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Activity of Sera from Patients with Lyme Disease Against *Borrelia burgdorferi*

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Sera from patients with Lyme disease were evaluated for their ability to kill *Borrelia burgdorferi* in vivo and in vitro. Separate groups of C3H mice received sera from seropositive humans with early- or late-stage Lyme disease or from seronegative controls. Eighteen to 24 hours after passive transfer of sera, the mice were challenged with 100,000 low-passage *B. burgdorferi* strain B31 or CA287 organisms. Sera from subjects with late-stage Lyme disease protected the mice against infection after challenge with *B. burgdorferi*, but sera from subjects with early-stage Lyme disease were not protective. Late-stage sera also inhibited the growth of *B. burgdorferi* in microcultures on Barbour-Stoenner-Kelly media better than early-stage sera. Immunoblot analysis revealed that the protective properties of late-stage sera were associated with a response of antibodies to multiple proteins. This response included strong reactivity with the outer-surface proteins A and B, which was lacking in early-stage sera.

Lyme disease evokes a complex immune response in naturally infected humans as well as in experimentally infected laboratory animals [1, 2]. Currently available data support the concept that humoral factors provide only partial protection against local, cutaneous, and disseminated *Borrelia burgdorferi* infection in humans. This concept is based on the well-established observations that individuals who contract Lyme disease can have spirochetemia [3, 4] during certain stages of active infection and that their infection can progress through various stages of extracutaneous disease, despite the formation of abundant quantities of antibodies to *B. burgdorferi*.

In spite of these findings, it has been shown that vaccine formulations consisting of dead [5] or purified recombinant [6] antigen preparations of *B. burgdorferi* induce the formation of antibodies that can prevent borrelial infection in challenged laboratory animals. Furthermore, results from passive immunization experiments have demonstrated that serum from vaccinated donors [6–8], as well as from infected hosts [9], can confer protection in recipient mice against systemic *B. burgdorferi* infection and its manifestations. Related in vitro studies [10, 11] have shown that immune sera from various *Borrelia*-infected hosts can inhibit the growth of *B. burgdorferi* organisms.

With the foregoing considerations in mind, we reexamined the borreliacidal activity and potential protective effects of sera from patients with well-defined Lyme disease. Our results demonstrate that sera from patients with late-stage disease was more effective than sera from patients with early-stage disease in killing *B. burgdorferi* both in vitro and in vivo. In addition, on the basis of results of immunoblot analysis, late-stage sera recognized a greater spectrum of borrelial proteins than did early-stage sera.

Materials and Methods

Animals. C3H mice (3–4 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). All mice were housed in an air-filtered environment maintained at 20°C ± 2°C. Mice were kept in separate cages for the infectivity experiments.

Bacteria and bacterial cultures. The tick-derived *B. burgdorferi* strains B31 and CA287 were obtained through the courtesy of Tom Schwan (Rocky Mountain Laboratories, Hamilton, MT). Both strains were maintained as low-passage organisms (<15) in Barbour-Stoenner-Kelly (BSK) media as previously described [4, 10]. Organisms used in the borreliacidal assay and for the infectivity experiments (see below) were diluted to the appropriate concentration with use of BSK media.

Patient sera. Serum samples were obtained from patients with erythema migrans (EM) alone (early-stage Lyme disease) or from those with arthritis of 6–18 months' duration (late-stage Lyme disease). All cases of borreliosis satisfied the surveillance case definition for EM or arthritis due to *B. burgdorferi* infection of the Centers for Disease Control and Prevention [12]. Sera were obtained from all patients just before administration of conventional antibiotic therapy. Control sera were obtained from 10 patients with syphilis (seropositive by both rapid plasma reagin and specific treponemal antibody tests) and from a group of healthy individuals living in an area in which Lyme disease is endemic.

