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DRUG BIOCHEMISTRY

EFFECT OF NICOTINAMIDE AND ITS TWO DERIVATIVES ON THE GENERATION OF REACTIVE OXYGEN SPECIES IN HUMAN MONOCYTES COOPERATING WITH PLATELETS

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Abstract: The study was designed to demonstrate the relationship between the activity of human normal monocytes and blood platelets, to determine the metabolic activity of normal monocytes and monocytes cooperating with blood platelets in respect of their generation of reactive oxygen species (ROS) and to study the response of cooperating cells to nicotinamide (NA), 1-methylnicotinamide (MNA^+) and 1-methyl-N`-hydroxymethylnicotinamide ($MNAF^+$). The ability of those potential antiinflammatory compounds to inhibit oxygen respiratory burst was also assessed. Measurements were carried out by luminol chemiluminometry. The results of the measurements were compared to the data acquired for aspirin (ASA). The results obtained showed that MNAF inhibited oxygen burst in monocytes cooperating with platelets, whereas the two other compounds, NA and MNA, did not cause inhibition of oxygen burst *in vitro* at a statistically significant level.

Keywords: nicotinamide, monocytes, plateles, inflammation, reactive oxygen species

The entire population of microorganisms inhabiting both the external and internal surfaces of a healthy organism is called normal microflora. When factors affecting the balance of microflora, such as mechanical injuries, diminished immunity, a change in diet or climate come into play, the disturbance of balance between micro- and macroorganism takes place, leading to the development of infection and inflammation. The characteristic symptoms of inflammation are erythema (*rubor*), pain (*dolor*), edema (*tumor*), elevated body temperature (*calor*) and dysfunction of organs (*functio laesa*) (1). Immune system cells of different types, both sedentary and tissue-infiltrating, are involved in the process of inflammation.

The major mediators of inflammation are amines and biogenic amino acids (histamine and serotonin), bradykinin, platelet-activating factor (PAF), fragments of the complement (mainly C3a and C5a), some cytokines, and arachidonic acid derivatives.

We observe the following pathological responses to antigen: an excessively prolonged response, a response inadequate to the degree of exposure, or a response to the organism`s own antigens. Stimulation of the immune system, originating from a chronic inflammatory state, can negatively affect quality of life or can cause a disease, cripplehood, and may even sometimes lead to death. Therefore, studies focusing on potential antiinflammatory drugs are important for prevention of such harmful consequences of immune system impairment (2, 3).

Monocytes are the biggest leukocytes of diameter up to 20 μm. They are formed in the bone marrow and then move into the bloodstream where they circulate for ca. 24 h. Afterwards, they leave the circulatory system and further differentiate in body tissues where they develop into macrophages. Monocytes together with macrophages constitute a system whose basic biological role is phagocytosis. Receptors of the stable fragment of immunoglobulin G can be detected on the surface of monocytes. In monocyte's Golgi apparatus the presence of peroxidase can be demonstrated. Peroxidase plays an important role in phagocytosis. Monocyte lysosomes contain a number of hydrolytic enzymes, e.g., arginase, deoxyribonuclease, elastase, β-glucuronidase, glucosidase, cathepsins, collagenase, lipases, lysozyme and sulfatases.

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Studies that examine the metabolic role of oxygen, confirm its functional metabolic significance, but also its toxicity. The toxicity is caused by formation of reactive oxygen species (ROS) such as, hyperoxide anion radical ($O^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen, hydrogen peroxide and many others. ROS play a number of important functions in the organism, but under some conditions they can also bring harmful consequences.

At the moment of their conctact with a pathogen, phagocytizing cells produce ROS to protect the organism against pathogenic factors. On such occasions, the presence of ROS in the place of invasion plays an important protective role. However, the uncontrolled increase in the concentrations of reactive oxygen metabolites, known as oxidative stress, may have serious negative consequences (4, 5). As a result of hyperoxide anion radical dismutation, hydrogen peroxide is formed. A million neutrophilic granulocytes, stimulated with a chemotactic peptide, produce at least 5.3 pmol of hyperoxide anion radicals and 6.0 pmol of hydrogen peroxide per second (6).

