Lyme Borreliosis in Rhesus Macaques: Effects of Corticosteroids on Spirochetal Load and Isotype Switching of Anti-Borrelia burgdorferi Antibody

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Experimental *Borrelia burgdorferi* infection of rhesus monkeys is an excellent model of Lyme disease and closely parallels the infection in humans. Little is known about the interaction of host immunity with the spirochete in patients with chronic infection. We hypothesized that rapid development of anti-*B. burgdorferi* antibody in immunocompetent nonhuman primates (NHPs) is the major determinant of the reduction of the spirochetal load in Lyme borreliosis. This hypothesis was tested by measurement of the spirochetal load by PCR in association with characterization of the anti-*B. burgdorferi* humoral immune response in immunocompetent NHPs versus that in corticosteroid-treated NHPs. Although anti-*B. burgdorferi* immunoglobulin G (IgG) antibody was effectively inhibited in dexamethasone (Dex)-treated NHPs, anti-*B. burgdorferi* IgM antibody levels continued to rise after the first month and reached levels in excess of IgM levels in immunocompetent NHPs. This vigorous production of anti-*B. burgdorferi* IgM antibodies was also studied in vitro by measurement of antibody produced by *B. burgdorferi*-stimulated peripheral blood mononuclear cells. Despite these high IgM antispirochetal antibodies in Dex-treated NHPs, spirochetal loads were much higher in these animals. These data indicate that Dex treatment results in interference with isotype switching in this model and provide evidence that anti-*B. burgdorferi* IgG antibody is much more effective than IgM antibody in decreasing the spirochetal load in infected animals.

*Borrelia burgdorferi* enters the host via a tick bite, followed by growth through the skin and then dissemination to target organs. The host immune response to *B. burgdorferi* during Lyme borreliosis results in both protection, by limitation of spirochetal growth, and inflammation, which can be detrimental in that it causes both symptoms and tissue damage (26).

The host immune response to *B. burgdorferi* has been studied extensively, mostly by in vitro techniques. Cytokines, many of which are produced by Th1 cells, contribute to inflammation (14). Recent work has focused on protective aspects of the immune response important in the development of vaccines (11, 24, 25).

The arm of the immune response believed to be most important in protection and clearance is humoral immunity. This has been investigated in a number of animal models of experimental Lyme borreliosis, including hamsters (13), mice (3, 10, 17), and dogs (27). Vaccine efficacy is thought to be due to the development of protective antibody, and a variety of proteins of *B. burgdorferi* appear to have protective effects including outer surface protein A (OspA) (8), OspB (9), OspC (17), and decorin binding protein A (5). Clearance of spirochetes naturally occurs with antibody directed against a variety of *B. burgdorferi* epitopes; the isotype and immunoblot pattern optimal for clearance of active infection by antibody are unknown.

Also, little is known about how the immune response reacts with the spirochete in generating either helpful or harmful immunity. Since the skin is involved early in infection, early immune responses are probably driven by antigen-presenting cells (APCs) and lymphocytes in the skin and draining lymph nodes. This local immune response and the development of high antibody-titers in the serum likely limit spirochetal growth and dissemination.

We hypothesized that interference with humoral immunity to *B. burgdorferi* would result in higher spirochetal loads in target organs. We also hypothesized that B cells circulating in the peripheral blood can produce anti-*B. burgdorferi* antibody and that in vitro antibody production by these B cells would mirror the antibody amplitude and isotype in the serum. We tested these hypotheses with the rhesus monkey model of Lyme borreliosis.

MATERIALS AND METHODS

NHPs and spirochetes. The four adult rhesus macaques (*Macaca mulatta*; two males and two females) used in this study were housed, cared for, and anesthetized and underwent cisternal punctures as described previously (20). This housing and care were in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (29) in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. Prior to initiation, the study was reviewed and approved by the New Jersey Medical School Animal Care and Use Committee.

