ARTHRITIS & RHEUMATISM Volume 36
Number 11, November 1993, pp 1621–1626

PERSISTENCE OF *BORRELIA BURGDORFERI* IN LIGAMENTOUS TISSUE FROM A PATIENT WITH CHRONIC LYME BORRELIOSIS

THOMAS HÄUPL, GABRIELE HAHN, MICHAEL RITTIG, ANDREAS KRAUSE, CHRISTOPH SCHOERNER, ULRICH SCHÖNHERR, JOACHIM R. KALDEN, and GERD R. BURMESTER

Objective. **To document the persistence of *Borrelia burgdorferi* in ligamentous tissue samples obtained from a woman with chronic Lyme borreliosis.**

Methods. **Spirochetes were isolated from samples of ligamentous tissue, and the spirochetes were characterized antigenetically and by molecular biology techniques. The ligamentous tissue was examined by electron microscopy. Humoral and cellular immune responses were analyzed.**

Results. **Choroiditis was the first recognized manifestation of Lyme disease in this patient. Despite antibiotic therapy, there was progression to a chronic stage, with multisystem manifestations. The initially significant immune system activation was followed by a loss of the specific humoral immune response and a decrease in the cellular immune response to *B burgdorferi* over the course of the disease. "Trigger finger" developed, and a portion of the flexor retinaculum obtained at surgery was cultured. Viable spirochetes were identified. Ultramorphologically, the spirochetes were situated between collagen fibers and along fibroblasts, some of which were deeply invaginated by these organisms. The cultured bacteria were identified as *B burgdorferi* by reactions with specific immune sera and monoclonal antibodies, and by polymerase chain reaction amplification and Southern blot hybridization techniques.**

Conclusion. **To our knowledge, this is the first report of the isolation of *B burgdorferi* from ligamentous tissue. This suggests that tendon tissues serve as a specific site of spirochete residence in human hosts.**

From the Institute of Clinical Immunology and Rheumatology, Department of Medicine III, the Institutes of Anatomy I, Clinical and Molecular Virology, and Clinical Microbiology, and the Department of Ophthalmology, University of Erlangen-Nuremberg, Erlangen, Germany.

Dr. Burmester's work was supported by the Federal Ministry of Research and Technology (BMFT) grant 01 KI 9104-I (*Borrelia burgdorferi*-Infecktion). Dr. Häupl is a fellow of the "Graduierten-Kolleg Infektion, Entzündung und Autoimmunität" of the "DFG" at the University of Erlangen-Nuremberg.

Thomas Häupl, MD: Institute of Clinical Immunology and Rheumatology, Department of Medicine III; Gabriele Hahn, MD: Institute of Clinical and Molecular Virology; Michael Rittig, MD: Institute of Anatomy I; Andreas Krause, MD: Institute of Clinical Immunology and Rheumatology, Department of Medicine III; Christoph Schoerner, MD: Institute of Clinical Microbiology; Ulrich Schönherr, MD: Department of Ophthalmology; Joachim R. Kalden, MD: Professor of Medicine, Institute of Clinical Immunology and Rheumatology, Department of Medicine III; Gerd R. Burmester, MD: Professor of Rheumatology and Clinical Immunology, Institute of Clinical Immunology and Rheumatology, Department of Medicine III.

Address reprint requests to Gerd R. Burmester, MD, Department of Medicine III, Krankenhausstrasse 12, 91054 Erlangen, Germany.

Submitted for publication December 23, 1992; accepted in revised form April 15, 1993.

Lyme disease is a multisystemic infectious disease caused by *Borrelia burgdorferi*. If it is not treated in the early stages, it may evolve to a chronic infection. *B burgdorferi* has been cultured from blood, cerebrospinal fluid (CSF), and several organs of patients with late-stage manifestations, even at the time of a clinical remission and after antibiotic therapy (1–3). The genetic background (4) or immune phenomena, such as cross-reactive antibodies against bacterial flagellin and a human axonal protein (5), may influence susceptibility to chronic Lyme borreliosis. However, the successful isolation of *B burgdorferi* from typical lesional sites of stages II and III disease suggests the chronicity is due to persistence of the microbe rather than "autoaggressive" mechanisms alone. Here, we describe a patient with chronic Lyme disease in whom