Blood platelets, alias thrombocytes, are non-nucleated granular cells, disc-like in shape, with a diameter of 2–4 μm. Their number, under normal conditions, ranges from 150 000 to 400 000 per 1 μL in the circulating blood of an adult human. Thrombocytes participate in the process of blood clotting. In the locus of damage of the vascular wall activation of blood platelets occurs, consisting of release of cytokines, followed by formation of a platelet "plug" which inhibits bleeding. On their surface, platelets accelerate the conversion of prothrombin into thrombin; the latter plays a role in the formation of a fibrin clot by retaining therein the platelet plug and thus forming a more durable clot. Activation of platelets may be induced by the contact with leucocytes, predominantly neutrophils stimulated by an inflammatory process (7). Platelets initiate and maintain inflammatory processes by releasing: a platelet-activating factor (PAF), platelet-derived growth factor (PDGF), selectin P, transforming growth factor β (TGFβ), platelet factor 4 (PF4), β-thromboglobulin, interleukin-1 (IL-1), prostaglandins (PGs) (8, 9). Platelets are capable of activating phagocytosis, increasing lysosome degranulation and potentiating the cytotoxicity of neutrophils and monocytes (10, 11).

During the inflammatory process, the blood platelets in the organism are activated and affect other morphotic components of blood. This in turn triggers, among others, an interaction between platelets and neutrophils and monocytes (12). Platelets and monocytes can form platelet-monocyte aggregates, mainly *via* platelet P-selectin and its ligand PSGL-1, but also *via* fibrinogen bridges between glycoprotein Gp IIb/IIIa on blood platelets and the corresponding integrin receptors Mac-1 present on monocytes. A fusion of this type may facilitate interactions between cells (13). Mediators effecting cell activation may be involved in these interactions. The monocyte activation is expressed by degranulation, an oxygen burst, chemotaxis or phagocytosis. All these processes may be affected by blood platelets through their release of such compounds as PDGF, TGFβ, interleukins and thromboglobulins, as well as selectin P and PF4. Like neutrophils, also monocytes exert an effect on blood platelets, among others by releasing PAF, monokines, interleukins and cachectins. Based on some reports it is assumed that cells of the immune system may also participate in TXA_2 biosynthesis by providing blood platelets with arachidonic acid (2, 14). The phenomenon of cooperation between monocytes and blood platelets has not been studied in detail so far.

The aim of the present study was:

- to assess the metabolic activity of normal monocytes in respect of their generation of ROS;
- to determine a possible relationship between the activity of normal human monocytes and blood platelets, and to estimate the ability of these cells to generate ROS under *in vitro* conditions;
- to study the response of cooperating cells in the presence of nicotinamide, 1-methylnicotinamide and 1-methyl-N`-hydroxymethylnicotinamide;
- to make a preliminary assessment of the selected compounds as potential antiinflammatory drugs.

EXPERIMENTAL

Drugs and chemicals

Nicotinamide and its derivatives

The tested compounds, i.e., β-pyridinecarboxylic acid amide (NA), 1-methylnicotinamide (MNA^+) and 1-methyl-N'-hydroxymethylnicotinamide ($MNAF^+$) were obtained from the Institute of Applied Radiation Chemistry of the Technical University of Łódź. The chemical structures of these compounds are presented in Figure1. For the purpose of measurement, the following concentrations of the preparations in question were used: 1, 5, 10, 50, 100, 500 and 1000 μM. They were prepared as described previously (15, 16). The aspirin (acetylsalicylic acid, ASA, Bayer) samples were obtained from the Department of Experimental

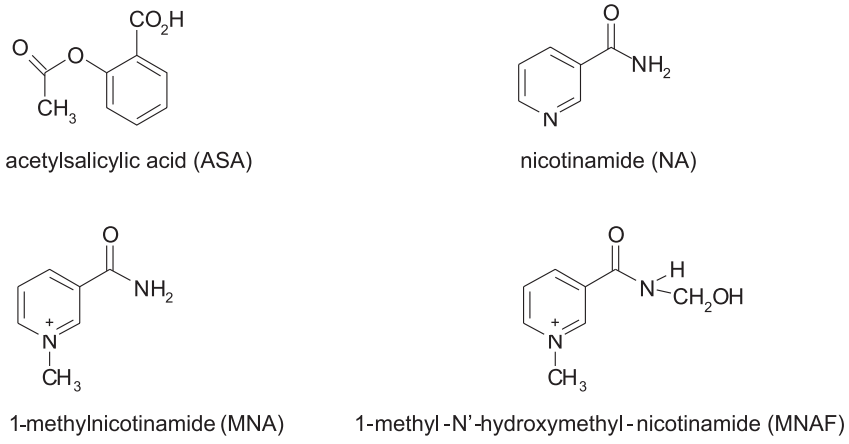

Figure 1. Chemical structure of the examined compounds

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Latex (polystyrene particles) of diameter 0.9 μm came from the Institute of Catalysis and Physicochemistry, Polish Academy of Sciences, Kraków, Poland. A 10% of standard latex suspension in 10 μL was used for activation of cells.

