Evaluation of serum levels of nitric oxide and its biomarkers in patients with Lyme borreliosis

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ABSTRACT

Purpose: In response to penetration by the spirochete *Borrelia burgdorferi*, neutrophils trigger the mechanisms of intracellular killing, including the generation of reactive oxygen and nitrogen species, e.g. superoxide anion radical and nitric oxide (NO). Overproduction of these reactive molecules leads to oxidative stress which contributes to lipid peroxidation and protein nitration. The major products of these processes include malonyldialdehyde (MDA) and nitrotyrosine, the biomarkers of NO action in the *in vivo* conditions. The study objective was to assess the serum levels of total nitric oxide and its biomarkers in patients with Lyme borreliosis before treatment, and to elucidate the relationship between these parameters.

Materials and Methods: Clinical types of the disease were considered: early (Erythema migrans - EM) and late (Lyme arthritis - LA). The serum level of total NO was determined by using Griess' reaction, MDA was measured by spectrophometry according to Buege and Aust, whereas nitrotyrosine by the immunoenzymatic ELISA method.

Results: The study revealed significantly higher serum levels of NO, MDA and nitrotyrosine in the two study groups of patients as compared to healthy people. The analysis of the results with respect to various types of borreliosis showed significantly higher levels of malonyldialdehyde in patients with EM as compared to those with LA. Moreover, a correlation was noted between the serum levels of total NO and nitrotyrosine in the early and late type of borreliosis.

Conclusions: High serum levels of total NO, MDA and nitrotyrosine observed in patients with Lyme borreliosis indicate on enhancement of lipid peroxidation and protein nitration, which in consequence may enhance the inflammatory process in patients with Lyme borreliosis.

Key words: Nitric oxide (NO), malonyldialdehyde (MDA), nitrotyrosine, Lyme borreliosis

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INTRODUCTION

Lyme borreliosis is a multiorgan disease transmitted by ticks of the genus *Ixodes* that carry the spirochete *Borrelia burgdorferi* [1]. This is a gram negative anaerobic bacterium, 0.3-0.5 μm in diameter and 20-30 μm long. The spiral shape and the presence of flagella promote its enhanced mobility and easy movement within the infected organism. Even when the spirochete lacks or has too few components that condition its growth, it is able to produce endospores, such as cysts (spheroplasts, L forms) or the so called blebs (gemmae) [2, 3].

The outer cell membrane of *Borrelia burgdorferi* contains proteins that differ in molecular weight, including surface antigens - OspA (30-32 kDa), -B (34-36 kDa) and -C (22-24 kDa) [2, 4]. *Borrelia burgdorferi* are able to modify immunological response through e.g. activation of lymphocytes, endothelial cells, neutrophils and macrophages. A major role in the immune response to the presence of the spirochete has been ascribed to reactive oxygen and nitrogen species, including superoxide anion radical and nitric oxide (NO). There is evidence that, in the bone marrow-derived macrophages, the OspA and OspB lipoproteins stimulate the production of superoxide anion radical and activate the inducible nitric oxide synthase (iNOS), responsible for the synthesis of a large amount of NO [2, 5, 6].

Many clinical and experimental findings have demonstrated that the role of nitric oxide in the functioning of the immune response depends on its concentration. At high concentrations, NO shows increased toxicity towards viruses, parasites and numerous bacteria, including *Borrelia burgdorferi* [6, 7, 8]. The cytotoxic effect of NO towards the spirochete indicates its significance for bacterial growth monitoring in the *in vivo* conditions.

The excessive production of reactive oxygen and nitrogen species leads to oxidative stress which contributes, among other things, to lipid peroxidation. This process, occurring in cascades, incessantly provides free radicals that initiate further peroxidation. Aldehydes, e.g. malonyldialdehyde (MDA), are among many compounds produced through peroxidation of polyunsaturated acids [9]. These compounds are less reactive than free radicals and can therefore diffuse over considerable distances in the cell, thus acting as "secondary transmitters" of injury induced by reactive oxygen and nitrogen species. Aldehydes modify physical properties of cell membranes, disturbing the hydrophobic nature of the lipid interior and impairing the two-layer membrane structure. The membranes can be discontinued, their fluidity and permeability can change, depolarisation or even lysis may occur. By affecting cell structures, aldehydes affect their normal functioning and cause dysfunction within the respective organs [10-12].