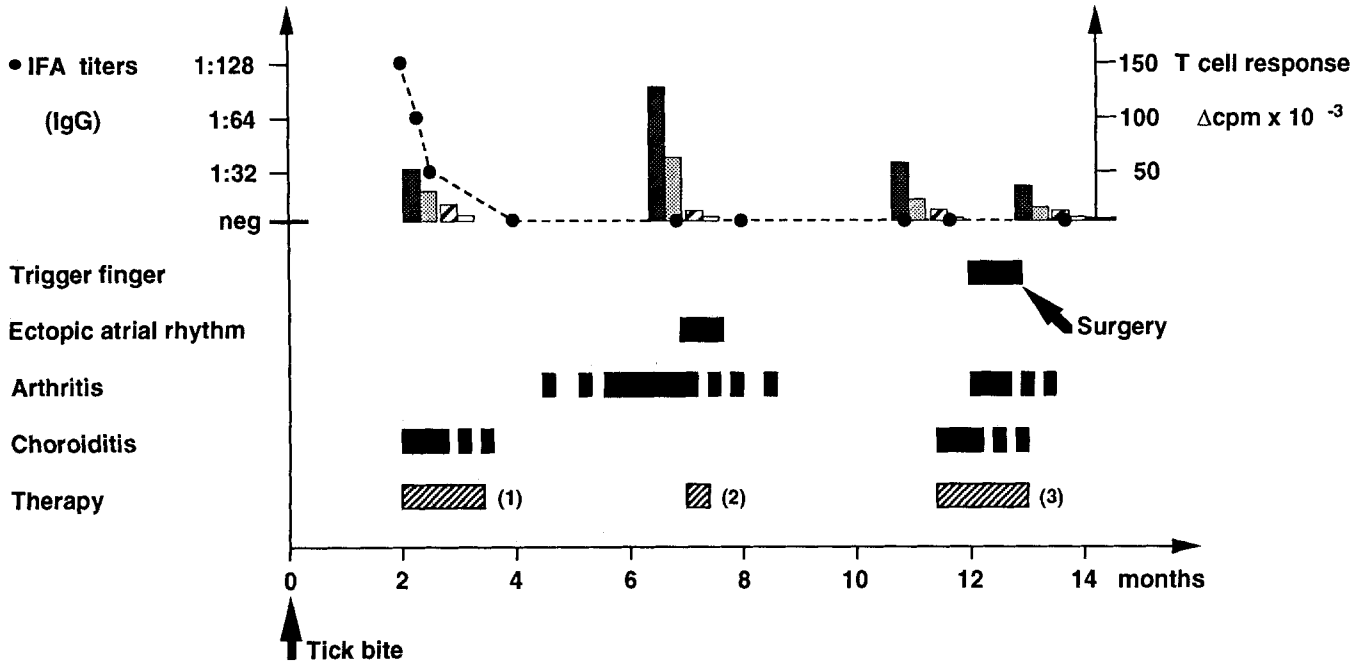


Figure 1. Course of clinical manifestations, therapeutic regimens, and specific immune responses in a patient with Lyme borreliosis. Antibiotic therapy consisted of 200 mg of doxycycline/day for the first course (**1**), 2 gm of ceftriaxone/day for the second course (**2**), and 300 mg of roxithromycin, 1,600 mg of sulfamethoxazole, and 320 mg of trimethoprim daily for the third course (**3**). Peripheral blood T cells were stimulated with whole *Borrelia burgdorferi* strain PKo 2-85 (▩) or with the 31-kd outer surface protein A (OspA) (▨). Stimulation with flagellin resulted in 22,000 Δcpm in the first assay, declining to 2,500 Δcpm subsequently. Cells from normal blood donors were run with each assay (▨ = stimulation with *B burgdorferi* strain PKo 2-85; □ = stimulation with OspA); mean values of all the controls were 13,400 Δcpm for *B burgdorferi*, 3,400 Δcpm for OspA, 10,000 Δcpm for the 42-kd protein, and 112,000 Δcpm for tetanus toxoid. Immunofluorescence assay (IFA) titers are plotted at the top.

B burgdorferi was cultured from samples of the flexor retinaculum.

