INFECTION AND IMMUNITY, Jan. 1981, p. 465-469
0019-9567/81/010465-05$02.00/0

Vol. 31, No. 1

In Vivo Evidence that an Intact Lytic Complement Pathway Is Not Essential for Successful Removal of Circulating *Borrelia turicatae* from Mouse Blood

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Complement component C5-deficient mice were found to be able to eliminate blood-borne *Borrelia turicatae* as effectively as normocomplementemic control animals. The absence of C5 and the resultant loss of hemolytic complement activity were confirmed for the deficient mouse strains used. Immunofluorescent staining of complement component C3 on blood-derived borreliae could be readily accomplished as early as 2 days before spirochetal elimination from all mice tested. These observations would suggest that C3 deposition was occurring, even though the terminal lytic steps were blocked in the complement-deficient mice. We propose that spirochetolysis is not requisite for successful removal of borreliae from the circulation of mice.

Relapsing fever borreliae are pathogenic spirochetes that are primarily transmitted to mammalian hosts via specific arthropod vectors (6). The human disease includes episodes of spirochetemia and fever followed by elimination of spirochetes with the concomitant establishment of a transient afebrile period. Untreated persons suffering from the louse-transmitted disease generally have 1 to 3 relapses (18), whereas the tick-transmitted borreliae produce 5 to 12 relapses (11).

The mechanism by which circulating borreliae are eliminated from mammalian hosts remains unclear. Various laboratories have reported that passive transfer of immune serum could delay the onset of or afford complete protection against borrelemia in laboratory animals (2, 4, 14). Nuttall (14) reviewed the early studies which demonstrated that borreliae could be lysed in the presence of immune serum. Felsenfeld firmly believed that borreliae were killed by complement-mediated lysis since immune serum-sensitized borreliae were readily lysed by nonimmune guinea pig plasma (7-9). Support of this notion comes from the report that patient complement titers are lower than normal during spirochetemia and the ensuing crisis periods than during convalescence (10). Additional support of the suggestion that in vivo lysis of borreliae has the potential to occur arises from the finding that immunoglobulin M, a potent activator of the classical complement pathway, is protective when passively transferred to nonimmune mice (2).

An opposing school of thought contends that phagocytes play an essential role in the elimination of viable blood-borne borreliae (12). Wolman and Wolman (20) reported that carbon blockade afforded susceptibility of otherwise resistant rabbits and guinea pigs to Ethiopian strains of *Borrelia recurrentis*. Adler and Asbel (1) demonstrated that immune serum-treated as well as untreated borreliae avidly bound to the surface of human polymorphonuclear cells and monocytes. More recently, it has been reported that [^{3}H]thymidine-labeled *Borrelia hermsii* cells would associate with human polymorphonuclear cells in the presence or absence of immune serum (P. J. Spagnudo, T. Butler, E. H. Block, and C. Santoro, Clin. Res. **27**: 698, 1979). Additionally, antibiotic-debilitated *Borrelia recurrentis* cells appear to be ingested by human polymorphonuclear cells in vitro (3).

Rodents are the primary host for *Borrelia turicatae*. We set out to determine whether an intact lytic complement pathway was essential for the successful elimination of borreliae. This paper presents our observation that the course of borrelemia in complement-deficient mice does not differ from that observed in normocomplementemic mice.

MATERIALS AND METHODS

Organism. *B. turicatae* phase variants M2007AN104 and M2007AN134 were obtained by single cell inoculation (17) of a series of outbred mice with parental stock spirochetes isolated from the tick *Ornithodoros turicatae*. The ticks were generously supplied by Willy Burgdorfer (Rocky Mountain Laboratory, Hamilton, Mont.). The strains were dispensed into 0.5-ml lots and stored in liquid nitrogen. Individual lots were periodically revived and maintained by 24-h animal passage for a maximum of five successive transfers. *B. turicata* cells from these passages served as inocula for the experimental animals.

