Studies of the Components of Pro- and Antioxidative Systems in Blood of Patients Suffering from Rheumatoid Arthritis

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The aim of the study was to investigate the level of some prooxidants and the activity of certain antioxidant enzymes in the blood of patients suffering from rheumatoid arthritis (RA). For this purpose we determined the level of nitric oxide (NO) and lipid peroxidation measured as malondialdehyde (MDA) in blood, as well as the activity of the erythrocyte antioxidant enzyme, superoxide dismutase (SOD), and of the whole blood enzyme glutatione peroxidase (GPO). The concentration of Zn in serum was also determined. The results revealed a significantly increased level of NO in the blood serum of patients with RA, while the content of MDA was not considerably elevated as compared to control. Interestingly, there was a positive correlation (r = 0.52) between NO and MDA levels in the blood of the patients. The activity of SOD in erythrocytes of RA patients was by 24.3% lower as compared to control. The level of Zn was also diminished by 27.6% in the group of patients as compared to control, as was the activity of another antioxidant enzyme, GPO (by 39.6%). The present study revealed that increased levels of free radical concentration and lipid peroxidation in RA patients were not accompanied by significant changes in the activity of antioxidant enzymes.

Key words: antioxidants, glutathione peroxidase, malondialdehyde, nitric oxide, prooxidants, rheumatoid arthritis, superoxide dismutase, zinc

INTRODUCTION

Oxidative stress defined as an imbalance between oxidants and antioxidants potentially leading to damage has been associated with a number of morbid states in humans (1). Several studies suggest that oxidative stress plays a significant role in the pathogenesis of rheumatic diseases, too (2–4). It was shown that in inflammatory arthritis and osteoarthritis (OA) joint cartilage damage together with bone damage is induced by inflammatory cytokines and other mediators such as enzymes, nitric oxide (NO) and other highly reactive oxygen species (ROS: superoxide radical, hydrogen peroxide, hydroxyl radical) produced by activated neutrophils, chondrocytes, lymphocytes, macrophages, endothelial cells (3, 5, 6). By several observations, a role of NO as well as of other ROS in chondrocyte apoptosis and matrix loss was suggested (7). In addition to the existing opinion about the role of ROS in the pathogenesis of systemic lupus erythematosus (SLE), recent findings support the hypothesis that cytokine-activated endothelial cells and an increased inducible NO synthase activity play a central role in the development of vascular injury that characterizes SLE (8).

In accordance with enhanced oxidation, patients with rheumatoid arthritis (RA) and other rheumatic diseases exhibit abnormalities consistent with injury of membranes by lipid peroxidation. All ROS attack polyunsaturated fatty acids in the membrane lipids, causing lipid peroxidation which may lead to a damage of cell structure and function. Decomposition of peroxidized lipids yields a variety of end products, including malondialdehyde (MDA), elevated levels of which have been reported in the serum and synovial fluid of patients suffering from RA (2, 9). It has been shown that MDA levels are reliable indicators of ROS molecular reaction.

As is well known, in order to prevent the damage caused by ROS, multiple enzymatic and nonenzymatic defence systems called antioxidants are present in human cells and tissues. It has been considered that imbalance between ROS and antioxidants may be a cause of destruction of tissues and may impact immunological response. In case of rheumatic diseases it seems to be associated with a

reduced capacity of enzymatic and nonenzymatic (vitamins E and C, etc.) antioxidant systems to convert ROS into more inert species. This in part can be explained by a beneficial effect observed following treatment of equine and human OA with superoxide dismutase (SOD), an intracellular antioxidant (10). The hypothesis exists that a low antioxidant level is a risk factor for RA (3). In case of OA antioxidants could play a great role in preventing its progression (8).

Glutathione peroxidase (GPO) and SOD are important members of the antioxidant team that have been shown to destroy ROS and other free radicals by enzymatic mechanisms (12). Some observations suggest that erythrocytes could also be important in handling the ROS generated by activated neutrophils in the surrounding medium. However, the protective efficiency of erythrocytes against lipid peroxidation would depend on the balance between oxidant species and the availability of antioxidant defences (13, 14).

Since ROS and antioxidant defence mechanisms appear to act in concert rather than alone, the aim of this study was to investigate the level of representatives of prooxidants (NO, MDA) and the activity of antioxidant enzymes (SOD and GPO) in the blood of patients suffering from RA in comparison with those in the healthy control group. The concentration of Zn in the serum of patients and controls was also determined, because it is present in one form of SOD.

