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Borrelia burgdorferi Antigens That Are Targeted by Antibody-Dependent, Complement-Mediated Killing in the Rhesus Monkey

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We identified surface antigens of *Borrelia burgdorferi* that are targeted by antibody-dependent, complement-mediated killing (ADCK) in the rhesus monkey. For this purpose, we had available serum samples from three animals infected with *B. burgdorferi* JD1 by needle inoculation and from two monkeys that were infected with the same *B. burgdorferi* strain by *Ixodes scapularis* tick bite. Sera from monkeys from the first group contained antibodies to OspA and OspB detectable by Western blot (immunoblot) using whole *B. burgdorferi* antigens, whereas serum samples from animals in the second group did not. The targeting of OspA and OspB by functional antibodies was demonstrated directly by showing that ADCK was partially inhibited when antibodies were preincubated with an excess of soluble recombinant OspA or OspB. Simultaneous addition of OspA and OspB did not result in an additive inhibitory effect on ADCK, a result that suggests that the epitopes on OspA and that on OspB targeted by antibody in this mechanism are the same, or at least cross-reacting. The targeting of non-OspA, non-OspB surface antigens was inferred from the fact that sera from tick-inoculated animals, which did not contain detectable anti-OspA or anti-OspB antibodies, were able to effect ADCK. This killing effect was not inhibitable by the addition of recombinant OspA or OspB or both proteins together. We also showed that both immunoglobulin G and M antibodies participate in the ADCK mechanism in the rhesus monkey. Rhesus complement does not kill *B. burgdorferi* in vitro in the absence of antibody, and antibody alone is effective in killing only at serum dilutions lower than 1:15. However, such "complement-independent" antibodies were not present in all bleeds. Two main conclusions may be drawn from the analysis of our results. First, both OspA and OspB are targeted by the ADCK mechanism in the rhesus monkey. Second, one or more *B. burgdorferi* surface antigens that are neither OspA nor OspB also participate in ADCK.

Binding of antibody to the surface of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, is known to effect killing of this spirochete in vitro by at least two mechanisms. One of these mechanisms requires antibody to facilitate the interaction of the bacterial membrane with the complement C5b-9 membrane attack complex. This complex is originated from the C5 convertase of the classical pathway (13). The other mechanism, thus far poorly understood, is complement independent and requires only antibody (4, 22).

In a normal immune-competent host, it is unlikely that anti-*B. burgdorferi* antibody may attach itself to the spirochete in an environment where complement is not present. Therefore, it is appropriate to postulate that antibody-dependent, complement-mediated killing (ADCK) and not killing mediated solely by antibody is an, or the main, effector mechanism of *B. burgdorferi* in vivo. We are interested in identifying the molecular targets of the ADCK mechanism. The underlying assumption is that these surface antigens are a key to understanding protective immunity against *B. burgdorferi* infection and perhaps also the pathogenesis of Lyme borreliosis. For this purpose, we have utilized the rhesus monkey (*Macaca mulatta*) model.

We had shown previously that *B. burgdorferi* infection in the rhesus monkey mimics signs which appear during the early and early-disseminated phases of human Lyme disease, including erythema migrans, conjunctivitis, fever, and splenomegaly. Moreover, both the time course and the specificity spectrum of the serum antibody response to *B. burgdorferi* in rhesus monkeys were shown to be very similar to their counterparts in humans (16). It has also been shown by our group that chronic signs of the infection become manifest in this model, including arthritis and neurologic changes (20).

To characterize the rhesus monkey model, we had infected animals either by the bite of *B. burgdorferi*-infected *Ixodes scapularis* nymphs or by needle inoculation. As we analyzed the antigens bound by serum antibodies obtained from each of these two groups of animals, we noted that sera from needle-inoculated (NI) animals had antibodies recognizing the outer surface proteins A and B (OspA and OspB), whereas serum samples from tick-inoculated (TI) animals did not (16). This differential antibody response has been observed also in other animals, including dogs and rodents (8, 9, 21, 23). Rhesus monkey serum samples with and without anti-OspA and/or anti-OspB antibodies, as detected by Western blotting (immunoblotting), provided us with antibody reagents to begin an investigation of the antigenic targets of ADCK in this animal model.

MATERIALS AND METHODS

Organisms. *B. burgdoferi* JD1 (passage 3) was cultured in Barbour-Stoenner-Kelly medium (BSK II medium) by standard procedures, as previously described (16), up to a concentration of 10^6 cells per ml and stored in liquid nitrogen.

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Animals and experimental infection. Six male, 2-year-old rhesus monkeys (*M. mulatta*) of Chinese origin, part of a group of animals employed previously (16), were used in this study. Three animals (J743, J748, and K167) were infected by needle inoculation with 4×10^8 cultured *B. burgdorferi* organisms suspended in 3.1 ml of RPMI 1640 medium. The inoculum was divided and delivered intraperitoneally (2.0 ml), subcutaneously (1.0 ml), and intradermally (0.1 ml) at a site on the lower right abdominal quadrant. Animal J743 from this group was given only intraperitoneal (3.0-ml) and intradermal (0.1-ml) inoculations. Two animals (J307 and J677) were infected by bites of *I. scapularis* nymphal ticks infected with *B. burgdorferi* JD1 by using tick capsules located dorsolaterally on the cranial thorax as described previously (16). One animal (J206) was exposed to the bites of uninfected ticks and used as a control.

