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HOST RESPONSE AND INFLAMMATION

Depletion of Complement Enhances the Clearance of *Brucella abortus* in Mice

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ABSTRACT Brucellosis is a bacterial disease of animals and humans. *Brucella abortus* barely activates the innate immune system at the onset of infection, and this bacterium is resistant to the microbicidal action of complement. Since complement stands as the first line of defense during bacterial invasions, we explored the role of complement in *B. abortus* infections. *Brucella abortus*-infected mice depleted of complement with cobra venom factor (CVF) showed the same survival rate as mice in the control group. The complement-depleted mice readily eliminated *B. abortus* from the spleen and did so more efficiently than the infected controls after 7 days of infection. The levels of the proinflammatory cytokines tumor necrosis factor alpha and interleukin-6 (IL-6) remained within background levels in complement-depleted *B. abortus*-infected mice. In contrast, the levels of the immune activator cytokine gamma interferon and the regulatory cytokine IL-10 were significantly increased. No significant histopathological changes in the liver and spleen were observed between the complement-depleted *B. abortus*-infected mice and the corresponding controls. The action exerted by *Brucella* on the immune system in the absence of complement may correspond to a broader phenomenon that involves several components of innate immunity.

KEYWORDS *Brucella*, *Brucella abortus*, brucellosis, complement, cobra venom factor, innate immunity

Brucella organisms are facultative extracellular-intracellular bacteria of animals and humans that establish chronic infections (1). *Brucella abortus* avoids activating the innate immune system at the onset of the infection (2–5). This phenomenon is due to the absence and modification of the *Brucella* pathogen-associated molecular patterns, which result in the weak activation of pattern recognition receptors (2, 3, 5–8). The lack of obvious clinical signs and symptoms at early stages of *Brucella* infections in animals and humans is in accordance with this model (5, 9).

The absence of polymorphonuclear leukocytes (PMNs) promotes the elimination of *B. abortus* in mice by favoring the Th1 over the Th2 immune response (4). This supports the proposal that innate immune recognition is hampered at the onset of the infection and that PMNs play a regulatory role in the adaptive immune response in brucellosis (4).

The complement system is a cascade of various elements of innate immunity and the first line of defense against microorganisms. Complement activation has three main functions: opsonization of invaders, amplification of the immune response through

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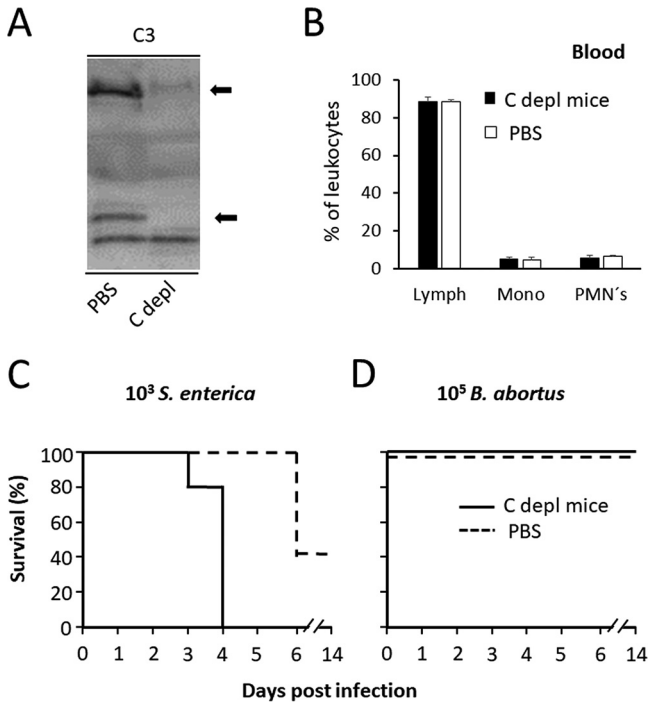