Nitrotyrosine is another very important oxidative stress marker generated through nitration of tyrosine phenol groups in tissues and blood proteins. The major nitrating agents include nitric oxide and strongly toxic peroxynitrite (V) which is a combination of NO and superoxide. Nitrotyrosine produced in this process can lead to the loss of biological functions of proteins, which may have pathological consequences. It has been suggested that the level of nitrotyrosine can be a useful marker for nitric oxide-mediated tissue injury in the *in vivo* conditions. Moreover, due to a longer half-life, nitrotyrosine can be regarded as a better marker of increased NO production than nitric oxide metabolites [13, 14].

Taking into account the bidirectional effects of NO, the study objective was to assess the serum levels of total nitric oxide and its biomarkers, malonyldialdehyde and nitrotyrosine, in patients with Lyme borreliosis, and to elucidate the relationships between these parameters in order to extend the knowledge on the potential role of NO and its biomarkers. A change in the level of nitric oxide and its biomarkers may have a substantial effect on defence reactions of the organism in response to the action of such pathogens as *Borrelia burgdorferi*, and can disturb the immune response to this pathogen.

MATERIALS AND METHODS

The study included 42 patients treated in the Department of Infectious Diseases and Neuroinfection of Medical University of Bialystok (Poland) diagnosed with Lyme borreliosis before treatment. 21 subjects (7 females and 14 males) were diagnosed with early stage (Erythema migrans - EM). The diagnosis of the disease was based on the patients's epidemiological history and the characteristic clinical picture. 21 subjects (15 females and 6 males) were diagnosed with late stage (Lyme arthritis - LA) of the disease. The diagnosis of of the disease was confirmed by epidemiological anamnesis, clinical manifestation and serological examinations results (detection of anti-B. *burgdorferi* IgM and IgG antibodies in the ezyme-linked immunosorbent assay [ELISA] method. In all cases, results of the ELISA test were confirmed by western blot testing.

Control subjects (n=15/males) were healthy people, aged from 25 – 40 years.

None of the patients and control subject had concomitant diseases such as diabetes mellitus, rheumatoid arthritis or other.

The study was approved by the Ethics Committee of the Medical University of Bialystok (R-I-002/107/2011).

Blood Sampling

Patients with Lyme disease and healthy control subjects were recruited into the study after obtaining their informed consent. Blood samples were taken from each patients before treatment. Five milliliters of fasting blood was collected by venous arm puncture under aseptic conditions. Serum was obtained by centrifugation at 2000×g for 5 min of blood samples taken without anticoagulant. Serum was kept at 20°C until the analysis date.

Determination of total NO (NO_3^-/NO_2^-) concentration in serum

Nitrite and nitrate are stable final products of NO metabolism and may be used as indirect markers of NO presence. Total NO concentration is commonly determined as a sum of nitrite and nitrate concentrations. NO concentration was determined using an indirect method based on measurement of nitrite concentration in serum according to Griess's reaction [15]. In the samples analyzed, nitrate were reduced to nitrite in the presence of cadmium (Sigma-Aldrich, Steinheim, Germany), and then converted to nitric acid that gave a color reaction with Griess's reagent (Sigma-Aldrich). Nitrite concentrations were determined by spectrophotometric analysis at 540 nm (UVN-340 ASYS Hitech GmbH microplate reader; Biogenet, Eugendorf, Austria) with reference to a standard curve. NO products were expressed as μmol/ml.

Determination of MDA concentration in serum

The evaluation of MDA in serum was done by the method of Buege and Aust [16]. The reaction is based on fusion of lipids oxidation products with thiobarbituric acid leading to creation of colored product. In the first step, 200 μl of 50% trichloroacetic acid (TCA) (Sigma-Aldrich) was added to 200 μl of serum, and then mixed until precipitation of proteins and centrifugated at 5000×g for 5 minutes. Following addition of 160 μl of thiobarbituric acid to 160 μl of supernatant collected after centrifugation, samples were incubated in a water bath at 90°C. After 30 minutes the colored product produced by the reaction of thiobarbituric acid with MDA was measured colorimetrically at 533 nm using DU SERIES 600 spectrophotometer (BECKMAN, USA). The obtained results were expressed as nmol/ml.