Source of complement. Blood samples were collected from uninfected, normal, anesthetized rhesus macaques by femoral venipuncture and allowed to clot at room temperature for 30 to 45 min. Clotted blood was then kept at 4°C for 2 h. After centrifugation of the samples at 800 × *g* for 20 min, the sera were decanted and stored in small aliquots at −70°C until used. The animals chosen as a source of complement for the study were selected from a group of normal rhesus macaques after it was determined that their sera did not contain cross-reactive anti-*B. burgdorferi* antibodies, as determined by Western blot using whole *B. burgdorferi* antigens.

In vitro bactericidal assay. Frozen samples of *B. burgdorferi* were quickly thawed at 37°C, cultured until they reached mid-log phase (approximately 3 days; 1×10^7 to 2×10^7 spirochetes per ml), centrifuged at 8,000 × *g* for 20 min, and resuspended in BSK II medium, and the organisms were counted. The complement-mediated killing assay was carried out with 96-well tissue culture plates. A total of 5×10^5 to 6×10^5 spirochetes in 25 μl of BSK II medium was added to each well containing 50 μl of heat-inactivated (56°C, 30 min) serum samples serially diluted in the same medium. The plates were incubated at 34°C for 20 min before the addition of 25 μl of complement (normal monkey serum). After 5 h of incubation at 34°C, the total numbers of dead (nonmotile) and live (motile) bacteria were determined by dark-field microscopy. Equivalence between nonmotility and death was determined by quantifying in solid phase, spirochetes that had been subjected to ADCK titrations. Viable spirochetes were counted by their ability to form colonies on solid agar, by a procedure described previously (22). Three wells from an ADCK titration series that had contained initially the same number of viable HB19 spirochetes (5×10^5 to 6×10^5) were chosen so that one contained 100% nonmotile spirochetes, the second contained 46% nonmotile spirochetes, and the third contained 0% nonmotile organisms. A volume of 100 μl of a 1,000-fold dilution of BSK II medium from each well was mixed with 1.5 ml of 1% low-gelling-point agarose (Sigma Chemical Co., St. Louis, Mo.) in BSK II medium. Three such tubes were prepared for each of the initial microtiter plate wells. Each of the nine tubes was poured onto a 6-cm-diameter petri dish that contained 1% LE agarose (Sigma) in BSK II medium, and colonies were allowed to grow for 10 days at 34°C in a candle jar. The well that had contained 0% nonmotile spirochetes yielded 346 ± 15 colonies (mean number of colonies in three plates ± standard deviation), the well that had contained 46% nonmotile organisms yielded 187 ± 71 colonies, and the well with 100% nonmotile organisms yielded no colonies. Plating efficiency is routinely about 68%. The 50% killing titer ($ADCK_{50}$) was defined as the serum dilution at which 50% of the spirochetes were killed after 5 h of incubation with antibody and complement.

Western blot analysis. Antigen preparations were electrophoresed in 15% acrylamide mini-gels (10 by 10 by 0.1 cm) with a 5% acrylamide stacking gel. Twenty microliters of lysate containing 7×10^8 solubilized bacteria or 25 μg of protein (measured by optical density at 280 nm) was dispensed per track (the whole preparative track is equal to 16 single tracks; therefore, 400 μg of protein was loaded onto each preparative gel). Electrophoresis was performed with a mini-gel apparatus (Integrated Separation Systems, Hyde Park, Mass.) at a constant current of 23 mA with the buffers of Laemmli (14). For immunoblotting, the proteins in the polyacrylamide gels were electrotransferred to nitrocellulose paper (Schleicher & Schuell, Keene, N.H.) overnight at a constant voltage of 22 V in a Mighty Small transfer unit (Hoefer Scientific Instruments, San Francisco, Calif.) as described by Towbin et al. (28). Efficiency of transfer was assessed by staining part of the nitrocellulose with colloidal gold (Integrated Separation Systems). Nitrocellulose membranes were blocked with 3% fat-free powdered milk (Carnation) prepared in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Integrated Separation Systems) (PBS-T) for 2 h at room temperature. After the blocking step, the membranes were mounted in a Miniblotter 45 (Immunetics, Cambridge, Mass.) according to the manufacturer's instructions, and 110 μl of each serum sample diluted 1/50 with PBS-T was introduced into the Miniblotter's channels and allowed to interact with the nitrocellulose membrane for 1 h at room temperature on a rocking platform. After the incubation, the manifold system was used to wash the membranes with PBS-T. At this point, the Miniblotter was disassembled and the blot was taken out. The rest of the incubation steps were performed in small trays. After the wash, the membranes were incubated for 1 h with biotinylated anti-human immunoglobulin M (IgM) (μ-chain-specific) and IgG (γ-chain-specific) antibodies (Vector Laboratories, Burlingame, Calif.) diluted 1/200 in PBS-T. Biotinylated antibodies were probed with an avidin-biotinylated horseradish peroxidase complex (Vector) prepared according to the manufacturer's instructions. The reagent 4-chloro-1-naphthol (Sigma) was used as a chromogen. The color reaction was stopped by washing the membranes with distilled water. Mouse monoclonal antibodies (MAbs) H5332, H6831, and H9724 (Symbicom, Umea, Sweden) and anti-p39 MAb (kindly provided by Barbara Johnson, Centers for Disease Control and Prevention, Ft. Collins, Col.) were used to identify OspA, OspB, flagellin, and p39 antigens, respectively. Bound MAbs were detected with biotinylated anti-mouse IgG (heavy plus light chain) antibodies.

