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Subclinical Lyme borreliosis is common in south-eastern Sweden and may be distinguished from Lyme neuroborreliosis by sex, age and specific immune marker patterns

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ABSTRACT

Background: Determinants of a subclinical course of Lyme borreliosis (LB) remain largely unknown. The aim of this study was to assess the extent, sex and age profiles of subclinical *Borrelia* seroconversion in a LB endemic area in Sweden and to map blood cellular *Borrelia*-specific immune marker patterns in individuals with a previous subclinical LB course compared with patients previously diagnosed with Lyme neuroborreliosis (LNB).
Methods: A large group of 1113 healthy blood donors was screened for multiple IgG anti-*Borrelia* antibodies and asked to complete a health inquiry regarding previous LB. A group of subjects with anti-*Borrelia*-specific IgG antibodies but no previous history of LB (subclinical LB, n = 60) was identified together with 22 cases of previous LNB. Whole *Borrelia* spirochetes, strains *B. afzelii* ACA1 and *B. garinii* Ip90, were used for *ex vivo* whole blood stimulations, whereas outer surface protein enriched fractions of the same strains were used for stimulation of peripheral blood mononuclear cells (PBMCs). An extensive panel of immune markers was analysed in the supernatants after stimulation using multiplex bead arrays, and *Borrelia*-specific secretion was determined by subtracting the spontaneous secretion.
Results: A total of 125/1113 blood donors reported previous clinical LB. In contrast, 66 donors denied previous LB but showed multiple IgG anti-*Borrelia* antibodies; these were defined as subclinical subjects, of whom 60 were available for further studies. The subclinical subjects consisted of significantly more men and had a younger age compared with the LNB patients ($p \leq 0.01$). Discriminant analysis revealed a distinct pattern of sex, age and PBMC *B. garinii*-specific levels of IL-10, IL-17A and CCL20 discriminating subclinical subjects from LNB patients.
Conclusions: This study confirms that subclinical *Borrelia* seroconversion is common in south-eastern Sweden. The findings further suggest that male sex, younger age together with *B. gariniii* induced levels of IL-10, IL-17A and CCL20 may be associated with a subclinical course.

1. Introduction

Lyme borreliosis (LB) is the most common tick-borne infection in North America and Europe. LB is caused by a group of spirochetes, *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.). Genospecies of the bacteria that are known to cause human disease include *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), *B. afzelii* and *B. garinii* and rarely other species such as *B. bissettiae, B. spielmanii* and *B. valaisiana* (Rauter and Hartung, 2005; Stanek et al., 2011; Strle and Stanek, 2009). *B. burgdorferi* s.s. constitutes the only known genospecies to cause LB in North America (Piesman and Gern, 2004). Clinically, LB can present a number of different manifestations of which localized skin infection, erythema migrans, is the most common in Europe as well as in North America. Lyme neuroborreliosis (LNB) has been reported as the second most common clinical manifestation (Berglund et al., 1995; Strle and Stanek, 2009). There is evidence that the different genospecies are more or less associated with the various clinical manifestations. For example, *B. burgdorferi* s.s. is often associated with arthritis, particularly in North America, whereas *B. afzelii* is associated with skin manifestations of LB and *B. garinii* with neurological symptoms, *i.e.*, LNB (Lünemann et al.,

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2001; Ohlenbusch et al., 1996; Rauter and Hartung, 2005).

Although infection with *B. burgdorferi* s.l. may cause clinical infections with symptoms and signs, *B. burgdorferi* s.l. infection may also be subclinical in a considerable proportion of cases, especially in Europe but also in the USA (Ekerfelt et al., 2001; Fahrer et al., 1991; Gustafson et al., 1990; Strle and Stanek, 2009). Even if the three main *Borrelia* genospecies show more or less organotropism, determinants for the individual clinical outcome, including a subclinical course, remain largely unknown. In a study comparing children with a history of previous LB and children with a positive *Borrelia* serology without a history of previous clinical LB, representing subclinical seroconversion, similar immune responsiveness to *B. burgdorferi* s.l. was observed (Skogman et al., 2012). We hypothesize that the clinical outcome is determined by the individual, its genetic predisposition and the interplay between the tick, the infecting *Borrelia* strain and the sum of immune responses evoked, including both innate and adaptive immunity. Furthermore, we believe that the characteristics of this immune response may be determined analyzing *Borrelia*-induced cyto- and chemokine patterns rather than analyzing single cytokines one at a time.

The aim of this study was to screen blood donors in an LB-endemic area for signs of previous subclinical *Borrelia* seroconversion, and to characterize the *Borrelia*-induced immune response pattern in these subclinical individuals compared with patients previously diagnosed with LNB.

2. Materials and methods

2.1. Study subjects

2.1.1. Blood donors

A total of 1126 healthy blood donors in Kalmar County in Sweden were consecutively included in the study in the spring of 2012 when scheduled for routine blood donation. Blood donor sera were collected together with health inquiries including information on previous tick exposure, previous history of LB and the treatment thereof. A complete set of health inquiries and blood sera was obtained from 1113 blood donors. Blood donors were placed in three groups depending on whether they answered yes, no or undetermined to a previous history of LB. To ensure a higher level of certainty of *Borrelia* exposure, the blood donors answering yes to a previous history of LB also needed a clinical diagnosis to be placed in this group.

