Published in final edited form as:

Immunol Res. 2011 December ; 51(2-3): 249–256. doi:10.1007/s12026-011-8260-8.

B1b lymphocyte-derived antibodies control *Borrelia hermsii* independent of Fcα/μreceptor and in the absence of host cell contact

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Abstract

The critical role of IgM in controlling pathogen burden has been demonstrated in a variety of infection models. In the murine model of *Borrelia hermsii* infection, IgM is necessary and sufficient for the rapid clearance of bacteremia. Convalescent, but not naïve, B1b cells generate a specific IgM response against *B. hermsii*, but the mechanism of IgM-mediated protection is unknown. Here we show that neither Fcα/μR, a high-affinity receptor for IgM, nor IgM-dependent complement-activation are required for controlling *B. hermsii*. Bacteria in diffusion chambers with a pore size impermeable to cells were killed when diffusion chambers were implanted either into convalescent or passively immunized mice. Furthermore, adoptively transferred convalescent B1b cells in Rag1^{-/-} mice produced specific IgM that also cleared *B. hermsii* in diffusion chambers independent of complement. These results demonstrate that IgM-mediated clearance of *B. hermsii* does not require opsonophagocytosis and indicate that a mechanism for *in vivo* B1b cell-mediated protection is through the generation of bactericidal IgM.

Keywords

B1b cells; IgM; Complement; *Borrelia hermsii*; Fcα/μR

Introduction

IgM is important for the early control of infection. Natural IgM is generated spontaneously in the absence of antigenic stimulation and can contribute to early defense by limiting the initial pathogen burden [1, 2]. IgM is also generated in an antigen-specific manner during the early phases of the immune response to specific pathogens. Natural IgM as well as antigen-induced IgM have been demonstrated in protective immunity to a number of pathogens, such as *Streptococcus pneumoniae* and influenza virus [3, 4].

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The vital role of IgM in the rapid control of bacteremia due to infection with relapsing fever bacteria is well defined [5, 6]. To study the role of IgM in controlling bacteremia we have been utilizing the murine model of infection by *Borrelia hermsii*, a relapsing fever spirochete that causes recurrent episodes of high-level ($\sim 10^8$ bacteria/ml blood) bacteremia associated with the outgrowth of individual serotypes [7, 8]. These serotypes are defined by the transient expression of antigenically distinct major surface proteins of *B. hermsii*, the variable major proteins (Vmbs) [8]. Control of each wave of bacteremia is concurrent with the rapid production of specific IgM [9–14]. Mice that are deficient in the secreted form of IgM – but not other isotypes – exhibit persistent bacteremia that is identical to that found in mice that lack B cell development [14]. In contrast, mice deficient in activation-induced cytidine deaminase – that produce only IgM but no other antibody isotypes – control infection indistinguishably from wildtype mice [15]. These results demonstrate that IgM is required and sufficient for the resolution of *B. hermsii* bacteremia.

Concurrent with the resolution *B. hermsii* infection there is an expansion of B1b lymphocytes (IgM^{hi}, IgD^{lo}, CD23⁺, CD5⁺, Mac1⁺), a minor subset of mature B cells that are abundant in the peritoneal cavity [14, 15]. Reconstitution of immunodeficient *Rag1*^{−/−} mice (lacking T and B cells) with expanded B1b cells from convalescent mice confers complete immunity, whereas reconstitution with naïve B1b cells provides only partial protection, demonstrating that convalescent B1b cells have acquired immunological memory [15]. Like conventional memory B cells, the reconstituted population persists but does not spontaneously secrete *B. hermsii*-specific antibodies; rather the B1b cells maintain quiescence. Upon a subsequent exposure to antigen, however, B1b cells rapidly differentiate into antigen-specific IgM secreting plasma cells, resulting in a specific antibody response that is faster and of a greater magnitude than that of naïve B1b cells [15]. Although a *B. hermsii* antigen recognized by B1b cell-derived IgM has been defined [15, 16], it is not clear how B1b cell-derived IgM controls *B. hermsii*.