FIG 1 *B. abortus* is not lethal in complement-depleted mice. Groups of BALB/c mice were chronically depleted of complement by means of repeated injections of CVF. (A) Serum C3 detection by Western blotting was used to determine the depletion of complement (Cdepl) in CVF-injected mice. Arrows indicate the position of the C3 fraction and its fragments. (B) Percentage of leukocyte types in complement-depleted mice ($n = 5$) and PBS-injected control mice ($n = 5$). Lymph, lymphocytes; Mono, monocytes. (C) Survival percentage of PBS-injected control mice ($n = 7$) and complement-depleted mice ($n = 7$) after infection with *S. enterica*. (D) Survival percentage of PBS-injected control mice ($n = 7$) and complement-depleted mice ($n = 7$) after infection with *B. abortus* 2308W. Black and white bars represent the median ± interquartile range (IQR). The Mann-Whitney U test showed no significant differences. Data are representative of those from at least three independent experiments.

recruitment of inflammatory cells, and directing of the killing of microorganisms (10). Pathogens have developed different strategies to evade complement killing or activation (11, 12). Virulent *Brucella* organisms resist the killing action of complement and induce low levels of activation of the complement cascade (13–15). In spite of this, the role of complement during brucellosis *in vivo* has not been explored. Here, we demonstrate that the removal of complement promotes the elimination of *B. abortus* in mice at early times of infection, suggesting a regulatory role of this system during brucellosis.

RESULTS AND DISCUSSION

***B. abortus* does not kill mice depleted of complement with CVF.** C3 knockout mice display innate and diverse long-lasting immunological defects (16–18) that may hamper the examination of the role of complement in brucellosis. To circumvent this, we have used the cobra venom factor (CVF) complement depletion model. CVF is a three-chain protein that structurally resembles C3c, one of the degradation products of C3b, but is unable to form a C3/C5 convertase (19). After intraperitoneal (i.p.) injection of CVF, the C3 component was not detected in the serum of mice (Fig. 1A). Complement depletion did not alter the leukocyte count or cell profiles in mice (Fig. 1B). We recorded reduced survival rates for complement-depleted mice infected with *Salmonella enterica* in comparison to those for the infected control group (Fig. 1C). This observation is consistent with previous works showing that CVF complement depletion *in vivo* exacerbates bacterial infections and lethality (20, 21). In contrast, complement-depleted mice infected with *B. abortus* displayed the same survival rate as *B. abortus*-infected control mice (Fig. 1D). Complement-depleted mice infected with *B. abortus*