Blood donor sera were screened for IgG antibodies specific for several borrelial antigens using a commercial multiplex kit, recomBead Borrelia IgG (Mikrogen GmbH, Neuried, Germany) (Anonymous, 2012). Analyses were performed according to the manufacturer's instructions, whereby each specific IgG antibody-antigen reaction corresponds to 4 points and a low-level reaction corresponds to 1 point. The results of the individual antigen reactivities were added to a final sum. According to the manufacturer, a total sum of 8 or more points was regarded as a positive test result. To ensure an even higher specificity and to rule out crossreactive antibodies, a sum of at least 12 points was interpreted as a positive test result and used as an inclusion criterion for blood donors in this study. Blood donors who denied a previous LB with serological test results of at least 12 points (n = 66) were regarded as previously subclinically *Borrelia* seroconverted individuals, hereafter referred to as subclinical subjects. All 66 subclinical subjects were invited for follow-up blood sampling according to the study protocol. Six subclinical subjects did not continue their participation in the study, among which four did not attend the follow-up visit, one was not reachable to schedule follow-up sampling, and one was excluded due to the onset of chronic myeloid leukaemia. Blood sera, heparinized blood and health inquiries were collected in the spring of 2013 from the remaining 60 subclinical subjects. Blood sera were re-analysed for multiple IgG anti-*Borrelia* antibodies using the same commercial multiplex kit, recomBead Borrelia IgG (Mikrogen), used previously. Analyses and scoring were performed according to the manufacturer's instructions.

2.1.2. Patients with previous Lyme neuroborreliosis

From the laboratory database in Kalmar County, 40 patients with laboratory results consistent with LNB were identified between 1 January 2008 and 30 June 2012 and asked if they wanted to participate in the study. Inclusion criteria were lumbar puncture with the presence of intrathecal IgM and/or IgG *Borrelia* antibodies in cerebrospinal fluid analysed with IDEIA™ Lyme Neuroborreliosis (Oxoid Limited, Hampshire, UK) together with pleocytosis of the cerebrospinal fluid (defined as a total leucocyte count $> 5 \times 10^6$/L). Twenty-two patients agreed to participate, and blood sera, heparinized blood and health inquiries were collected simultaneously with subclinical subjects in the spring of 2013 and in a similar manner.

2.2. Stimulation and analysis procedures

2.2.1. Whole blood

Borrelia spirochetes: *B. afzelii* strain ACA1 and *B. garinii* strain Ip90 spirochetes (kindly provided by professor Sven Bergström, Department of Microbiology, University of Umeå) were grown in Barbour Stoenner Kelly II (BSKII) medium supplemented with 7% rabbit serum (Department of Microbiology, University of Umeå, Sweden) at 37 °C without CO_2. Spirochetes were counted in a Bürcher chamber using phase-contrast microscopy. Upon stimulation, the spirochetes were diluted in RPMI 1640 (Gibco, Paisley, United Kingdom) with 10% human AB serum (Department of Transfusion Medicine, University Hospital, Linköping, Sweden) to a density of 200×10^6/mL.

Whole blood stimulation: Whole blood was collected in sodium-heparin vacuum tubes (Becton, Dickinson and company, Swindon, UK) from 60 subclinical subjects and 22 LNB patients. Five millilitres of heparinized blood was washed twice with sterile phosphate-buffered saline (PBS) and centrifuged at 400 x g for 10 min at room temperature (RT). The supernatant was then removed, and the cell pellet was suspended in 10 mL of RPMI 1640 with 10% human AB serum. Two millilitres of the cell suspension was added to three wells in a six-well culture plate (Prima plate, Falcon, Fischer Scientific, Gothenburg, Sweden). To two wells, 20×10^6 spirochetes of strains ACA1 and Ip90 were added. Control cells from each subject were cultured with only RPMI 1640 with 10% human AB serum. The cells were cultured at 37 °C with 5% CO_2 and 95% relative humidity for 24 h. The supernatant was collected after centrifugation at $1000 \times g$ for 10 min and stored at −70 °C until further analysis.

Luminex analyses of whole blood supernatants: Supernatants from *Borrelia*-stimulated whole blood were thawed and centrifuged at $2000 \times g$ for 10 min at 7 °C to avoid clogging during analysis. The supernatants were analysed for the presence of interferon gamma (IFN-γ), granulocyte macrophage colony-stimulation factor (GM-CSF), tumour necrosis factor (TNF), lymphotoxin-α (LT-α), chemokine (C−C motif) ligand (CCL) 20 (macrophage inflammatory protein, MIP-3α), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-17F, IL-21, IL-22, IL-23, (IL-17E)-IL-25, IL-27, IL-28a, IL-31, and IL-33 (Milliplex MAP Human Th17 Magnetic Bead 25-plex, #HTH17MAG-14K, Millipore Corporation, Billerica, MA, USA), CCL17 (thymus and activation regulated chemokine, TARC) (Milliplex MAP Human Cytokine/Chemokine panel II 1-plex, #HCYP2MAG-62K, Millipore Corporation), chemokine (C-X-C motif) ligand (CXCL) 9 and CXCL11 (interferon-inducible T-cell alpha chemoattractant, ITAC) (Milliplex MAP Human Cytokine/Chemokine magnetic bead panel III 2-plex, #HCYP3MAG-63K, Millipore Corporation), chemokine (C-X3-C motif) ligand (CX3CL) 1 (fractalkine), IFNα2, CXCL1-3 (growth-regulated protein, Gro), CCL22 (macrophage-derived chemokine, MDC), CXCL8/IL-8 and CXCL10 (interferon gamma-induced protein, IP 10) (Milliplex MAP Human Cytokine/Chemokine Magnetic Bead 6-plex, #HCYTOMAG-60K, Millipore Corporation). All procedures were performed according to the manufacturer's instructions, and the samples were analysed on a Luminex 200™. The median fluorescent intensity (MFI) was analysed with xPONENT 3.1 (Luminex Corporation, Austin,