Two nonhuman primates (NHPs), designated PAX219 and Z1, respectively, were treated orally with dexamethasone at a dosage considered low to moderate for rhesus macaques (2 mg/kg of body weight/day for 1 week and then 1 mg/kg/day) for 10 weeks after infection; these NHPs are referred to as immunosuppressed (IS1 and IS2, respectively). The other two NHPs, designated E680 and Z33, respectively, did not receive dexamethasone and are referred to as im-

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necompotent (IC1 and IC2, respectively). Blood was obtained for baseline serum analysis at three times prior to infection. NHPs were necropsied 10 weeks postinfection (p.i.), after euthanasia with ketamine, xylazine, and pentobarbital. Prior to necropsy, the NHPs were perfused with 2 to 4 liters of normal saline. One million cells of B. burgdorferi were added to each well. Data were shown in Fig. 1 and 2 for IS1, IS2, and IC1. Adequate blood from IC2 could not be obtained for these studies.

DNA extraction from organs. Tissue samples were processed by DNA extraction, as follows. The tissue was minced with a scalpel. The minced tissue was brought to a final volume of 0.5 ml with a solution containing 10% sodium dodecyl sulfate and proteinase K. After an overnight incubation at 37°C, the tissue suspension was mixed 10 times and vortexed twice with phenol-chloroform extraction with chloroform was performed to remove phenol. One-ninth of the volume of chloroform (pH 5.4 to 6.0; Sigma Chemical) was then added to the aqueous mixture, followed by the addition of 2 volumes of ice-cold 100% ethanol (Warner-Graham, Cockeyville, Md.). The DNA was precipitated overnight at −20°C and then spun at 14,000 rpm in a Sorval SS-34 rotor for 20 min. The pellet was dried and resuspended in 0.2 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 7.6]). Proteinase K was then inactivated by boiling the samples for 5 min. The samples were read on a spectrophotometer at optical densities (ODs) of 260 and 280, and reextraction was performed if the ratio of the OD at 260 OD at 280 was less than 2. The DNA concentration was calculated on the basis of the assumption that 1 OD unit at 260 represents a concentration of 50 μg/ml.

Concentration of competitive PCR internal standards. Construction of competitive PCR internal standards was carried out as described in the PCR mimic construction kit (Clontech Laboratories, Inc., Palo Alto, Calif.); and 125 U of Taq DNA polymerase (Life Technologies, Grand Island, NY). The amplification was achieved with a DNA thermal cycler (Perkin-Elmer Cetus 480). After denaturation at 94°C for 4 min, the amplification was conducted for 35 cycles at 94°C for 45 s for denaturation, 60°C for 45 s for annealing, and 72°C for 2 min for elongation. The final cycle was followed by a single period at 72°C for 7 min. The PCR products were inactivated in the UV reaction chamber and then electrophoresed on a 2% agarose gel and stained with ethidium bromide. The bands were visualized with a UV transilluminator and photographed with a Polaroid camera.
20). A total of 200 μl (per well) of a 10-mg/ml solution of streptavidin in assay buffer (PBS, 0.5% gelatin, 0.15% Tween 20) was then added, and the plate was incubated at 37°C for 1 h. The plate was washed with PBS wash buffer and was then used for the ELISA.

(ii) Digoxigenin detection. A hybridization solution was prepared by adding 10 pmol of an internal biotinylated probe for OspB (synthesized and labeled by Lofstrand Labs Limited, Gaithersburg, Md.) per ml to a hybridization buffer (0.15 M NaCl, 0.015 M sodium citrate, 20 mM HEPES, 2 mM EDTA, 0.15% Tween 20). A total of 490 μl of hybridization solution was added to 10 μl of PCR products that had been inactivated in the UV reaction chamber and denatured at 94°C for 5 min. A total of 200 μl of the samples was then added to the well of a coated plate. The plate was incubated at 37°C for 3 h. After the plate was washed three times with washing buffer, 200 μl of peroxidase-labeled anti-digoxigenin antibody, diluted 1:5,000 in assay buffer, was added to each well and the plate was incubated for 30 minutes at 37°C. After the plate was washed, 200 μl of 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) substrate solution was added, the color was allowed to develop fully, and the plate was read on an ELISA plate reader at 405 nm. Samples were considered positive if the OD readings were greater than the mean plus 3.5 standard deviations for four negative samples (two water controls and DNA samples from uninfected brains). Use of these cutoffs resulted in positive signals for less than 1% of samples of DNA from uninfected NHPs.

