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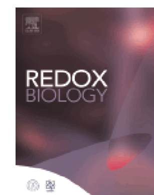

Research Paper

New insights into Lyme disease

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ABSTRACT

Lyme borreliosis is transmitted through the bite of a tick that is infected by the bacterial spirochete *Borrelia burgdorferi*. Clinical manifestation of the disease can lead to heart conditions, neurological disorders, and inflammatory disorders. Oxidative stress has been implicated in the pathogenesis of many human diseases. The aim of this study was to investigate the mechanisms of oxidative stress and intracellular communication in Lyme borreliosis patients. Mitochondrial superoxide and cytosolic ionized calcium was measured in peripheral blood mononuclear cells (PBMCs) of Lyme borreliosis patients and healthy controls. Mitochondrial superoxide levels were significantly higher ($p < 0.0001$) in Lyme borreliosis patients ($n = 32$) as compared to healthy controls ($n = 30$). Significantly low ($p < 0.0001$) levels of cytosolic ionized calcium were also observed in Lyme borreliosis patients ($n = 11$) when compared to healthy controls ($n = 11$). These results indicate that there is an imbalance of reactive oxygen species and cytosolic calcium in Lyme borreliosis patients. The results further suggest that oxidative stress and interrupted intracellular communication may ultimately contribute to a condition of mitochondrial dysfunction in the immune cells of Lyme borreliosis patients.

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Introduction

In North America, *Borrelia burgdorferi* is the predominant bacterial species responsible for infection leading to the emerging health threat of Lyme borreliosis (Lyme disease) [1,2]. In 2013 there were more than twenty-five thousand new cases of Lyme borreliosis reported by the Centers for Disease Control and Prevention (CDC) across the United States [3]. The currently accepted practice for clinical diagnosis of Lyme disease is using the two-tier testing of ELISA and Western Blot analysis [4]. These tests are limited in both sensitivity and specificity, often providing both false negative and false positive results [5–8]. To overcome this limitation, our laboratory has developed an enhanced T cell-based immunospot assay which bridges the gap between the ability to detect humoral immunity and cellular immunity to *B. burgdorferi*. We have been able to simultaneously increase the number of true positive diagnoses while decreasing the number of false positive and negative results [9].

When an infected tick bites a host, *B. burgdorferi* is transmitted through the infected tick's saliva. Once transferred, *B. burgdorferi* stimulates the host's immune system to activate a localized inflammatory response [10]. Consequently the infection often presents itself by the presence of a “bulls-eye” rash called erythema migrans (EM) within 3–30 days post infection [11]. Once infected, *B. burgdorferi* disseminates and causes a variety of immunological and inflammatory reactions throughout the body. Early manifestations of infection can lead to heart complications (e.g. carditis, dizziness, palpitations), neurological disorders (e.g. Bell's and/or cranial nerve palsy, peripheral neuropathy), and other inflammatory disorders (e.g. head and neck aches (meningitis), arthritis) [11]. If treatment is ineffective (Post-Treatment Lyme Disease Syndrome) or if infected individuals remain undiagnosed and untreated, some symptoms can persist for months to years. These symptoms may include muscular pains, arthritis, neurological disorders, fatigue, etc. [10,11].

In order to combat an infection the host's immune cells will generate reactive oxygen species (ROS) through NADPH Oxidase (NOX; producing superoxide anion radical), and nitric oxide synthase (NOS; producing nitric oxide) [12]. The predominant generator of ROS within the cells is the mitochondria and it is believed that the major contributor to cellular oxidative damage is

Abbreviations: PBMCs, peripheral blood mononuclear cells; HBSS, Hanks' balanced salt solution; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; CDC, centers for disease control and prevention; ELISA, enzyme-linked immunosorbent assay; EM, erythema migrans; fMLP, formyl-methionyl-leucyl-ribose; IL, interleukin; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma; TGF β, transforming growth factor beta; NF-κB, nuclear factor Kappa-light-chain-enhancer of activated B cells

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mitochondrial superoxide [13]. Upon the generation of superoxide ($O_2^{-\bullet}$) and nitric oxide (NO), the reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$) can be formed. An example of this process is the neutrophil defense mechanism of oxidative burst which results in the mobilization of calcium and activation of NADPH oxidase leading to the subsequent generation of superoxide. Superoxide dismutase (SOD) then converts superoxide to hydrogen peroxide (H_2O_2), which is bactericidal [14]. These reactive species are normally kept in balance by endogenous antioxidant enzymes such as SOD and glutathione peroxidase which converts H_2O_2 to water [15]. However, if an imbalance occurs between ROS/RNS and the antioxidant enzymes, oxidative stress will ensue causing a toxic environment that can lead to damage of DNA, protein, and lipids [16]. In addition to creating a toxic environment for pathogens, ROS and RNS activate NF-κB. One of the major roles of the NF-κB pathway is generation of pro-inflammatory cytokines such as Interleukins 1 & 6, TNFα, and IFNγ [17]. Individuals infected by *B. burgdorferi* present with significantly increased levels of TNFα in their sera and synovial fluid [18,19]. This observation is similar to what is found in patients diagnosed with rheumatoid, suppurative, and reactive arthritis [19]. A number of studies have shown that in vitro stimulation of an infected individuals immune cells by either *B. burgdorferi* or associated proteins results in an induction of pro-inflammatory cytokines (IL-1β, IL-6, IL-17, IL-23, TNFα, and TGF β) [19–22]. Importantly, studies have shown that during an active Lyme infection or *with* in vitro stimulation with *B. burgdorferi*, both NOS and ROS are generated [22,23].

