INFECTION AND IMMUNITY, Oct. 1991, p. 3815–3818
0019-9567/91/103815-04$02.00/0

Vol. 59, No. 10

Depletion of Complement and Effects on Passive Transfer of Resistance to Infection with *Borrelia burgdorferi*

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Received 10 April 1991/Accepted 19 July 1991

When irradiated hamsters are passively immunized with immune serum before challenge with *Borrelia burgdorferi*, they are completely protected from arthritis and infection. The complement dependency of this protection was addressed by treating hamsters with cobra venom factor. Depletion of complement abrogated the ability of immune serum obtained 1 and 10 weeks after infection to confer complete protection. By contrast, depletion of complement had no effect on the ability of 3-week immune serum to confer protection. These results suggest that complement-dependent, and possibly complement-independent, antibodies are important for preventing the induction of Lyme arthritis.

We showed previously that inbred, immunocompetent LSH hamsters develop histologically demonstrable arthritis after injection of *Borrelia burgdorferi* into the hind paws (9, 22). When hamsters are irradiated and infected with the Lyme disease spirochete, the hind paws become severely inflamed (22). The prolonged inflammation results in destructive and erosive bone changes (9) that resemble Lyme arthritis in humans (6). The irradiated-hamster model of Lyme arthritis has proven useful for monitoring the humoral immune responses of immunocompetent hamsters infected with *B. burgdorferi* (23, 24).

We showed that serum obtained from immunocompetent hamsters infected with the Lyme disease spirochete could confer complete protection on recipient hamsters challenged with the same isolate (23, 24). Recipients of immune serum failed to develop swelling of the hind paws or histologic evidence of arthritis, and, most importantly, no spirochetes were recovered from their tissues. These studies (23, 24) and others (7, 10–13, 21) have firmly established a role for antibody in protection against infection with *B. burgdorferi*.

The role of complement in antibody-mediated resistance to infection with *B. burgdorferi* needs to be defined. Cobra venom factor (CVF), a decomplementing agent, has been used frequently to define the role of complement in microbial infections (31). CVF combines with factor B to form a complex which cleaves C3, leading to its depletion and, to a lesser extent, to depletion of the terminal complement proteins C5 through C9 (30, 31). In this report, we treated irradiated hamsters with CVF and immune serum and determined their susceptibility to infection with *B. burgdorferi* by monitoring the induction of Lyme arthritis.

Inbred LSH/Ss Lak hamsters, 6 to 8 weeks old, were obtained from the National Institutes of Health (Bethesda, Md.) and our breeding facility. Hamsters weighing 60 to 100 g were housed three or four per cage at an ambient temperature of 21°C. Groups of hamsters were exposed to 600 rads of gamma radiation with a cobalt 60 irradiator (Picker Corp., Cleveland, Ohio). Hamsters survived this level of radiation without reconstitution with normal bone marrow cells.

B. burgdorferi 297 was obtained from Russell C. Johnson (University of Minnesota, Minneapolis). The isolate was originally obtained from human spinal fluid (26) and has been maintained by passage in modified Barbour-Stoenner-Kelly medium (BSK) (4) and in hamsters (10–14, 22). The hamster-passed spirochetes were grown in BSK at 35°C for 5 days. The suspension of *B. burgdorferi* was adjusted with fresh BSK to contain approximately 10^7 organisms per ml. One-milliliter samples were then dispensed in vials, which were sealed and stored in liquid nitrogen until use.

A frozen vial containing a suspension of *B. burgdorferi* was thawed and used to inoculate fresh BSK. The culture was grown for 5 days at 31°C and diluted with BSK to contain 5×10^6 organisms per ml. The number of spirochetes per milliliter was quantitated by dark-field microscopy. Spirochetes that had undergone fewer than four in vitro passages were used in all experiments.

Groups of five or more nonirradiated LSH hamsters were injected subcutaneously in each hind paw with 0.2 ml of the suspension of *B. burgdorferi* described above. The hamsters were mildly anesthetized by inhalation of ether contained in a nose-and-mouth cup and bled by intracardiac puncture at 1, 3, and 10 weeks after infection. The blood was allowed to clot, and the serum was separated by centrifugation at 500 × *g*, pooled, divided into 1-ml amounts, and frozen at −20°C until use. Concomitantly, pooled normal hamster serum was obtained from noninfected normal hamsters.

