

# Role of reactive oxygen species (ROS) in patients with erythema migrans, an early manifestation of Lyme borreliosis

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**key words:** Lyme borreliosis, erythema migrans, reactive oxygen species, antioxidants

## SUMMARY

**Background:** Lyme borreliosis is a tick-transmitted, chronic, zoonotic disease caused by *Borrelia burgdorferi* spirochete. The clinical picture of Lyme disease is characterized by the variety of tissue and organ involvement and differing severity of symptoms. One of the pathogenic symptoms of early Lyme disease is a skin lesion called erythema migrans.

**Material and methods:** The purpose of our research was to estimate the parameters of the antioxidant system and the concentration of lipid peroxidation products in the plasma of patients with erythema migrans (EM). The parameters measured included the activity levels of superoxide dismutase (SOD) according to Sykes, glutathione reductase (GSSG-R) according to Mize and Langdon, glutathione peroxidase (GSH-Px) according to Paglia and Valentine; the concentrations of malondialdehyde (MDA) were examined by means of a Bioxytech LPO-586 kit. The total sulphhydryl groups (-SH) according to Ellman and reduced glutathione (GSH) were measured using a Bioxytech GSH-400 test in plasma samples collected from 20 patients with EM aged from 19 to 50, taken before (examination 1) and after (examination 2) therapy with amoxicycline. The control group consisted of 8 healthy people.

**Results:** The results of our examinations prove that beta-lactamase antibiotic therapy brings non-enzymatic antioxidant parameters to control values, though the treatment causes no change in enzymatic antioxidant parameters, resulting in the further activation of free radicals.

**Conclusions:** In patients with Erythema migrans, the decreased capability to reduce lipid superoxidants leads to maintaining a high concentration of membrane lipid peroxidation products.

## BACKGROUND

Lyme borreliosis (tick spirochaetosis, Lyme disease) is a tick-transmitted, chronic, multisystem, zoonotic disease of worldwide distribution, caused by *Borrelia burgdorferi* spirochete, and in Europe also by *Borrelia garini* and *Borrelia afzeli*. Ticks of the Ixodes type are main carriers of *B. burgdorferi*. In Europe, *I. ricinus* and *I. persulcatus* are main carriers of *B. burgdorferi*, whereas in the United States *I. dammini* is present in the North-eastern states

and *I. pacificus* in the Western states. *I. neotomae*, *I. spinipalpis*, *I. hexagonus*, and *I. granulatus* ticks are of less importance in the transmission of *B. burgdorferi* [1–4].

Research done in Poland by Siński et al. revealed *B. burgdorferi* infection in 3.5% to 58.33% of ticks, depending on the region of Poland [3]. Wegner et al. documented infection in 11.5% of the *I. ricinus* ticks in the region of Olsztyn, and in 4.0% to 15.6% in the region of Białystok [5].

Received: 2000.08.28

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Accepted: 2001.09.28

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The tests performed by many authors, both in Poland and elsewhere, show that *B. burgdorferi* is commonly present in high risk groups, especially among forestry employees and farmers. Panciewicz et al. in 1993–1997 examined 1466 forestry employees from the northeastern region of Poland, and found *B. burgdorferi* antibodies in 23.81% of their subjects [6]. Hulsse C. von Stenglin found *B. burgdorferi* infection in 7.8% of the ticks in the Mecklenbug-Vorpommern region of Germany, and detected *B. burgdorferi* antibodies in 31.4% of forestry employees [7]. According to Rath et al, 53% of forestry workers reported tick bites, but erythema migrans appeared after the bite only in 8% of these persons. IgG antibodies to *B. burgdorferi* (immunoblot assay) were found in 18% of the persons examined [8]. Burek et al. discovered IgG antibodies to *B. burgdorferi* in 9.7% of their total research population, but in the group coming from a high risk region the figure rose to 44%, as against 8% in the group from a low risk region; moreover, antibodies were found in 42.9% of forestry workers [9].