CASE REPORT

The patient, a 48-year old woman, presented with progressive disturbance of the central vision in her right eye. Ophthalmoscopy demonstrated multifocal choroiditis, with 1 focus involving the macula lutea. The patient reported that 2 months previously, a tick had bitten her left lower leg, near the ankle, and she had experienced occipital headaches and a macular skin lesion. Serologic tests performed at our university revealed a positive IgG antibody titer against *B burgdorferi*. Other infectious diseases, particularly toxoplasmosis, were excluded by laboratory evaluations. The patient was treated with 200 mg/day of doxycycline (orally) for 6 weeks (Figure 1). The visual disturbance was ameliorated, the inflammatory foci of the choroid diminished, and scar tissue formed.

Four weeks after the end of antibiotic therapy, the patient began to experience brief episodes of an asymmetric arthritis, primarily involving the metacarpophalangeal (MCP) and proximal interphalangeal joints. Routine electrocardiography (EKG) revealed negative P waves, with an ectopic atrial pacemaker that had not been present on an EKG performed at gall bladder surgery 1 year previously. On ophthalmoscopic examination of the eye fundus, there was no evidence of a recurrence of the choroiditis.

Tests for antinuclear antibodies, immune complexes (by C1q binding assay and polyethylene glycol precipitation), rheumatoid factor (RF), immunoglobulins, serum complement levels, and C-reactive protein levels all gave normal or negative results. HLA phenotyping revealed HLA–A24/26;B7/27;Bw4/6;Cw2/7;DR15(DR2 split);DQ1. Despite the presence of the HLA–B27 antigen, there was no evidence of any typical manifestation of a seronegative spondylarthropathy.

Therapy was started with 2 gm of ceftriaxone, intravenously, for 14 days (Figure 1). Within the next 4 weeks, the ectopic atrial rhythm converted to a

normal sinus rhythm, and the arthritis disappeared. After 2 months free of clinical symptoms, the visual disturbances recurred. Ophthalmoscopy showed reactivation of the initial foci of choroiditis. Analysis of CSF demonstrated normal levels of albumin and IgG, no disturbance of the blood–CSF barrier, negative findings on immunofluorescence (IF) analysis for *B burgdorferi*–specific antibodies, and a normal cell count; thus, there was no evidence of an inflammatory process involving the central nervous system.

Antibiotic therapy with a combination of 300 mg of roxithromycin, 1,600 mg of sulfamethoxazole, and 320 mg of trimethoprim per day, which has been described as effective in several cases of advanced Lyme borreliosis (6,7), was initiated. However, tenosynovitis and mild arthralgia of the patient's hands occurred. Despite concurrent antibiotic therapy, "trigger thumb" developed within 2 weeks, accompanied by pronounced pain of the MCP joint, and surgical splitting of the flexor retinaculum was performed (Figure 1).

After exsanguination of the patient's right arm, surgery was performed in a bloodless field. The macroscopic appearance was typical of "trigger finger." A specimen of the altered ligament was obtained, with particular attention to avoiding surface contamination of the tissue sample. The specimen was rinsed several times in saline and medium, and was placed in culture with modified BSK medium. The patient's postoperative course was without complications, and normal functioning of the operated thumb was achieved. Six weeks after the course of antibiotics, the choroid foci were scarring. Unfortunately, the patient had an irreversible, 70% reduction of vision in the right eye. Approximately 3 weeks later, the arthralgia also disappeared. Currently, after approximately 2½ years of followup, there has been no evidence of reactivation of the Lyme borreliosis.

METHODS AND RESULTS

Immunofluorescence assay and findings. The IF assay was performed as described earlier, using the German isolate of *B burgdorferi*, PKo 2-85 (3,8). Serum samples were preabsorbed with *Treponema phagedenis* lyophilysate (Behringwerke, Marburg, Germany). For the detection of specific IgM antibodies, a second absorption step with RF absorbent (Behringwerke) was performed. Using this technique, titers in control sera were <1:16. In parallel, the serum samples were analyzed using a commercial *B burgdorferi* enzyme-linked immunosorbent assay (ELISA) kit (Viramed, Munich, Germany), with a protein preparation of B31 *Borrelia* as antigen. A positive, a negative, and a borderline control specimen served as internal standards. The ratio between the optical density of the serum samples versus that of the borderline specimen was used to quantitate the results. Ratios >1.0 were considered positive; those <1.0 were negative.