We also analysed the correlation of serum level of prooxidant indices (NO and MDA) and Zn with the activities of GPO and SOD as well as their correlation with disease activity, mean disease duration and patient's age.

MATERIALS AND METHODS

Blood samples were obtained from 22 hospitalised patients suffering from RA. All patients were women, mean age 53.6 ± 11.2. RA patients had an active disease according to the EULAR criteria (mean activity 5.3 ± 0.86), and mean disease duration was 2.08 ± 1.78 years. The patient and healthy control (10 persons) groups were comparable with respect to age and gender.

NO was determined photometrically via its oxidation products nitrite and nitrate on microtiter plates. The nitrate present in the serum was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the enzyme nitrate reductase. The nitrite formed reacted with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye. The dye was measured on the basis of its absorbance in visible range at 550 nm. The results were calculated from the calibration curve using the standard solutions. For determination of NO, a test combination from Boehringer Mannheim was used.

For the quantitative evaluation of lipid peroxides, their transformation into a coloured compound under the effect of thiobarbituric acid (TBA) was used. MDA, the final product of fatty acids peroxidation, reacts with TBA with the formation of a coloured complex, which was determined spectrophotometrically (Marcel-PRO spectrophotometer) at wavelengths of 530 nm and 580 nm. The MDA concentration was calculated by regression (15).

SOD was determined in erythrocytes by the colorometric method, using a Ransod SOD pack (Randox). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The colour was measured on the basis of its absorbance at 505 nm. The superoxide dismutase activity was then measured by the degree of inhibition of the described reaction. Heparinized whole blood samples were used. Then 0.5 ml of whole blood was centrifuged for 10 min at 3000 rpm and aspirated off the plasma. The erythrocytes were washed four times with 3 ml of 0.9% NaCl solution and centrifuged for 10 min at 3000 rpm after each wash. The washed centrifuged erythrocytes then were made up to 2.0 ml with redistilled water, mixed and left to stand at +4 °C for 15 min. The lysate was diluted with 0.01 mmol/l phosphate buffer pH 7.0, so that the % inhibition fell between 30% and 60%.

Glutathione peroxidase (GPO) was determined in heparinized whole blood using a Ransel GPO pack (Randox). The method of GPO determination is based on the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH the oxidised glutathione is immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$. A decrease in absorbance at 340 nm was measured.

The preparation of serum samples for determination of Zn is described by de Blas et al. [16]. The serum was separated within 30 min of blood collection and stored frozen (–20 °C) until required for analysis. A sample of 0.2 ml serum was placed in a digestion tube, and 0.1 ml 65% HNO_3 and 0.1 ml 95% H_2SO_4 were added. The digestion tubes were placed in oven and heated gently until the froth disappeared. After that the temperature was raised to 130–150 °C until the solution became dark (*ca* 1 h). The samples were cooled to room tempera-

ture, 0.1 ml 65% $HClO_4$ was added, and heating was resumed until white fumes began to appear, but not above 180 °C. After acid digestion, the samples were not diluted further. The concentration of Zn in the serum was determined and calculated by the standard addition method, using an atomic absorption spectrophotometer.

The results were statistically analysed by the Statistic for Windows program.

RESULTS AND DISCUSSION

The results revealed that patients with RA had an almost two and a half times higher level of NO in blood serum than did control group (28.07 ± 2.70 and 11.58 ± 1.93 μmol/l, respectively). Unfortunately, as is shown in Fig. 1 (A, B), the distribution curves of NO data of the patient and control groups were not very different. While changes in the content of NO in RA patients were significant, the level of another representative of prooxidants, MDA, in blood was not considerably elevated as compared to control (3.68 ± 0.57 and 4.18 ± 1.15 nmol/l, respectively). Interestingly, a positive correlation (r = 0.52) existed between NO and MDA levels in the blood of RA patients. Similarly, the blood NO and MDA levels showed a positive although insignificant correlation with the age of patients, duration of illness and disease activity.

From these results and data reported by other researchers (2, 4, 15, 18) it is clear that elevated NO and (to a less extent) MDA levels in the blood of patients ill with RA and other rheumatic diseases are indicators of increased ROS production. The latter could be responsible for cartilage degradation, joint damage and changes in immunologic reactivity in RA patients. In addition, a direct positive correlation observed by us between NO and MDA levels in the blood of RA patients could imply an interrelationship among individual prooxidants, which might certainly influence the final outcome of the disease. It could be very important in those cases that excessive prooxidant production is not inhibited by the combined activities of various antioxidants present in blood and other tissues.