Phosphate buffered saline (PBS) of the following composition was used: monobasic sodium phosphate (0.2 M), dibasic sodium phosphate (0.2 M) and sodium chloride (0.9%) adjusted to pH 7.4.

All chemicals used in assays were of the highest purity available on the market.

Preparatione of buffer for chemiluminescence

The following reagents were mixed: 5 mL of a luminol solution (0.0125 g of luminol, Sigma; 97% pure by HPLC + 1.785 g $Na_2HPO_4 \times 12\ H_2O$ + 50 mL of destilled water), 1 mL of the Krebs-Ringer phosphate buffer, pH 7.4 concentrated fivefold, 3.8 mL of distilled water, 200 mL of glucose solution at a concentration of 50 mg/mL. The buffer was protected from the light at a temperature of 4°C.

Chemiluminescence measurements

Chemiluminometry is a method used to assess the functional state of immune system cells by measuring their chemiluminescence (17, 18). It consists in measuring the emitted light with scintillation counters or luminometers equipped with a photomultiplier. The number of photons emitted during the chemiluminescence of the samples studied was measured with the microplate chemiluminometer Berthold MicroLumat LB 96 P. The results can be obtained in the form of curves showing changes in luminescence intensity *versus* time (19).

The results were recorded with a computer running WinGlow program (10, 20). The software allows chemiluminescence experiment to be displayed on the basis of the following parameters: the integration of the emmision curve; the maximum value of the peak height in relative luminescence units (RLU)/s and the time of reaching the maximum peak in minutes.

Biological material

Blood collected from healthy donors was centrifuged to obtain platelet-rich plasma (PRP). Blood platelets were obtained after two-fold PRP centrifugation in the presence of prostaglandin J_2 (PGJ_2), (1 μL of prostaglandin per 2 mL of PRP). The centrifugation was performed under the following conditions: 10 min, 1000 × g at room temperature with Eppendorf Centrifuge 5804 R. After the first centrifugation, the supernatant was decanted and 2 mL of PBS were added to the yielded deposit and mixed; afterwards, the tube was supplemented with PBS up to the previous volume and the appropriate amount of PGJ_2 was added. The mixture was centrifuged again for 10 min at 800 × g. After the second centrifugation, the supernatant was decanted and 3 mL of PBS were added to the final deposit. The counting of platelets was performed as follows: 10 μL of the platelet suspension was diluted 20-fold in a 3% polocaine. A few drops of the solution containing the platelets were put onto Thoma counting chamber and left for 10 min. Then the platelets were counted under a microscope.

The monocytes were isolated from blood collected from healthy donors, the same as for platelets.

The cells were isolated in the Department of Immunology of the Jagiellonian University. For a preliminary isolation of monocytes, the JE-6B apparatus *Elutriator System* was used. The monocyte suspension was placed in an Eppendorf tube, supplemented with PBS and centrifuged (the Eppendorf Lo Bind Tubes Centrifuge 5415R) for 5 min at a temperature of 4°C, 2000 rpm. The supernatant was decanted, and the monocyte deposit was supplemented with PBS and re-centrifuged twice under the same conditions. After the third centrifugation, the supernatant was decanted and the deposit was supplemented with PBS to obtain 5 millions monocytes per 1 mL volume. The isolated cells were kept on ice since an increase in temperature is a factor activating monocytes and leading to a decrease in their activity.

RESULTS

Samples containing cells or cells with tested compounds but without activator were used as neg-

