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NOTES

The Spirochete *Borrelia crocidurae* Causes Erythrocyte Rosetting during Relapsing Fever

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Several species of the genus *Borrelia* exhibit antigenic variation of variable major proteins on their surface during relapsing fever. We have investigated the African relapsing fever species *Borrelia crocidurae* during infections in mice and compared it with the thoroughly studied North American species *Borrelia hermsii*. A major difference between the two species is that *B. crocidurae* can bind and become completely covered with erythrocytes. In addition, *B. crocidurae* causes a prolonged spirochetemia which coincides with a delayed appearance of antiborrelial antibodies. We show that the antibody response against an unrelated antigen is not delayed and that antibiotic treatment, which dissociates rosettes and inhibits the spirochetes, also leads to an early antibody response. Taken together, the erythrocyte aggregation and prolonged spirochetemia hint at a new mode of immune evasion where erythrocyte-covered spirochetes may avoid contact with the phagocytic cells and B cells of the immune system, thereby delaying the onset of a specific immune response.

Relapsing fever is a disease caused by spirochetes that belong to the genus *Borrelia*. The borreliae are transmitted from one vertebrate host to another by the bite of soft-shelled ticks (*Argasidae*) or lice (*Pediculus humanus*) (11). Therefore, presence in the blood of a host is a prerequisite for transmission of the bacteria. Antigenic variation is the characteristic virulence mechanism by which relapsing fever *Borrelia* species are able to persist in a mammalian host. Several other pathogenic microorganisms employ antigenic variation of surface proteins. The most familiar are the etiologic agent of sleeping sickness, *Trypanosoma brucei* (7), and the malaria parasite *Plasmodium falciparum*. In addition to antigenic variation the malaria parasite displays an erythrocyte (RBC)-rosetting mechanism (20). Antigenic variation among *Borrelia* species has mainly been studied in spirochetes of the North American species *Borrelia hermsii*, whose capacity to periodically express new variable major proteins, Vmp, on their surface leads to the typical symptoms of relapsing fever (5, 9). The clinical manifestations during relapsing fever are very diverse, and different species of relapsing fever *Borrelia* cause different symptoms in different mammals (11, 12). The African relapsing fever species *Borrelia crocidurae* displays antigenic variation which is very similar to that of *B. hermsii*. After infection of a mammalian host with one serotype the bacteria grow and a spirochetemia develops whereby numerous bacteria are visible in the blood. A few of these bacteria switch to express a new, antigenically distinct, Vmp protein. When the immune reaction against the primary infecting serotype occurs the spirochetemia subsides and no spirochetes are detected in the blood of the host, but eventually a second spirochetemia appears with borreliae of the new serotype (9).

During growth of *B. crocidurae* in mice we observed that RBCs aggregated around the spirochetes, as reported previously (17). This aggregation does not occur during *B. hermsii* spirochetemias. The RBC aggregates are reminiscent of the rosettes formed by *Plasmodium*-infected RBCs, although the mechanism behind the aggregation is probably different (20). In the present study, we have examined the ability of two relapsing fever *Borrelia* spirochetes to adhere to and aggregate RBCs. We have also investigated if the immunogenicity of the Vmp or a general suppression of the immune response is responsible for the longer spirochetemias in the *B. crocidurae* infection.