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Lyme disease serology. All serum samples were assayed for reactivity by using the Lyme Stat System (Whittaker MA Bioproducts, Walkersville, MD), which is a commercially available polyvalent ELISA kit designed to measure antibodies to *Borrelia.* An in-house immunoblotting technique [13] was used for the separate determination of reactivities of IgM and IgG antibodies to *B. burgdorferi* antigens prepared from strain B31.

Inhibition of borrelial growth. Patient sera were tested for inhibitory activity by a slight modification of the previously described methodology for an in vitro borreliacidal assay [10] measuring the inhibitory effects of sera from immune rats. Each well of flat-bottomed 96-well microculture plates (Costar, Cambridge, MA) contained 50 μL of borrelial suspension (final concentration, 2×10^7 spirochetes/mL) and 130 μL of BSK media. Twenty microliters of undiluted test sera or various dilutions of the serum samples was added to designated wells (in triplicate). Therefore, each well contained 1×10^6 borreliae in a final volume of 200 μL.

To maintain complement activity, sera were not subjected to heat inactivation. Cultures were kept airtight by putting the plates into sealed plastic bags followed by incubation at 33°C for 18–24 hours. At the end of the 18- to 24-hour incubation period, the number of spirochetes in each separate test well was counted microscopically [4]. The percentage of inhibition of growth was calculated as follows: [1 − (number of motile borreliae in early- or late-stage sera/number of motile borreliae in control sera)] × 100.

Passive transfer experiments. Separate groups of recipient mice were injected iv or ip with 0.5 mL of serum from patients with Lyme disease or healthy controls. These test sera were not pooled; the serum samples were taken from 10 seropositive patients with EM, 10 seropositive patients with arthritis, and 10 seronegative healthy controls with no history of Lyme disease. Eighteen to 24 hours later, recipient mice were challenged intradermally (id) at one site in the abdominal area with 100,000 low-passage *B. burgdorferi* strain B31 or CA287 organisms.

Six to seven days after the challenge, each mouse was killed, and the presence of viable *Borrelia,* in either blood or the urinary bladder, was detected to ascertain the degree of infectivity in each of the challenged mice. On the day of killing, blood was collected via a cardiac puncture into heparinized syringes, and 0.4 mL of the collected blood was added to 6.0 mL of BSK media in screw-capped tubes.

Extracts of excised urinary bladders were prepared by mincing the bladders finely with the use of scissors and forceps and suspending them in a small volume (0.3–0.4 mL) of BSK media. Each of the extract suspensions was added to separate screw-capped tubes containing 6 mL of BSK media. These tubes, along with those containing blood, were incubated at 33°C. Three to six weeks later, or when color changes were noted in the media, the cultures were examined microscopically (phase contrast and/or fluorescence) for motile live spirochetes as previously described [4] after staining with the fluorochrome acridine orange.

Table 1. Results of ELISA of serum samples from patients with Lyme disease and controls that were used in borreliacidal and passive immunization experiments.

Sera	No. of ELISA-positive samples	Index titer*
Samples from healthy controls (n = 10)	0	<0.8
Samples from controls with syphilis (n = 10)	0	<0.8
Samples from patients with early-stage Lyme disease (n = 10)	10	3.2–5.9
Samples from patients with late-stage Lyme disease (n = 10)	10	4.7–7.8

* Index titers of antibodies to *Borrelia burgdorferi* that are in accordance with specified criteria of the ELISA's manufacturer (Whittaker MA Bioproducts, Walkersville, MD): <0.8 = negative; 0.8–1.0 = borderline; >1.0 = positive.

