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The Absence of Linear Plasmid 25 or 28-1 of *Borrelia burgdorferi* Dramatically Alters the Kinetics of Experimental Infection via Distinct Mechanisms

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The 25-kb linear plasmid lp25 and one of the 28-kb linear plasmids (lp28-1) are required for experimental infection in *Borrelia burgdorferi*, the etiologic agent of Lyme disease. The loss of these plasmids either eliminates infectivity (lp25) or significantly increases the 50% infective dose during a 2-week infection period (lp28-1). This study assessed the kinetics of bacterial dissemination in C3H/HeN mice infected with *B. burgdorferi* lacking either lp25 or lp28-1, as well as their wild-type parent, and tracked the development of specific borrelial antibodies over a 3-week period. The results indicated that the wild type and the lp28-1$^-$ strains were able to disseminate throughout the host, whereas the lp25$^-$ strain was cleared within 48 h of inoculation. While the wild-type *B. burgdorferi* persisted in tissues for the duration of the study, the lp28-1$^-$ mutant began clearing at day 8, with no detectable bacteria present by day 18. As expected, the wild-type strain persisted in C3H/HeN mice despite a strong humoral response; however, the lp28-1$^-$ mutant was cleared coincidently with the development of a modest immunoglobulin M response. The lp28-1$^-$ mutant was able to disseminate and persist in C3H-*scid* mice at a level indistinguishable from that of wild-type cells, confirming that acquired immunity was required for clearance in C3H/HeN mice. Thus, within an immunocompetent host, lp28-1-encoded proteins are not required for dissemination but are essential for persistence associated with Lyme borreliosis.

Lyme disease or Lyme borreliosis is a multisystemic disorder transmitted by ticks of the genus *Ixodes* infected with the spirochetal bacterium *Borrelia burgdorferi* sensu lato, composed of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (1, 8, 16). Lyme disease is the leading zoonotic infection in the United States, accounting for 95% of all reported vector-borne illness (10). The completed *B. burgdorferi* sensu stricto strain B31 annotated sequence revealed an unusual genome containing a linear chromosome of 910 kb in size as well as several extrachromosomal elements, including 12 linear plasmids of sizes ranging from 5 to 56 kb and 9 circular plasmids with sizes between 9 and 32 kb (9, 14).

Early studies had demonstrated a correlation between plasmid content and infectivity but were hindered by the plethora of plasmid species in *B. burgdorferi* and the inherent difficulty in resolving the many linear and circular species (24, 29–31). As such, prior to the information gained from the *B. burgdorferi* strain B31 genome sequence, it was difficult to definitively link a specific plasmid to a defect in infectivity. Exploiting the completed genome sequence of *B. burgdorferi*, we designed oligonucleotide primers that could be used in conjunction with PCR to specifically catalog the presence of plasmids from clonal isolates of *B. burgdorferi* (17). In our quest to link infectivity to any of the plasmids, we determined, along with others, that two linear plasmids, 25 and 28 kb in size (designated lp25 and lp28-1, respectively), were required for maximal infectivity in the mouse model of infection, suggesting that these plasmids encode proteins important for survival of *B. burgdorferi* in the mammalian host (17, 27). In addition, McDowell et al. demonstrated that the loss of infectivity of *B. burgdorferi* does not always correlate with plasmid content, indicating that other plasmid-independent mechanisms are involved in pathogenesis (22).

An initial report indicated that cells lacking lp25 were completely noninfectious, whereas cells missing lp28-1 were still infectious, as *B. burgdorferi* could be isolated from joint tissue 14 days following inoculation (17). It was speculated that cells missing lp28-1 might also prove to be defective in persistence in this tissue in a prolonged experimental infection (17). Furthermore, it is not known how the absence of lp25 and lp28-1 affects the kinetics of visceral dissemination, and no serological analysis was evaluated to determine the humoral response to the mutant strains. As such, the role of host immune mechanisms in the accelerated clearance of the mutants relative to their wild-type parent has not been characterized.