Observation of distinct RBC binding phenotypes. One significant difference between the infection of BALB/c mice (Bomholtgård, Bomholtgård, Denmark) with *B. hermsii* HS1 serotype 7 (ATCC 35209) versus *B. crocidurae* serotype C2 (cloned from the strain collection of Alan G. Barbour, Irvine, Calif.) is the apparent RBC binding capacity of *B. crocidurae*. We found that *B. crocidurae* binds to RBCs during the spirochetemia so that each bacterium is completely covered by RBCs (Fig. 1A). The aggregation of RBCs around *B. crocidurae* can be reconstituted in vitro by mixing culture-grown *B. crocidurae* with diluted blood on a microscopic slide (Fig. 1B). The borreliae were grown in BSKII medium at 34°C for 48 h (3). The bacteria were centrifuged, and the bacterial pellet was resuspended in blood from an uninfected mouse and diluted 1:10 in phosphate-buffered saline (PBS) to a bacterial titer of 10^8 spirochetes · ml^{-1}. The spirochete-blood mixture was placed on a microscope slide and covered with a cover slip that was sealed along the edges with nail polish. The sealed samples were incubated at 22 or 37°C for 30 min before microscopic examination. Aggregates formed when the spirochete-blood mixture was incubated at 37° but not at 22°C (Fig. 1B and C, respectively). The North American relapsing fever species *B. hermsii* does not aggregate RBC in vivo, and it did not bind to the cells in vitro at either of the temperatures tested. The in vitro aggregation assay was also applied to mononuclear cells (MNCs). The separation of MNCs was performed with a Lymphoprep kit according to the protocol of the vendor (Nycomed, Oslo, Norway). The cells were diluted in PBS to 10^6 cells · ml^{-1}. At 22°C neither *B. hermsii* nor *B. crocidurae* bound

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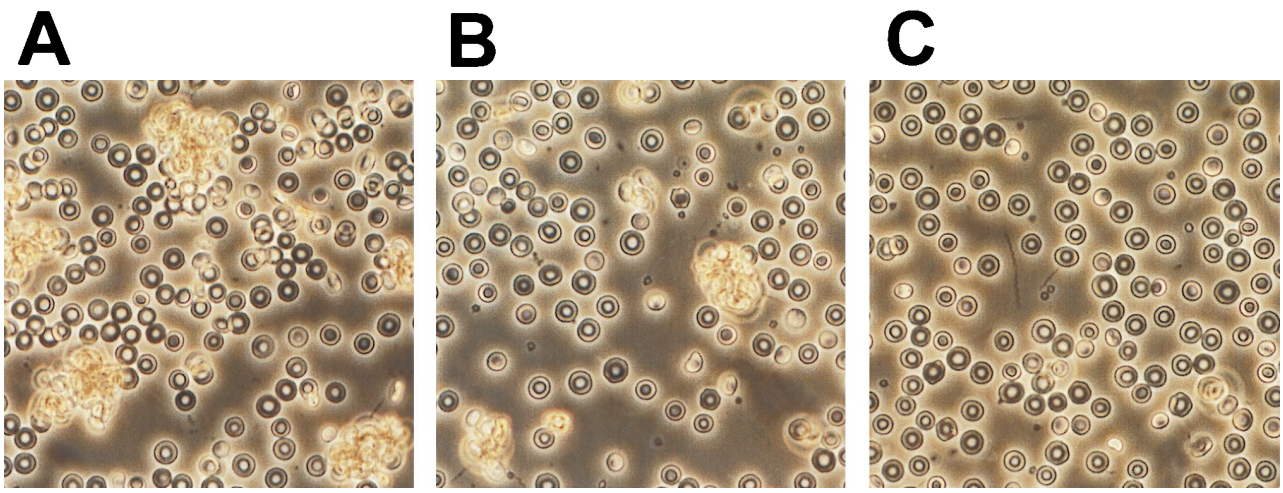

FIG. 1. Photographs depicting the interaction between *B. crocidurae* serotype C2 and mouse RBCs. (A) Aggregation of RBC in vivo. The photographed sample was taken early during the massive spirochetemia. (B) Reconstitution of RBC aggregation in vitro after incubation at 37°C for 30 min. (C) No RBC aggregation was seen after incubation at 22°C in vitro.