The pentameric structure of IgM is known to efficiently induce the classical complement pathway (11). Despite this, C3^{−/−} and C5^{−/−} mice control relapsing fever *Borrelia* infection as efficiently as wildtype mice demonstrating that antigen-specific IgM eliminates its targets independently of complement [5, 13, 17, 18]. Although IgM is not typically known as an opsonin, a high-affinity Fcα/μ receptor for IgM has been identified. This receptor is expressed on macrophages and B cells as well as on non-hematopoietic tissues in organs such as kidney and intestine [19–21]. Furthermore, Fcα/μR has been demonstrated to facilitate the phagocytosis of IgM-coated *Staphylococcus aureus in vitro*, suggesting a role for it in the clearance of IgM-coated pathogens [19]. In support of this, specific antibody-coated *B. hermsii* bacteria are engulfed by neutrophils in a complement-independent manner, indicating that antibody-mediated opsonophagocytosis is a potential mechanism of clearance of *B. hermsii* [22]. Nevertheless, it is unknown whether Fcα/μR-mediated endocytosis plays a role in facilitating IgM-mediated control of *B. hermsii in vivo*.

In addition to promoting opsonophagocytosis and complement activation, antibodies of various isotypes have been shown to exert direct anti-bacterial activities. These antibodies appear to exert these effects by catalyzing the production of oxidative molecules [23–25], interfering with pathogen iron uptake [26, 27], or by directly damaging the bacterial

membrane [5, 18]. Among the IgM antibodies that have been described to be bactericidal, CB515 recognizes a Vmp expressed by a Spanish relapsing fever *Borrelia* species, lyses bacteria *in vitro*, and confers partial protection in complement-deficient mice *in vivo* [18]. The scFv fragments of CB515 retain the lytic activity of the parent monoclonal, demonstrating that the bactericidal activity resides in the variable region of the antibody [28].

In this study, we examined the mechanism of B1b cell-derived antibody in control of *B. hermsii* infection *in vivo*. Here we show that the IgM-mediated control of *B. hermsii* bacteremia is independent of Fcα/μR and C3 and does not require innate or adaptive immune cell contact. Using a diffusion chamber model to examine host-pathogen interactions, we demonstrate that B1b cells contribute to protective immunity by producing *B. hermsii*-specific antibodies that are directly bactericidal.

Materials and Methods

Mice.

The Institutional Animal Care and Use Committee approved these studies. Mice were housed in micro-isolator cages with free access to autoclaved food and water, and were maintained in a specific pathogen-free facility at Thomas Jefferson University. C57BL/6J (B6), Balb/cJ, and C57BL/6J-Rag1^{tm1Mom/J} (Rag1^{-/-}) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Fcα/μR^{-/-} mice were described previously [20]. C3-deficient mice on a C57BL6 background (C3^{-/-}) were provided by Robert Eisenberg, University of Pennsylvania.

Infections and cobra venom factor treatment.

Mice were infected intravenously (i.v.) via the tail vein with 5×10^4 bacteria of the fully virulent *B. hermsii* strain DAH-p1 (from the blood of an infected mouse), and bacteremia was monitored by dark-field microscopy. To deplete C3, mice were treated i.p. with 30 g (14.0 units) cobra venom factor (CVF) (Quidel, San Diego, CA) one day prior to infection and again three days post infection as described [29]. This dose of CVF is sufficient to maintain serum C3 levels below 5% of normal levels for four days [30]. At three days post-infection, mice were treated with the same dose of CVF to maintain C3 depletion.

Passive immunizations.

To obtain convalescent immune serum, blood was obtained via cardiac puncture from wildtype mice that had been infected (>60 days post infection) with *B. hermsii*. To initiate clotting, blood was pooled and incubated at 37°C for 1 hr. After clotting, blood was centrifuged at 1000 x g, and serum was collected and filtered through 0.22 μm PES membranes (Millipore, Billerica, MA). Passive immunizations were performed by injecting mice with 250 μl serum via the tail vein.

Enzyme-linked immunosorbent assay (ELISA).

IgM levels were measured with ELISA kits according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). *B. hermsii*-specific IgM was determined by coating

96-well plates (ICN Biomedicals Inc., Aurora, OH) with *in vivo* grown *B. hermsii* DAH (10^5 bacteria/well). Plates were washed and blocked with 2% BSA in PBS pH 7.2 for 2 hr at room temperature. Blood samples of immunized mice were diluted 1:250 and samples were centrifuged ($16,000 \times g$ for 10 min.). Bound IgM was measured using HRP-conjugated goat anti-mouse IgM. Specific antibody levels were converted to ng/ μ l equivalents using IgM standards.