Determination of nitrotyrosine concentration in serum

The nitrotyrosine level in serum was assessed by sandwich enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Nitrotyrosine ELISA kit, Hycult biotechnology b.v., PB Uden, Netherlands). Before performing the assay, serums were diluted 10 times with dilution buffer (protein stabilized phosphate buffered saline, containing 2-chloroacetamide as preservative). Nitrotyrosine standards or serums (100 μl) were pipetted into an antibody-coated 96-well plate and incubated at room temperature for 1 hour. The wells were then washed four times with wash buffer (containing Tween-20), 100 μl of anti-human nitrotyrosine antibody was added, and the samples were again incubated for 1 hour at room temperature. The plate was washed four times, 100 μl of streptavidin-peroxidase conjugated was then applied for 1 h at room temperature. After a final washing, 100 μl of tetramethylbenzidine (TMB) substrate was added and allowed to develop colour for 20–30 minutes in the dark at room temperature. After stopping the reaction with stop solution (containing citric acid, 2.0 M.) (100 μl), absorbance was read at 450 nm with a UVN-340 ASYS Hitech GmbH microplate reader (Biogenet). The sample concentration was calculated from the standard curve. The obtained results were expressed as nmol/l.

Statistical evaluation

The data obtained were subjected to statistical analysis using Microsoft Excel calculation sheet and STATISTICA version 9.1 (StatSoft, Inc., Tulsa, OK, USA). Data are presented as median and ranges (minimum and maximum). Data distribution normality was determined using Kolmogorov-Smirnov test. Since the data were not normally distributed, for comparison of variations between assayed groups, U-Mann-Whitney nonparametric tests were applied to unrelated results. A statistical significance level of $p<0.05$ was assumed. The correlation between variables was evaluated using Spearman's rank coefficient (r).

RESULTS

1. Total concentration of NO in the serum of patients with Lyme borreliosis and control group.

The serum levels of total nitric oxide in patients with Lyme disease were significantly higher as compared to the control group (Fig.1). No statistically significant differences were noted in the levels of total NO between patients with Erythema migrans and Lyme arthritis.

2. Malonyldialdehyde level in the serum of patients with Lyme borreliosis and control group.

The serum levels of malonyldialdehyde in patients with Lyme disease were found to be significantly higher than those in control group (Fig.2).
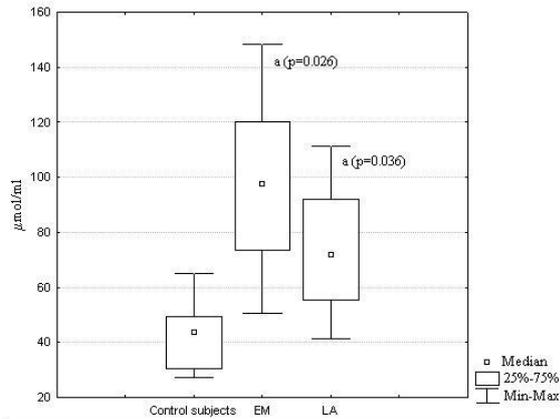
Figure 1. Concentrations of total NO in the serum of patients with Lyme disease. a - statistical differences with control subjects (p<0.05).

The analysis of the results with respect to various types of borreliosis showed significantly increased levels of MDA in patients with Erythema migrans as compared to those with Lyme arthritis.
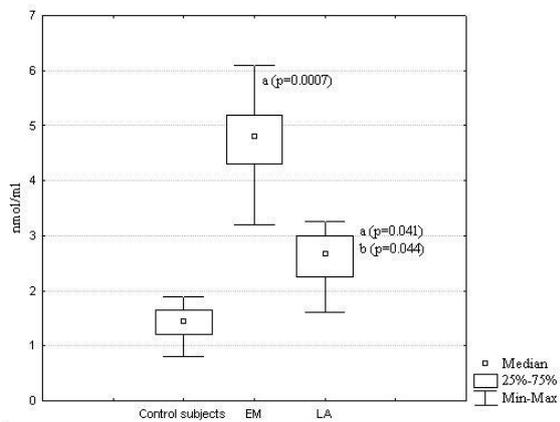

Figure 2. Serum levels of MDA in patients with Lyme borreliosis. a - statistical differences with control subjects (p<0.05); b - statistical differences between patients with Erythema migrans and patients with Lyme arthritis (p<0.05).