IF analysis revealed positive titers of IgG, but not IgM, antibody directed against *B burgdorferi* only during the early stage of infection when the patient presented with the first episode of choroiditis. Analysis by ELISA revealed comparable results, with specific IgG ratios of 1.15 at the onset of disease and 0.85 in the later stage. The IgG titer rapidly decreased within a few weeks after the first antibiotic therapy, and remained negative in both the IF and ELISA evaluations, despite progression of the disease.

Immunoblot analysis and findings. For immunoblot analysis, we used a method previously described (9), in which lysed specimens of whole *B burgdorferi* strain PKo 2-85 and LW2, the isolate from the patient (see below) (protein concentration 100 μg/ml), were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5 μg of protein per lane) in a 10% gel. Proteins were transferred to nitrocellulose and incubated with the sera at a dilution of 1:100, which we had previously found yielded optimum results. Bound immunoglobulins were visualized by application of peroxidase-conjugated reagents. Polyclonal antisera from patients with Lyme disease and monoclonal antibodies against outer surface protein A (OspA) and flagellin (the latter kindly provided by M. D. Kramer, Institute of Immunology and Serology, University of Heidelberg, Germany) were used to compare the isolated spirochete LW2 and the *B burgdorferi* strain PKo 2-85 by immunoblot.

All serum samples from the patient were tested against protein preparations of both the LW2 and the PKo 2-85 strain, on immunoblots. There were no significant differences in reactivity to the isolates. Upon repeated analysis of several consecutive serum samples, only nonspecific faint bands (75 kd, 41 kd, and 15 kd) were revealed, demonstrating a pattern different from that found in typical patients with stage III disease (10).

Preparation of mononuclear cells and results of lymphocyte proliferation assay. Peripheral blood mononuclear cell (PBMC) separation and antigen stimula-

tion were performed as described previously (8,9). Freshly isolated cells (10^5/well) were stimulated in triplicate with either 10^5 or 10^6 PKo 2-85 *B burgdorferi* per well, 10 μg/ml of recombinant OspA, 10 μg/ml recombinant flagellin (41-kd protein) from *B burgdorferi* (both expressed and purified as described elsewhere [9]), or 7 μg/well of *T phagedenis* lyophilysate. Previous studies had shown these to be optimal concentrations (8,9). Control wells received either medium alone or tetanus toxoid (10 μg/ml; Behringwerke). Samples from normal blood donors were run in each assay to exclude any nonspecific response. It has been clearly documented by appropriate separation experiments (9) that reactivity to *Borrelia* antigens under these conditions is T cell derived.

Stimulation of the patient's PBMC with the 2 strains of *B burgdorferi* as well as with OspA resulted in significantly elevated ^{3}H-thymidine uptake at all times tested (compared with normal PBMC) (Figure 1). Tetanus toxoid induced high levels of ^{3}H-thymidine uptake, between 143,000 and 181,000 Δcounts per minute (stimulation values in the presence of antigen minus those in the absence of antigen). In the earlier disease stages and prior to ceftriaxone therapy, proliferation to *Borrelia* antigens corresponded well to the clinical course, despite negative findings on IF and ELISA testing, reaching peak values at peak disease activity, as manifested by intermittent arthritis and cardiac involvement (Figure 1). After the ceftriaxone therapy and a 2-month symptom-free period, PBMC proliferation decreased, but during a period of minor inflammatory reactivation of the disease, was still elevated. The patient's symptoms at that time were choroiditis, mild arthralgia, and the development of "trigger finger." *B burgdorferi* was isolated from ligament samples.