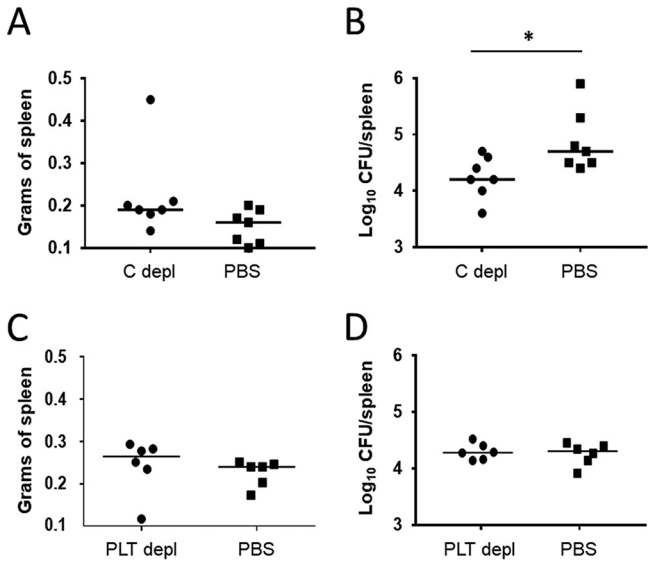

FIG 2 The absence of complement favors the removal of *B. abortus*. (A) Groups of seven BALB/c mice were depleted of complement or injected with PBS and then infected with 0.1 ml of *B. abortus* 2308W at 10^6 CFU/ml by the i.p. route. At 7 days postinfection, the spleen weight and bacterial counts were determined for each group. (B) To ensure that CVF did not prime an adjuvant immune response, promoting bacterial clearance, groups of seven BALB/c mice were treated with repeated i.p. injections of 80 μg of aspercetin or with PBS and then infected with 0.1 ml of *B. abortus* 2308W at 10^6 CFU/ml by the i.p. route. (C and D) At 7 days postinfection the spleen weight (C) and bacterial counts (D) were determined for each group. The median is represented by a line. Statistical significance was calculated by the Mann-Whitney U test. *, $P < 0.05$. Data are representative of those from at least three independent experiments.

did not show any behavior changes or weight loss throughout the duration of the experiment.

The absence of complement favors the removal of *B. abortus*. In order to determine if the acute absence of complement alters the course of *Brucella* infection, we performed bacterial counts in the spleen. As demonstrated in Fig. 2A, the spleen weights were not statistically significantly different between the complement-depleted and control *B. abortus*-infected mice (Fig. 2A). In contrast, the number of bacteria in the spleens of complement-depleted infected mice was significantly lower than that in the spleens of the control infected group (Fig. 2B). As shown in Fig. 2C and D, we did not detect differences in spleen weights or bacterial clearance between the aspercetin-treated and *B. abortus*-infected mice and the phosphate-buffered saline (PBS)-treated control mice. This result is consistent with our previous experience demonstrating that the repeated administration of a sole foreign immunogenic protein does not alter the course of *B. abortus* infection in mice (22), therefore ruling out an adjuvant effect by the early induction of the adaptive immune response.

Complement-depleted *B. abortus*-infected mice produce higher levels of pro-inflammatory cytokines. In order to evaluate the proinflammatory response of complement-depleted *Brucella*-infected mice after 7 days of infection, quantitation of cytokines was performed (Fig. 3). The amounts of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) remained close to the background levels. In contrast, the quantities of gamma interferon (IFN-γ) as well as the regulatory cytokine IL-10 were significantly higher in the infected complement-depleted mice (Fig. 3). This cytokine profile is similar to the one induced in *Brucella*-infected mice depleted of PMNs (4).

Complement depletion does not influence the pathology in *B. abortus*-infected mice. As expected, *S. enterica*, used for comparative purposes, induced a severe acute inflammation in the spleen and liver of complement-depleted mice at 7 days of infection (Fig. 4A). In contrast, *B. abortus*-infected complement-depleted mice demonstrated chronic inflammation with granuloma formation (Fig. 4B) indistinguishable from