However, the specific mechanisms of how these reactive species interact with and change intracellular communication of immune cells during an infection by *B. burgdorferi* are still unknown. In a previous study we addressed antigen specific T cell response to *B. burgdorferi* by measuring release of IFNγ [9]. The goal of this study was to explore the immune stimulated, inflammatory response to the oxidative stress state in PBMCs of Lyme borreliosis patients. To accomplish this we compared levels of mitochondrial superoxide and cytosolic ionized calcium in Lyme borreliosis patients with those in healthy controls.

Materials and methods

Reagents

Unless otherwise stated, all reagents were purchased from Sigma Aldrich (St. Louis, MO).

Clinical study population

Healthy control subjects in this study were either healthy adults without known inflammatory conditions or history of *Borrelia* infection. Subjects suspected for Lyme borreliosis infection were classified by CDC surveillance definition of Lyme disease, including clinical signs and symptoms, history of possible exposure to infected blacklegged ticks, with or without a positive antibody response to B. burgdorferi by ELISA and Western Blot, interpreted according to CDC and the Infectious Disease Society of America (IDSA) criteria [11]. In addition, any subject known to be on antibiotic therapy was omitted from this study. Study subjects were tested further to confirm their negativity or positivity of *B. burgdorferi* infection by a Lyme ELISpot assay [9]. Collection of blood, isolation of PBMCs, and determination of infection by *B. burgdorferi* were all performed as described previously [9]. All individuals gave their informed consent. The studies were performed following a protocol approved by the internal clinical ethics committee.

Mitochondrial superoxide

Levels of mitochondrial superoxide, in PBMCs, were measured using the fluorogenic dye MitoSOXTM Red (Life technologies; Eugen, OR). Measurements were made following the manufacturer's suggested protocol with slight modifications. Isolated PBMCs were incubated with MitoSOXTM Red for 10 min at 37 °C with 5% CO_2 at a ratio of 5 fmol MitoSOX per Cell, in Hanks' Balanced Salt Solution (HBSS). Cells were then washed once in HBSS at room temperature with centrifugation at 250*g* for 5 min followed by resuspension in HBSS. Fluorescence was measured at 37 °C with an excitation λ of 510 nm and an emission λ at 595 nm.

Cytosolic ionized calcium

Cytosolic ionized calcium (Ca_i^{2+}) levels were measured in PBMCs by using the calcium specific fluorogenic dye Fura2 AM ester (Molecular Probes/Life technologies; Eugen, OR). Measurements were made following the manufacturer's suggested protocol and for details read work of Carruthers et al. [27]. Briefly, isolated PBMCs were incubated with Fura2 for 30 min at room temperature at a ratio of 0.5 fmol Fura2 per Cell, in HBSS. Cells were then washed three times in HBSS, at room temperature, with centrifugation at 250*g* for 5 min, once the washes were completed cells were resuspended in HBSS and incubated at room temperature for 10 min to allow de-esterification of AM ester by intracellular esterase. Cells were washed three times again as before to remove any dye that may have leaked. Fluorescence was measured at room temperature with an excitation λ of either 335 nm (Ca_i^{2+} Bound Fura2) or 363 nm (Ca_i^{2+} Free Fura2) and an emission λ being read at 510 nm. Calibrators were used for each subject to measure the minimal (Ca_i^{2+} Free Fura2) and maximal (Ca_i^{2+} Bound Fura2) signals. The calibration for measuring the minimal level was achieved by post-de-esterification addition of 10 mM EGTA and 0.05% Triton X100. The calibration for measuring the maximal level was achieved by post-de-esterification addition of 5 μM Ionomycin and 20 mM Calcium. Assuming the K_d of Ca_i^{2+} -Fura2 at room temperature in the cytosol to be 143 nM the equation suggested by the manufacturer was used to determine [Ca_i^{2+}].

Statistical analysis

Student's *t* test was used to compare results of healthy controls with Lyme borreliosis patients. A *p*-value of < 0.05 was considered statistically significant. The analyses were done by Prism 6.0 analysis software (GraphPad Software Inc., La Jolla, CA).

Results and discussion

Excessive mitochondrial superoxide is believed to be one of the main contributors to oxidative stress and damage within the cell. Oxidative stress can damage DNA, proteins, and lipids, this damage has been proposed to contribute to diseases and disorders such as cancer, Parkinson's, Alzheimer's, atherosclerosis, chronic fatigue syndrome, and possibly Lyme borreliosis [12,17]. Our goal was to assess if mitochondrial superoxide is a contributing factor to oxidative stress within PBMCs of Lyme borreliosis patients (Table 1A). We measured the level of mitochondrial superoxide in PBMCs using the mitochondrial targeted and superoxide specific fluorogenic dye MitoSOXTM Red. Significantly higher levels of mitochondrial superoxide were observed in Lyme borreliosis patients when compared to healthy controls (Fig. 1).

A feed forward-cycle during pathophysiological conditions has been proposed by Dikalov et al. in which they suggest that “NADPH oxidases increase mitochondrial ROS, which further

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