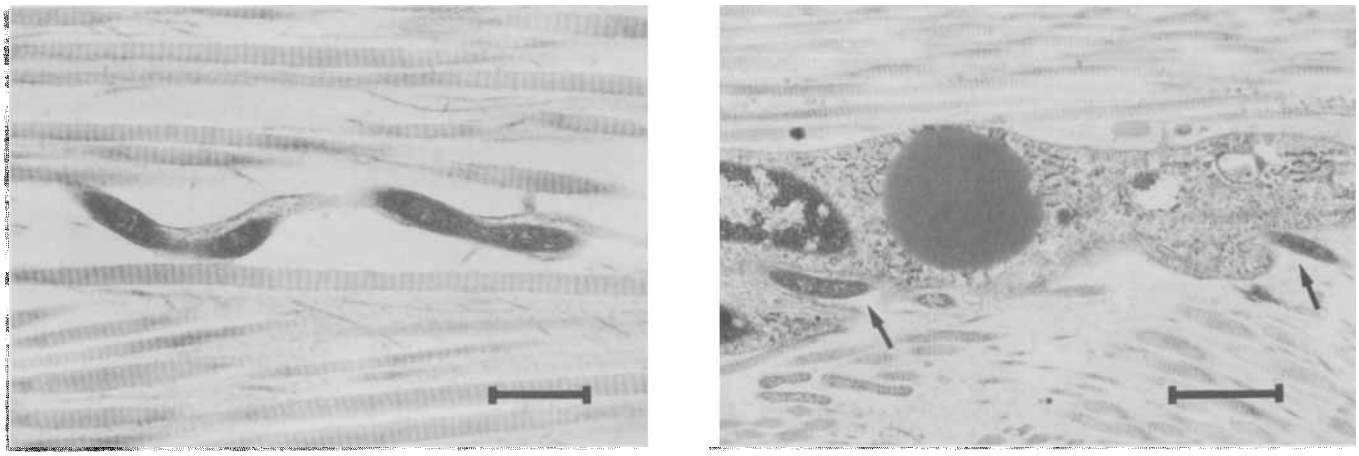
Isolation and characterization of *B burgdorferi* strain LW2. Tissue samples from the flexor retinaculum of the patient's right thumb were taken during surgery for the "trigger finger." Samples were cultured at 37°C under microaerophilic conditions, in modified BSK medium. Cultures were evaluated weekly for spirochetes in the supernatant, as detected by darkfield microscopy. After 3 weeks, viable spirochetes were seen. This particular spirochete, designated LW2, was used as antigen for immunoblot. *B burgdorferi*–specific immune sera and monoclonal antibodies against OspA and flagellin stained protein bands in a pattern comparable to that of the PKo 2-85 bacterial antigen (data not shown). An aliquot of this supernatant was examined for *B burgdorferi*–specific gene sequences by polymerase chain reaction (PCR) amplification and Southern blot.

PCR and Southern blot techniques. Cultured spirochetes from the patient's tissue specimen were investigated by standard PCR procedures (11). We used primers which amplified a 276-basepair segment (kb-ladder; Gibco BRL Life Technologies, Munich, Germany) of the 41-kd flagellin protein of *B burgdorferi* (primer 5′-TTCAGGGTCTCAAGCGTCTTGGACT-3′; reverse primer 5′-GCATTTTCAATTTTAGCAAGTGATG-3′) (12,13). The amplification protocol consisted of 40 cycles: 1-minute denaturation at 94°C, 30-second annealing at 50°C, and 1-minute extension at 67°C.

An amplification product, which could be hybridized by Southern blot technique with the ^{32}P-γ-ATP–labeled probe 5′-CTCTGGTGAGGGAGCTCAAACTGCTCAGGCTGCACCGGTTCAAGAGGGT-3′ (13) using Hybond-N nylon-blotting membranes (Amersham, Amersham, UK), was obtained. The amplimer, which was characterized by Picken (13), is derived from the central, *nonhomologous* region of the flagellin gene. It has been shown to be both specific for *B burgdorferi* and discriminatory for 3 groups of this spirochete, based on the nucleic acid sequence. Water, synovial tissue from a patient with rheumatoid arthritis, and *B burgdorferi* strain PKo 2-85 were used as negative and positive controls for the studies.

Electron microscopy techniques and results. Transmission electron microscopy was performed on the ligament tissues. The specimen was removed from the culture medium and prepared and analyzed as described previously (14). Semithin sections were cut from the plastic block for the light microscopic evaluation. Thin sections were cut from selected areas and placed onto copper grids (Polysciences, St. Goar, Germany). After counterstaining with 10% uranyl acetate, followed by 2.8% lead citrate (both from Merck, Darmstadt, Germany), sections were studied using Siemens EM 101 (Munich, Germany) and Zeiss EM 902 (Wetzlar, Germany) electron microscopes.

The ligament tissue was found to be heavily infiltrated by spirochetes. Some of the organisms lay between unaltered collagen fibers (Figure 2A); others were closely attached to the cell surface of the fibroblasts (Figure 2B). There were numerous fibroblasts deeply invaginated by the spirochetes, thereby creating membrane-bound cavities. These cavities appeared as vacuoles in transverse tissue sections.