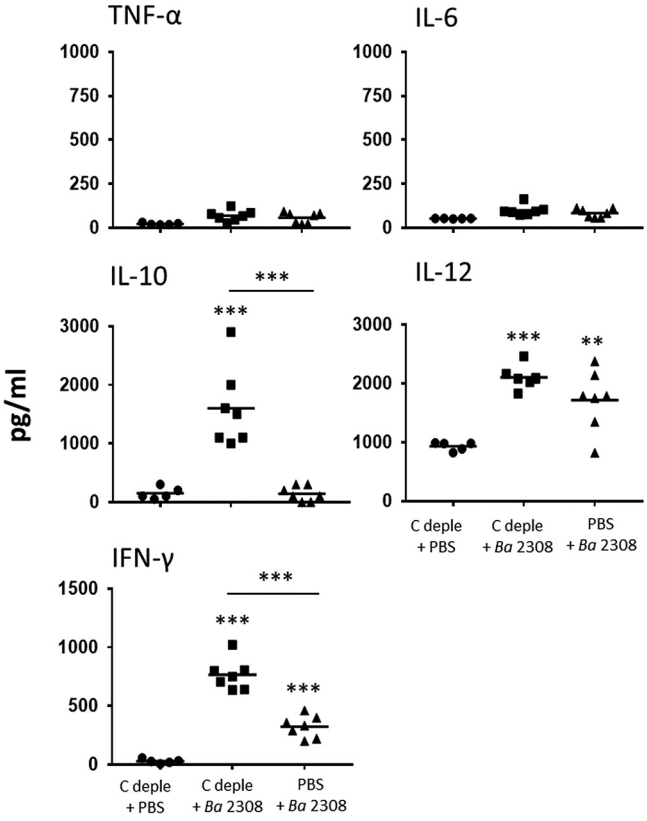

FIG 3 Complement-depleted *B. abortus*-infected mice produce higher levels of proinflammatory cytokines. Cytokine levels in sera from complement-depleted ($n = 7$) and control ($n = 7$) mice were determined by ELISA after 7 days of infection with 0.1 ml of *B. abortus* 2308W (*Ba* 2308) at 10^6 CFU/ml by the i.p. route. A group of noninfected complement-depleted mice ($n = 5$) was used as a control. The mean is represented as a line. Asterisks above the bars indicate statistically significant differences relative to complement-depleted noninfected mice. Statistical significance was calculated by ANOVA and Tukey's multiple-comparison test. **, $P < 0.01$; ***, $P < 0.001$. Data are representative of those from at least three independent experiments.

that in *B. abortus*-infected mice not depleted of complement (Fig. 4A). Although little vasodilation and hyperemia were observed in the liver and spleen of noninfected C3-depleted mice, the histopathological effect observed in the *B. abortus*-infected mice was not due to the action of CVF (Fig. 4A).

At the onset of infection, *Brucella* follows a stealthy strategy, taking advantage of an immunological window to invade the host cells and disperse throughout the reticuloendothelial system (1, 3, 5). During infection, *Brucella* interacts with a variety of serum proteins, such as antibodies and complement components, that selectively bind to the bacterium surface (22). Regardless of this, *Brucella* resists the killing action of complement and does not fully activate the complement cascade (13–15). Interestingly, these two complement activities vary depending upon the animal species: human serum opsonizes better and is more bactericidal than mouse serum (22). In spite of this, complement still plays a relevant role *in vivo* in the mouse model during the acute phase of *Brucella* infection, as shown here. From our results, we propose that *Brucella* hampers the opsonizing, killing, and chemotactic functions of complement by C3 convertase modulation and does so independently if activation is related to the alternative pathway at the onset of the infection or if activation is related to the classical pathway in later stages to sustain persistence.

It is worth noting the parallelism observed between the complement depletion model, used in this work, and the PMN depletion model, used previously during *B. abortus* infections (4). Indeed, in both murine models, the cytokine profile is similar and *B. abortus* is more efficiently eliminated from the spleen. It seems that in both systems,