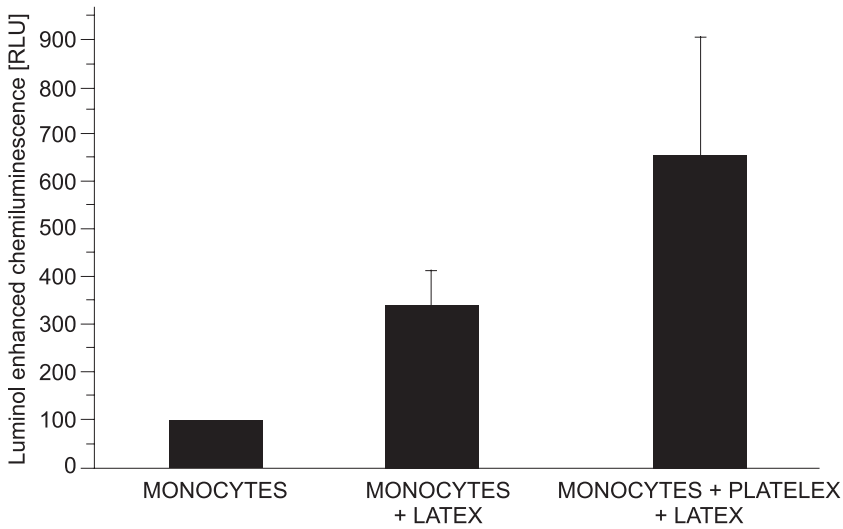

Figure 2. Control experiments – activation of monocytes and monocytes with platelets by latex

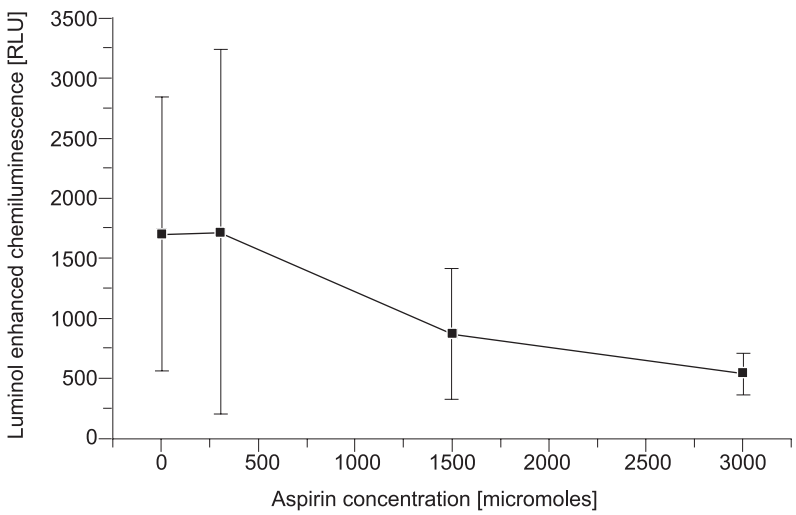

Figure 3. The effect of aspirin (ASA) on the activated human monocytes cooperating with blood platelets

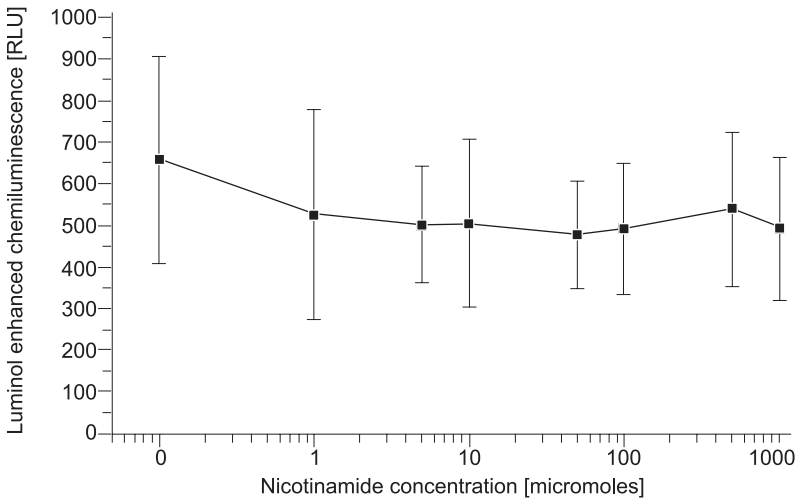

Figure 4. The effect of nicotinamide (NA) on the activated human monocytes cooperating with blood platelets