qt-PCR-ELISA. The DNA extracts from samples that were positive by the screening analysis described above were further analyzed by quantitative PCR-ELISA (qt-PCR-ELISA). At least two samples, and usually three or four samples, were obtained from each tissue of animal PAX219 (IS1) tested. A range of concentrations of OspB competitor plasmid was added to aliquots containing a total of 500 ng of DNA from the tissue samples, and this mixture of wild-type and competitor DNA was subjected to PCR. In the ELISA, 200 μl of PCR product was analyzed for hybridization to the unique wild-type internal sequence, and an equal volume of PCR product was analyzed for the unique competitor internal sequence. ODs from the ELISA reader were transferred to an SPSS spreadsheet, and regression analyses were performed. A negatively sloped curve of the OD versus the concentration of competitor was generated from the wild-type DNA hybridization data, and a positively sloped curve was generated from the competitor DNA data. The x-axis intercept of the intersection of these two curves yielded a value for the copy number of the sample. Standard errors of the mean were determined for multiple determinations for the same DNA as well as of the same tissue; standard errors of the mean averaged 11 and 17%, respectively.

RESULTS

Anti-B. burgdorferi antibody response in serum. In immuno-
competent NHPs, there was a brisk rise in serum IgM levels
within the first weeks of infection, followed by a decline in IgM
levels beginning in the 5th week (Fig. 1). As the IgM levels
were declining, IgG levels rose and continued to rise to the end of the study.

In immunosuppressed NHPs, IgM antibody levels continued to rise throughout the experiment so that by week 6 levels were higher than those in immunocompetent NHPs and continued to rise. IgG antibodies barely rose in the immunosuppressed NHPs and were low at the time of the necropsy.

**Anti-*B. burgdorferi* antibody levels in CSF.** As in previous studies, the time of appearance of anti-*B. burgdorferi* antibody in CSF was delayed relative to the time of appearance in serum. Levels of antibody in CSF in immunosuppressed NHPs were low throughout the study, while these levels in immunocompetent NHPs were positive by 5 weeks p.i. and remained high (Fig. 2).

**Immunoblotting of sera.** (i) **Anti-*B. burgdorferi* IgM.** In IS1 IgM reactivity did not appear until 5 weeks after infection, at which time bands appeared at 37, 39, 41, 58, 60, and 66 kDa and a new band appeared at 93 kDa (Fig. 3A). This pattern persisted. In IS1, weak reactivity was present at 31, 39, 60, and 66 kDa at 3 weeks and a strong 41-kDa band appeared at 6 weeks. This pattern persisted. In IC1 and IC2 there were strong early responses, with bands at present 31, 34, and 41 kDa at 3 weeks p.i.; these early responses faded. IC1 had no IgM bands by 9 weeks p.i., while IC2 had only a relatively weak 60-kDa band.

(ii) **Anti-*B. burgdorferi* IgG.** IS1 did not display IgG bands until 7 weeks p.i., at which time the predominant response was to p39 (Fig. 3B). The IgG response increased slightly from 7 to 9 weeks p.i. In contrast, IS2 displayed bands of 31, 34, and 39 kDa early after infection, despite a relatively low IgG response by ELISA. The sera from neither immunosuppressed NHP demonstrated positivity by the criteria of the Centers for Disease Control and Prevention (7). For IC1, the immunoblot was positive (7) at 3 weeks p.i. (bands of 21, 31, 34, 39, 58, and 60 kDa), with bands of 52 and 55 kDa appearing later. By 9 weeks p.i. there was fading of the 21-kDa band and appearance of the 66-kDa band. For in IC2 the immunoblot was also positive at 3 weeks, but there were not as many bands as for

![Figure 2](http://cvl.asm.org/)

**FIG. 2.** Anti-*B. burgdorferi* IgM (A) and IgG (B) antibody titers in CSF. The means of replicate values are shown, with the OD of the ELISA being 1,000 times the recorded value. The duplicate values were within 10% of the mean. The figure was generated from an Excel 1998 spreadsheet by using the Chart Wizard. •, IC1; ●, IC2; △, IS1; ○, IS2.
IC1; i.e., for IC2 there were bands of 31, 34, 39, 41, 55, and 60 kDa. By 5 weeks p.i. there were new bands at 52 and 66 kDa.