Animals. Swiss Webster mice were obtained from the BioLab Corp. (St. Paul, Minn.), DBA/2 mice were obtained from the University of Minnesota mouse colony, Minneapolis, and AKR/J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Animals were housed five to six individuals per cage at 18°C with 12-h controlled light periods and provided with food and water ad libitum. Only 6- to 8-week-old animals were used in these studies.

Enumeration of and inoculation with organism. Infected blood was diluted 5.5-fold in 0.05% saline to allow lysis of erythrocytes but maintain motility of spirochetes. Low densities of borreliae (ca. 1×10^3 to 5×10^5 cells per ml) were enumerated by a modified version of the method of Stoenner (19). Briefly, 1 μl of diluted blood was dispensed onto a microscope slide within a ring of petroleum jelly (inside diameter, ca. 1.5 cm). A cover glass was pressed onto the specimen to spread the fluid to a diameter of ca. 0.75 cm. The entire fluid-filled region was scanned by using the 10× objective (total magnification, ×125) of a Leitz dark-field microscope, and the total number of spirochetes was determined. High densities of borreliae (ca. 5×10^5 cells per ml and greater) were enumerated in triplicate by using a Petroff-Hausser counting chamber. Mice were inoculated with 10^5 blood-derived borreliae via the intraperitoneal route.

Confirmation of C5 deficiency and resultant loss of lytic capacity in AKR/J and DBA/2 mouse strains. Blood samples were obtained from complement-deficient and normocomplementemic mice by cardiac puncture using 1-ml tuberculin syringes equipped with heparinized (1 U) 25-gauge needles. The blood samples were immediately transferred to 12- by 75-mm plastic tubes (Falcon), and blood cells were sedimented by centrifugation at 1,500 × *g* for 3 min at 4°C with a Beckman TJ-6R refrigerated centrifuge. The plasma supernatant was immediately used in each of the two following immunodiffusion test systems. All immunodiffusion plates were prepared by using 3.0 ml of molten 1% agarose supplemented with 0.01 M ethylenediaminetetraacetic acid, pH 7.2. Single radial diffusion plates were additionally fortified with 1.0 ml of anti-human C5 (Kent Laboratory, Redmond, Wash.). Single and double diffusion plates were incubated with plasma samples from the deficient mice as well as from normocomplementemic mice and humans. A 10% bovine serum albumen sample served as a negative control. Incubation at 8 to 10°C was allowed to proceed for 72 h in a humidified chamber.

Plasma samples were also subjected to the hemolytic assay system first described by Rosenberg and Tachibana (16). Washed sheep erythrocytes were sensitized with a 1:50 dilution of goat anti-sheep erythrocyte antiserum (titer, 1:2,000; lot no. 3-9670; Microbiological Associates, Bethesda, Md.) for 5 min with constant agitation. The cells were washed twice in 0.01 M phosphate-buffered saline and resuspended to a final concentration of 2% erythrocytes (packed volume) in a barbital buffer containing 0.375 g of sodium diethylbarbital and 0.575 g of 5,5-diethylbarbituric acid per liter of water supplemented with 1.0 ml of 0.5 M $MgCl_2$ and 0.15 M $CaCl_2$. Samples of 50 μl of sensitized erythrocytes were dispensed into microtiter wells containing 50-μl samples of serially diluted complement. The samples were incubated for 2 h at 37°C.

Immunofluorescent detection of C3 on the surface of blood-borne borreliae. Borreliae-laden blood samples drawn 1 and 2 days before spirochetal elimination from the various mice used were smeared onto glass slides and air dried; the bacteria were fixed to the glass by exposure to acetone for 10 min. Lysed erythrocytes and nonfixed materials were removed by rinsing the dried slides in phosphate-buffered saline (pH 7.2). The slides were blotted dry and stained in subdued light with a 1:8 dilution of fluorescein-labeled (fluorescene/protein = 3.7 μg/mg) anti-mouse C3 (Cappel Laboratories, Downingtown, Pa.) for 30 min at room temperature in a humidified chamber. Stained slides were given two 10-min washes in phosphate-buffered saline before being rinsed with distilled water. The slides were viewed with a Leitz-Wetzler ultravion Kodak Ektachrome film (ASA 400) with 1-min exposure times.