Results

Patient samples and serology. The demographic and clinical features of the 10 patients with EM have been previously described [14]. These patients had EM for >7 days, and cultures for some of these patients were positive for *B. burgdorferi;* all 10 were seropositive for *B. burgdorferi* (table 1) on the basis of the ELISA results. Immunoblot analysis of sera from these patients with early-stage Lyme disease showed reactivity with several borrelial protein bands. The 10 patients with arthritis (late-stage Lyme disease) presented with a 6- to 18-month history of intermittent attacks of joint swelling and pain (most commonly of one knee). Joint fluid was obtained from most of these patients; elevated titers of antibodies to *Borrelia* comparable with those in the patients' sera were found in the joint fluid samples (table 1). Serum specimens from controls (healthy patients and patients with syphilis) were negative for antibodies to *Borrelia.*

To further characterize the specific antibody responses in this sample of patients with Lyme disease, separate serum samples from three patients with early-stage Lyme disease and four patients with late-stage Lyme disease underwent immunoblotting with solubilized strain B31 organisms (figure 1). Late-stage sera reacted with 15–20 borrelial proteins, while early-stage sera reacted with five to 10 borrelial proteins.

Irrespective of the reactive immunoglobulin (IgM or IgG), the most common proteins recognized collectively by all seven serum samples had molecular weights of 18 kD, 41 kD, and 60 kD. Only late-stage sera, however, reacted with outer-surface protein (OspA; 31 kD) and outer-surface protein B (OspB; 34

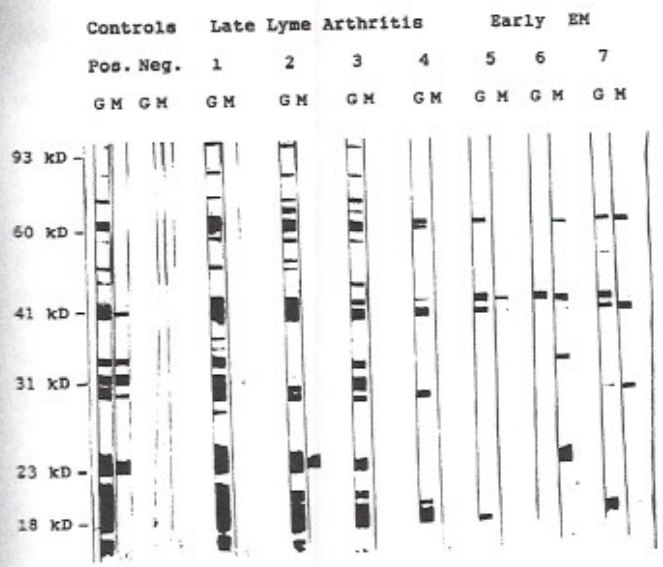

Figure 1. Immunoblots for sera from patients with Lyme disease. Sera were analyzed for their reaction to sonicated *Borrelia burgdorferi* strain B31 antigens subjected to electrophoresis; reactions are based on the recognition of antigens by alkaline phosphatase–conjugated human IgG (G) or IgM (M). *Lanes 1–4,* reactivity of sera from four patients with Lyme arthritis (late-stage Lyme disease); *lanes 5–7,* reactivity of sera from three patients with early EM (erythema migrans; early-stage Lyme disease). Pos. = positive; Neg. = negative.

kD). Reactivity was limited almost exclusively by IgG for late-stage sera, whereas the level of reactivity for early-stage sera was similar for both IgM and IgG (figure 1).

In vitro inhibitory activity of patient sera. *B. burgdorferi* strains B31 and CA287 were tested for their sensitivity to immune factors (such as antibodies) present in sera from patients with Lyme disease. When tested at a final concentration of 10%, most (90%–100%) of the late-stage sera inhibited the growth of strains B31 and CA287, whereas only 30%–40% of the early-stage sera had borreliacidal activity against the same two strains of *Borrelia* (table 2). Titration of selected inhibitory

Table 2. In vitro borreliacidal activity of serum samples from patients with Lyme disease and controls.

Sera	No. (%) with significant borreliacidal activity against Strain B31	Strain CA287
Samples from healthy controls (n = 10)	0	0
Samples from controls with syphilis (n = 10)	0	0
Samples from patients with early-stage Lyme disease (n = 10)	4 (40)	3 (30)
Samples from patients with late-stage Lyme disease (n = 10)	10 (100)	9 (90)

Table 3. Titers of antibody to *Borrelia burgdorferi* strains B31 and CA287 in serum samples from patients with early-stage Lyme disease (erythema migrans only) or late-stage Lyme disease (arthritis).