The pathomechanisms controlling the course of Lyme borreliosis have not yet been fully explained. It is known, however, that the accompanying inflammatory states are characterized by the increased stimulation of phagocytes, which leads to the increased generation of reactive oxygen species (ROS) [10,11]. The presence of ROS causes modifications to occur in low- and macromolecular, endo- and exogenous compounds [10]. It has been proved that *B. burgdorferi* induces O<sub>2</sub> generation, and that ROS derived from macrophages, mainly nitrous oxide (NO), take part in the inactivation of *B. burgdorferi* spirochete [12–15]. The balance between free radicals and antioxidants is the main factor in the defense against harmful processes at the cellular and tissue levels. According to current findings, excessive ROS production in the organism and imbalance between the concentrations of ROS and defense antioxidants may be related to such pathologic processes as intestinal inflammation, joint inflammation, local ischemic processes, cardiovascular diseases, multiple sclerosis, or central and peripheral nervous system diseases [16–26].

The prevalence of Lyme borreliosis among the population in the northeastern region of Poland and difficulties in the treatment and prognosis of the course of the disease have prompted us to evaluate selected parameters of the antioxidant system and lipid peroxidation products in patients with erythe-

ma migrans, an early manifestation of Lyme borreliosis.

## MATERIAL AND METHODS

Twenty patients aged from 21 to 66 years ( $x=41.9$ ) treated for Lyme borreliosis in the form of erythema migrans (EM) were enrolled in the study. The diagnosis of the disease was based on the patients' epidemiological history and the characteristic clinical picture. The examinations were carried out from July to September of 1998. All the patients reported for treatment within 2 to 4 weeks after a tick bite. Skin changes of 5 to 30 cm in diameter and characteristic of EM were found on the patients' skin, primarily on the extremities. Four weeks of therapy with Amoxycycline at a dosage of 2.0 g daily resulted in complete remission of pathological skin changes.

IgM class antibodies to *B. burgdorferi* were measured in serum by means of the ELISA method, using Borrelia recombinant IgG and IgM High Sensitivity kits from Biomedica (Austria) within 4 to 6 weeks after treatment.

The control group consisted of 8 healthy individuals (2 women and 6 men) aged 21 to 45 years ( $x=31$  years).

Enzyme activity and the concentration of micromolecular substances were assessed twice: before Amoxycycline treatment (examination 1) and after 4 weeks of therapy (examination 2).

The activity of Cu, Zn-SOD (EC. 1.15.1.1) was determined by the method of Misra and Fridovich [27] as modified by Sykes et al. [24]. A standard curve for SOD activity was made by using SOD from bovine erythrocytes (Sigma Biochemicals, St. Louis, Missouri, USA). One unit of SOD was defined as the amount of the enzyme needed to inhibit epinephrine oxidation to adrenochrome by 50%. Enzyme activity was expressed in units per mg of protein for liver and serum, or per mg of hemoglobin for erythrocytes.

Glutathione peroxidase (EC. 1.11.1.6) activity was measured in the liver, erythrocytes and serum spectrophotometrically using a technique based on Paglia and Valentine [28], whereas GSH formation was assayed by measuring the conversion of NADPH to NADP. Enzyme activity was expressed as micromoles of NADP/min per mg of protein for the liver and per mg of hemoglobin for erythrocytes.

The activity of glutathione reductase (EC. 1.6.4.2) was measured by the method of Mize and Langdon, which involves monitoring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm [29].

Sulfhydryl compounds were estimated according to Ellman using 5,5'-dithiobis (nitrobenzoic acid, DTNB) [30].

The glutathione (GSH) concentration was measured using a Bioxytech GSH-400 test. This method proceeds in two steps. The first step leads to the formation of substitution products between a patented reagent and all the mercaptans (GSH) present in the sample. The second step transforms specifically the substitution product obtained with GSH into a chromophoric thione whose maximum absorbency wavelength is 400 nm.

Lipid peroxidation in liver, erythrocytes and serum was assayed using a Bioxytech LPO-586 kit that measures MDA together with 4-hydroxyalkanal or MDA alone. The colorimetric assay uses a chromogenic reagent which reacts with the products mentioned above, generating a stable chromophore which is measured spectrophotometrically at 586 nm. This technique requires sample incubation at 45°C, thus avoiding undesirable artifacts.

### Statistical analysis

The results of our examinations were analyzed statistically by calculating the arithmetic mean and standard deviation for each variable. The evaluation of statistical significance was performed by means of the Wilcoxon test for two related and unrelated trials, since the variables were not subjected to normal distribution. The value  $p < 0.05$  was taken as the limit of statistical significance.