RESULTS AND DISCUSSION

Our intention was to determine whether an intact lytic complement pathway was essential for the in vivo elimination of blood-borne borreliae by mice. Because Nilsson and Müller-Eberhard (13) reported that the levels of late-acting murine complement components varied with the sex of the mouse sampled, the ability of female versus male outbred mice to eliminate circulating borreliae was compared. A representative sampling of data is presented in Fig. 1. There appeared to be no difference with regard to the ability of either sex to remove circulating borreliae during either the first peak or relapse.

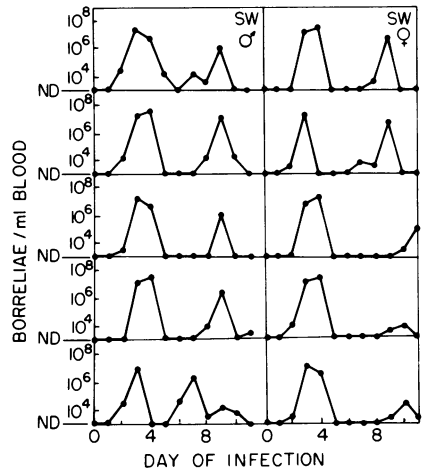

FIG. 1. *Comparison of borrelemia in male and female normocomplementemic animals. Mice were infected with 10^5 borreliae, and bacterial counts were made on a daily basis. The figure shows a representative sampling of data obtained from 50 female and 20 male animals. ND, Not detectable.*

Apparently, the submaximal complement levels of female mice did not adversely influence ability of the animals to remove efficiently circulating spirochetes relative to what was observed for male animals.

Normocomplementemic female and male mice displayed no differences with regard to the ability to eliminate circulating borreliae. A limited study comparing male and female C5-deficient mice also showed no differences with regard to the ability to remove circulating borreliae. We therefore opted to compare the ability of C5-deficient female mice with that of normal female mice in regard to their ability to eliminate borreliae (Fig. 2 and 3). Female AKR/J and DBA/2 mice eliminated *B. turicatae* with an observable efficiency comparable to that of the normocomplementemic animals. This observation is interesting as both inbred mouse strains used have been shown by immunochemical techniques to lack C5 (5, 13). Most recently, Patel and Minta (15) reported that the AKR/J mouse strain could not synthesize the proper messenger ribonucleic acid which would allow an in vitro translation system to produce detectable quantities of C5. Hence, these mice would not be expected to be able to lyse spirochetes by the complement pathway.

To confirm that the mice used were indeed C5 deficient, plasma samples were subjected to Ouchterlony and Mancini immunodiffusion analyses. Since Nilsson and Müller-Eberhard (13) used anti-human C5 antiserum to demonstrate that human C5 cross-reacts with mouse

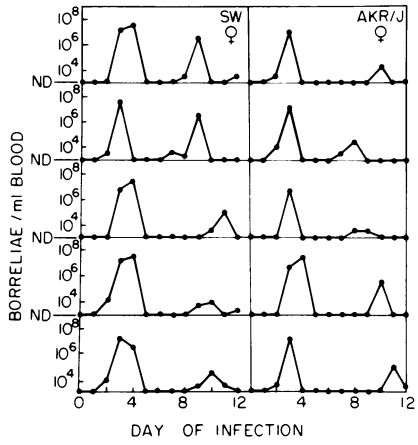

FIG. 2. *Comparison of borrelemia in C5-deficient and normocomplementemic female mice. Mice were infected with 10^5 borreliae, and bacterial counts were made on a daily basis. The figure shows a representative sampling of data obtained from 50 normocomplementemic mice and 25 AKR/J mice. ND, Not detectable.*