In this study, we tested the hypothesis that cells lacking plasmids lp25 and lp28-1 have distinct dissemination kinetics during the course of infection in the murine animal model of Lyme disease. Furthermore, we were interested in determining the role that adaptive immunity played in the clearance of the mutants, since the parent isolate is not affected by the robust humoral immune response observed following infection. The results obtained indicate that cells lacking lp25 do not survive in the host and are quickly eliminated. Moreover, these data demonstrate that lp28-1 is not required for disseminated in-

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fection in the mouse animal model of Lyme borreliosis but is essential for persistent infection by *B. burgdorferi*.

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* sensu stricto strain B31 was used exclusively in this study. The clonal derivatives of strain B31 used have been previously described: MSK5 contains all the plasmids, MSK10 is missing lp28-1, and ML23 lacks lp25 (17). All *B. burgdorferi* cultures were grown in 1% CO_2 at 32°C in BSK-II liquid medium lacking gelatin (2) and supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, Ark.).

Infection experiments. All animal procedures were done in accordance with recommended and approved protocols from the Texas A&M University Laboratory Animal Care Committee. Eight-week-old C3H/HeN (Charles River Laboratories, Wilmington, Mass.) or C3H/Smn.ClcrHsd-*scid* (C3H-*scid*) (Harlan Laboratories, Philadelphia, Pa.) female mice were separately infected by intradermal inoculation with either 10^3 or 10^5 bacteria of the three different *B. burgdorferi* B31 isolates tested: MSK5 (wild type), MSK10 (lp28-1$^-$), and ML23 (lp25$^-$). Mice were sacrificed at different time points, depending on the mouse strain used and the borrelial isolate used for infection. Immune-competent animals were sacrificed at 11 different time points (4 mice per time point): 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 21 days following intradermal inoculation with either MSK5 or MSK10. C3H/HeN mice infected with 10^5 ML23 organisms were sacrificed on days 1, 2, 4, 6, 8, 10, 12, and 14; mice infected with 10^3 ML23 organisms were sacrificed on days 1, 2, and 4 only. The *scid* mice were sacrificed as follows. MSK10-infected mice were sacrificed on days 4, 6, 8, 12, and 14 postinfection; MSK5-infected mice were sacrificed on days 7 and 14 postinfection, and ML23-infected mice were sacrificed at 7 days postinfection. For all infectivity studies, the spleen, left tibiotarsal joint, left inguinal lymph node, heart, bladder, and a piece of abdominal skin were removed aseptically, processed using sterile pestles, and placed in liquid BSK-II medium. Cultures were passaged 5 days later into fresh BSK-II medium to minimize toxicity associated with host tissue. Tubes were examined for borrelial growth every week thereafter and up to 21 days using dark field microscopy; tissue samples were considered positive if actively motile spirochetes were observed (after scanning approximately 100 fields).

Infection-derived sera. Serum from 4 mice per time point was obtained at days 1, 2, 4, 6, 8, 10, 12, 14, 18, and 21 postinfection by retro-orbital bleed prior to sacrifice and was processed and stored as previously described (15).

ELISA. Ninety-six-well enzyme-linked immunosorbent assay (ELISA) plates (Dynex Technologies Inc., Chantilly, Va.) were coated with 100 μl of in vitro-grown low-passage wild-type infectious *B. burgdorferi* total sonicate (25 μg/ml) per well in coating buffer (50 mM sodium carbonate, pH 9.6) and incubated at 4°C overnight. After three 3-min washes with 200 μl of phosphate-buffered saline (PBS)-Tween buffer (0.80 mM Na_2HPO_4, 137 mM NaCl, 0.27 mM KCl, 0.15 mM KH_2PO_4, and 0.5% Tween 20 [pH 7.4]), 200 μl of blocking buffer (PBS-Tween buffer supplemented with 3% bovine serum albumin) was applied to each well and incubated for 2 h at 37°C. The wells were washed three times with 200 μl of PBS-Tween buffer. Twofold serial dilutions of mouse serum, ranging from 1:200 to 1:25,600 (in 100-μl volumes), were placed in blocking buffer (PBS-Tween buffer supplemented with 1% bovine serum albumin) and added to each well, and the plates were incubated for 1 h at 37°C. Each serum sample from an individually infected mouse was assayed in triplicate at the time points indicated above. Serum samples from a given time point were not pooled. Normal mouse serum was diluted (starting at 1:200 and twofold serially diluted to 1:25,600) and assayed in triplicate to establish a baseline. After the primary antibody incubation, the wells were washed five times with PBS-Tween. Subsequently, 100 μl of goat anti-mouse immunoglobulin G (IgG) or goat anti-mouse IgM (Pierce Endogen, Rockford, Ill.), each conjugated to horseradish peroxidase and dissolved in blocking buffer, was diluted 1:5,000 and added to each well, and the plates were incubated at 37°C for 1 h. After five washes with PBS-Tween buffer, 100 μl of *o*-phenylenediamine dihydrochloride (Sigma Chemical, St. Louis, Mo.) was added. Development of the enzymatic reaction proceeded for 10 min. The plates were read at 490 nm in an ELISA plate reader (MR5000; Dynatech). The values for optical density at 490 nm obtained for each mouse at a given time point were averaged, and their standard deviation was determined. The titer for an individual mouse serum sample from a given time point was determined to be the reciprocal of the highest dilution that was greater than twice the average value obtained for the normal mouse serum sample at the same dilution value.