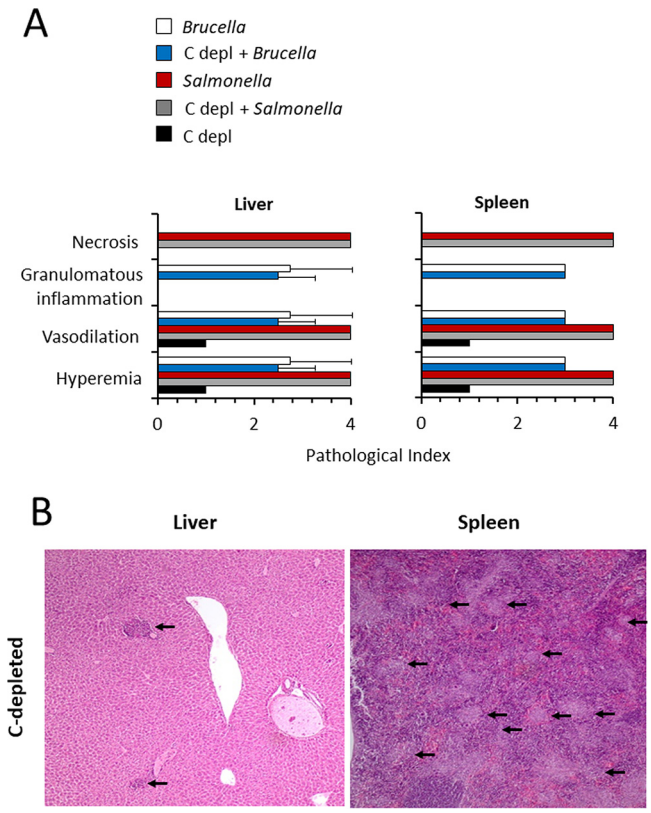

FIG 4 Complement-depleted *B. abortus*-infected mice show a histopathological profile similar to that of the control PBS-injected *B. abortus*-infected mice. (A) The semiquantitative pathological index was determined by histopathological observation of the spleen and liver from complement-depleted mice ($n = 7$) and control mice ($n = 7$) after 7 days of infection with 0.1 ml of 10^6 *B. abortus* bacteria or 3 days of infection with 10^3 *S. enterica* bacteria. (B) Histopathological examination of the liver and spleen of complement-depleted *B. abortus*-infected mice after 7 days of infection. The arrows indicate the characteristic granulomas induced by *Brucella* in the liver and spleen (35). Data are representative of those from at least three independent experiments.

the regulatory mechanism of the adaptive immune response during brucellosis is released after removal of complement or PMNs (4). This is not totally unexpected, since both elements cross talk during the innate immune response (23).

Bacterial infections inducing low inflammatory responses (as is the case in brucellosis) drive the production of IL-10 by naturally occurring regulatory T cells (nTregs) (24). In the course of the infection, this allows pathogens to escape from the immune control, preventing pathology and favoring pathogen persistence (24). It may be that the *Brucella*-infected complement-depleted mice induced adaptive nTregs with the ability to promote the synthesis of IFN-γ, IL-12, and IL-10. Moreover, it is well-known that complement depletion hampers the arrival of inflammatory cells, including PMNs, in injured animals (25). Therefore, complement and PMNs are key components of innate immunity, and the efficient removal of *B. abortus* from the spleen of complement-depleted mice and PMN-depleted mice may be due to the higher levels of production of IFN-γ and IL-12, which are the most relevant cytokines in brucellosis (26). Interestingly, mice lacking B cells, which are mainly elements of the adaptive immune response, also favor the removal of *Brucella* by increasing IFN-γ levels (27). This particular immune response may favor the positive balance of the Th1 immune response and the suppression of downstream pathologies, as seen for other microbes (28, 29). This proposal is in tune with our previous proposal resulting from findings obtained with the PMN depletion model (4). Although we do not know the mechanism exerted by *Brucella*, it seems that it corresponds to a broader phenomenon that involves several components of the innate immune system.

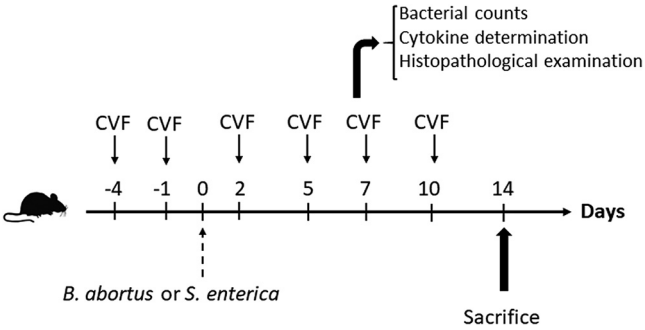

FIG 5 Protocol for depletion of complement with CVF in mice. CVF (0.8 μg/g body weight) was administered intraperitoneally at different times before and after bacterial infections (thin arrows). Bacteria were inoculated at time zero (broken arrow). Bacterial counts, cytokine estimation, and histopathological studies were performed at 7 days of infection (bent arrow). The end of the survival experiment is indicated with a thick arrow.