Diffusion chambers.

Mixed cellulose ester membranes (0.05 μ m pore size; Millipore) were adhered to lucite diffusion chamber rings (weight - 0.4 g; diameter - 14 mm; Width - 5 mm; Millipore) as described previously [31]. Diffusion chambers were loaded with 200 μ l of *in vivo*-adapted *B. hermsii* strain DAH in BSK-H medium (Sigma-Aldrich, St. Louis, MO) supplemented with 6% rabbit serum (Sigma-Aldrich) at 6.67×10^6 bacteria/ml (1.33×10^6 bacteria per chamber), and diffusion chambers were sealed. Mice were anesthetized with 3% isoflurane, and using aseptic technique, diffusion chambers were implanted in a subcutaneous pocket formed by making an incision of approximately 1.5 cm in the dorsal-anterior skin of each mouse. Diffusion chambers were removed after 24 hours, and contents were collected. Bacterial numbers in the chamber were measured by dark-field microscopy. Chamber contents were stored at -20°C until analyzed. In some experiments, mice were passively immunized with a) 250 μ l convalescent immune serum or b) such serum from mice treated with 14.0 units CVF one day prior to implantation as described above.

B1b cell reconstitution.

Rag1^{-/-} mice were reconstituted with 10^5 purified B1b cells from convalescent (i.e., resolved *B. hermsii* infection) mice as described [15]. Because *Rag1*^{-/-} mice reconstituted with convalescent B1b cells do not generate a specific IgM response without bacterial stimulation, they were infected i.v. with 5×10^4 bacteria of the *B. hermsii* DAH-p1 ten days after adoptive transfer of B1b cells.

Statistical analysis.

Statistics were performed using the Prism 5 software program (GraphPad Software, Inc., La Jolla, CA). To analyze statistical significance, two-tailed unpaired Student's *t*-test or two-way ANOVA were used as necessary.

Results

Clearance of *B. hermsii* does not require Fc α / μ R-mediated opsonophagocytosis.

Antibody has been shown to enhance opsonophagocytosis of *B. hermsii* by neutrophils *in vitro* [22]. Recently Fc α / μ R, a specific receptor for IgM, has been shown to mediate opsonophagocytosis of IgM-coated *S. aureus* [19]. Since antibodies – in particular IgM – are critical for the protection against *B. hermsii*, we infected mice deficient in Fc α / μ R to examine whether Fc α / μ R plays a role in IgM-mediated opsonophagocytosis. The magnitude and duration of *B. hermsii* bacteremia during the first episode in these mice were comparable to that of wildtype mice (Figure 1A). Moreover, passive immunization conferred protection to Fc α / μ R^{-/-} mice indicating that immune serum can control *B. hermsii* in an

Fc α / μ R-independent manner (Figure 1B). Consistent with the critical role for IgM in control of this bacterium, Fc α / μ R^{-/-} mice rapidly generated a *B. hermsii*-specific IgM response to a similar extent as in wildtype mice (Figure 1C). Although infection with *B. hermsii* does eventually elicit an IgG response, this does not occur until after the first wave of bacteremia is cleared. Thus, the IgM-mediated clearance of the first wave does not require Fc α / μ R.

Control of *B. hermsii* in the absence of Fc α / μ R-mediated opsonophagocytosis is independent of C3.

It is known that control of relapsing fever bacteremia occurs independently of the complement system, as bacterial clearance occurs in the absence of C3 and C5 [13]. These results suggest that complement either does not contribute to clearance or that other mechanisms such as opsonophagocytosis operate in a redundant fashion. To examine whether complement-mediated lysis or complement-mediated opsonophagocytosis plays a critical function in the absence of Fc α / μ R-mediated opsonophagocytosis, we depleted C3 in wildtype and Fc α / μ R^{-/-} mice with cobra venom factor (CVF). Despite a deficiency in both Fc α / μ R and C3, the magnitude of the bacteremia was similar in CVF-treated and-untreated wildtype and Fc α / μ R^{-/-} mice (Figure 2). These results indicate that the effector mechanism of *B. hermsii*-specific IgM is neither C3- nor Fc α / μ R-dependent.

Antibody-mediated control of *B. hermsii* does not require phagocytic cell contact.