Serum sample, titration	Titer* of antibody to Strain B31	Strain CA287
Early-stage serum		
1	125	125
2	62.5	62.5
3	62.5	31.3
4	31.3	NID
Late-stage serum		
1	2,000	1,000
2	1,000	500
3	500	500
4	500	NID

NOTE. NID = no inhibition detected.
* Reciprocal of the highest dilution of serum with significant borreliacidal activity.

sera revealed that levels of borreliacidal activity against strains B31 and CA287 in the presence of late-stage sera were higher than those in the presence of early-stage sera (table 3). Borreliacidal activity against both strains of *B. burgdorferi* was highly specific, with no inhibition of growth being detected when serum samples from healthy controls with no history of Lyme disease and serum samples from patients with syphilis were used (table 2).

Serum-mediated protection against borrelial infection. Three separate groups of recipient mice (20 animals per group) given early-stage sera, late-stage sera, or control sera were challenged id at one site with 100,000 *B. burgdorferi* strain B31 organisms. An additional three groups of recipient mice were challenged in parallel with 100,000 *B. burgdorferi* strain CA287 organisms. In all experiments, duplicate mice were infused with equal amounts of serum from an individual (described above). A total of 120 mice were analyzed for disseminated infection on the basis of isolation of *Borrelia* in separate cultures of peripheral blood and urinary bladder specimens.

As shown in figures 2 and 3, late-stage sera conferred complete protection against disseminated infection for both strains of *B. burgdorferi.* In marked contrast, 90% of the recipients of early-stage sera and all of the recipients of control sera had evidence of disseminated infection on the basis of the recovery of live borreliae from blood and urinary bladder specimens.

Discussion

There is a growing body of evidence based on passive transfer experiments that humoral factors, generated in response to borrelial infection or immunization against *Borrelia,* confer various levels of resistance to *B. burgdorferi* infection in