Inhibition of borreliacidal activity of serum by preincubation with recombinant OspA and OspB. Bactericidal assays were performed with sera preincubated (20 min, 34°C) with various concentrations of purified, nonlipidated recombinant OspA and/or OspB antigens from the B31 strain of *B. burgdorferi* (a gift from John Dunn, Brookhaven National Laboratory, Upton, N.Y.). The nonlipidated OspA provided by J. Dunn had been expressed from a DNA construct lacking the first 17 codons of the OspA gene, which code for an N-terminal signal sequence and a potential recognition site for lipoprotein signal peptidase II. The resulting water-soluble nonlipidated OspA was purified to homogeneity as described by Dunn et al. (6). OspB was cloned and purified to homogeneity by a similar procedure. Preliminary data for the deduced amino acid sequence of the JD1 OspA molecule indicate that it is over 96% identical to the OspA of strain N40 (14a). The latter OspA molecule, in turn, has a 99.27% amino acid sequence

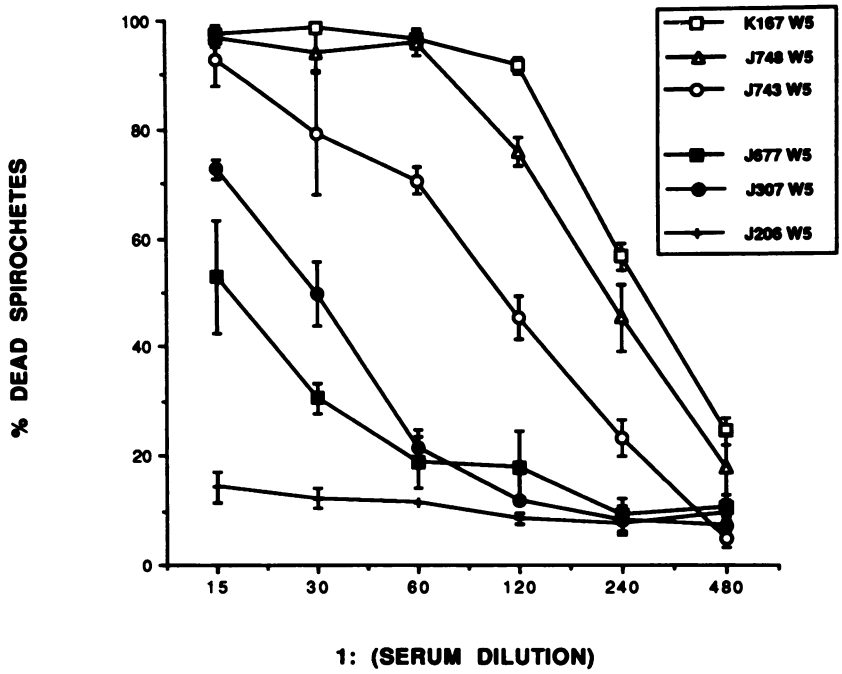

FIG. 1. ADCK of *B. burgdorferi* in vitro, assayed by using serum samples taken 5 weeks p.i. (W5) from rhesus monkeys infected with *B. burgdorferi* by needle inoculation (K167, J748, and J743) or by tick inoculation (J677 and J307) and from an uninfected rhesus macaque (J206). Spirochetes were processed for the ADCK assay as described in Materials and Methods. Error bars represent standard errors of the means of two determinations.

similarity with the OspA of B31 (29). We have assumed that the sequences of the OspB molecules from JD1 and B31 are similar to each other to the same extent.

Neutralization of borreliacidal activity with anti-human IgM and IgG antibodies. Monkey sera were preincubated for 20 min at 34°C with various concentrations of anti-human IgM (μ-chain-specific) or IgG (γ-chain-specific) antibodies (Vector Laboratories) and then used immediately in the borreliacidal assays.

Analysis of significance. Statistical significance of differences between mean $ADCK_{50}$s of TI and NI animals during the first 5 weeks postinoculation (p.i.) (see Fig. 2) and ADCK values for serum samples from NI animals in the presence and absence of added recombinant OspA or OspB were determined with the Student *t* test.