Since phagocytosis can be promoted by a variety of other mechanisms, we tested whether IgM-mediated control of *B. hermsii* *in vivo* requires cell contact. To test this, we adapted a diffusion chamber system originally devised to model host-pathogen interactions during parasite infections [31]. The pore size of these diffusion chambers (0.05 μ m) was large enough to allow free diffusion of IgM (300 Å) while restricting both the escape of *B. hermsii* (~0.25 $\times$ ~5.0 μ m) and the entrance of host cells. Each chamber contained 1.33×10^6 *in vivo*-adapted *B. hermsii* spirochetes in bacterial growth medium. A schematic of the diffusion chamber model is shown in Figure 3A. After 24 hours in naïve mice, the number of bacteria in each chamber was approximately threefold higher than the initial number. In contrast, the bacteria in diffusion chambers implanted in convalescent mice were nearly undetectable (Figure 3B).

The numbers of *B. hermsii* were also reduced significantly in diffusion chambers implanted in passively immunized mice (Figure 3B). Interestingly, the degree of bacterial killing in these mice was lower than what was observed in the diffusion chambers implanted in convalescent mice, suggesting that the amount of specific IgM present in the chamber had a direct effect on bacterial killing inside the diffusion chambers. Therefore, we examined specific IgM levels in the diffusion chambers taken from naïve mice, convalescent mice, and naïve mice that were given convalescent immune serum. As expected, we found that specific IgM had entered the diffusion chambers implanted in convalescent mice and passively immunized naïve mice. Furthermore, increasing amounts of specific IgM in each chamber inversely correlated with bacterial viability (Figure 3C). These results demonstrate that lysis of *B. hermsii* in diffusion chambers is IgM-dependent and does not require cell contact.

Killing of *B. hermsii* in diffusion chambers is C3-independent.

As described above, IgM controls *B. hermsii* independent of C3 or Fc α / μ R-mediated opsonophagocytosis. To test whether cell contact-independent killing of *B. hermsii* in diffusion chambers is also complement-independent, we depleted C3 in naïve and convalescent wildtype mice with CVF one day prior to implantation of diffusion chambers. As expected, bacterial growth was unhindered in naïve mice regardless of whether complement was depleted (Figure 4A). In contrast, there were no bacteria remaining in chambers removed from convalescent mice irrespective of the presence or depletion of C3 (Figure 4A).

To confirm that IgM is bactericidal *in vivo* independent of the complement system, we used mice deficient in both the alternative and classical complement pathways due to a targeted deletion in the C3 gene. Diffusion chambers containing 1.33×10^6 *B. hermsii* bacteria were implanted into convalescent wildtype and C3^{-/-} mice. In agreement with the above results, bacteria in diffusion chambers were killed in both wildtype and C3^{-/-} mice (Figure 4B). These results demonstrate that C3 is not required for antibody-mediated lysis of *B. hermsii* in the diffusion chambers.

B1b cell-derived antibodies are sufficient to kill *B. hermsii* in diffusion chambers.

To determine whether B1b-derived IgM is capable of clearing *B. hermsii* independently of opsonophagocytosis, we tested whether IgM generated by adoptively transferred convalescent B1b cells was able to kill *B. hermsii* in diffusion chambers. Rag1^{-/-} mice reconstituted with purified convalescent B1b cells and primed with *B. hermsii* generated a *B. hermsii*-specific IgM response (Figure 5A). To measure cell-independent killing by B1b-derived IgM, we implanted diffusion chambers containing 1.33×10^6 spirochetes in reconstituted Rag1^{-/-} mice at ten days post-infection. As expected, *B. hermsii* bacteria in diffusion chambers proliferated extensively when implanted in unreconstituted Rag1^{-/-} recipients. On the other hand, we observed a significant decrease in the number of live bacteria in diffusion chambers implanted in mice reconstituted with convalescent B1b cells (Figure 5B). These results demonstrate that IgM generated by B1b cells is directly bactericidal *in vivo*.

Discussion

IgM plays a critical role in immunity to a number of infections [2]. The production of specific IgM is required and sufficient to control bacteremia during relapsing fever infection [14, 15]. We have previously shown that B1b cells generate *B. hermsii*-specific IgM that is capable of controlling *B. hermsii* bacteremia. Here we show that the IgM-mediated control of *B. hermsii* bacteremia is independent of Fc α / μ R and C3 and does not require innate or adaptive immune cell contact. Our data indicate that the mechanism of B1b-mediated control of *B. hermsii* is through the secretion of bactericidal antibodies.