Hamsters, in groups of three, were injected intravenously in the sublingual vein with 0.4 ml of normal or immune serum at 3-day intervals (−3, 0, +3, and +6) for 9 days. Three days after the first injection, hamsters were irradiated and injected in each hind paw with 10^6 *B. burgdorferi* organisms. In some experiments, hamsters were injected intraperitoneally with 0.2 ml (20 U) of CVF on days −1, +2, +5, +8, and +11. The lyophilized CVF from *Naja haje* (Diamedix, Inc., Miami, Fla.) was reconstituted with sterile distilled water before use.

To confirm the effectiveness of the CVF treatment, total hemolytic complement levels in serum specimens from three hamsters were monitored 1, 2, 3, and 4 days after a single injection of 20 U of CVF by using a commercially available kit (Sigma Chemical Co., St. Louis, Mo.). A 1-ml sample of blood from each hamster was collected by intracardiac puncture before injection of CVF and daily thereafter for 4

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days. The blood was allowed to clot at room temperature for 1 h to prevent cold activation of complement (20) and centrifuged at 600 × *g* for 10 min at 4°C. The serum was removed and stored at −70°C until assayed. One day after administration of CVF, the 50% hemolytic complement activity was 2 ± 0.60 U/ml and remained depressed for 4 days (3.3 ± 2.0 U/ml), in comparison with the control (147.2 ± 24.4 U/ml). This quantity of CVF has also been shown to depress C3 levels in hamsters for 6 days (27).

Swelling of the hind paws of irradiated hamsters was used to evaluate the inflammatory response to infection. The volume of each hind paw was measured with a plethysmograph (Buxco Electronics, Sharon, Conn.) on days 1 to 16 after challenge with *B. burgdorferi*. Measurements were obtained by lightly anesthetizing the hamsters and carefully dipping a hind paw of each into a column of mercury up to the ankle and recording the amount (in milliliters) of mercury displaced. If mercury remained on the paws of hamsters, it was carefully removed with a mercury collector. The mean plethysmograph values for three hamsters (six paws) were obtained and used as an index of the severity of arthritic swelling. Mercury displacement was standardized with a volume calibrator.

The plethysmograph values obtained for irradiated hamsters were tested by analysis of variance. The Fisher least-significant-difference test (25) was used to examine pairs of means when a significant F ratio indicated reliable mean differences. The alpha level was set at 0.05 before the experiments were started.

Hamsters were sacrificed by inhalation of carbon dioxide 16 days after infection. The spleen and urinary bladder of each hamster were removed aseptically and homogenized separately with 1 ml of BSK in a sterile petri dish. These tissue suspensions (0.5 ml) were then inoculated into 4 ml of BSK and incubated at 31°C. Cultures were monitored weekly for spirochetes by dark-field microscopy.

In duplicate experiments, immune (1, 3, and 10 weeks after infection) hamster sera were heat inactivated at 56°C for 45 min and diluted 1:10 in BSK. Subsequently, a 3-day culture of *B. burgdorferi* was diluted to approximately 2 × 10^6 organisms per ml, and 50-μl aliquots were added to 1.5-ml microcentrifuge vials containing 100 μl of the heat-inactivated, diluted antisera. Sterile guinea pig serum (50 μl) with a complement activity of ~210 50% hemolytic complement units/ml was added, yielding a final dilution of antisera of 1:20. Heat-inactivated (56°C for 45 min) guinea pig serum (50 μl) was added to a second set of tubes set up in parallel.

All vials were gently vortexed and incubated at 31°C for 6 h. BSK (1 ml) was added to all vials, which were then incubated 4 to 7 days at 31°C. After incubation, 10-μl aliquots were removed in triplicate and the total number of live spirochetes was determined. Twenty-five random fields of each 10-μl aliquot were read at a magnification of ×400 by using dark-field microscopy. Live spirochetes were defined as those that were motile (15, 16).