RESULTS

Abilities of serum antibodies from TI and from NI animals to kill spirochetes by ADCK. While performing experiments to characterize the humoral response to *B. burgdorferi* infection in the rhesus monkey, we noticed that the titer of anti-*B. burgdorferi* antibody, as measured by ADCK, was higher in an animal that had been needle inoculated than that of an animal that had been infected by tick inoculation (16). To confirm this result, the experiment was repeated with serum samples obtained 5 weeks after infection from three NI and two TI animals and one uninfected animal. The results are shown in Fig. 1. The $ADCK_{50}$ was between 1:105 and 1:278 in the NI animals (K167, J748, and J743), whereas in the TI monkeys (J677 and J307) it was between 1:17 and 1:30. Serum from uninfected animal J206, which contained a low titer of antibodies to flagellin and to three other unidentified antigens (see Fig. 3), killed about 10 to 15% of the spirochetes at a 1:15 or lower dilution (Fig. 1). Incubation of spirochetes with BSK II medium in the absence of antibody also resulted in 5 to 10% killing, with or without added complement. At a dilution of 1:15, heat-inactivated serum samples obtained 5 weeks p.i. from NI animals K167 and J748, but not J743, and from TI animals J307 and J677 were found to contain antibodies able to kill between 20% (TI animals) and 35% (NI animals) of the spirochetes in the absence of added complement (data not shown). This phenomenon was not observed in any animal with serum samples drawn 3 weeks p.i.

$ADCK_{50}$ as a function of time after infection. Sera from NI animals contained antibodies that reacted with OspA and OspB on Western blots of whole *B. burgdorferi* antigens, whereas sera from TI monkeys did not (16). The higher $ADCK_{50}$s observed in the NI animals by week 5 p.i. could be a direct consequence of this fact. On the other hand, the NI animals had received a much larger inoculum of *B. burgdorferi* (4×10^8 spirochetes) than the TI animals could have received. Thus, the differences in the $ADCK_{50}$s observed 5 weeks p.i. could be due to a more slowly evolving antibody response in the TI animals, corresponding with the smaller antigen load received. We measured the $ADCK_{50}$s in all animals as a function of time after infection, over a 36-week period, to determine whether the $ADCK_{50}$s of animals in both groups converged upon a value similar to that which the NI animals had shown by week 5 p.i.

Significant differences ($P < 0.005$) in the ability of sera from TI and NI monkeys to kill *B. burgdorferi* by ADCK were apparent only during the first 8 weeks p.i. (Fig. 2). By week 4 p.i., the $ADCK_{50}$ for NI animals averaged about 1:200, whereas it was about 1:30 for the TI monkeys. However, from weeks 6 through 8 p.i., the $ADCK_{50}$s for the NI animals diminished precipitously to a value comparable to that for the TI animals, which was maintained thereafter throughout the

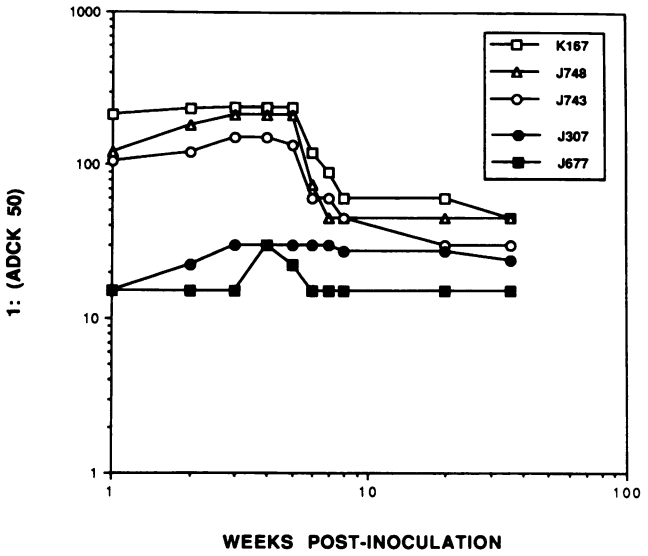

FIG. 2. $ADCK_{50}$ (reciprocal) of *B. burgdorferi* as a function of time after infection. Rhesus macaques were infected by needle inoculation (K167, J748, and J743) or by tick inoculation (J307 and J677).

36-week period investigated. Thus, the $ADCK_{50}$s of serum samples from TI animals did not increase with time; rather, the titer of killing antibody in the sera from the NI animals decreased. The results appeared to be the consequence of an antibody response to *B. burgdorferi* surface antigens that was transient in NI animals superimposed with a response that was long-lasting and present in both groups of monkeys. To explain the differences observed in the $ADCK_{50}$s for NI and TI animals, a longitudinal analysis of the antigens recognized by serum antibodies on Western blots was undertaken.

Antigens recognized by NI and TI monkeys as a function of time after infection. Western blot analysis of IgG serum antibody specificities as a function of time p.i. revealed that NI animals had anti-OspA and anti-OspB antibodies detectable between weeks 2 and 8 p.i., whereas TI monkeys did not show such antibodies at any time during the period studied. Otherwise, the antigen recognition patterns of antibodies from both groups of animals were indistinguishable from each other (Fig. 3).