**Table 1.** Activity of antioxidation parameters and peroxidation products (MDA) in patients with Erythema migrans before and after treatment and in healthy controls.

Enzymes examined	Examination 1	Examination 2	Controls
SOD (u/ml)	2.93±1.14	2.77±1.14	3.02±2.50
GSSG-R (u/ml)	26.46±11.88	29.57±5.28	24.87±10.73
GSH-Px (u/ml)	132.0±7.21	130.00±9.11	141.71±12.52
-SH (nmol/l)	152.52±19.58	183.30±53.22	198.83±65.02
GSH (nmol/l)	15.66±3.66	18.14±2.39	21.23±5.89
MDA (nmol/l)	15.66±4.21	14.21±3.99	4.76±3.13

## RESULTS

SOD activity in healthy controls was 3.02 u/ml, whereas in patients with erythema migrans, it was lower before treatment than in the controls, and decreased insignificantly after treatment, to 2.77 u/ml (Tables 1 and 2).

GSSG-R activity was insignificantly higher than in the controls, amounting to 26.46 u/ml in examination 1, before treatment. After 4 weeks' treatment with Amoxycycline this figure increased to 29.57 u/ml (Tables 1 and 2).

GSH-Px activity was insignificantly lower in both examination 1 and 2 in comparison with controls: 93.15% in examination 1, decreasing after treatment to 91.74% of the values found in controls (Tables 1 and 2).

The total concentration of SH-groups was significantly lower (76.71%) in examination 1 when compared to the concentration in the controls. After treatment, an insignificant increase to 183.3 nmol/l was observed in the total concentration of SH-groups, which again was significantly lower in comparison to the controls (92.19%) (Tables 1 and 2).

**Table 2.** Comparison of enzyme and micromolecular substance activity in patients with Erythema migrans before and after treatment in comparison with controls.

Enzymes examined		p=	p<
SOD	Test 1 - Test 2	0.72	0.8
	Test 1 - controls	0.0932	0.100
	Test 2 - controls	0.627	0.700
GSSG-R	Test 1 - Test 2	0.66	0.7
	Test 1 - controls	0.6437	0.700
	Test 2 - controls	0.381	0.400
GSH-Px	Test 1 - Test 2	0.75	0.8
	Test 1 - controls	0.0776	0.080
	Test 2 - controls	0.0336	0.05*
-SH	Test 1 - Test 2	0.02	0.05*
	Test 1 - controls	0.0119	0.02*
	Test 2 - controls	0.3393	0.400
GSH	Test 1 - Test 2	0.05	0.06
	Test 1 - controls	0.0076	0.008*
	Test 2 - controls	0.1675	0.200
MDA	Test 1 - Test 2	0.21	0.3
	Test 1 - controls	0.00001	0.0001*
	Test 2 - controls	0.00001	0.0001*

\* statistical significance  $p < 0.05$

The GSH concentration was 26.24% lower in comparison to controls in examination 1. After treatment, it increased insignificantly to 18.14 nmol/ml, and did not differ significantly when compared to healthy controls (Tables 1 and 2).

The mean MDA concentration was significantly higher in both examinations 1 and 2 than in the controls. In examination 1, before treatment, it was 328.99% of control value; after treatment it decreased insignificantly, but was still significantly higher than in the controls (298.53%).

## DISCUSSION

The skin lesion called erythema migrans is an early pathogenic symptom of Lyme disease, appearing from 10 days to several weeks after infection. Erythema migrans is present as a ring-like or homogeneous erythema at the site of *B. burgdorferi* spirochete penetration. Afterwards, it spreads concentrically in the intercellular matrix, which explains why erythema migrans is later so dispersed [4,31]

Patients with erythema migrans present with non-specific symptoms, such as malaise, fatigue, fever, cephalalgia, arthralgia and myalgia.

Neutrophils and macrophages belong to the most important elements of the host's defense system against the bacterial infection. The activation of neutrophils and macrophages greatly intensifies their metabolism, causing an increase in oxygen absorption, and in glucose, lipid, and protein metabolism, the production of proinflammatory cytokin, and the release of proteolytic enzymes from these cells, such as collagenase and elastase. Neutrophils are capable of producing active oxygen metabolites, which act as bactericidal agents, but when produced excessively they may cause harmful reactions [32].