Treatment of hamsters with CVF prevented the passive transfer of protection with immune sera obtained 1 and 10 weeks after infection, in contrast to results for hamsters not treated with CVF (Table 1). Spirochetes were recovered only from CVF-treated hamsters. In addition, CVF-treated hamsters receiving 1- and 10-week immune sera developed swelling of the hind paws on days 13 and 7, respectively (Fig. 1A and C). However, 3-week immune serum prevented the development of arthritis in hamsters with or without treatment with CVF (Fig. 1B). No spirochetes were recovered

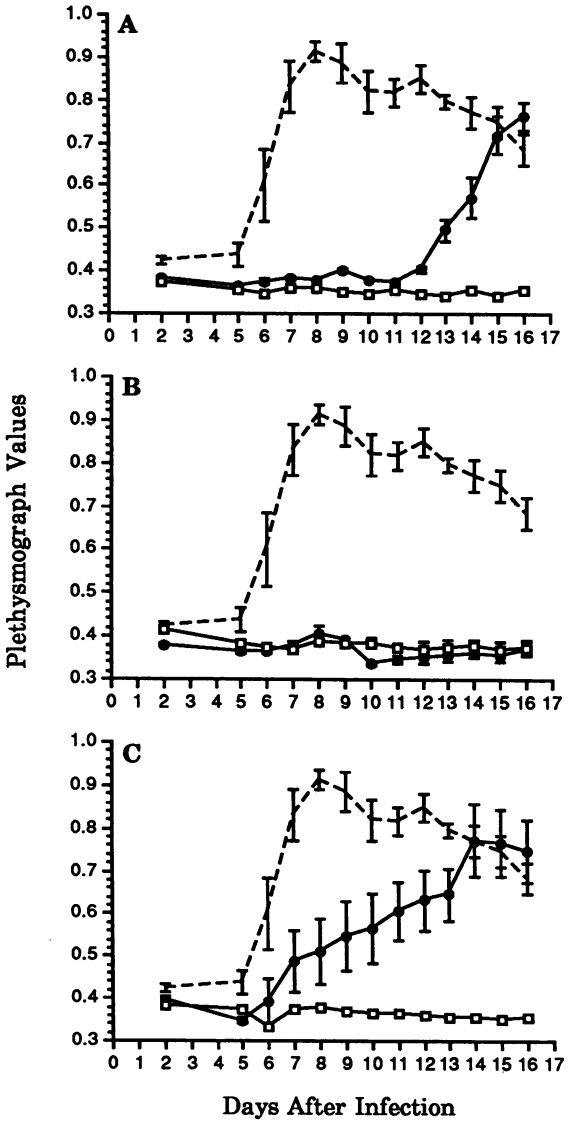

FIG. 1. Plethysmograph values (mean ± standard error) for hamsters administered normal serum (----) or CVF (●) and non-CVF-treated (□) hamsters passively immunized with 1-week (A), 3-week (B), or 10-week (C) immune serum and infected with *B. burgdorferi*.

TABLE 1. Effect of CVF on isolation of *B. burgdorferi* cells from tissues of hamsters administered immune or or normal serum

Serum type	No. culture positive/total[a]	
	With CVF	Without CVF
Normal	3/3	3/3
Immune		
1 wk	3/3	0/3
3 wk	0/3	0/3
10 wk	3/3	0/3

[a] Results are presented as number of culture-positive hamsters per total number of hamsters for which cultures were done. The spleen and urinary bladder were cultured in BSK.

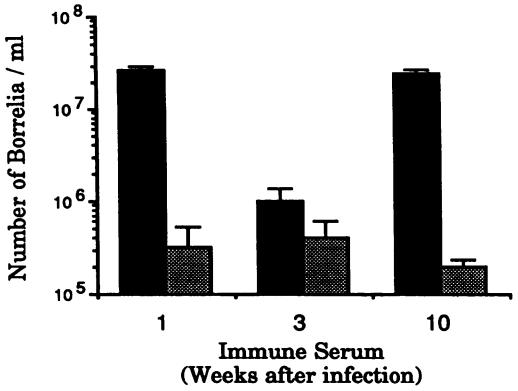

FIG. 2. Number of motile *B. burgdorferi* organisms per milliliter detected in 1-, 3-, and 10-week immune sera in the presence (stippled bars) or absence (solid bars) of complement after addition of 1 ml of BSK and incubation for 7 days.

from their tissues (Table 1). Similar results were obtained when experiments were performed with either one or four injections (0.4 ml each) of 3-week immune serum.

We also determined the effect of complement on the ability of immune serum to inhibit growth of *B. burgdorferi* in vitro. In the presence of complement, immune sera from weeks 1, 3, and 10 inhibited the growth of *B. burgdorferi* (Fig. 2). Only 3-week immune serum inhibited growth of *B. burgdorferi* in the absence of complement (Fig. 2).