OspA and OspB antigens bound by IgG antibodies were first detected by week 2 p.i. (Fig. 3), whereas IgM antibodies to both proteins were detected 1 week p.i. (not shown). The intensity of the OspA and OspB bands peaked between weeks 2 and 4 p.i. and then gradually declined. By week 8 p.i., the OspA and OspB bands were no longer detectable on the Western blots (Fig. 3). The temporal correlation between the elevated $ADCK_{50}$s and the presence of detectable anti-OspA and anti-OspB antibodies in the sera of NI animals was a strong indication that these two antigens were targeted by the ADCK mechanism.

Antigens that are targeted by the ADCK mechanism. To ascertain whether OspA and OspB were targeted by antibody participating in the ADCK mechanism, purified nonlipidated recombinant OspA and OspB were added to serum samples obtained 3 weeks p.i. from NI animals K167 and J748, at the appropriate dilutions, and incubated for 20 min at 34°C prior to the killing assay. Preincubation of serum from monkey K167 with 4 μg of OspA per well significantly inhibited killing of *B. burgdorferi* at serum dilutions of 1:240 and 1:480 ($P < 0.001$ at 1:240 and 1:480 for 8 μg of OspA added). Doubling the amount of OspA to 8 μg per well did not increase further the inhibition of ADCK (Table 1). Addition of increasing amounts of OspB in the range of 0.5 to 1.5 μg per well also diminished ADCK significantly at the same serum dilutions of 1:240 and 1:480 ($P < 0.001$ at 1:240 and $P < 0.025$ at 1:480, with 1.5 μg of OspB added) (Table 1). The effect of simultaneous addition of 4 μg of OspA per well and 1.5 μg of OspB per well was not additive (Table 1). Addition of either 4 μg of OspA or 1.5 μg of OspB to serum samples from animal J748 also inhibited significantly ADCK of *B. burgdorferi*, with this serum, at dilutions of 1:120 and 1:240 (Table 1). As before, the simultaneous addition of OspA and OspB did not result in further inhibition of ADCK beyond the level already achieved with either OspA or OspB (Table 1). As expected, ADCK levels with serum from a TI animal (J307) were unaltered in the presence of OspA or OspB or both proteins together (Table 2). Sera from NI animals reacted with both recombinant OspA and OspB on Western blots, whereas serum samples from TI animals did not (Fig. 3).

Immunoglobulin classes of the antibodies involved in the ADCK mechanism in the rhesus monkey. Serum samples obtained from rhesus monkey K167 at 1 and 4 weeks p.i. were diluted to 1:160 and incubated with 5 or 10 μg of anti-human IgM antibodies or with 15 or 30 μg of anti-human IgG antibodies prior to the ADCK assay. The rationale for choosing these two times was based on the fact that within the first week p.i. only IgM antibodies are detectable on Western blots of whole *B. burgdorferi* antigens, whereas by week 4 p.i. IgM antibodies have largely disappeared and IgG antibodies are prevalent (16). Hence, the involvement of IgM and IgG antibodies in ADCK could be assessed independently of each other. As expected, 1 week p.i. anti-IgM, but not anti-IgG, antibodies reduced the fraction of dead spirochetes to background values (5 to 10% with complement alone) (Fig. 4A), whereas by week 4 p.i. *B. burgdoferi* killing was abolished only by the addition of anti-IgG antibodies (Fig. 4B).

DISCUSSION

Two main conclusions may be drawn from our results. First, both OspA and OspB are targeted by ADCK of *B. burgdorferi* in the rhesus monkey. Second, one or more *B. burgdorferi* surface antigens that are neither OspA nor OspB also participate in the ADCK mechanism. The targeting of OspA and OspB by killing antibodies was demonstrated directly by the inhibition observed in the killing after the addition of recombinant OspA or OspB and indirectly by the temporal correlation between elevated $ADCK_{50}$s and the presence of detectable anti-OspA and -OspB antibodies in the sera of NI animals. Although it could be postulated that the inhibition observed was due to an *Escherichia coli* component that copurified with the recombinant OspA and OspB molecules, such a component also would have exerted its putative inhibitory effect in the experiment in which spirochetes were killed by ADCK with serum from TI animal J307 (Table 2). This serum does not have detectable anti-OspA or -OspB antibodies. Since no inhibition of ADCK was observed in the absence of anti-OspA or -OspB antibodies, a nonspecific inhibitory effect such as the one postulated above has to be ruled out.

The second conclusion, i.e., that killing of *B. burgdorferi* by antibody and complement in the rhesus monkey also proceeds through the binding of antibody to non-OspA, non-OspB (non-OspA/B) surface antigens, can be inferred from the fact that sera from TI animals, which do not contain detectable