MNCs. At 37°C binding was observed between the MNCs and the borrelial cells; however, the MNCs bound almost as well to *B. hermsii* as to *B. crocidurae*. In a competitive assay where equal numbers of RBCs and MNCs were incubated with *B. crocidurae*, a clear preference for RBC binding was evident. The RBCs formed rosettes around the *B. crocidurae* cells, and only a few individual MNCs were bound. The displacement of MNCs by RBCs already at a 1:1 ratio suggests that the binding of MNCs is negligible in vivo where the excess of RBCs to MNCs is of several orders of magnitude. The absence of in vitro aggregation of RBCs at 22°C may be a simple developmental strategy of the borreliae. This is the typical temperature of ticks, in which dissociation of the rosette could release the spirochetes for unimpeded development. We have routinely observed rosetting at all spirochetemic peaks in *B. crocidurae* infections of BALB/c and C3H/Tif mice, suggesting that the phenomenon is independent of antigenic variation and infected mouse strain. Despite the invariable observation of RBC rosetting around *B. crocidurae* whether blood samples are observed undiluted or diluted with 0.15 M NaCl, PBS, or BSKII culture medium, it is still possible that the rosetting is not an in vivo phenomenon. This may not be undisputedly resolved without microscopic observation of the rosettes in the bloodstream of a living mouse. However, an additional indication of the presence of RBC aggregation in vivo comes from observations of damage on highly vasculated tissues of mice infected with *B. crocidurae*. It is conceivable that the observed rosetting of RBC may clog the capillaries and thereby cause infarctions, petechiae, or more severe bleedings, explaining some of the clinical symptoms seen in *B. crocidurae* relapsing fever patients (2, 10). We are currently investigating the role of RBC rosetting in the lesions observed during *B. crocidurae* infection in mice.

Analysis of a delayed antibody response. BALB/c mice were inoculated with 10^6 borreliae by intraperitoneal injection. To monitor the developing spirochetemia, a drop of blood was taken from the tail vein of each mouse, placed on the edge of a drop of PBS with 0.5% bovine serum albumin, covered with a coverslip, and observed by microscopic examination at ×400 magnification. The samples were searched for spirochetes in the boundary between buffer and blood cells. If present, spirochetes could be observed between individual RBC or in RBC aggregates. The first spirochetemia appeared after approximately 2 days for both infections. The *B. crocidurae* spirochetemia, however, lasted for 4 to 5 days, whereas the *B. hermsii* spirochetemia lasted for only 1 day (Fig. 2). We suspect that the aggregation of RBCs around the bacteria might cause the prolonged spirochetemia during *B. crocidurae* infection, by delaying the onset of a specific immune reaction directed against the bacteria. We tested this hypothesis by infecting mice with an equal amount of *B. hermsii* or *B. crocidurae*. Presence of spirochetes in the blood was recorded (Fig. 2), and a 30-μl blood sample was collected from each mouse daily for 10 days postinfection. The blood was diluted 1:10 with PBS prior to serum preparation (13). The sera were then tested by Western blotting for the emergence of antibodies specific for the infecting agent. Total protein extracts from *B. hermsii* and *B. crocidurae* were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (MSI, Westboro, Mass.). After transfer, each membrane was cut into 5-mm-wide strips, and protein detection was performed as described by Jonsson et al. (15). As a positive control, one strip was incubated for 1 h in a 1:100

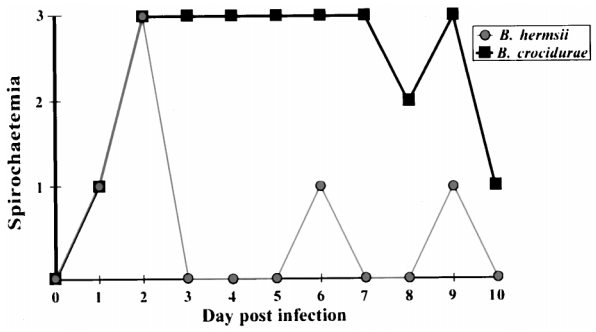

FIG. 2. Spirochetemia in mice. The development of spirochetemia in BALB/c mice infected with an equal amount (10^6 spirochetes) of *B. hermsii* serotype 7 (A) or *B. crocidurae* serotype C2 (B). The spirochetemia was graded in four groups as follows: 3, one or more spirochetes in each field of vision in the studied sample; 2, one spirochete in every field of vision to one spirochete in 10 fields of vision; 1, less than one spirochete in 10 fields of vision and 0, no spirochete found in the sample.