3. Nitrotyrosine level in the serum of patients with Lyme borreliosis and control group.

Serum levels of nitrotyrosine in patients with Lyme borreliosis were significantly higher in comparison with the control group (Fig.3). No statistically significant differences were observed in the levels of nitrotyrosine between patients with Erythema migrans and Lyme arthritis.

4. Assessment of relationships between the study parameters.

A correlation was observed between the serum levels of total NO and nitrotyrosine in patients with Erythema migrans (r=0.74, p=0.0001) (Fig.4) and Lyme arthritis (r=0.7, p=0.0005) (Fig.5).

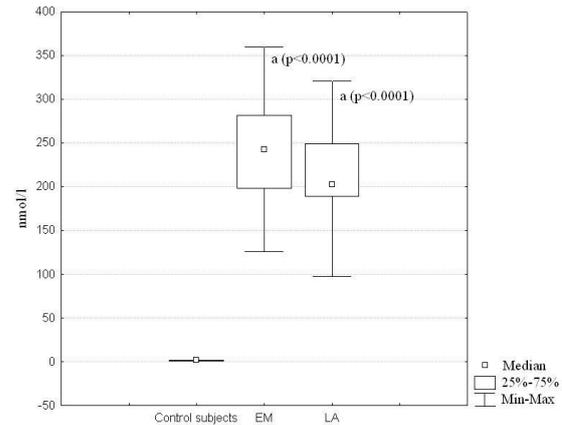**Figure 3.** Serum levels of nitrotyrosine in patients with Lyme borreliosis. a - statistical differences with control subjects (p<0.05)

However, no correlations were found between serum levels of total NO and MDA, and between MDA and nitrotyrosine in the two study groups of patients with Lyme borreliosis.
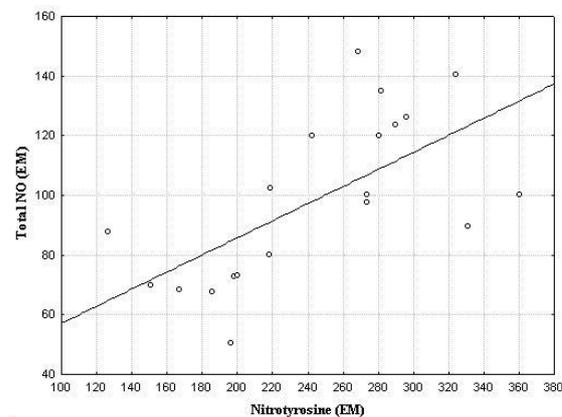

Figure 4. Correlations between total NO and nitrotyrosine concentrations in the serum of patients with EM.
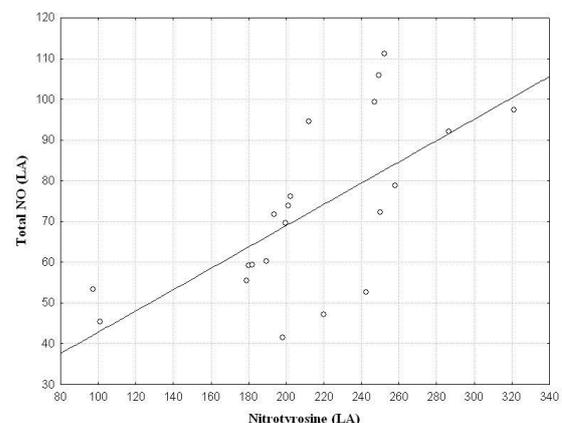

Figure 5. Correlations between total NO and nitrotyrosine concentrations in the serum of patients with LA.

DISCUSSION

Immunopathogenesis of Lyme borreliosis transmitted by the spirochete *Borrelia burgdorferi* is complicated and not yet fully elucidated. The in-depth understanding requires detailed knowledge of interactions between the spirocheta and the host. The studies conducted so far revealed that impairment of the host the non-specific immune mechanisms, associated with impaired migration, chemotaxis, as well as phagocytosis of neutrophils, occurs in the course of Lyme borreliosis [17].