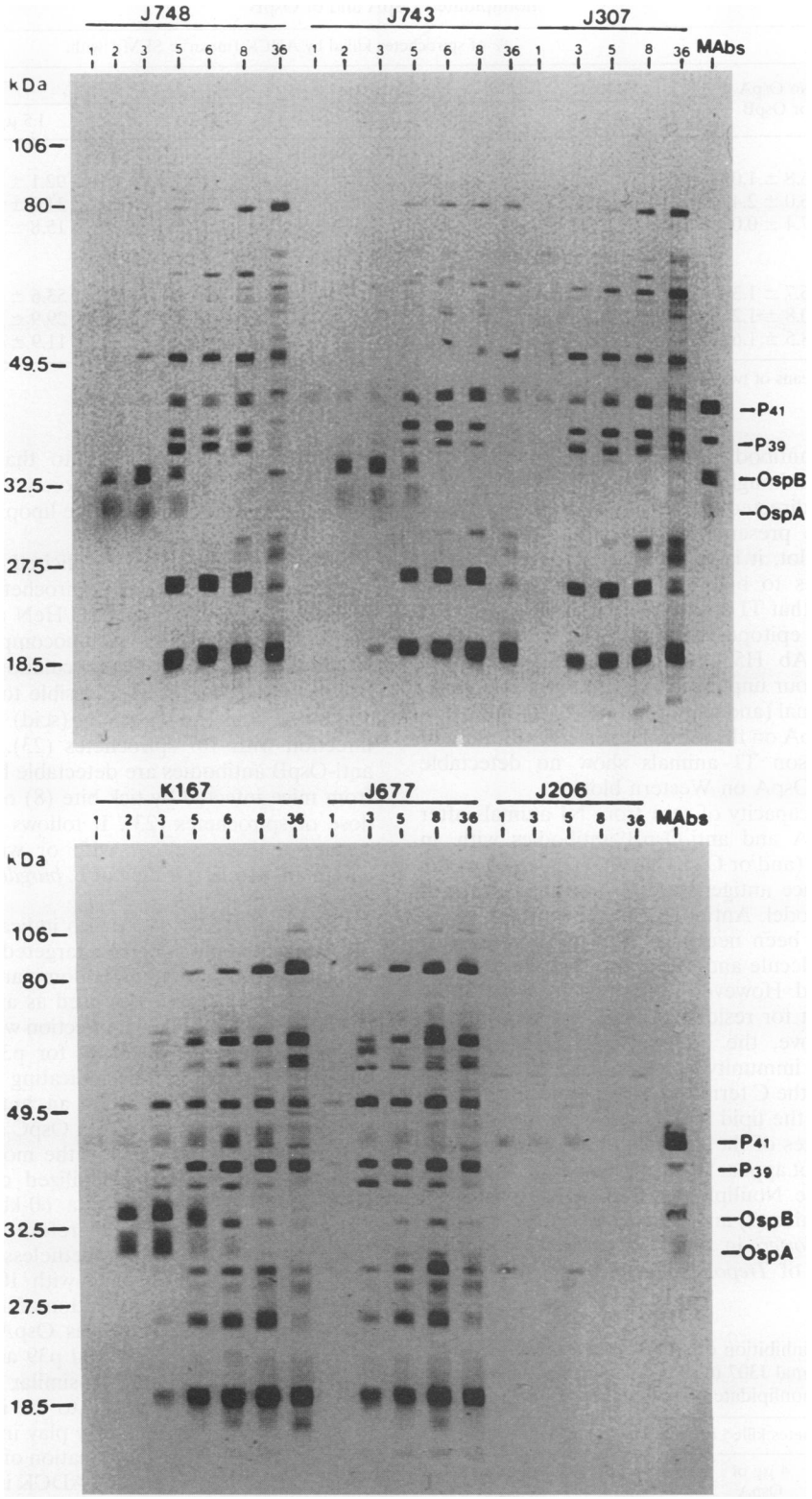

FIG. 3. Western blot analysis of *B. burgdorferi* antigens recognized by IgG antibody in serum samples from animals infected with *B. burgdorferi* JD1 by needle inoculation (K167, J748, and J743) or by tick inoculation (J677 and J307), as a function of time (weeks) p.i. (indicated above the lanes). J206 is a control (uninfected) animal. MAbs to p41 (flagellin), p39, OspA, and OspB were used to help identify the corresponding bands on the gels. Whole *B. burgdorferi* cells solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer were electrophoresed on 15% acrylamide gels and blotted onto nitrocellulose as described in Materials and Methods.

TABLE 1. Inhibition of ADCK of *B. burgdorferi* with sera from NI animals by addition of purified recombinant nonlipidated OspA and/or OspB

Animal and serum dilution	% of spirochetes killed by ADCK (mean ± SEM)[a] with:					
	No OspA or OspB	OspA		OspB		OspA (4 μg) + OspB (1.5 μg)
		4 μg	8 μg	0.5 μg	1.5 μg	
K167						
1:120	96.8 ± 1.0	90.0 ± 1.2	87.9 ± 2.0	96.0 ± 1.9	92.1 ± 2.3	90.7 ± 3.1
1:240	56.0 ± 2.4	36.3 ± 3.7	34.3 ± 2.4	40.1 ± 0.9	31.8 ± 3.3	38.4 ± 5.6
1:480	27.4 ± 0.0	ND	12.5 ± 2.6	ND	15.8 ± 3.2	12.3 ± 2.1
J748						
1:120	76.7 ± 1.3	55.7 ± 1.8	ND	ND	53.6 ± 3.1	52.8 ± 4.0
1:240	40.8 ± 1.2	27.7 ± 1.2	ND	ND	29.9 ± 2.0	25.7 ± 1.7
1:480	18.5 ± 1.6	14.4 ± 1.0	ND	ND	11.9 ± 0.2	11.4 ± 0.5