RESULTS

Kinetics of infection in immunocompetent C3H/HeN mice. Previous observations indicated that *B. burgdorferi* lacking lp25 or lp28-1 was significantly attenuated in the mouse model of infection (17, 27). Specifically, lp25 mutants were never recovered from mouse tissues, whereas lp28-1 mutants were found only in joint tissue. Based on these observations, we speculated that lp28-1 mutants had a tropism for joint tissue. However, since all prior infection studies were limited to a single terminal 2-week sample, we were interested in determining the kinetics of dissemination to all tissues, including joints, for the mutants relative to that for their wild-type parent. To address this, we infected mice with the parent strain, infected the lp25 and lp28-1 mutants at two different inocula, and sacrificed animals at days 1 and 2 and then every 2 days to day 18. A final sample was taken also at day 21. Tissue samples were cultivated in BSK-II medium and were examined for borrelial growth by dark-field microscopy, and the cultures were considered positive if actively motile spirochetes were observed up to 3 weeks after the mice were sacrificed. The results shown in Fig. 1 indicate that MSK5, the parent strain, was able to disseminate to all the tissues tested by day 6 at the low inoculum (10^3 organisms) (Fig. 1A) and by day 4 at the large inoculum (10^5 organisms) (Fig. 1B). All tissues tested remained infected for the duration of the study for MSK5 samples. MSK10, the isolate lacking lp28-1, was able to disseminate to all the tissues by day 6 at the low inoculum (Fig. 1C) and by day 4 at the high inoculum (Fig. 1D). When the low inoculum was used, the number of infected tissues started to decline at day 10, and by day 18, no organisms could be recovered (Fig. 1C). At the high inoculum, the number of tissues infected with MSK10 started to decline at day 8 until no organisms could be recovered by day 18 (Fig. 1D). The ML23 isolate, lacking lp25, was recovered 24 h after infection in the either the skin or, additionally, the lymph node and spleen at an inoculum of 10^3 or 10^5 organisms, respectively (Fig. 1E and F). No samples were culture positive for ML23 in any tissue at either inoculum following 48 h of infection (Fig. 1E and F).

Measurement of immunoglobulin levels. Previous studies have shown that passive transfer of antisera leads to protection against *B. burgdorferi* infection, indicating that humoral mechanisms are sufficient for the clearance observed (4, 11, 34). To determine if the differences in the clearance of the mutants correlates with the development of the humoral response observed, the levels of both IgM and IgG were measured for four separate mice at each of the aforementioned time points by ELISAs (Table 1), and a range of titers was then determined for each time point. At an inoculum of 10^3 organisms, IgM levels could be detected for both MSK5 (wild type) and MSK10 (lp28-1$^-$), with levels that peaked at day 14 for MSK5 (with titers ranging from 1:3,200 to 1:12,800) and at day 10 for MSK10 (with titers ranging from undetectable levels up to 1:3,200) (Table 1). At the higher inoculum, the IgM levels peaked at day 12 and day 10 with an endpoint titer varying from 1:1,600 to 1:3,200 for MSK5 and from 1:1,600 to 1:6,400 for MSK10, respectively (Table 1). When mice were infected with an inoculum of 10^3 MSK5 organisms, IgG was detected by day 12 and rose to endpoint titers ranging from 1:6,400 to 1:12,800 by day 16, whereas only sparse levels of IgG were