High serum level of total NO in patients with Lyme disease, both in its early type (EM) and late type (LA), is a cause of the reduced activity of these cells. The available data suggest that the presence of NO can lead to functional alterations in these cells, associated with the ability to migrate and eliminate pathogens via phagocytosis. Insufficient activity of neutrophils can be compensated by the increased production of proinflammatory cytokines, e.g. IL–1, IL-6 and TNF–α, under the influence of NO [18, 19, 20]. Overexpression of these inflammatory mediators can promote a chronic inflammatory process observed in patients with borreliosis. In early localized Lyme disease clinically evident inflammation is limited to the skin. In Lyme arthritis joint symptoms are frequently accompanied by systemic symptoms, indicating that the response of the body is not limited to intra-articular space [21, 22].

The excess of NO seems to play a special role in LA. It has been shown that chondrocytes producing NO in the joints, along with eicosanoids and cytokines, activate collagenase directly or indirectly, decrease synthesis of collagen and proteoglycans, inhibit proliferation of chondrocytes and induce their apoptosis, which in consequence leads to the degradation of the articular cartilage [23]. However, we found no differences in the level of NO between patients with LA and EM.

On the other hand, we noted higher NO levels in patients with EM, which was accompanied by similar alterations in MDA levels.

Higher serum levels of MDA in patients with Erythema migrans as compared to Lyme arthritis may result from reduced total antioxidative potential of the peripheral blood in the early stage of EM, which cannot prevent uncontrolled free-radical oxidation [9, 24].

This has been confirmed by the results reported by Pancewicz et al. from patients with Erythema migrans. The authors showed high serum levels of MDA in EM patients as compared to the control group, with a simultaneous decrease in the activity of SOD and GSH-Px enzymes [25].

The increased serum levels of MDA in patients with EM and LA suggest enhancement of lipid peroxidation in both groups of patients. Lack of a direct correlation between NO and MDA indicates the involvement of other factors in MDA production in this group of patients.

Similar results were obtained by Luczaj et al. who observed a high level of MDA in plasma of LA patients [26].

There are reports regarding presence of high concentrations of MDA in serum as well as in cerebrospinal fluid in patients with neuroborreliosis in the form of meningitis [27].

High serum levels of MDA in EM and LA patients may cause morphological and functional changes in a number of cells, leading to dysfunctions of such organs as liver, kidneys or lungs [9, 24, 28, 29]. It is believed that MDA exerts cytotoxic, mutagenic and carcinogenic effects. MDA reaction with nucleobases of the nucleic acids triggers disintegration of the cell nucleus, inhibition of mitosis and synthesis of DNA, RNA as well as proteins. Moreover, the increased level of MDA may lead to the deposition of lipofuscin pigments inside the cell, and finally to its death [24, 30, 31].

The presence of considerable amounts of NO in the serum of patients with Lyme borreliosis may lead to high levels of nitrotyrosine, which is confirmed by the direct correlation revealed between NO and nitrotyrosine.

Tyrosine nitration is usually irreversible and may inhibit its phosphorylation and increase protein sensitivity to the action of proteolytic enzymes [32].

High serum levels of total NO accompanied by increased concentration of nitrotyrosine in patients with EM and LA indicate on important role of NO in the process of nitration in patients with Lyme borreliosis. Increase in NO and nitrotyrosine serum levels, may enhance the inflammatory process in EM and LA patients [32, 33].

In the current literature there are few reports regarding concentrations of nitrotyrosine in disease entities caused by bacteria. Kwiatkowska et al. showed, inter alia, high concentrations of nitrotyrosine in serum of patients infected with Mycobacterium tuberculosis [34].

CONCLUSIONS

High serum levels of NO, MDA and nitrotyrosine observed in patients with Lyme borreliosis before treatment indicate on the enhancement of lipid peroxidation and protein nitration, which in consequence may enhance the inflammatory process in Lyme borreliosis patients. Further studies extended by the assessment of NO, MDA and nitrotyrosine after treatment might show their potential clinical diagnostic and prognostic values in Lyme borreliosis.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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