mL) on chemotaxis of isolated human neutrophils treated for 1 h with the drug and in absence of chemoattractant, no difference with untreated control was noted. Figure 6B shows the effect of the studied agent in the same conditions of above but in presence of chemoattractant, the drug caused an inhibiting dose-dependent effect comprised between 25% and 42%. Figure 6C shows the effect of Glutoxim used as chemoattractant in place of fMLP at the same concentration as above; we observed a chemoattracting dose-dependent activity that was 20% and 30% greater than control for concentration of 1 and 6 μg/mL respectively.

DISCUSSION

Glutoxim is a therapeutic agent classified as immunomodulator for its effect on proliferation and stimulation of immunocompetent cells involving

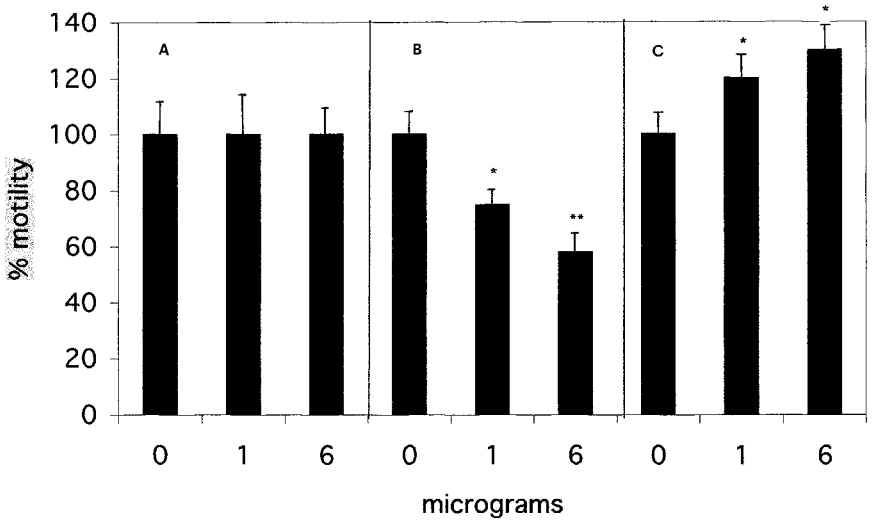

Figure 6. Effect of Glutoxim on neutrophil chemotaxis. A. Cells were preincubated for 1 h with 1 or 6 μg/mL of the drug and then tested for chemotaxis without chemoattractant. B. Cells were preincubated for 1 h with 1 or 6 μg/mL of the drug then tested for chemotaxis in presence of chemoattractant. C. Untreated cells were tested for chemotaxis applying 1 or 6 μg/mL of Glutoxim as chemoattractant. Values are the means ± standard deviation for three or more separate experiments. $*P \leq 0.05$; $**P \leq 0.01$ significantly different from control.

redox-state regulation,[1–3] and in human neutrophils the drug was effective on all the functions studied. Respiratory burst of human neutrophils was studied in whole blood, the agent showed high stimulating effect on O_2^- production in resting cells, on the contrary inhibiting effect in cells stimulated with zymosan or PMA. This demonstrated the immunomodulating effect of the studied agent in neutrophils and, at the same time, its selective interference on enzyme system regulating O_2^- generation represented by NADPH-oxidase and associated proteins. In resting cells the enzyme system is located at the plasma membrane and associated proteins are segregated into cytoplasmic and plasma membrane compartments, the NADPH-oxidase became active when cytosolic proteins translocate from cytosol to the membrane during assembly of the functional enzyme complex. The high generation of O_2^- caused by the drug in resting neutrophils was probably determined in cytosol inducing translocation of associated proteins there located (Fig. 1). Particulate neutrophil stimulation (zymosan) puts in action all steps leading to O_2^- production, and chemical stimulation (PMA) activates directly proteinkinase c bypassing membrane steps. The results obtained (Fig. 2) did not showed significant difference in neutrophils stimulated with particulate or chemical agent regarding superoxide anion production. This behavior, independently on nature of the stimulus, suggested an effect of the drug on terminal steps of cytosol superoxide

production. The O_2^- production inhibition in cells preincubated with the studied agent and stimulated with zymosan or PMA showed interference between drug and stimulants confirming cytosolic action of the Glutoxim likely being proteinkinase c activation leading to formation of flavocytochrome b558 that is the central component of the NADPH-oxidase. In whole blood we did not observe a dose-dependent effect but repetition of the experiment on isolated neutrophils showed the dose-dependence (data not shown); evidently such complex system as whole blood neutralized the drug excess. Concentration of 3 μg/mL showed higher activity and corresponds to the recommended average therapeutic IV dose. The hypochlorous production, as expected, was enhanced by the drug in resting cells (Fig. 3), and, vice versa, after stimulation it was inhibited by the preincubation with Glutoxim (Fig. 4). In fact the hypochlorous generation is directly dependent on O_2^- production since superoxide anion is rapidly converted to secondary toxic oxygen species such hydrogen peroxide (H_2O_2) and hydroxil radical (OH) that in presence of the mieloperoxidase in neutrophils determines the HOCl production. The studied agent showed dose-dependent stimulating or inhibiting effect on HOCl production except at the lower dose (1 μg/mL). It showed inhibiting effect also on nitric oxide production after LPS stimulation on phagocyte cells such as RAW 264.7 (Fig. 5). The NO generation is the product of the five-electron oxidation of one of the chemically equivalent guanidine nitrogens of L-arginine by nitric oxide synthase (NOS),[12] therefore, completely independent from superoxide anion production. Simultaneous inhibition of two independent enzyme systems, NADPH-oxidase and NOS, suggests competition or interference of the drug with NADPH that is directly essential for O_2^- generation, as well as for NO production since NOS require NADPH-dependent flavoproteins flavin-adenine-dinucleotide (FAD) and flavin-mononucleotide (FMN) to be active.