A B

Figure 2. Electron micrographs of the cultured ligament section, showing **A,** a spirochete between unaltered collagen fibers, and **B,** invagination of a fibroblast by spirochetes (**arrows**). Bar = 10 μm.

DISCUSSION

The patient whose case is presented herein had relapsing Lyme borreliosis, with choroiditis, arthritis, carditis, and tendinitis. The humoral immune response correlated with neither the cellular reactivity in vitro nor the clinical activity of the disease manifestations. Repeated antibiotic treatment was necessary to stop the progression of disease, but obviously did not completely eliminate *B burgdorferi* from all sites of infection. This was confirmed by the culture of viable *B burgdorferi* from a ligament sample obtained surgically. This organism characterized by molecular biology studies by our group, was subsequently evaluated in a genomic comparison study of other isolates. In that study, Wallich et al identified the genogroup AAA (flagellin type A, heat shock protein [HSP] 60 type A, and HSP 70 type A), and OspA genotype I (15). Electron microscopy of the ligament revealed spirochetes situated between collagen fibers or associated with fibroblasts, deeply invaginating these cells. This is the first time that *B burgdorferi* was isolated from human ligamentous material.

These data indicate that vital *B burgdorferi* persisted (a) despite several courses of antibiotic therapy, (b) even when clinical symptoms subsided, and (c) even when no humoral immune response was detectable by ELISA or by IF. Therefore, the hypothesis may be raised that an inadequate immune response as well as an evasion into immunologically privileged sites may be the mechanisms of microbial persistence in patients with chronic Lyme borreliosis.

The specific humoral and cellular immune responses to *B burgdorferi*, which were elevated during early disease manifestations, apparently were not sufficient to eliminate the pathogen. In the later stage, these specific immune responses became discordant, with negative humoral and positive cellular immunity, as has been described in another cohort with chronic disease (16). Interestingly, the cellular immune responses were also directed against the surface protein OspA during each recurrence of clinical symptoms, even though anti-OspA antibodies were not detectable by immunoblot. Interpretation of this dissociation of the humoral and cellular immune responses is difficult and requires further investigation. Initial experiments with T cell clones in patients with chronic Lyme disease (17) suggest that selective activation of a T cell subset may occur, producing a restricted pattern of cytokines which are incompetent to activate B cells.

Even in the presence of an ineffective immune response, antibiotic therapy should have eradicated the spirochetes and stopped the disease progression in our patient. However, several of the treatment regimens recommended in the then-current literature, including combination therapies which have been described as effective in several refractory cases of advanced-stage disease (6,7), did not eliminate the pathogen. Of interest, our patient showed the DR15 (split of DR2) HLA type (possibly even homozygous), which has been shown to be associated with a poor response to antibiotic therapy in chronic *B burgdorferi* infection (4). Possible explanations for the persistence

of *Borrelia* are that spirochetes either develop resistance to the antibiotics (though not experimentally documented so far) or escape into sites at which drug levels are ineffective. The detection of spirochetes between collagen fibers of bradytrophic dense connective tissue supports the second hypothesis. Moreover, the motility of spirochetes has been shown to be enhanced in fluids as viscous as the extracellular matrix (18). The hypothesis of evasion supports the use of more aggressive therapy as described in recent reports (19), in which 3–4 weeks of intravenous antibiotics was suggested as first-line treatment when systemic manifestations develop, such as the choroiditis in our patient.

Although our electron microscopic studies were of a subcultured ligament specimen, and therefore in vitro effects cannot be excluded, it is of great interest that spirochetes penetrated into the extracellular matrix without causing apparent destruction. Spirochetes had also deeply invaginated into fibroblasts, thereby suggesting transcellular passage. Penetration of cell monolayers by *B burgdorferi* has been demonstrated (20). In conclusion, an inappropriate immune response as well as the evasion of *B burgdorferi* into specific sites that are only slightly accessible to antibiotics and immunologic attack, may be mechanisms that lead to chronic infection with *B burgdorferi*.

ACKNOWLEDGMENT

The authors thank Dr. Reinhard Wallich for helpful discussions and for kindly providing the OspA material.