Suhonen et al. examined oxidative burst, calcium mobilization and phagocytosis induced by *B. burgdorferi* proper, *B. afzeli* and *B. garini*. They proved that each genotype of *B. burgdorferi* induces all neutrophil functions, depending on the complement. The CR3 (CD11b) integrin was shown to have a role in the oxidative burst and calcium mobilization induced by *B. burgdorferi* [15].

Modolell et al. examined *B. burgdorferi*'s interaction with murine macrophages derived from bone marrow. They found that this interaction caused microorganized phagocytosis, the induction of ni-

tric oxide (NO) and oxygen free radicals, and the elimination of spirochete. The phagocytosis of *B. burgdorferi* by macrophages and the generation of NO and free oxygen radicals was intensified through spirochete opsonization with monoclonal antibodies. The addition of NO-synthesis specific inhibitors to macrophage and spirochete cultures, together or separately, caused only a partial reduction in the effector cell-killing potential. The data they obtained suggest that NO and oxygen free radicals are essential but insufficient for the complete elimination of *B. burgdorferi* by macrophages. The authors concluded that the defense against *B. burgdorferi* infection is associated with the humoral response, and that specific antibodies play an important role in the spirochete control mediated by macrophages [14].

Harter L. et al. stated that mRNA regulation of nitric oxide (NO) synthetasis, one of the free radicals, was part of the host's immune response to *B. burgdorferi* infection. They suggested that NO may play a role in arthritis in dogs [33].

Georgili et al. have proved that all *B. burgdorferi* strains have a different susceptibility to elimination through phagocytosis; however, all of them provoked oxidative burst [13].

Active oxygen metabolites, especially the hydroxyl radical, exhibit a high level of chemical activity, and in they organism they interact with proteins, lipids, carbohydrates and nucleinic acids, which causes changes in cell function and structure. However, the excessive release of these compounds by the cell may cause tissue damage. An excess of reactive oxygen species activates the mechanisms which immobilize them by means of enzymatic superoxide dismutase, catalase and glutathione peroxidation. The activity of oxygen reactive species is harmful when the antioxidant system is disturbed or when it is unable to eliminate the results of ROS activity [10,11,18].

The excess of ROS together with the inefficiency of the antioxidant mechanisms intensifies lipid peroxidation, causing a decrease in cell membrane flow, damage to the nucleinic acid structure, and enzyme inactivation. Lipid peroxidation is an avalanche process, continually supplying free radicals that initiate further peroxidation reactions. The products of lipid peroxidation and their reactions with other cell components cause changes in cell membrane properties, resulting in cell homeostasis disorder, which leads to cell death [10,11,18,32].

The results obtained in our studies indicate that there are changes in the serum antioxidant system of patients with Lyme borreliosis presenting in the form of erythema migrans. The activity of antioxidant enzymes (SOD and GSH-Px) in patients before treatment was significantly lower than in the controls. After treatment, despite complete remission of pathological changes, a further decrease was observed in the activity of these enzymes. The concentration of SH- and GSH- group micromolecular substances, which was significantly lower before treatment when compared to controls, increased after treatment, and did not differ significantly from the values of healthy individuals. The MDA concentration was significantly higher both before and after treatment than the values found in the controls.

These results indicate that beta-lactamase antibiotic therapy brings non-enzymatic antioxidant parameters to control values, though no change is observed in the enzymatic antioxidant parameters, resulting in the further activation of free radicals. This may suggest an ongoing asymptomatic pathological process, leading to the appearance of late borreliosis symptoms.

A constant decrease in the activation of glutathion peroxidase indicates a decreased capability to reduce lipid superoxidants, thus maintaining a high concentration of membrane lipid peroxidation products.

## CONCLUSIONS

1. In Lyme borreliosis presenting as erythema migrans, changes in the antioxidant system are observed in the plasma of patients, i.e. a decrease in the activity of SOD and GSH-Px antioxidant enzymes, a decrease in the concentration of antioxidant-SH and GSH non-enzymatic parameters, and an increase in MDA concentration.
2. Beta-lactamase antibiotic therapy brings non-enzymatic antioxidant parameters to control values, though it has no influence on enzymatic antioxidant parameters.
3. In erythema migrans, the decreased ability to reduce lipid superoxidants leads to maintaining a high concentration of membrane lipid peroxidation products.

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