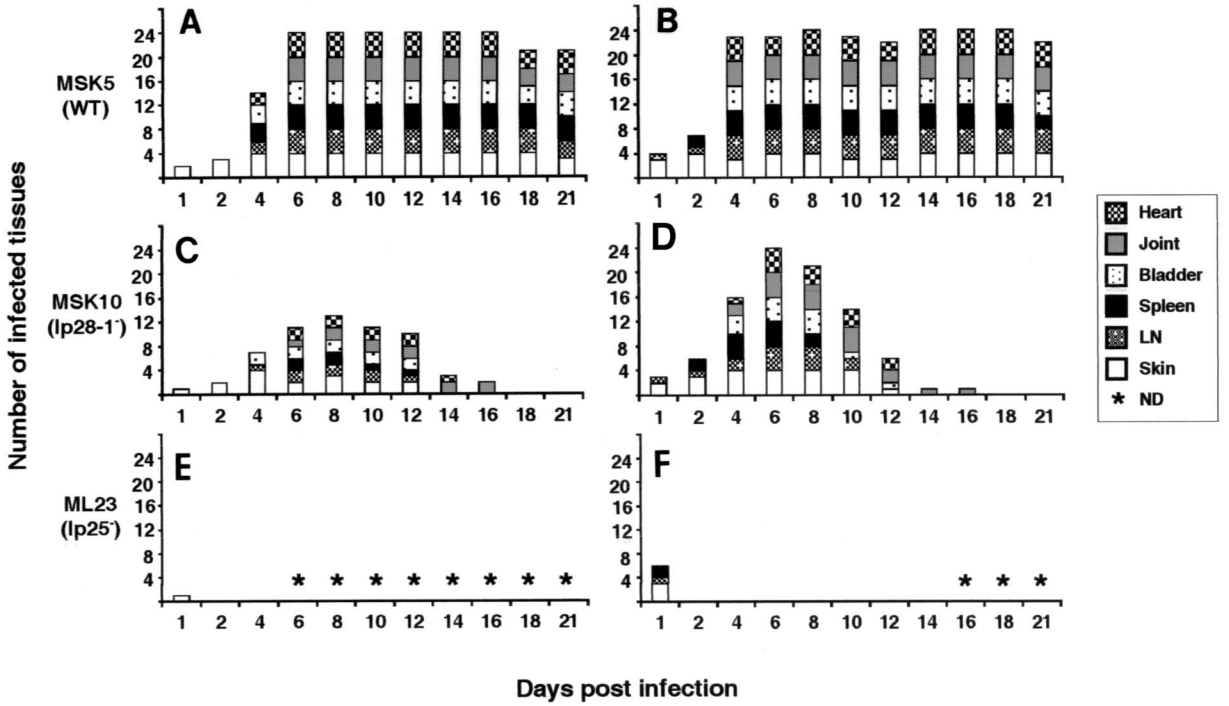

FIG. 1. Kinetics of bacterial dissemination in C3H/HeN mice infected with either wild-type *B. burgdorferi* cells (MSK5) at an inoculum of 10^3 (A) or 10^5 (B) organisms, lp28-1$^-$ cells (MSK10) at an inoculum of 10^3 (C) or 10^5 (D) organisms, or lp25$^-$ cells (ML23) at an inoculum of 10^3 (E) or 10^5 (F) organisms. Groups of four mice were sacrificed per time point indicated. The *y* axis indicates the total number of tissues tested that were infected, and each patterned box represents a specific tissue. The value shown for an individual tissue indicates the number of positive animals out of the four animals infected. LN, lymph node; ND, not determined.