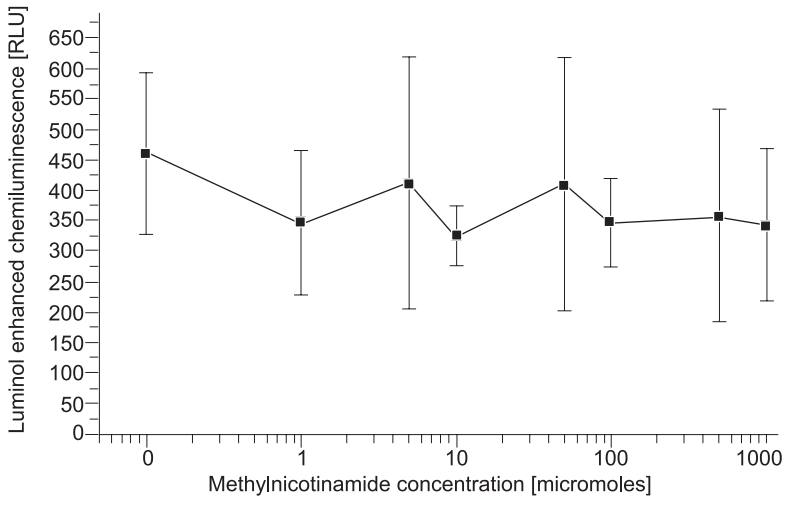

Figure 5. The effect of 1-methylnicotinamide (MNA^+) on the activated human monocytes cooperating with blood platelets

ative control, whereas monocytes activated with polystyrene particles served as positive control. The chemiluminescence levels for samples containing only blood platelets were close to zero. The blood platelets stimulated with polystyrene particles showed chemiluminescene values that were a few per cent higher compared to the values for platelets without an activator. Monocytes activated by latex particles resulted in an increase in chemiluminescence level, and additionally the presence of platelets in monocyte suspension displayed a more considerable increase in chemiluminescence after stimulation by latex, as it is shown in Figure. 2. The results presented in Figure 3 show that the addition of aspirin to activated human monocytes cooperating with blood platelets decreased the generation of radical oxygen species which was expressed as significant lower chemiluminescence (CL) level.

The results for NA, MNA^+ and $MNAF^+$ are shown in Figures 4, 5, and 6, respectevely. The obtained results were compared to the data acquired for ASA (Fig. 3).

The addition of NA to monocytes cooperating with blood platelets caused a slight decrease in CL

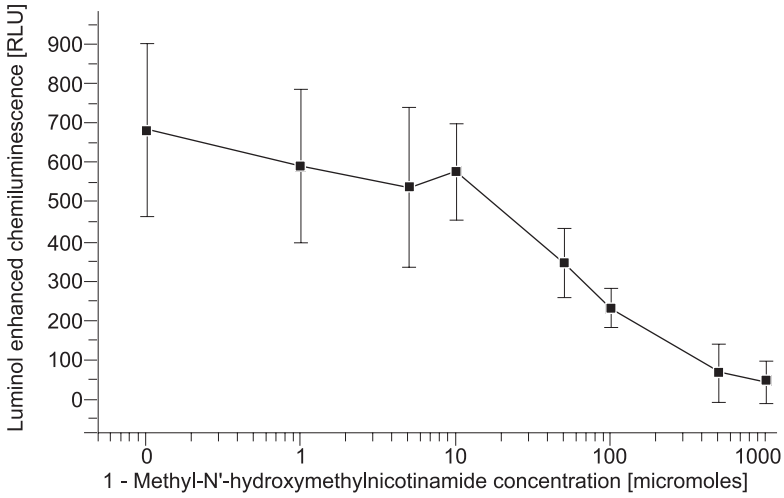

Figure 6. The effect of 1-methyl-N'-hydroxymethylnicotinamide ($MNAF^+$) on the activated human monocytes cooperating with blood platelets

levels (Fig. 4). The use of MNA^+ to stimulate the latex-activated monocytes cooperating with blood platelets yielded results similar to those obtained with NA (Fig. 5) and Figure 6 demonstrates that the addition of $MNAF^+$ to the cooperating monocytes with blood platelets produced results analogous to those shown for aspirin (Fig. 3). It was observed that $MNAF^+$ strongly inhibited the generation of ROS by monocytes which was expressed by low chemiluminescence level.

DISCUSSION AND CONCLUSION

During an inflammatory process, complex interactions of monocytes and neutrophils with blood platelets take place in the organism, however, these interactions have not been studied in detail. The available literature provides evidence of diverse impact of blood platelets on chemotaxis, phagocytosis, degranulation and the oxygen metabolism of blood cells. Similarly, neutrophils as well as monocytes can influence the adhesion, aggregation and secretion of different agents from blood platelets (13).

Human monocyte and blood platelets were used to carry out the studies under *in vitro* conditions. The cells were activated with latex (polystyrene particles). Its considerable impact on cells causes their activation, expressed as stimulation of a number of biochemical reactions. When supplemented with that activator, monocytes showed a considerable increase in CL both when used alone and in cooperation with blood platelets. Based on the results of experiments conducted by other researchers (19, 21), it may be assumed that the cause of the increased chemiluminescence was the acceleration of the formation of ROS.