**In vitro antibody production.** PBMCs produced anti-*B. burgdorferi* antibody in vitro, stimulated by *Borrelia* antigens present on *B. burgdorferi*-pulsed antigen APCs, as shown in Fig. 4. The pattern of antibody production mirrored that present in serum, i.e., low levels of predominantly IgM in IS1 and IS2 and substantial levels of immunoglobulins of both isotypes in IC1 and IC2.

**Spirochetal dissemination and load.** (i) Immunocompetent NHPs (IC1 and IC2). As in previous studies (19), the spirochetal level was low, but detectable, in immunocompetent NHPs. The level was near the sensitivity of the PCR technique used, i.e., about five copies of *B. burgdorferi* DNA per 500 ng of DNA extracted from tissue. Screening PCR-ELISAs resulted in positivity for skeletal muscle, nerve, dura mater, brachial plexus, heart, aorta, and bladder tissue samples. Only 7 of 17 (41%) brain stem and spinal cord samples tested were positive. When these samples were tested by the qt-PCR-ELISA technique, a less sensitive technique than the screening PCR-ELISA, all were below the limit of 100 copies (per 500 ng of extracted DNA) necessary for meaningful quantitation by using the qt-PCR-ELISA.

(ii) Immunosuppressed NHPs (IS1 and IS2). Screening PCR-ELISA readily detected *B. burgdorferi* DNA in almost all subtentorial structures of the CNS tested (11 of 12 [92%]), as well as nerve, muscle, bladder, and cardiac tissues in dexamethasone-treated NHPs. In one of these NHPs, PAX219, DNA specimens were analyzed by qt-PCR-ELISA (Table 1). Only one sample from these animals was below the cutoff able to be quantified, i.e., 100 copies. The remaining tissues displayed a range of spirochetal loads ranging from (hundreds of copies) in CNS tissue, to thousands of copies in nerve tissue, to tens of thousands of copies in skeletal and cardiac muscle tissue.

(iii) Uninfected NHPs. Tissues from uninfected NHPs served as negative controls. Screening PCR-ELISAs were negative 9 for 26 of these specimens.

**DISCUSSION**

Lyme borreliosis is a chronic infection in humans which is increasing in prevalence and which results in significant morbidity. The NHP model of Lyme borreliosis consists of chronic inflammation in the CNS, heart, skin, nerve, and muscle, associated with high levels of antibody in serum and CSF, and a strong cellular immune response to spirochetal antigens. This model, which closely parallels the disease in humans (6, 20), provides an opportunity to fill major gaps in our knowledge about Lyme disease in humans.

How spirochetes are cleared in patients with chronic infection is not well understood. A substantial body of work, primarily with experimental models of Lyme borreliosis, has shown that antibody is important in protection (2) but is not completely efficient in clearance of infection, since low-grade infection persists, despite high levels of antibody in the serum (21). Th2-associated cytokines, such as interleukin-4 (IL-4) (4) and IL-6 (18), are important cytokines in the antispirochetal response, probably through their role in the development of...
the antiborrelia antibody response. In our study, the spirochetal load was much higher in NHPs whose anti-\textit{B. burgdorferi} antibody response was blunted by dexamethasone than in immunocompetent animals.

The mechanisms of impaired immunity induced by corticosteroid administration are complex and not completely understood. Many cells have intracellular steroid receptors complexed to a heat shock protein, hsp90. Steroids displace hsp90 from the receptor, allowing the receptor to enter the nucleus and bind to the gene regulatory sequences. Corticosteroids have effects on a wide variety of cellular processes and cell types. Thus, the precise steroid effect or combination of effects that increased spirochetal loads in the corticosteroid-treated NHPs in this study was not determined. However, given previous studies that have demonstrated the importance of anti-\textit{B. burgdorferi} antibodies in protection from infection, it is likely that the much lower level of specific antibody in the dexamethasone-treated NHPs contributed significantly to the higher spirochetal loads in those animals. Any combination of the lymphoid cells critical to antibody generation (APCs, helper T cells, or B cells) could have been affected.