Although antibodies tend to exert their protective function through the activation of the complement cascade or via Fc receptor engagement, some cause direct damage to pathogens in a variety of other ways. For example, a *Pseudomonas aeruginosa* LPS-specific IgG alters

cell wall permeability and causes the formation of cell wall vesicular structures, elongation, and inhibition of cell division [32]. The bactericidal IgG CB2, specific for OspB of *Borrelia burgdorferi*, induces the formation of 2.8 – 4.4 nm pores in the outer membrane of the bacterium [33]. Paradoxically, this antibody does not induce lysis of OspB-expressing *E. coli*, indicating that IgG-induced lysis is dependent on the cell membrane structure of *B. burgdorferi* or the expression of another *B. burgdorferi* protein. In fact, the lytic activity of CB2 depends on cholesterol domains in the outer membrane of *B. burgdorferi*, suggesting that CB2 alters the fluid structure of the bacterial outer membrane [34]. In addition, *B. burgdorferi* has been shown to harbor a prophage holin or holin-like system containing the genes *blyA* and *blyB* on the conserved circular plasmid cp32 [35]. Transformation of *E. coli* with *blyA* induces cell membrane damage that is dependent on a host cytolysin ClyA acting in *trans*, demonstrating that the gene product of *blyA* is functional [36]. Remarkably, treatment of *B. burgdorferi* spirochetes with sublethal quantities of CB2 induced the expression of cp32 gene products BlyA and BlyB, suggesting that the bactericidal activity of CB2 may be dependent on the induced expression of bacteriophage gene products [37]. Since bactericidal activity of the V region of an IgM specific for a Vmp expressed by a related relapsing fever *Borrelia* species results in direct lysis of the bacterial outer membrane [18], it is possible that B1b-derived IgM also kills *B. hermsii* in a similar fashion.

Relapsing fever spirochetes achieve extremely high bacterial numbers ($\sim 10^8$ / ml blood) within 2–3 days. In the entire body the bacterial numbers can be on the order of hundreds of billions. Because of the bacterial tropism to blood and the high bacterial burden, the number of phagocytic cells needed to contain the infection may not be an achievable option by the host immune system. While complement activation could control the infection through bacterial lysis, the amount of complement-derived split products can have a detrimental effect on the host. Therefore the control of high bacterial burden by direct bactericidal activity of antibodies could play a role in host defense. Our data indicate that B1b cells can generate such an antibody response very rapidly to control *B. hermsii* bacteremia. Since B1b cells have been shown to play a role in controlling a number of bacterial pathogens including *Streptococcus pneumoniae* and non-typhoidal *Salmonella* [4, 15, 38], it is tempting to speculate whether these pathogens are also controlled by bactericidal antibodies derived from B1b cells.

In the absence of all other B cell types, convalescent B1b cells are sufficient to provide protection from *B. hermsii* infection in immunodeficient mice. We have shown that FhbA, an outer surface protein of *B. hermsii* is specifically recognized by the IgM secreted by convalescent B1b cells [16]. The previously described anti-*Borrelia* antibodies recognize major surface bacterial proteins such as OspB and Vmps rather than FhbA. Although the present study indicates that B1b cells secrete *B. hermsii*-specific IgM that is capable of leading to direct bacterial lysis, the biochemical basis for the antibody-mediated lysis of *B. hermsii* is unknown. The generation of B1b-derived monoclonal antibodies to FhbA of *B. hermsii* and the characterization of such antibodies would provide a better insight on how B1b cell-derived antibodies exert their antibacterial activity in the complete absence of a cell-contact.

Acknowledgements

We thank Jessica Hess and Sandra Bonne-Année for assistance with diffusion chamber experiments, Dr. Robert Eisenberg for providing C3^{-/-} mice and Dr. Utpal Pal for reviewing the manuscript. This work was supported by NIH grant R01 AI065750 to KRA and 1R56AI076345 to DA.

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Figure 1. $Fc\alpha/\mu$ Receptor is not required for clearance of *B. hermsii*.