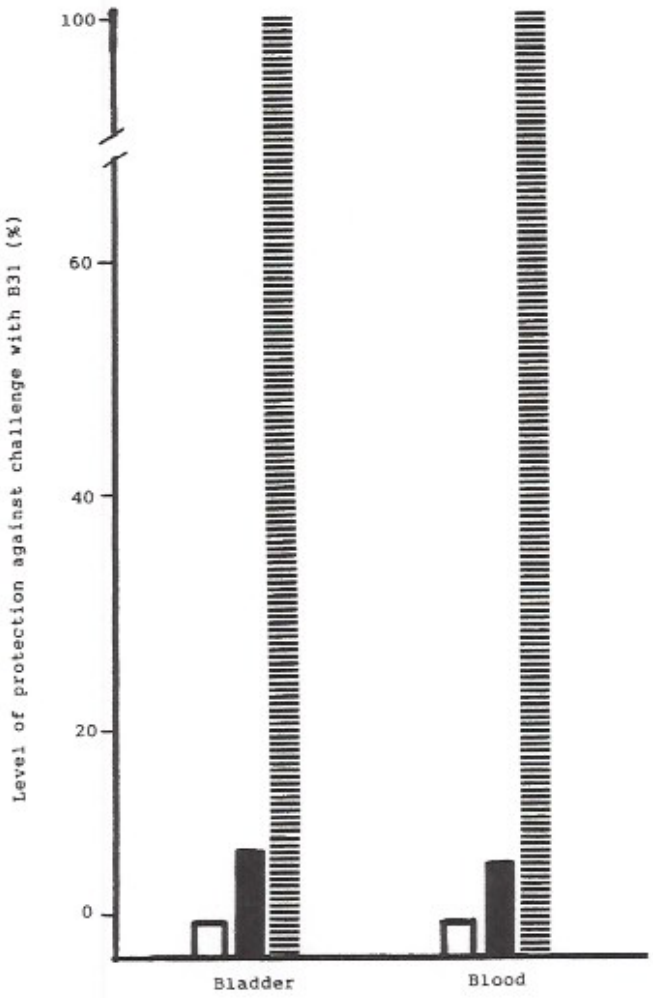

Figure 2. Levels of protection against infection after challenge with *Borrelia burgdorferi* strain B31 that were observed in C3H mice infused with high-titer sera from patients with early-stage Lyme disease (erythema migrans only) (solid bars), patients with late-stage Lyme disease (hatched bars), or controls (healthy patients or patients with syphilis) (open bars).

experimental animals [6–9]. The data presented here (i.e., sera from patients with late-stage Lyme disease provide a high level of protection against borrelial infection in mice challenged with two distinct strains of *B. burgdorferi*) are in close agreement with those of some of these earlier studies [6–9].

The ability of immune sera to provide complete protection was evident on the basis of the lack of dissemination of organisms from the primary challenge site to the bloodstream and urinary bladder. Infectivity tests of blood and urinary bladder specimens from mice challenged with sera from healthy controls and patients with EM revealed that significant numbers of borreliae were recovered from these sites, which were beyond the original focus of challenge organisms introduced id. These findings correlated well with the in vitro data (tables 2 and 3) showing that the maximal levels of growth inhibition occurred in the presence of late-stage sera.

To our knowledge, only one study [9] has reported evidence that human immune sera and selected isolated immunoglobulins are active in vivo in the expression of immunity against *Borrelia*. However, in this study by Fikrig et al. [9], mice receiving late-stage sera from two patients were found to be only partially protected against disseminated infection, but this limited protection was attributable, at least in part, to the host response to OspA or OspB.

In support of this latter finding, we observed in recent passive immunization experiments (C. S. Pavia, unpublished data) that