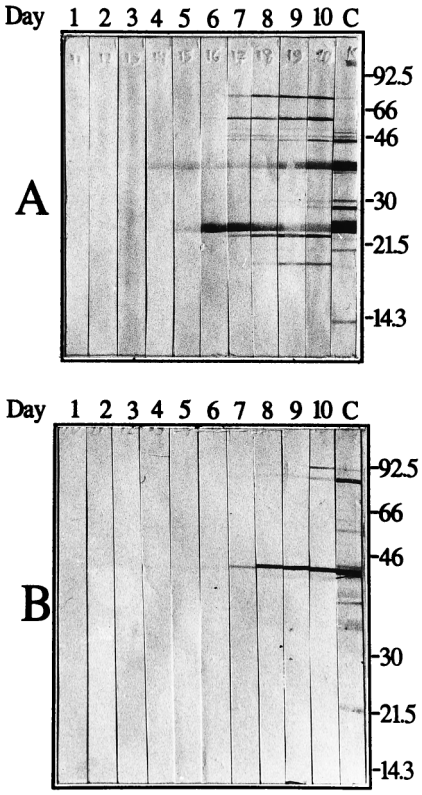

FIG. 3. Western blot showing the emergence of a specific immune response against the infecting *Borrelia* species. (A) Daily serum samples from a mouse infected with *B. hermsii* serotype 7 reacting against protein extract of *B. hermsii* serotype 7 cells. (B) Serum from a mouse infected with *B. crocidurae* serotype C2 reacting against protein extract of *B. crocidurae* serotype C2 cells. Numbers at the top of each panel correspond to day postinfection. The rightmost lane, marked C, includes a positive control antiserum. Molecular mass standards in kilodaltons are indicated at the right.

dilution of the corresponding mouse anti-*Borrelia* serum, raised as previously described (19). The procedure was followed by the binding of an alkaline phosphatase-conjugated rabbit anti-mouse antibody (Dako, Älusjö, Sweden), diluted 1:1,000 in 2.5% nonfat milk–PBS. Bound secondary antibody was visualized by detection of alkaline phosphatase activity. Western blot analysis revealed the presence of specific antibodies against *B. hermsii* 4 days after infection, 2 to 3 days after the start of the visible spirochetemia (Fig. 2 and 3A). The mice infected with *B. crocidurae* developed specific antibodies at least 7 days after infection, despite the development of the spirochetemia no later than 2 days after infection (Fig. 2 and 3B). This corresponds to a delay in the onset of an immune reaction of 3 days compared to that for *B. hermsii* infection. Enzyme-linked immunosorbent assay (ELISA) results from the same sera, performed as described by Bunikis et al. (8), agree with the Western blot data inasmuch as a sharp increase of *B. hermsii*-specific antibodies was apparent from day 3 until day 6. The amount of *B. crocidurae*-specific antibodies increased only moderately and reached a plateau at a low level already on day 4 (Fig. 4A).

Investigation of Vmp immunogenicity. The delayed immune response in *B. crocidurae* infections may have several explanations. If the rosetting and the effect on the immune system are unconnected, the delayed immune response could be accounted for by a lower immunogenicity of the surface molecules of *B. crocidurae*. To investigate the differences between *B. hermsii* Vmp7 and *B. crocidurae* VmpC2, mice were injected with 1 μg of protein extracts enriched for Vmp proteins (6) of *B. hermsii* serotype 7 and *B. crocidurae* serotype C2. Serum samples were collected daily for 10 days. Both proteins induced specific antibodies by 2 days postinoculation, as detected by Western blotting (data not shown). The immunogenicity of the Vmp proteins was explored further by treating mice infected with *B. hermsii* serotype 7 or *B. crocidurae* serotype C2 with the bacteriostatic antibiotic tetracycline at a concentration of 0.6 g/liter of drinking water as the mice became spirochetemic on day 2 (19). As previously, serum samples were collected daily for 10 days. When the antibiotic inhibits *B. crocidurae* the spirochetes are no longer able to aggregate RBCs, rendering them accessible to recognition and attack by the immune system. The immune responses in mice infected with *B. hermsii* were almost indistinguishable between the mice that were not treated and the mice that were treated with tetracycline (compare Fig. 3A with Fig. 5A and Fig. 4A with Fig. 4B). Specific antibodies were detectable from day 4 postinfection, 2 days after the start of the spirochetemia. In contrast, for *B. crocidurae* there was a clear difference between the antibiotic-treated and untreated mice. With tetracycline treatment, specific antibodies appeared 3 days after infection (Fig. 5B), 1 day after emergence of the spirochetemia. Without treatment, the immune response appeared 7 days postinfection. The corresponding ELISA confirmed that while there is an apparent