detected in mice infected with 10^3 MSK10 organisms (Table 1). With an inoculum dose of 10^5 MSK5 organisms, IgG levels were detectable by day 14, with all four mice having titers ranging from 1:1,600 to 1:6,400 by day 16 (Table 1). In contrast, mice infected with 10^5 MSK10 organisms showed a reduced response relative to their wild-type *B. burgdorferi* parent (MSK5), exhibiting a detectable IgG response at day 10 (with titers varying from undetectable levels up to 1:400) (Table 1) which peaked by day 21 (titers ranged from 1:400 to 1:3,200) (Table 1). Serum from ML23 (lp25$^-$)-infected animals was tested at days 1, 2, and 4 and every other day to day 14 for the inoculums of 10^3 and 10^5 organisms, respectively (data not shown). No IgM or IgG antibody titer was detected for either inoculum (data not shown). Taken together, these results in-

TABLE 1. Range of IgM and IgG antibody titers elicited by infection with *B. burgdorferi* strain B31 isolates MSK5 (wild type) and MSK10 (lp28-1$^-$)[a]

Days postinfection	MSK5 (wild type)				MSK10 (lp28-1$^-$)			
	IgM antibody titer with inoculum of:		IgG antibody titer with inoculum of:		IgM antibody titer with inoculum of:		IgG antibody titer with inoculum of:	
	10^3 cells	10^5 cells	10^3 cells	10^5 cells	10^3 cells	10^5 cells	10^3 cells	10^5 cells
1	—[b]	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—
4	0–800[c]	—	—	—	—	—	—	—
6	0–800[c]	—	—	—	—	—	—	—
8	—	800–3,200	—	—	—	800–3,200	—	—
10	1,600–3,200	1,600–3,200	—	—	0–3,200[c]	1,600–6,400	—	0–400[c]
12	1,600–6,400	1,600–3,200	0–1,600[c]	—	0–200[c]	800–3,200	0–200[c]	0–800[c]
14	3,200–12,800	1,600–3,200	3,200[d]	1,600[d]	0–1,600[c]	200–400	—	0–800[c]
16	6,400[d]	800–1,600	6,400–2,800	1,600–6,400	0–1,600[c]	0–1,600[c]	0–3,200[c]	200–1,600
18	0–3,200[c]	400–1,600	0–12,800[c]	0–6,400[c]	0–400[c]	0–1,600[c]	0–800[c]	800–1,600
21	0–800[c]	200–1,600	0–2,800[c]	0–12,800[c]	—	0–400[c]	0–200[c]	400–3,200

[a] Four mice were sacrificed per time point per inoculum size (either 10^3 or 10^5 organisms). Antibody titers were calculated for each individual mouse and are listed as the range observed for four animals per time point.
[b] —, no detectable antibody titer for any of the four mice at the time point and inoculum given.
[c] At least one of the 4 mice tested had no detectable antibody titer at this time point.
[d] All four mice had the same titer at the time point and inoculum.