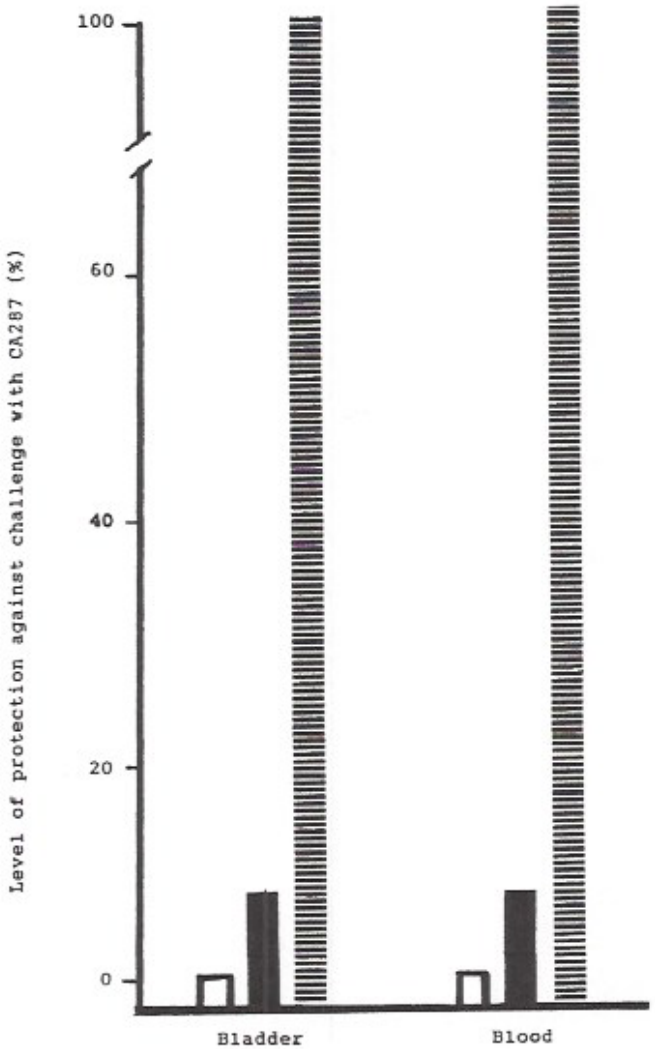

Figure 3. Levels of protection against infection after challenge with *Borrelia burgdorferi* strain CA287 that were observed in C3H mice infused with high-titer sera from patients with early-stage Lyme disease (erythema migrans only) (solid bars), patients with late-stage Lyme disease (hatched bars), or controls (healthy patients or patients with syphilis) (open bars).

fractions of immune sera from patients with Lyme disease that were enriched for human immunoglobulins were fully protective against borrelial infection, while removal of human IgG resulted in the loss of in vivo protection. Further analysis of our human sera by immunoblotting (figure 1) revealed immune reactivity with numerous borrelial proteins similar to what was previously reported by other investigators who used different antigen preparations [14, 15]. Our additional key finding that the protective serum samples reacted strongly with OspA and OspB lends further support to the important protective role of antibodies to OspA or OspB. In addition, along these lines, it was shown [9] that human immune sera depleted of antibodies to OspA or OspB were not protective in passively immunized and challenged recipient mice [9].

Although the aforementioned studies indicate the importance of OspA and OspB as key immunogens, the possible role of other borrelial antigens as significant elicitors of a protective host response should not be discounted. In this regard, it was shown that serum from mice inoculated with low doses of *B. burgdorferi* (which leads to little or no production of antibody to OspA) was still capable of conferring immunity to challenged recipients similar to sera from mice with antibodies to OspA [16].

Such divergent findings are not necessarily surprising and could be attributed to a number of factors: differences in the amount and source (homologous vs. heterologous) of sera used as the product for transfer of immunity; use of different strains of mice (C3H mice vs. mice with severe combined immunodeficiency) as either donors or recipients; use of numerous different isolates of *Borrelia* (*B. burgdorferi sensu stricto* vs. *Borrelia garinii* or *Borrelia afzelii*) as the infectious or disease-causing source of organisms; and differences in the challenge method (and in the dose) (low vs. high) and route (ip vs. id or tick bite) of the challenge inoculum.

Although advances have been made in our knowledge about immunity to Lyme disease [2, 17], we still do not understand how pathogenic *B. burgdorferi* organisms evade the host's immunologic defenses. As a possible explanation for the protracted nature of borrelial infections, we found evidence of a very weak inhibitory effect against *B. burgdorferi* both in vitro and in vivo on the part of sera from patients with early-stage Lyme disease. This phenomenon occurred despite high titers of antibody in early-stage sera that were nearly comparable with those in late-stage sera; the weak effect of early-stage sera led to an almost unimpeded growth of *Borrelia* at the site of EM on the skin along with dissemination of spirochetes to the bloodstream and other key tissue sites (brain, joints, and heart).

Although there is mounting evidence favoring a limited protective role for early-stage sera, our findings (coupled with those obtained so far by other investigators using the technique of passive immunization [6–9]) indicate that *B. burgdorferi* can be effectively neutralized in vivo by virtue of a late-developing polyclonal antibody response. With this information in hand, at least one vaccine candidate has been formulated with use of recombinant-derived OspA in an attempt to prevent the development of Lyme disease [6, 18]. However, it should be noted that other borrelial antigens (such as outer-surface protein C [OspC]) may be involved in the protective immune response, especially since antibodies to this component are readily detectable during both early- and late-stage Lyme disease [14, 15].

Despite the apparent strong immunogenicity of recombinant OspC, the use of this protein as a possible vaccine has been inconsistent so far since protection (based on passive and active immunity studies [2]) can be induced in some experimentally infected animal hosts but not in others. Nonetheless, such studies do provide key insights toward the development and testing of these as well as other possible vaccine candidates [19].

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