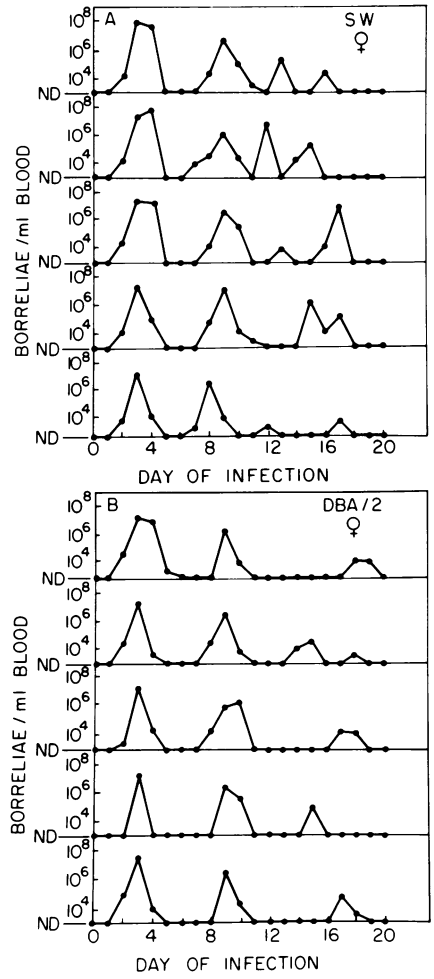

FIG. 3. *Comparison of borrelemia in C5-deficient and normocomplementemic female mice. Mice were infected with 10^5 borreliae, and bacterial counts were made on a daily basis. The figure shows a representative sampling of data obtained from (A) 50 normocomplementemic and (B) 20 DBA/2 mice. ND, Not detectable.*

C5 (formerly protein MuBl), anti-human C5 antiserum was used in demonstrating the presence of this mouse complement component. Although human C5 and C5 in normocomplementemic mouse plasma were immunologically cross-reactive, no such cross-reaction could be observed with plasma obtained from either AKR/J or DBA/2 mice (Fig. 4). A parallel experiment substituting anti-mouse C3 for anti-human C5 shows that the AKR/J and DBA/2 mouse plasma samples contained the earlier complement component.

By utilizing the Mancini technique, it can be observed that not even the faintest precipitation

zone is present when plasma proteins have an opportunity to diffuse directly into an antiserum containing agar medium (Fig. 5). This observation, plus that noted for Fig. 4, confirms the C5-deficient nature of our animals, and is in agreement with results established by others (5, 13, 15).

The ability of mouse plasma to lyse sensitized sheep erythrocytes is related to the detectability of C5 in the plasma samples tested (Table 1). As can be ascertained from the data presented, neither the AKR/J nor the DBA/2 mouse plasma samples were capable of mediating the lysis of sensitized erythrocytes via complement activation, whereas the Swiss Webster strain plasma samples had this property. This would suggest that although the Swiss Webster mouse strain possesses the potential to lyse borreliae in vivo, it is probably not an essential event for elimination of *B. turicatae*. Both of the C5-deficient mouse strains used could readily eliminate circulating spirochetes even though their plasma lacked a component necessary for lysis to occur.

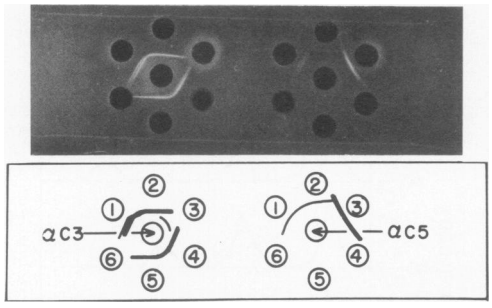

FIG. 4. *Double immunodiffusion analysis of mouse plasma samples from various sources. The center wells contained either anti-human C5 or anti-mouse C3, and the peripheral wells contained plasma obtained from (1) male Swiss Webster mice, (2) female Swiss Webster mice, (3) male human control, (4) female AKR/J mice, (5) female DBA/2 mice, and (6) bovine serum albumin control. Plasma samples from male DBA/2 and AKR/J mice gave identical results to those depicted in this figure for female mice.*