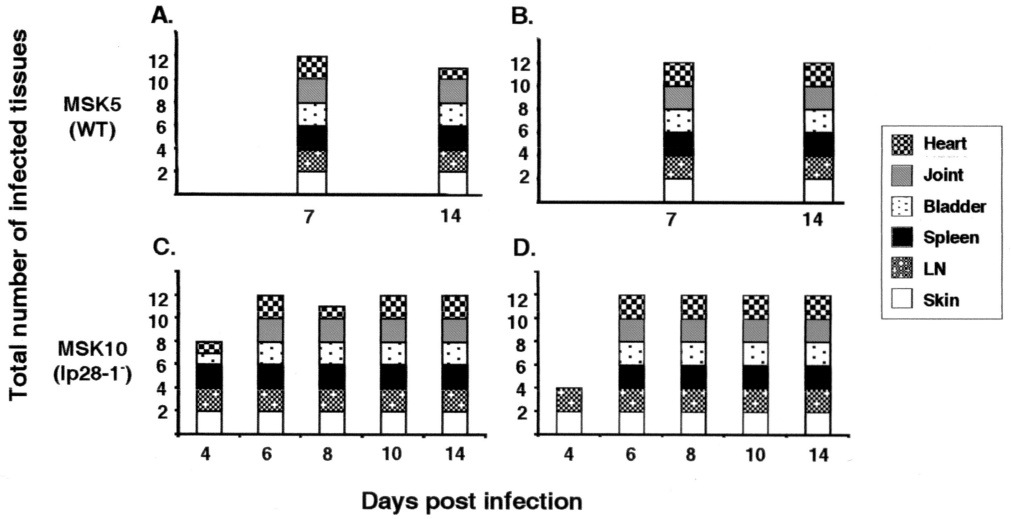

FIG. 2. Kinetics of bacterial dissemination in C3H *scid* mice infected with 10^3 (A) or 10^5 (B) wild-type *B. burgdorferi* cells (MSK5) or 10^3 (C) or 10^5 (D) lp28-1$^-$ cells (MSK10). Two mice were sacrificed per time point indicated. The *y* axis indicates the total number of tissues tested that were infected, and each patterned box represents a specific tissue. The value shown for an individual tissue indicates the number of positive animals out of the two animals infected. LN, lymph node.

dicate a dose dependence of the humoral response for IgG in both MSK5 and MSK10, as titers took longer to develop at the lower inoculum dose; no such correlation was found for the IgM response (Table 1). As previously reported (3–5, 7), the wild-type infectious isolate elicits a strong humoral response yet still disseminates and persists inside the host (Fig. 1 and Table 1), indicating that wild-type *B. burgdorferi* is able to avoid antibody-based clearance mechanisms due to immune-protected niches it occupies in the host and/or to antigenic evasive mechanisms, including both antigen variation and potential phase-variable mechanisms (20, 21, 32, 33). In contrast, MSK10 cells are cleared coincidently with the appearance of *B. burgdorferi*-specific IgM, suggesting that the absence of lp28-1 puts *B. burgdorferi* at significant risk of humoral-response-based clearance mechanisms.

Kinetics of infection in C3H/HeN *scid* mice. Because the bacterial clearance from tissues in the MSK10 (lp28-1$^-$)-infected animals correlated with the development of an adaptive immune response (i.e., specific antibodies), we tested the ability of the lp28-1 mutant to colonize mice lacking active immunity. To address this, the infection kinetics experiment described above for immunocompetent mice was repeated with C3H *scid* mice. If the lp28-1$^-$ cells are cleared from the animals in an antibody-dependent manner, then the MSK10 isolate should have a pattern of infection in *scid* mice similar to that of wild-type parent strain MSK5 in immunocompetent mice. The same infection protocol used for immune-competent mice was used to infect the *scid* mice except that the time points varied (see Materials and Methods for details). As positive controls, we infected *scid* mice with 10^3 and 10^5 MSK5 organisms and found that they demonstrated similar infectivity kinetics relative to C3H/HeN mice (Fig. 2A and B, respectively). Results shown in Fig. 2C and D indicated that MSK10 was able to disseminate and persist in all the tissues tested just as wild-type organisms do in immunocompetent mice, regardless of the infectious dose (compare Fig. 1A and B with Fig. 2C and D). As with the immunocompetent mice, the lp25$^-$ cells did not survive in *scid* mice (data not shown). These results support the hypothesis that the lp28-1$^-$ mutant organisms are cleared by the adaptive immune response and also rule out the possibility that innate immune mechanisms (i.e., dendritic cells, natural killer cells, complement, etc.) are responsible for the clearance of MSK10 observed in immunocompetent animals, since these modalities are operative in the *scid* mice as well.

DISCUSSION

We have previously shown that *B. burgdorferi* B31 lacking plasmid lp28-1 can be recovered from the joints 2 weeks after infection; however, when plasmid lp25 is missing, organisms cannot be recovered from mice infected with inocula as high as 10^5 bacteria (17). We suspected that immune mechanisms might explain the differential clearance observed for the lp25$^-$ and lp28-1$^-$ isolates. To test this hypothesis, we conducted a kinetic infection experiment over a period of 3 weeks to compare the dissemination in the mouse model of infection of the mutant strains relative to that of their isogenic parent and determine the differential humoral response to each strain tested. The results reported here indicate that *B. burgdorferi* strain B31 lacking lp25 does not survive in mice, as organisms are not detectable in host tissue 24 to 48 h after intradermal inoculation. In contrast, cells of *B. burgdorferi* strain B31 lacking lp28-1 disseminate to all tissues tested but are cleared starting at day 8 or day 10, depending on the inoculum (Fig. 1C and D) and coincidently with the appearance of a *B. burgdorferi*-specific IgM response (Table 1), until the spirochetes are eliminated from all tissues by day 18 (Fig. 1C and D).