We assumed at the initiation of the experiment that anti-\textit{B. burgdorferi} IgM antibody production in the dexamethasone-treated NHPs would be impaired similarly to IgG isotype production. However, the IgM titers in these animals continued to climb through the course of the experiment, surpassed the levels in the immunocompetent animals, and remained high. The IgM antibody produced in the immunosuppressed NHPs, although quantitatively high, did not have the complexity of the IgG response in immunocompetent NHPs. That is, IgM antibody, when tested by immunoblotting, bound predominantly to the 39- and 41-kDa proteins of the spirochete, while the IgG antibody response at the same time p.i. in the immunocompetent NHPs bound to a broader spectrum of spirochetal proteins. This fact, plus the inability of IgM to penetrate significantly into tissue and its relatively poor ability to fix complement, may explain to some extent the relative inability of IgM to limit spirochetal growth.

We tested an assay for in vitro production of antibody. The measurement of in vitro antibody production has proved helpful in learning about production of antibody to other pathogens such as \textit{Bordetella pertussis} \cite{10a}. We hoped that the in vitro antibody produced would reflect the level and isotype of antibody detectable in serum, and this proved to be true. This high degree of correlation of the level of antibody present in the supernatants of PBMCs to that present in the serum of the infected NHPs is important. It allows this system to be used in the future for dissection of conditions necessary for antibody production, such as important cytokines, APCs, peptides of \textit{B. burgdorferi}, helper T cells, and B-cell populations. For example, IL-6 is a major cytokine produced by mononuclear cells after stimulation with \textit{B. burgdorferi} \cite{18, 28}, plays a role in

![FIG. 4. In vitro antibody production. In vitro production of IgM in IS1 and IS2 and both IgM and IgG in IC1 is shown as function of the number of weeks p.i. The data shown represent those for samples taken from wells of the cultures on day 6. M, IgM; G, IgG.](http://cvl.asm.org/)
the largest concentration being found in skeletal muscle. This infected NHPs mirrored the levels of production of antibody in the sera of the antibody in vitro by these peripheral blood B cells in this study. Although most antibody to NHPs was confirmed in the measurement of anti-
model of Lyme borreliosis. The impaired isotype switch in the dexamethasone-treated NHPs was in the measurement of anti-B. burgdorferi antibody levels in vitro. The level of production of specific antibody in vitro by these peripheral blood B cells in this study mirrored the levels of production of antibody in the sera of the infected NHPs.

The spirochetal load in immunocompetent NHPs was very low, at or near the sensitivity of the detection of the PCR assay, i.e., 5 to 50 copies per 500 ng of tissue DNA. This level was below the level necessary for meaningful data from the quantitative assay, the qt-PCR-ELISA. The spirochetal load in the dexamethasone-treated NHPs was considerably higher, with the largest concentration being found in skeletal muscle. This tropism of the spirochete to skeletal muscle was unexpected and may have relevance to certain clinical symptoms that occur in infected humans, such as myalgia and fatigue. The dissemination of N40Br, a B. burgdorferi sensu stricto strain, to multiple sites was similar to that observed in our previous studies with NHPs (19). Since in our previous studies we had documented that culture of tissue in this model had a very low yield, tissue culture was not performed in this study to assess spirochetal load. This broad dissemination of sensu stricto strains in nonrodent vertebrates was also seen in a recent study dealing with naturally infected dogs (12); Borrelia garinii isolates, in contrast, appeared to have specific and restricted tropism to the liver. More studies of spirochetal load and tissue tropism in this infection in higher vertebrates are necessary.

In human infections, corticosteroids are sometimes used for the treatment of clinical syndromes such as facial paralysis or arthritis, when these problems are thought to be idiopathic. The data presented above demonstrate the interplay between host immunity and spirochetal infection in Lyme borreliosis and the importance of a competent immune response in limiting the spirochetal load. Since the diagnosis of Lyme borreliosis may sometimes be difficult to make, there is a risk in areas of endemicity of using corticosteroids as therapy in inflammatory syndromes that could represent Lyme disease.

REFERENCES

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