The role of complement in resistance to infection with *B. burgdorferi* has not previously been addressed. Kochi and Johnson (15) showed in vitro that complement mediates killing of *B. burgdorferi* through the classical pathway. We have now addressed the role of complement in resistance to *B. burgdorferi* by treating hamsters with CVF. CVF treatment has proven useful for studying the role of complement in other bacterial infections (27, 31). CVF combines with factor B to form a stable C3 convertase (30, 31), leading to depletion of C3 with a resultant decrease in total hemolytic complement activity. In this report, we depleted 95% or more of complement activity by injecting hamsters every 3 days with 20 U of CVF.

When hamsters were depleted of complement by CVF treatment, two alterations in host resistance to *B. burgdorferi* occurred. First, depletion of complement abrogated the ability of recipients of 1- and 10-week immune sera to resist infection with *B. burgdorferi*. In the absence of CVF treatment, immune serum conferred complete protection on recipients challenged with the Lyme disease spirochete. These latter results confirmed our previous findings on the ability of passively administered serum to protect recipients from infection (23). Second, recipients of 3-week immune serum, with or without CVF treatment, were resistant to infection with *B. burgdorferi*. Arthritis failed to develop, and no spirochetes were recovered from their tissues. These results argue that complement plays a major role in the development of an effective serum-mediated borreliacidal response. These results also suggest that complement-independent antibodies may be involved, especially at week 3 after infection.

Our observation that depletion of complement abrogates the ability of recipients of 1- and 10-week immune sera to resist infection with *B. burgdorferi* but delays the onset of arthritis, together with the observation that the onset and duration of arthritis are dependent on the inoculum of viable *B. burgdorferi* (23), suggests that 1-week immune serum reduced the number of viable spirochetes to a greater extent than 10-week immune serum. The class of antibody present in 1- and 10-week immune sera may explain the differences in the time of onset of arthritis. Immune serum from week 1 contains predominantly borreliacidal immunoglobulin M (IgM), while 10-week immune serum contains predominantly borreliacidal IgG (unpublished data). Pentameric IgM may bind residual C3 present in hamsters more efficiently after CVF treatment and therefore may delay the onset of arthritis. In addition, the delay in swelling of the hind paws after administration of 1-week immune serum is apparently not due to the presence of more-protective antibody in 1-week serum, because we showed previously (24) by passive transfer of diluted immune serum that 1-week immune serum had less borreliacidal activity than did 10-week immune serum.

While CVF treatment abrogated the protective abilities of 1- and 10-week immune sera, it had no effect on the ability of 3-week serum to confer complete protection. In the absence of complement, the growth of *B. burgdorferi* was also inhibited in vitro with 3-week immune serum. These results suggest that hamsters transiently develop complement-independent protective antibodies after infection with *B. burgdorferi*. Similarly, Pavia et al. (18) demonstrated that immune rat serum retained borreliacidal activity despite heat inactivation. Newman and Johnson (17) also demonstrated that C5-deficient mice could clear *Borrelia turicatae* in the absence of an antibody-dependent lytic mechanism. Complement-independent antibodies could prevent the induction of Lyme arthritis by promoting phagocytosis and elimination of *B. burgdorferi*. Several reports (1–3, 8, 19, 29) have shown that polymorphonuclear leukocytes, monocytes, and macrophages can phagocytize *B. burgdorferi*. Peterson et al. (19) and Benach et al. (2, 28) have shown that opsonization of *B. burgdorferi* facilitates phagocytosis. It is known that hamster IgG1 has a strong affinity for macrophages and does not bind complement (5). Opsonization of spirochetes with IgG1 and binding to phagocytic cells would, therefore, result in clearance of the Lyme disease spirochete. Even if IgG1 antibody is not directly involved in the killing of *B. burgdorferi*, it could promote destruction and elimination of spirochetes.

In conclusion, a picture of antibody-mediated resistance to infection with *B. burgdorferi* is beginning to emerge. We (23, 24) and others (7, 10–13, 21) have shown that serum and antibodies can confer protection against infection with the Lyme disease spirochete on animals. These results suggest that complement-dependent, and possibly complement-independent, antibodies are important for preventing the induction of Lyme arthritis. These results have practical implications for developing an effective vaccine against *B. burgdorferi* and suggest that a potential Lyme disease vaccine should induce complement-dependent antibodies to protect against infection with *B. burgdorferi*.

This work was supported by funds from Public Health Service grant AI-22199 from the National Institute of Allergy and Infectious Diseases.

We thank Dean D. Manning for reviewing the manuscript.

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