Since our previous study was limited to a single 2-week time point, we did not know the timing in which the mutants were cleared from the host. Interestingly, the lp28-1 mutant disseminated to all tissues tested, whereas the lp25 mutant was recovered only from the skin, lymph nodes, or spleen (depending on inoculum size) 24 h following infection but not at the 48-h time point, indicating that the lp25 mutant is not able to survive within the

host (Fig. 1E and F). Based on these results, and in conjunction with serological analysis, we determined that clearance of the lp28-1 mutant was superimposable with the peak of a *B. burgdorferi*-specific IgM response (Fig. 1 and Table 1). No detectable humoral response was detected for the lp25 mutant. Thus, the absence of either lp25 or lp28-1 puts these mutants at risk of clearance from the host due to distinct mechanisms.

In the case of the lp25 mutants, their inability to survive within the infected host suggests that they are either cleared by innate immune mechanisms or have a metabolic defect rendering them incapable of replication within the murine host (and presumably other mammals). Our attempts to address the innate immune mechanisms, specifically, complement sensitivity, have been inconclusive, since all *B. burgdorferi* B31 isolates used in this study demonstrated complete resistance to guinea pig complement (data not shown) despite controls indicating that the complement utilized was active (data not shown). Careful analysis of the open reading frames on lp25 indicated that the plasmid contains several paralogous genes found on all *B. burgdorferi* plasmids that are involved in plasmid partitioning to daughter cells and additional loci that either encode small peptides, contain frameshifts, or are pseudogenes (9, 14). Only two genes on lp25 are unique to this plasmid. One encodes a type IV restriction-modification enzyme that has recently been shown to function as a transformation barrier, since strains lacking lp25 are transformable with plasmid DNA, whereas the parental isolate were not (18). The other gene unique to lp25 encodes a nicotinamidase. The nicotinamidase is involved in the biochemical pathway that converts nicotinamide to nicotinate, both of which serve as precursors in the synthesis of intracellular NAD. Because *B. burgdorferi* does not encode cytochromes and thus does not utilize oxidative phosphorylation for energy production, substrate level phosphorylation via the glycolytic pathway is important in generating intracellular ATP (14). Thus, de novo synthesis of NAD via the nicotinamide-scavenging pathway is important for homeostasis in *B. burgdorferi*. Recently Purser et al. demonstrated that the nicotinamidase gene was sufficient to complement the lp25 noninfectious phenotype, indicating that the NAD biosynthetic pathway is essential for the survival of *B. burgdorferi* in a mammalian host (26). Furthermore, our kinetic studies indicate that the requirement of the nicotinamidase gene is absolute, since *B. burgdorferi* lacking this enzyme is unable to survive in the mammalian host 24 to 48 h following inoculation (Fig. 1E and F).

In contrast to the cells lacking lp25, lp28-1 mutants were able to disseminate to all tissues tested but were cleared coincidently with the appearance of an IgM response specific for *B. burgdorferi* (Fig. 1 and Table 1), indicating that the lack of lp28-1 abrogated the ability of the mutant to evade the host humoral immune response and persist like the parent isolate. The parent strain exhibited the well-characterized robust humoral immune response that is insufficient in clearing *B. burgdorferi* from the host (4, 5), indicating that this immune response-evasive activity is directly or indirectly associated with proteins that are encoded by lp28-1 (Fig. 1 and 2). The most likely candidate is the lp28-1-encoded antigenic variant VlsE (32). The *vls* locus contains 15 silent cassettes and an expression site near the 3′ telomeric end that comprise roughly one-third of the entire plasmid. Previous studies have shown that the silent cassettes are sources for multiple nonreciprocal recombination events with the expressed locus that give rise to large numbers of distinct antigenic variants during infection (21, 32, 33).