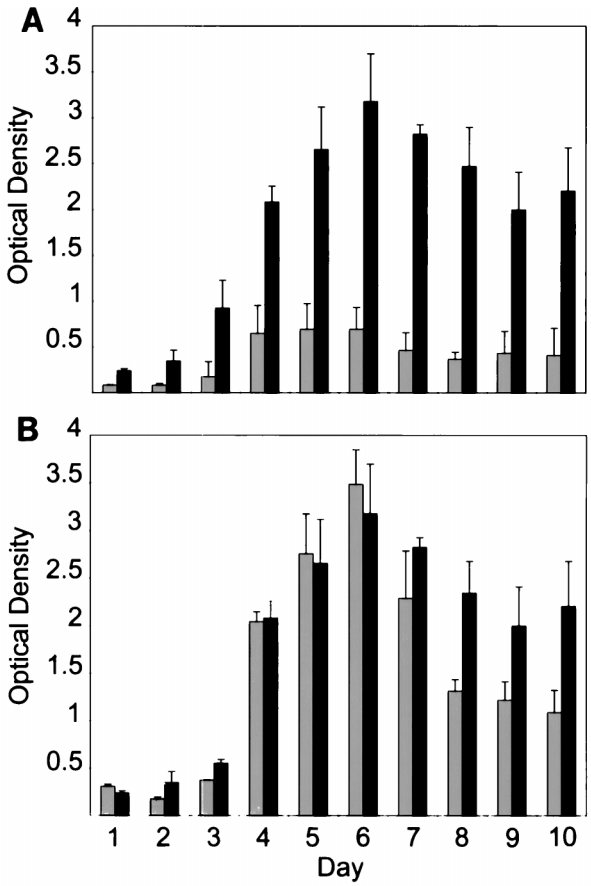

FIG. 4. Serologic responses (immunoglobulin M-ELISA) of mice infected with *B. hermsii* serotype 7 or *B. crocidurae* serotype C2. ELISA of total protein from *B. hermsii* serotype 7 (black bars) or *B. crocidurae* serotype C2 (grey bars) and either untreated (A) or treated with tetracycline (B) at day 2 postinfection. Error bars indicate standard deviation of the means.

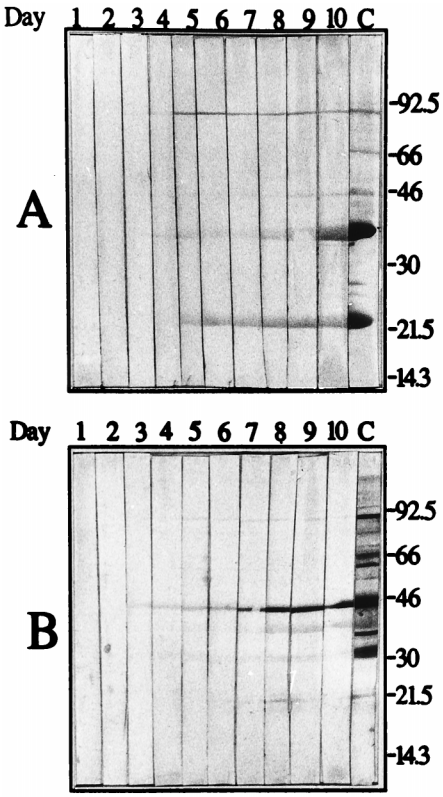

FIG. 5. Western blot analysis of serum from mice infected with *Borrelia* species after treatment with tetracycline. (A) Reactions of serum samples from a mouse infected with *B. hermsii* serotype 7 against protein extract of *B. hermsii* serotype 7 cells. (B) Reactions of sera collected daily from a mouse infected with *B. crocidurae* serotype C2 and tested against protein extract of *B. crocidurae* serotype C2 cells. The mice were treated with tetracycline at day 2 postinfection. Numbers at the top of each panel correspond to day postinfection. The rightmost lane, marked C, includes a positive control antiserum. Molecular mass standards in kilodaltons are indicated on the right.