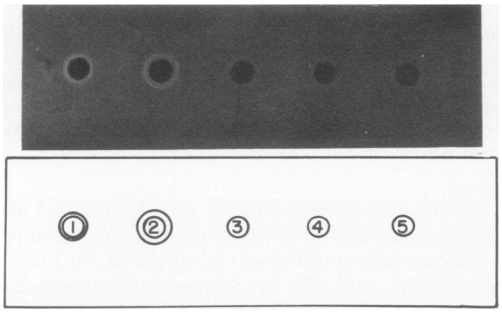

FIG. 5. *Single radial immunodiffusion analysis of relative C5 concentration in plasma samples from various mouse strains. The agarose medium contained anti-human C5, and the wells contained plasma samples obtained from (1) female Swiss Webster mice, (2) male Swiss Webster mice, (3) female AKR/J mice, (4) female DBA/2 mice, and (5) bovine serum albumen control. Plasma samples from male AKR/J and DBA/2 mice gave results identical to those depicted in this figure for female mice.*

TABLE 1. *Minimal hemolytic units (MHU) of complement in various plasma samples*[a]

Source	Sex	Sample size	MHU range
Guinea pig		Commercial pooled	512–1,024
Swiss Webster mouse	M	20	32–64
	F	20	8–32
AKR/J mouse	M/F	20	0
DBA/2 mouse	M/F	15	0
No complement			0

[a] All samples were removed from experimentally infected male (M) and female (F) animals after borrelemia had subsided. The values reported here were comparable with those obtained from a limited sampling of uninfected animals. The MHU values represent the minimum amount of complement required to completely lyse a standard volume of sensitized erythrocytes.

As a final point, we wished to demonstrate that complement deposition onto borreliae was occurring in situ. It was decided to examine blood-borne borreliae for the presence of surface-bound C3. C3 was present on the surface of borreliae as early as 2 days before their elimination from mouse blood (Fig. 6).

Our study thus shows that the terminal steps of complement activation, initiated by complement component C5, do not appear to be requisite for the elimination of borreliae in vivo. However, these data do not preclude the possibility that the earlier products of complement activation are important for the removal of circulating borreliae. It is known that cleavage products of native C3 can serve as opsonins for phagocytic events when deposited on the surface of phagocytizable matter. Further tests will thus be required to determine whether C3 activation is necessary for the elimination of circulating *B. turicatae* from mice. At present, our data indicate that lysis is not essential for the in vivo elimination of borreliae by mice.

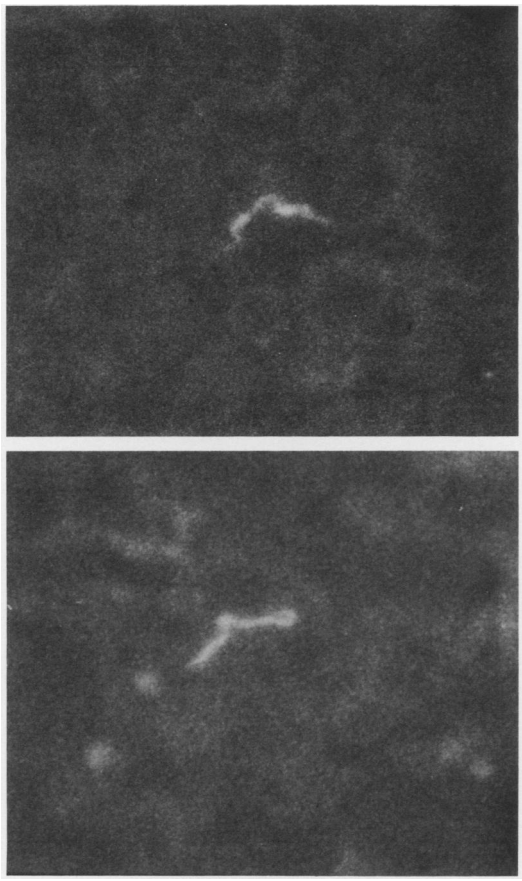

FIG. 6. *Micrographs of fluorescein-conjugated anti-mouse C3 antibody stained borreliae. The borreliae shown in these pictures were obtained from an infected female Swiss Webster mouse (top) 2 days before elimination and (bottom) 1 day before elimination. The staining patterns shown here are representative of patterns observed with borreliae obtained from all mouse strains used. A total of three animals from each group were sampled.*

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-06589 from the National Institute of Allergy and Infectious Diseases.

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