(A). Balb/cJ or $Fc\alpha/\mu R^{-/-}$ mice were infected i.p. with *B. hermsii*, and bacteremia was monitored by dark-field microscopy of blood specimens. Each plot represents an individual mouse. Dotted lines represent limit of detection. (B). One day prior to infection, serum from convalescent (80 days post-infection) wildtype mice was passively transferred either into Balb/cJ or $Fc\alpha/\mu R^{-/-}$ mice. Bacteremia was monitored by dark-field microscopy. Dotted lines represent the limit of detection. (C). *B. hermsii*-specific IgM was measured by ELISA. Data points represent mean SD. Kinetics of anti-*B. hermsii* IgM responses were not statistically significant between wildtype and $Fc\alpha/\mu R^{-/-}$ mice.

Figure 2. Fcα/μR- and C3-mediated opsonophagocytosis are not required for clearance of *B. hermsii*.

(A) Balb/cJ or (B) Fcα/μR^{-/-} mice were either treated or not treated with 14.0 units of cobra venom factor (CVF) to deplete complement component C3 on the days indicated (arrows). Mice were infected with *B. hermsii*, and bacteremia was monitored by dark-field microscopy of blood. Each plot represents an individual mouse. Dotted lines represent limit of detection.

Figure 3. Antibody-mediated lysis in diffusion chambers.

(A) Diffusion chamber model. *B. hermsii* (size: $\sim 0.25 \mu\text{m} \times \sim 5.0 \mu\text{m}$) and eukaryotic cells are unable to pass through the $0.05 \mu\text{m}$ pore size membrane of the diffusion chambers. IgM can passively diffuse through the chamber membrane. (B) Diffusion chambers containing 1.33×10^6 *B. hermsii* were implanted into groups of naïve, convalescent or passively immunized mice ($n = 4-7$ per group). Diffusion chambers were removed after 24 hours and live bacteria in the diffusion chambers were enumerated by dark-field microscopy. Data represent mean $\pm$ SD. The dotted line represents the starting number of bacteria in each chamber. Data were analyzed by two-tailed unpaired Student's *t* test, with significance reached at $p < 0.05$. (C) *B. hermsii*-specific IgM in diffusion chambers was measured by ELISA, and specific IgM values were matched with chamber bacterial density. Non-linear regression analysis measuring the effect of specific IgM concentration on bacterial viability was performed.

Figure 4. Lysis of *B. hermsii* in diffusion chambers is C3-independent.

(A) Cobra venom factor (CVF, 14.0 units) was given to naïve (n=4) or convalescent (n=5) B6 mice one day prior to implantation of diffusion chambers containing *B. hermsii*. Control groups of naïve (n=3) or convalescent (n=3) mice were given Dulbecco's phosphate-buffered saline. (B) Diffusion chambers containing 1.33×10^6 *B. hermsii* were implanted into naïve B6 mice (n=4), convalescent B6 mice (n=4), or convalescent C3^{-/-} mice (n=3). For A and B, diffusion chambers were removed from mice after 24 hours, and bacteria in the diffusion chambers were counted by dark-field microscopy. Mean numbers of bacteria $\pm$ SD are shown. (N.D.—not detectable). The dotted line indicates starting number of bacteria in each chamber. Data were analyzed by two-tailed unpaired Student's *t* test, with significance reached at $p < 0.05$.

Figure 5. B1b cell-derived IgM is sufficient to kill *B. hermsii* in diffusion chambers.

Rag1^{-/-} mice were either reconstituted or not with B1b cells (10^5 cells per mouse) from convalescent B6 mice. After 10 days, mice were infected with *B. hermsii* in order to stimulate IgM production. (A) *B. hermsii*-specific IgM in non-reconstituted (n=4) or B1b-reconstituted mice (n=4) was determined by ELISA. Means $\pm$ SD are shown. Data were analyzed by two-way ANOVA, with significance reached at $p < 0.05$. (B) On day 10 post-infection, diffusion chambers containing *B. hermsii* were implanted into mice from each group. Twenty-four hours later diffusion chambers were removed, and live bacteria in the diffusion chambers were counted by dark-field microscopy. The dotted line represents the starting number of *B. hermsii* in each chamber. Data were analyzed by two-tailed unpaired Student's *t* test, with significance reached at $p < 0.05$.