Chemotaxis of neutrophils was significantly affected by the drug that exerted chemoattracting effect on untreated cells when added in the lower compartment of the Boyden chamber instead of fMLP (Fig. 6C), and inhibited fMLP-induced chemotaxis of pretreated cells (Fig. 6B); on the contrary, that is the 1 h pretreatment with the drug did not modify random neutrophil migration (Fig. 6A). These data clearly show that Glutoxim has all the characteristics of a chemoattractant and not only increases neutrophil chemotaxis but inhibits chemoattractive effect of fMLP likely through a heterologous desensitization of surface receptors common to other chemotactic factors.[13–15]

These studies demonstrate, in conclusion, that neutrophil functions are deeply affected by Glutoxim that enhances O_2^- and HOCl generation; inhibits O_2^-, HOCl and NO production after stimulation; increases chemotaxis decreasing the fMLP-induced one. All found effects might be determined by impact of the studied agent on proteinkinase system that regulates reactive oxygen species and NO generation as well as some steps of

chemotaxis confirming the drug immunomodulating effect in neutrophils as well.

REFERENCES

1. Kozhemyakin, L.; Balazovski, M. Glutamed MH. Metabolic Immunomodulator and Haemopoietic Factor, Russian National Congress ''Man and drug,'' Moskow, April 21–25, 1999, 576.
2. Kozhemyakin, L.; Balazovski, M.; Belokhvostov, A. Induction of Apoptosis via RAS-Signalling Pathway by the New Drug Class-Thiopoietins. Int. Congress Chemotherapy, Birmingham, U.K., July 4–7, 1999, Abs 21th.
3. Kozhemyakin, L.; Ketlinskaya, O.; Romanova, S.; Belokhvostov, A. New Generation Drugs at Treatment of Viral Hepatitis: Differentiated Effect on Normal and Virus-Infected Cells. ExConsilio **1999**, *3* (1), 59.
4. Smirnov, A.; Kozhemyakin, L.; Levashov, F.; Orlov, S. Therapy with Containing Sulfur Oligopeptide in Patients with Small Cells Lung Tumor, III Russian Oncology Conference, St. Petersbourg, November 29–December 01, 1999; 140.
5. Kozhemyakin, L.; Rudakov, O.; Semiglasov, V.; Paltnev, R. Perspectives in the use of Metabolic Immunomodulators of Glutoxim Group in Breast Cancer Therapy, III Russian Oncology Conference, St. Petersbourg, November 29–December 01, 1999; 196.
6. Haslett, C.; Guthrie, L.; Kopaniak, M.; Johnston, R.; Henson, P. Modulation of Multiple Neutrophil Functions by Preparative Methods or Trace Concentrations of Bacterial Lipopolysaccharide. Am. J. Pathol. **1985**, *119*, 101.
7. Bellavite, P.; Dri, P.; Berton, G.; Zabucchi, G. The Measurement of Superoxide Anion Production by Granulocytes in whole Blood: A Clinical Test for Evaluation of Phagocyte Function and Serum Opsonic Capacity. Eur. J. Clin. Invest. **1983**, *13*, 363.
8. Weiss, S.J.; Klein, R.; Slivka, A.; Wie, M. Chlorination of Taurine by Human Neutrophils: Evidence for Hypochlorous Acid Generation. J. Clin. Invest. **1982**, *70*, 598.
9. Miles, A.M.; Owens, M.W.; Milligan, S.; Johnson, G.G.; Fields, J.Z.; Ing, T.S.; Kottapalli, U.; Keshavarizan, A.; Grishana, M.B. Nitric Oxide Synthase in Circulating vs. Extravasated Polymorphonuclear Leukocytes. J. Leuk. Biol. **1995**, *58*, 616.
10. Boyden, S.U. The Chemotactic Effect of Mixtures of Antibody and Antigen on Polymorphonuclear Leukocytes. J. Exp. Med. **1962**, *115*, 453.
11. Zigmond, S.H.; Hirsh, J.G. Leukocyte Locomotion and Chemotaxis: New Methods for Evaluation and Demonstration of a Cell-Derived Chemotactic Factor. J. Exp. Med. **1973**, *137*, 387.
12. Stuehr, D.J.; Griffith, O.W. Mammalian Nitric Oxide Synthases. Adv. Enzymol. Mol. Relat. Areas Biol. **1992**, *65*, 287.
13. Blackwood, R.A.; Sartiala, K.T.; Kwoh, E.E.; Transue, A.T.; Brower, R.C. Unidirectional Heterologous Receptor Desensitization Between Both the fMLP and C5a Receptor and IL-8 Receptor. J. Leuk. Biol. **1996**, *60*, 88.

14. O'Flaherty, J.T.; Kreutzer, D.L.; Showell, H.J.; Vitkauskas, G.; Becker, E.L.; Ward, P.A. Selective Neutrophil Desensitization to Chemotactic Factors. J. Cell Biol. **1979**, *80*, 564.
15. Kew, R.R.; Peng, T.; DiMartino, S.J.; Madhawan, D.; Weinman, S.J.; Cheng, D.; Prossnitz, E.R. Undifferentiated U937 Cells Transfected with Chemoattractant Receptors: A Model System to Investigate Chemotactic Mechanism and Receptor Surface/Function Relationships. J. Leuk. Biol. **1997**, *61*, 329.