The crystal structure of VlsE shows that the variable domains reside on the surface of the molecule where one would expect opsonogenic antibodies to bind (12). Based on the high degree of complexity associated with *vls* recombinational variants, the antibody response of the host to these variable domains is incapable of keeping pace with the heterogeneity of the VlsE variants produced per unit organism in a given population. As such, each individual *B. burgdorferi* cell will express a unique VlsE variant that, when processed by the host, will yield an immune response that is incapable of neutralizing the antigenically distinct VlsE molecules produced subsequently (21, 32, 33).

Our results demonstrate that mutants lacking lp28-1 alone are unable to persist in the face of a modest and variable humoral immune response generated by the host based on the low antibody titers observed following infection with the lp28-1$^-$ mutant (Table 1). Furthermore, the importance of the acquired immune response against the lp28-1 mutant as a clearance mechanism was corroborated by the results obtained using infected *scid* mice (Fig. 2). *scid* mice are deficient in T and B lymphocyte production and as such cannot generate either a cellular or humoral response to pathogens. When *scid* mice were infected with the lp28-1 mutant, we found that the infectivity profiles were indistinguishable from the parent strain with *B. burgdorferi* cultivated from all tissues tested, whether lp28-1 was present or not (Fig. 2), indicating that the clearance of the lp28-1 isolate in immunocompetent mice required an adaptive immune response. The fact that the clearance of the lp28-1 mutant correlated with the peak of IgM response in the immunocompetent mouse suggests that the basis of the differential clearance of the lp28-1 mutant seen in the C3H/HeN mice is due to humoral immune mechanisms (Fig. 1 and 2 and Table 1).

Our contention that the clearance mechanism observed for the lp28-1 mutant is due to humoral immune mechanisms is supported by studies demonstrating that T-cell depletion does not alter the course of experimental Lyme borreliosis (23). Furthermore, passive immunizations from both immunocompetent and T-cell-deficient mice provide protection against experimental infection, thus corroborating the importance of antibodies in the clearance of *B. burgdorferi* (4, 6, 11). Recently, Philipp et al. suggested that VlsE might be a T-cell-independent antigen that could directly stimulate B cells to produce antibodies (25). Such a response would be absent in the lp28-1 mutants lacking the *vls* system. If one assumes that the basis for the lp28-1$^-$ phenotype is directly linked to VlsE, then wild-type cells containing VlsE are capable of modulating the humoral immune response via antigenic variants that accumulate over time. Alternatively, because VlsE is an immunodominant antigen (19), the potent humoral response it engenders may override the production of antibodies against other potentially protective antigens. Thus, in the case of the lp28-1 mutant, the absence of VlsE allows for efficient processing of antigens via conventional T-cell-dependent immune responses that results in the development of antibodies that neutralize and kill *vls*-lacking *B. burgdorferi* organisms within the infected mammal. As such, it may be possible to use the lp28-1 mutant to

identify non-Vls antigens that may prevent colonization of infectious *B. burgdorferi* and thus confer protection. This approach would require high-titer borrelicidal antibodies that would neutralize infectious *B. burgdorferi* prior to the induction of the Vls-specific T-cell-independent response.

While it is possible that genes other than *vls* and its cassettes on lp28-1 are responsible for the phenotype of the lp28-1 mutant, this seems unlikely, inasmuch as most of these loci (20 out of 32) are either paralogues found on other borrelial plasmids, frameshifted genes, or pseudogenes (9, 14). In fact, only 12 loci encode novel products. Eleven are polypeptides of 99 amino acids or less that have no known functional homologues; the remaining unique locus is the *vls* system. Furthermore, the significant dedication of such a large component of this plasmid (>8 kb) to *vls* provides further support that this system is a key component of host immune evasion and the overall pathogenic strategy of *B. burgdorferi*. Nevertheless, until genetic knockouts are generated that specifically inactivate the *vlsE* expression site, or other equivalent genetic studies that exclude the effect of the other non-*vls* genes on lp28-1 are performed, the net effect of antigenic variation as the lone lp28-1-specific pathogenic mechanism used by *B. burgdorferi* will remain unresolved. With the recent advent of improved genetic systems for infectious *B. burgdorferi* mutagenesis (13, 28), the ability to conduct such studies is certainly forthcoming.

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