difference in immune response between *B. hermsii* and *B. crocidurae* in the absence of tetracycline (Fig. 4A), the immune reactions to *B. hermsii* and *B. crocidurae* are virtually identical upon inhibition of the bacteria early during the spirochetemia (Fig. 4B). The reason for the early immune response to *B. crocidurae* upon tetracycline treatment may be due to increased lysis. However, the identical immune reactions to *B. hermsii* with or without tetracycline treatment (Fig. 4) contradicts this, assuming a similar effect of tetracycline on *B. hermsii* and *B. crocidurae*. This is in conflict with the hypothesis that a lower immunogenicity of *B. crocidurae* causes the differences in timing of the immune responses. Similarities of the immune reactions upon interruption of *B. crocidurae* rosetting with antibiotics further imply an involvement of rosetting in the delay of the immune response.

Investigation of an immunosuppressing activity. To assess whether the protracted infection could be explained by a general immune suppression of the host by *B. crocidurae*, an unrelated antigen was injected into spirochetemic mice. Healthy mice were infected with *B. crocidurae* serotype C2 or *B. hermsii* serotype 7. At day 2, when all mice were spirochetemic, each mouse received an intraperitoneal injection of 10 μg of placental alkaline phosphatase (PLAP), purified as described elsewhere (14). Serum samples collected daily from day 1 to day 10 postinfection were used in Western blot assays. A 1:100 dilution of the H7 monoclonal antibody raised against PLAP was used as a positive control (16). Bound monoclonal antibody was detected by incubation with peroxidase-conjugated goat anti-mouse antibody diluted 1:1,000 in 2.5% nonfat milk–PBS. Peroxidase activity was detected with an enhanced chemiluminescence kit (ECL; Amersham, Buckinghamshire, England) and recorded on photographic film. A specific immune response to PLAP became detectable by Western blotting 1 day after injection for uninfected mice and for mice infected with *B. hermsii* serotype 7 or *B. crocidurae* serotype C2 (data not shown). The simultaneous appearance of an immune response against the unrelated antigen PLAP in all mice argues against a general immunosuppressing activity of *B. crocidurae*.

B. crocidurae persists in the bloodstream for a long time without eliciting an evident immune response. This does not seem to be attributable to a particularly low antigenicity of the surface proteins of the bacteria. Neither is there an apparent reduction of the immune response in the host. A potential explanation for the protracted infection is that *B. crocidurae* may impose a more specific immunosuppressing activity during close interaction with the cells of the host's immune system. This suppression seems to be dependent on live bacteria, since it is lost upon blocking protein synthesis of *B. crocidurae* with tetracycline. The assumption that the rosetting and delayed immune response are connected presents an alternative explanation that has been suggested to be involved in malaria infection (1).

It has been shown previously that relapsing fever borreliae are killed by the humoral immune response of the host (4, 18). The initiation of such a response requires the ingestion of bacteria or their antigens by antigen-presenting cells, whose subsequent interaction with B cells exhibiting the correct antiborrelial antibody specificities leads to the proliferation of those B cells and hence to production of anti-*Borrelia* antibodies. It is possible that binding of RBC to the surface of *B. crocidurae* can exclude direct interaction of the bacterium with the cells of the immune system. This exclusion would lead to the delayed appearance of a specific immune response during the infection. The RBCs cannot, however, protect the *Borrelia* from the binding of specific soluble antibodies. This implies that the presence of sufficient numbers of dead or non-RBC binding borreliae in the host eventually would lead to the generation of a delayed immune reaction, which then results in a rapid killing of the borreliae. This immune exclusion, by the aggregation of RBCs around the bacteria, is the favored interpretation of our research group. Experiments designed to elucidate the role of the rosettes in the duration of the infection are currently under way.

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