Our study was aimed to analyze interactions between monocytes and blood platelets in the process of generation of ROS by monocytes. In samples containing both monocytes and platelets, the CL value was considerably higher than the value recorded for activated monocytes or blood platelets separately. The present results confirm earlier reports that the cooperation of monocytes with blood platelets potentiates chemiluminescence which originates from an increase in the number of generated ROS. It was also observed that the CL value of blood platelets stimulated with polystyrene particles was slightly higher (by a few per cent) than the CL value of unactivated cells. The above findings seem to confirm that blood platelets are not equipped with a mechanism of oxygen killing. The weak light emission by blood platelets alone has permitted us to propose that the increased CL value of monocyte and blood platelets aggregates originates mainly from monocytes, and to a limited extent from blood platelets.

The addition of aspirin (ASA) to cooperating monocytes and blood platelets led to a decrease in the CL value of the studied cells. Aspirin is a commonly used non-steroid antiinflammatory (NSAI) drug. It inhibits cyclooxygenase (COX)-1 activity by hindering the generation of prostanoids in the

first phase of their biosynthesis from arachidonic acid (2, 22), which accounts for the observed decrease in CL. The obtained results clearly indicate that the rise in drug concentration is accompanied by a decrease in CL. When used in higher doses, aspirin causes a decrease in the activity of monocytes generating ROS. The CL of the cell aggregates under study, stimulated with a high dose of the drug, was lower than the CL of unactivated monocytes.

The addition of $MNAF^+$ to the cooperating monocytes and blood platelets produced a result analogous to that shown for aspirin. It was observed that $MNAF^+$ inhibited the generation of ROS by monocytes. Like in the case of ASA, the higher was the dose of the substance, the lower was the activity of monocytes cooperating with blood platelets. $MNAF^+$ concentrations higher than 0.5 mM caused a drop in the CL values of the cells examined below the value of the CL of unactivated monocytes.

The addition of NA, commonly known as vitamin B_3 to monocytes cooperating with blood platelets, caused a decrease in CL value. In contrast to the previously discussed substances, NA – independently of the dose used – decreased the CL of the examined cells. However, the obtained CL values never fell below those of the CL of unactivated monocytes. NA is recognized for its antiinflammatory action; it is commonly used and supplied to the organism with food. Therefore, further studies are necessary to estimate the natural levels of NA present in monocytes.

The use of MNA^+ to stimulate the latex-activated monocytes cooperating with blood platelets yielded results very close to those obtained with nicotinamide. The only difference was that the degree of inhibition of monocyte activity in their generation of oxygen free radicals was inconsiderably larger than in the case of nicotinamide.

The results shown for the respective measurements, concerning the effect of the same chemical compound – a nicotinamide derivative – on CL, differ considerably, which leads to an increase in variance value. The latter phenomenon is most probably due to considerable individual differences in cell donors. Under normal conditions, the response to an antigen depends to a large extent on the individual's genetic predispositions, physical condition, lifestyle, diet and a number of other factors. Also the response to drugs depends upon all the above-mentioned factors which may significantly modify the efficiency of drugs, their activity time, the rate of elimination from the organism and other variables.

Our results originating from all the experiments demonstrated an activated state of monocytes, expressed as the ability of those cells to respond to an external stimulus by increasing the numer of generated ROS. The evidence for the existence of a high functional potential of monocytes came from their response to stimulation with polystyrene particles. The study showed that, like aspirin, $MNAF^+$ inhibited the generation of ROS by the cooperating monocytes and blood platelets, and that substances such as nicotinamide and methylnicotinamide decreased the activity of human monocytes.

The results obtained in the experimental series with nicotinamide and its derivatives are promising. They have demonstrated the possibility of using these compounds in the future as a basis for the production of non-steroidal antiinflammatory drugs which could be used in rheumatic diseases, or as analgesic drugs in degenerative joint diseases, parodontosis, sports injuries, menstrual colic and others. There have also been an increasing number of reports stating that these drugs may be useful in the prophylaxis of colorectal carcinoma and other neoplasms, as well as in the prevention of Alzheimer`s disease and the therapy of imminent premature child birth. Nonetheless, further studies should be carried out before introducing these compounds into clinical practice.

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