[a] Standard errors of the means of two or more independent determinations. ND, not determined.

anti-OspA or -OspB antibodies, can nonetheless effect the ADCK mechanism. Although it is formally possible that anti-OspA antibodies directed exclusively to conformational epitopes of OspA are present in TI animals and are not detected by Western blot, it is very unlikely that NI animals would make antibodies to both linear and conformational epitopes of OspA and that TI animals would make antibodies only to conformational epitopes. Moreover, the blot shown in Fig. 3 shows that MAb H5332, which is able to kill *B. burgdorferi* by ADCK (our unpublished data) and must therefore bind to the functional (and conformational) (5) epitope of OspA, does bind to OspA on the blots. Hence, denaturation of OspA is not the reason TI animals show no detectable antibody responses to OspA on Western blots.

The residual killing capacity of sera from NI animals after inhibition of anti-OspA and anti-OspB antibodies with an excess of soluble OspA (and/or OspB) further underscores the notion that other surface antigens are also targeted by antibodies in the rhesus model. Antibodies to the lipid moiety of OspA would not have been neutralized by the nonlipidated recombinant OspA molecule and could also contribute to the residual killing observed. However, we do not think that these antibodies may account for residual ADCK, for two reasons. (i) As mentioned above, the epitope(s) of OspA that is targeted by protective immunity in the mouse is conformational and is located at the C terminus of the molecule (5), not the N terminus, where the lipid moiety is placed. Despite the conformational attributes of the epitope, absence of the lipid moiety in OspA does not appear to alter the epitope specificity of the antibody response. Nonlipidated OspA elicits protective antibody responses in mice (7) and antibody responses capable of ADCK of *B. burgdorferi* in monkeys (18). (ii) TmpC, a membrane lipoprotein of *Treponema pallidum* which is lipidated in a manner similar to that of OspA, elicits only antibodies to the peptide moiety of the lipoprotein when rabbits are immunized with the lipoprotein or infected with *T. pallidum* (25).

TABLE 2. Absence of inhibition of ADCK of *B. burgdorferi* with serum from TI animal J307 in the presence of purified recombinant nonlipidated OspA and/or OspB

Serum dilution	% of spirochetes killed by ADCK (mean ± SEM)[a] with:			
	No OspA or OspB	4 μg of OspA	1.5 μg of OspB	4 μg of OspA + 1.5 μg of OspB
1:15	72.6 ± 1.8	74.4 ± 0.2	71.6 ± 2.9	74.6 ± 0.6
1:30	49.8 ± 11.8	53.0 ± 8.1	42.9 ± 2.9	46.8 ± 5.2
1:60	21.4 ± 5.6	23.4 ± 4.5	24.3 ± 5.1	20.7 ± 4.3
1:120	11.7 ± 0.6	11.1 ± 1.5	12.5 ± 0.9	10.8 ± 1.1

[a] Standard errors of the means of two or more independent determinations.

In the mouse, antibodies elicited by inoculation with very low doses of *B. burgdorferi* spirochetes (≤10) were shown to passively protect normal C3H/HeN animals (3). With serum antibodies produced in immunocompetent mice infected with *B. burgdorferi* either by tick inoculation or by a similar low-dose needle inoculation, it was possible to passively protect severe combined immune deficient (scid) mice from a challenge infection with 10^4 spirochetes (23). Neither anti-OspA nor anti-OspB antibodies are detectable by Western blot in serum from mice infected by tick bite (8) or by injection with a low dose of spirochetes (23). It follows that antibodies to other surface antigens, either with or without the assistance of complement, effect killing of *B. burgdorferi* in the mouse model as well.

Other lines of evidence also indicate that OspA and OspB are not the only antigens targeted by protective immune responses. OspC, an immunodominant 22-kDa surface protein (31) of *B. burgdorferi*, was used as an immunogen to protect gerbils against a challenge infection with the spirochete (19). A human MAb with specificity for p39 was shown to kill *B. burgdorferi* in vitro, thus indicating that this molecule is a surface antigen targeted by an antibody-mediated effector mechanism in vitro (27). Both OspC and p39 also were shown indirectly to be protective in the mouse. A vaccine prepared with an adjuvant and lyophilized cells of a mutant of *B. burgdorferi* 297 that lacked a 60-kb plasmid encoding the OspA/B operon and was therefore unable to express either surface protein was able nonetheless to induce 100% protection in hamsters challenged with 10^6 wild-type spirochetes. Conversely, a similar preparation of a nonvirulent form of this bacterial strain that expresses OspA and OspB, but whose expressed forms of OspC and p39 are nonimmunogenic, did not elicit protection under similar conditions (10). Taken together, these results attest to the important role that non-OspA/B surface antigens may play in the immunoprophylaxis of Lyme disease. The identification of the non-OspA/B surface antigens that are targeted by ADCK in the rhesus monkey is in progress.