It was shown by us that the activity of SOD in the erythrocytes of RA patients was by 24.36% lower, but insignificantly as compared to control. These findings are in good agreement with the data of other researchers (2). Some authors have reported a significant decrease in erythrocyte SOD activity in RA patients (17). As is shown in Fig. 2 (A, B), the histograms and the shape of distribution curves of SOD activity were a little different in RA and con-

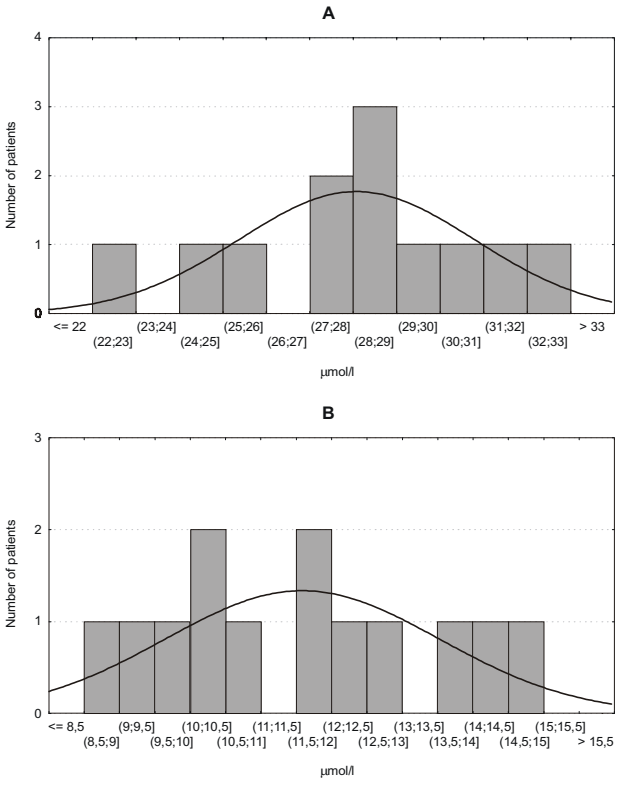

Fig. 1. NO histograms and distribution curves of RA and control group

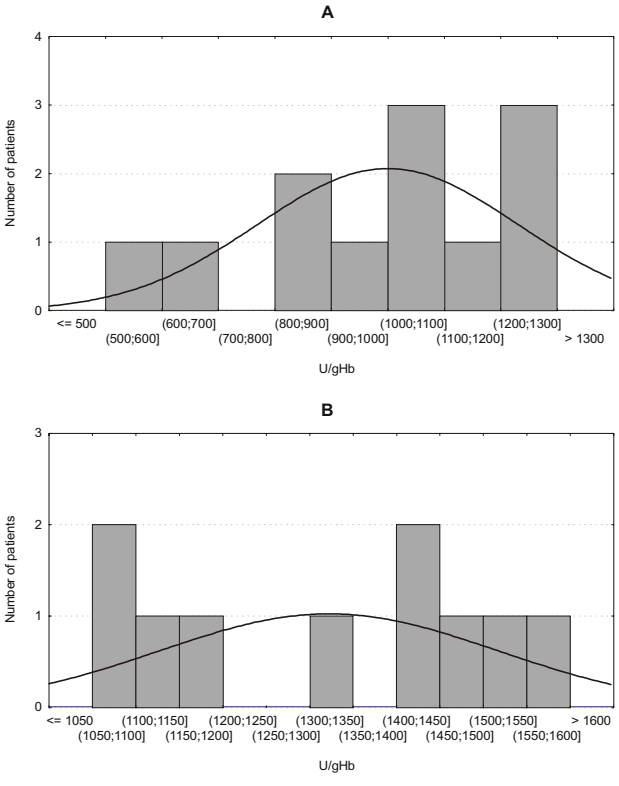

Fig. 2. SOD histograms and distribution curves of RA and control group

trol groups. The correlation of SOD activity with the age of patients, disease activity as well as with the duration of illness was imperceptible.