MATERIALS AND METHODS

Ethics statement. The protocols for mouse experimentation were approved by the Comité Institucional para el Cuido y Uso de los Animales of the Universidad de Costa Rica (CICUA-019-16, CICUA-067-17). These protocols were in agreement with the corresponding law, Ley de Bienestar de los Animales, of Costa Rica (Law 9458 on animal welfare). Mice were housed in the Animal Building of the Veterinary Medicine School, Universidad Nacional, Costa Rica. All of the animals were kept in cages with water and food *ad libitum* under biosafety containment conditions before and during the experiment.

Mouse strains and infection protocols. CVF was purified from the venom of the *Naja naja* cobra as described elsewhere (30). Female BALB/c mice (weight, 18 to 21 g) were depleted of complement by intraperitoneal (i.p.) injection of CVF at 0.8 μg/g body weight/mouse in 0.1 ml PBS (20) following the protocol described in Fig. 5. Controls and complement-depleted mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU/ml of the virulent strain *B. abortus* 2308W (31) or 10^3 CFU of *Salmonella enterica* serovar Typhimurium strain SL1344 (Fig. 5). A group of mice was injected with CVF only to ensure that the effects seen in infected mice were not only due to the CVF injection. Controls were injected with 0.1 ml sterile PBS. Complement depletion was confirmed by the absence of the C3 protein by Western blotting using polyclonal C3 antibody (catalog number ab14232; Abcam) in combination with goat anti-mouse IgG (H+L)-horseradish peroxidase for detection (Thermo Fisher Scientific). Complement depletion was continued for 7 and 10 days after infection, as indicated in the time course protocol presented in Fig. 5.

Heat-inactivated CVF is not a suitable antigenic control. Like many other proteins related to complement, the CVF protein is thermolabile and breaks down into many small peptides with low immunogenicity. Therefore, to ensure that the immune response adjuvant effect against a foreign protein was not promoting *B. abortus* clearance, a second group of mice was treated with aspercetin, a low-molecular-weight protein from the venom of the *Bothrops asper* snake that depletes platelets via the de Hageman factor (32). Briefly, BALB/c mice were depleted through the i.p. injection of 80 μg of purified aspercetin protein in 0.1 ml PBS. Then, platelet-depleted mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU/ml of virulent *Brucella abortus* 2308W at 6 h after depletion. Platelet-depleted mice were again i.p. injected with the same dose of aspercetin at 24 and 48 h postinfection. Controls were injected with 0.1 ml sterile PBS. A group of mice was injected with aspercetin only to ensure that the effects seen in infected mice were not due to the injection of aspercetin. Platelet depletion was confirmed with a VetScan HM5 hematology analyzer (Abaxis). Mice were sacrificed at 7 days postinfection to perform CFU counts.

Bacterial counts, cytokine quantitation, and histopathology. Spleens were collected, weighed, and homogenized in sterile PBS, and bacterial counts were estimated as described elsewhere (33). Blood was collected from the mice, and serum was kept at −80°C until it was used. Murine levels of IL-6, IL-10, IL-12, IFN-γ, and TNF-α were measured using mouse enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go! kits (Affymetrix; eBioscience) according to the manufacturer's specifications. A pool of serum from healthy BALB/c mice was used for background estimation. For histopathological studies, the spleens and livers of the mice were fixed in 10% neutral buffered formalin, processed, stained with hematoxylin and eosin or Giemsa stain as described elsewhere (34), and then observed under a microscope.

Statistical analysis. We used GraphPad Prism software (La Jolla, CA, USA) to statistically analyze our results. The Mann-Whitney test was used for comparison of the cell profiles of mice that had been complement depleted with CVF and control mice, as well as the spleen weights and CFU of complement-depleted or platelet-depleted and control *Brucella*-infected mice. Analysis of variance (ANOVA) followed by Tukey's multiple-comparison test was used for comparison of cytokine levels among mice complement depleted with CVF, complement-depleted mice, and control *Brucella*-infected mice. *P* values of <0.05 were considered to represent a statistically significant difference.

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