As expected, both IgG and IgM antibodies were able to effect the ADCK mechanism. Complement alone (normal serum), added either to BSK II medium or to heat-inactivated sera from uninfected animals, did not cause significant killing of spirochetes. Thus, both in humans (12) and in rhesus

FIG. 4. Inhibition of ADCK of *B. burgdorferi* by addition of anti-IgG or anti-IgM antibodies to serum samples from rhesus macaque K167 obtained 1 (A) and 4 (B) weeks p.i.

monkeys, *B. burgdorferi* is resistant to the nonspecific bactericidal activity of normal serum. It should be noted, however, that we did not use serum dilutions lower than 1:15. Perhaps coincidentially, this is also the dilution above which nonspecific killing mediated by normal hamster serum was no longer detectable (15). On one occasion (5 weeks p.i.), killing in the absence of added complement was detectable. The effect was attainable only at a serum dilution of 1:15, not at higher dilutions, and was not found by week 3 p.i. A similar phenomenon was observed by Schmitz et al. (24), who noted that resistance to a challenge infection with *B. burgdorferi* could be passively transferred to irradiated hamsters pretreated with cobra venom factor with heat-inactivated serum obtained from infected hamsters 3 weeks p.i. To interpret these results, a better understanding of the mechanism of killing by antibody alone is required (4, 22).

Two of our results are puzzling and have interesting implications. The first is the nonadditiveness of the inhibitory effects of OspA and OspB on ADCK, which was evidenced when the two antigens were simultaneously present in the wells used for the assay. The second is the short duration of the antibody response to OspA and OspB (8 to 10 weeks) in NI animals, in the face of a persistent response to diverse antigens, including the non-OspA/B surface antigens targeted by the ADCK mechanism.

That the inhibitory effects of OspA and OspB on ADCK

were nonadditive implies that the epitope(s) on OspA and that on OspB targeted by antibody in this mechanism are the same, or at least cross-reacting, and that anti-ADCK epitope antibodies were completely bound by the added amounts of either OspA or OspB. This hypothesis is testable, but we have not performed the cross-adsorption experiments required to prove it. The cross-reactive epitopes on OspA and OspB could both be conformational and thus have no ostensible representation(s) in the primary amino acid sequence of the molecules.

A transient antibody response to OspA and OspB in mice of three different strains, AKR/N, C3H/HeJ, and DBA/2, also has been documented (8). Six weeks after the subcutaneous inoculation of 2×10^7 *B. burgdorferi* spirochetes, the latest time point for which data are available, antibodies to OspB were no longer detectable on Western blots, whereas anti-OspA antibodies had decreased significantly in avidity compared with those on Western blots at week 4 p.i. (8). As in the rhesus monkey, this ephemeral anti-OspA and -OspB response was in contrast with a relatively invariant response to other *B. burgdorferi* antigens.

The early decrease in detectable anti-OspA and -OspB antibodies, together with the inability of these antigens to elicit a detectable antibody response when *B. burgdorferi* is administered by tick bite or by injection of low numbers of organisms, indicates that they are either poorly immunogenic, at least as presented to the host's immune system by living spirochetes, or that the antibody response to them is actively and specifically suppressed. Alternatively, it is conceivable that antibodies to OspA and OspB are eventually complexed by these abundant antigens, which are known to be sloughed off together with membranous blebs (30). Indeed, in human infections with *B. burgdorferi*, anti-OspA antibodies have been found early after infection only in the form of immune complexes (26). However, OspC also appears to be present in membrane blebs (30), yet the antibody response to this molecule is quite evident in TI rhesus monkeys over prolonged periods after infection (1). One additional, tantalizing possibility is that the expression of the OspA/B operon is interrupted upon infection and is restored only in in vitro culture, within the tick vector, or in certain organs or tissular compartments within the vertebrate host. As a consequence, an antibody response to OspA or OspB would be detectable only if a large dose of spirochetes, including dead ones, were administered. Since OspA is a notoriously polymorphic antigen (2, 11) which, in extremis, may not be expressed at all (22), it is imperative to identify the other surface antigens targeted by the ADCK mechanism, in pursuit of more-conserved yet equally effective immunogens to compose a second-generation vaccine against Lyme borreliosis (17).

The pathogenesis of Lyme disease is insufficiently understood. Hence, the possibility that misdirected anti-*B. burgdorferi* immune responses contribute to Lyme arthritis or to neuroborreliosis cannot be discarded. A cautious approach to the immunoprophylaxis of Lyme disease should include studies of vaccine safety in animal models. The rhesus monkey, whose taxonomic proximity to the human and ability to mimic the signs of both early and late *B. burgdorferi* infection are unparalleled by other animal models of Lyme disease, lends itself well to the study of safety, immunogenicity, and efficacy of candidate vaccines (16, 18, 20). Since recombinant OspA is considered a major candidate for a molecularly defined vaccine against Lyme disease, the fact that this molecule and OspB are targeted by the ADCK mechanism in the rhesus monkey is of obvious relevance, for it entails that the functional epitope(s) of OspA (or OspB) are immunogenic in this animal.

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