The results of Zn determination in serum showed that its level diminished by 27.6% in the group of patients suffering from RA as compared to control (74.86 ± 19.75 and 102.09 ± 25.00, respectively). These data could be somehow connected with changes in the SOD level, because the total enzyme activity is influenced by the presence of Zn along with Cu and Mn. Unfortunately, the correlation between the SOD activity and Zn content in RA patients, although positive, was very weak ($r = 0.25$) and insignificant. As in the cases with SOD activity, a correlation of Zn content with the patient's age as well as with disease activity and duration was insignificant.

The activity of another antioxidant enzyme, GPO, in the blood of RA patients was also diminished by 39.6% as compared to control. GPO activity histograms and distribution curves of RA and control groups are presented in Fig. 3 (A, B). One can see that they are not identical. Interestingly, in the case of GPO there was a definite negative correlation ($r = -0.66$, $p < 0.01$) between the duration of illness and the activity of this enzyme.

Thus, our data showed that in RA patients as compared to control the levels of prooxidants were increased, accompanied by suppressed activities of the antioxidative enzymes SOD, GPO and a blood lower level of Zn.

So, data of the present study show that increased levels of free radical concentration and lipid peroxidation (measured in blood as NO and MDA, respectively) in RA patients were not accompanied by significant changes in the activity of the antioxidant enzymes SOD and GPO. These results suggest that the increased oxidant stress present in RA is not compensated by the protective action of free radical scavenging enzymes.

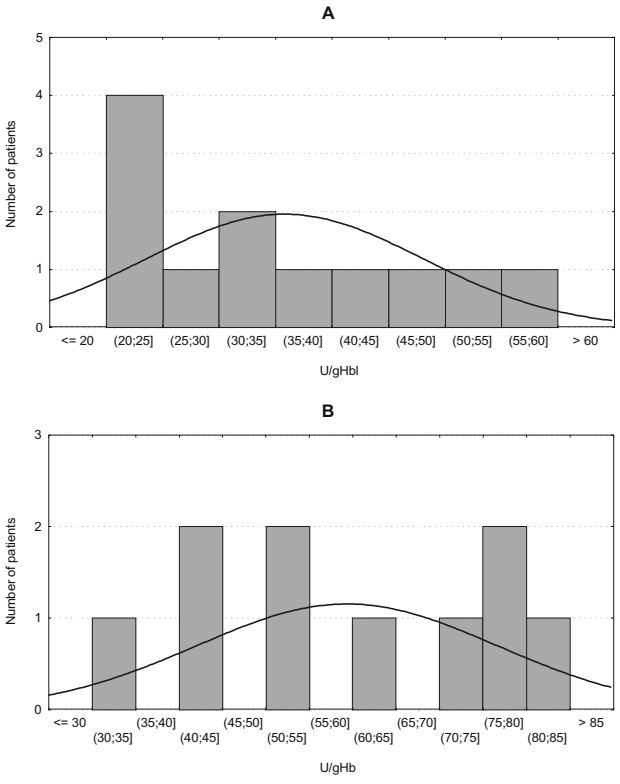

Fig. 3. GPO histograms and distribution curves of RA and control group

Received 10 January 2002
Accepted 7 March 2002

References

1. Sies H. What is oxidative stress? In: Keancy JF. Oxidative stress and vascular disease. Boston: Kluwer Academic Publishers, 2000: 1–8.
2. Jasvinder K, Pramod L, Anil KJ. Correlation between blood antioxidant levels and lipid peroxidation in rheumatoid arthritis. Clin Biochem 1997; 30: 351–5.
3. Heliövaara M, Knekt P, Aho K, Aaram RK, Alfthan G, Aromaa A. Serum antioxidants and risk of rheumatoid arthritis. Ann Rheum Dis 1994; 53: 51–3.
4. Lisitsina TA, Ivanova MM, Durnev AD. Active forms of oxygen and pathogenesis of rheumatic arthritis and systemic lupus erythematosus. Ter Arch 1994; 66: 82–7.
5. Pelletier JP, Mineau F, Fernandes JC, Duval N, Martel-Pelletier J. Diaacerhein and rhein reduce the interleukin 1 beta stimulated inducible nitric oxide synthesis level and activity while stimulating cyclooxigenase-2 synthesis in human osteoarthritic chondrocytes. J Rheumatol 1998; 25: 2417–24.
6. Michael JD, Shanlin FU, Hongjie W, Roger TD. Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Radic Biol 1999; 27: 1151–63.
7. Hashimoto S, Takahaski K, Amiel D, Coutts RD, Lotz M. Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. Arthr Rheum 1998; 41: 1126–74.
8. Belmont HM, Levartovsky D, Goel A, Amin AR, Skovron ML, Buvon J et al. Increased nitric oxide production accompanied by the up-regulation of inducible nitric oxide in vascular endothelium from patients with systemic lupus erythematosus. Arthr Rheum 1997; 40: 1810–20.
9. Lunec J, Halloran SP, Whithe AG, Dormandy TL. Free radical oxidation (peroxidation) products in serum and synovial fluid in rheumatoid arthritis. J Rheumatol 1981; 8: 233–45.
10. Menander-Huber K. Double-blind controled clinical trials with bovine copper–zinc superoxide dismutase (Orgotein). In: Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Eds. Bannister WH and Bannister JV. Elsevier, New York, 1980: 408–23.
11. McAlindon TE, Jacques P, Jhang Y, Hannan MT et al. Do antioxidant micronutrients protect against the