REFERENCES

1. Preac-Mursic K, Pfister HW, Wilske B, Gross B, Baumann A, Prokop J: Survival of Borrelia burgdorferi in antibiotically treated patients with Lyme borreliosis. Infection 17:355–359, 1989
2. Steere AC, Grodzicki RL, Kornblatt AN, Craft JE, Barbour AG, Burgdorfer W, Schmid GP, Johnson E, Malawista SE: The spirochetal etiology of Lyme disease. N Engl J Med 308:733–742, 1983
3. Preac-Mursic V, Wilske B, Schierz G: European Borrelia burgdorferi isolated from humans and ticks: culture conditions and antibiotic susceptibility. Zentralbl Bakteriol Mikrobiol Hyg A 263:112–118, 1986
4. Steere AC, Dwyer E, Winchester R: Association of chronic Lyme arthritis with HLA-DR4 and HLA-DR2 alleles. N Engl J Med 323:219–223, 1990
5. Sigal LH, Dal ZZ, Stein S, Lackland HJ: B. burgdorferi's flagellin cross-reactivity with human axonal protein p67: location of the epitope defined by monoclonal antibody H9724 (abstract), Fifth International Conference on Lyme Borreliosis. Arlington, VA, 1992
6. Gasser R, Dusleag J: Oral treatment of late borreliosis with roxithromycin plus co-trimoxazole. Lancet 336:1189–1190, 1990
7. Pedersen LM, Friis-Moller A: Late treatment of chronic Lyme arthritis. Lancet 337:241, 1991
8. Krause A, Brade V, Schoerner C, Solbach W, Kalden JR, Burmester GR: T cell proliferation induced by Borrelia burgdorferi in patients with Lyme borreliosis: autologous serum required for optimum stimulation. Arthritis Rheum 34:393–402, 1990
9. Krause A, Burmester GR, Rensing A, Schoerner C, Schaible UE, Simon MM, Herzer P, Kramer MD, Wallich R: Cellular immune reactivity to recombinant OspA and flagellin from Borrelia burgdorferi in patients with Lyme borreliosis: complexity of humoral and cellular immune responses. J Clin Invest 90:1077–1084, 1992
10. Zöller L, Burkard S, Schäfer H: Validity of Western immunoblot band patterns in the serodiagnosis of Lyme borreliosis. J Clin Microbiol 29:174–182, 1991
11. Mullis KB, Faloona FA: Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155: 335–350, 1987
12. Wallich R, Moter SE, Simon MM, Ebnet K, Heiberger A, Kramer M: The Borrelia burgdorferi flagellum-associated 41-kilodalton antigen (flagellin): molecular cloning, expression, and amplification of the gene. Infect Immun 58:1711–1719, 1990
13. Picken RN: Polymerase chain reaction primers and probes derived from flagellin gene sequences for specific detection of the agent of Lyme disease and North American relapsing fever. J Clin Microbiol 30:99–114, 1992
14. Rittig M, Krause A, Häupl T, Schaible UE, Modolell M, Kramer MD, Lütjen-Drecoll E, Simon MM, Burmester GR: Coiling phagocytosis is the preferential phagocytotic mechanism for Borrelia burgdorferi. Infect Immun 60:4205–4212, 1992
15. Wallich R, Helmes C, Schaible U, Lobet Y, Moter S, Kramer M, Simon M: Evaluation of genetic divergence among Borrelia burgdorferi isolates by use of OspA, fla, HSP60 and HSP70 gene probes. Infect Immun 60:4856–4866, 1992
16. Dattwyler RJ, Volkman DJ, Luft BJ, Halperin JJ, Thomas J, Golightly MG: Seronegative Lyme disease: dissociation of specific T- and B-lymphocyte responses to Borrelia burgdorferi. N Engl J Med 319:1441–1446, 1988
17. Yssel H, Shanafelt M-C, Soderberg C, Schneider PV, Anzola J, Peltz G: Borrelia burgdorferi activates a T helper type 1-like T cell subset in Lyme arthritis. J Exp Med 174:593–601, 1991
18. Kimsey RB, Spielman A: Motility of Lyme disease spirochetes in fluids as viscous as the extracellular matrix. J Infect Dis 162:1205–1208, 1990
19. Rahn DW, Malawista SE: Lyme disease: recommendations for diagnosis and treatment. Ann Intern Med 114:472–481, 1991
20. Comstock LE, Thomas DD: Penetration of endothelial cell monolayers by Borrelia burgdorferi. Infect Immun 57:1626–1628, 1989