development and progression of knee osteoarthritis? Arthr Rheum 1996; 39: 648–55.
12. Cadmas E. Biochemistry of oxygen toxicity. Ann Rev Biochem 1989; 80: 1486–91.
13. Cimen MY, Cimen OB, Kacmez M, Ozturk HS et al. Oxidant/antioxidant status of the erythrocytes from patients with rheumatoid arthritis. Clin Rheumatol 2000; 19: 275–7.
14. Kaajachumpol S, Vanichapuntu M, Verasertniyone O et al. Levels of plasma lipid peroxide products and antioxidant status in rheumatoid arthritis. Southeast Asian J Trop Med Public Health 2000; 31: 335–8.
15. Gavrilov VB, Gavrilova AR, Mazhut LM. Analysis of the procedures for estimation of lipid peroxidation products using thiobarbituric acid test. Vop Med Chim 1987; 1: 118–22.
16. de Blas OJ, Rodriguez RS, Mendez JH. Determination of zinc in serum, blood and ultrafilterate fluid from patients on hemofiltration by graphite furnace/atomic absorption spectroscopy or flow injection analysis/atomic absorption spectroscopy. J AOAC International 1994; 77: 722–7.
17. Bandorf JC, Brown DH, Hazelton RA, Mcneil CJ, Sturrock RD, Smith WE. Serum copper and erythrocyte superoxide dismutase in rheumatoid arthritis. Ann Rheum Dis 1982; 41: 458–62.
18. Krupovnickienė A, Vipartienė D, Valiukienė K. Study of malondialdehyde content, total antioxidant and catalase activities in rheumatic diseases. Acta Medica Lithuanica 1999; 4: 279–82.

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PRO- IR ANTIOKSIDACINĖS SISTEMOS KOMPONENTŲ TYRIMAS LIGONIŲ, SERGANČIŲ REUMATOIDINIU ARTRITU, KRAUJYJE

S a n t r a u k a

Tirti pro- ir antioksidacinės sistemos komponentų pokyčiai ligonių, sergančių reumatoidiniu artritu (RA), kraujyje. Tam tikslui buvo nustatyta azoto oksido (NO) ir malondialdehido (MDA) koncentracija kraujyje, superoksido dismutazės (SOD) aktyvumas eritrocituose ir gliutationo peroksidazės (GPO) aktyvumas kraujyje. Taip pat tirtas mikroelemento Zn kiekis kraujo serume. Gauti rezultatai parodė, kad NO koncentracija ligonių, sergančių RA, kraujo serume ryškiai padidėja, lyginant su kontroline grupe, kai tuo tarpu MDA kiekio skirtumas tarp abiejų grupių buvo nedidelis. Nustatyta teigiama koreliacija ($r = 0,52$) tarp NO ir MDA kiekių RA ligonių kraujo serume. Lyginant su kontroline grupe, eritrocitų SOD aktyvumas buvo mažesnis 24,3%, o GPO aktyvumas kraujo serume – 39,6%. Nustatyta sumažėjusi Zn koncentracija ligonių kraujo serume (27,6%), palyginus su kontroline grupe. Pateikti duomenys leidžia manyti, kad reumatoidiniu artritu sergančių ligonių kraujyje dėl nepakankamo antioksidantų aktyvumo padidėja laisvųjų radikalų koncentracija bei lipidų peroksidacija.

Raktažodžiai: antioksidatoriai, azoto oksidas, cinkas, gliutationo peroksidazė, malondialdehidas, prooksidatoriai, reumatoidinis artritas, superoksido dismutazė