Erythrocyte-Aggregating Relapsing Fever Spirochete *Borrelia crocidurae* Induces Formation of Microemboli

Alireza Shamaei-Tousi, Pierre Martin, Anders Bergh, Nils Burman, Thomas Brännström, and Sven Bergström

Department of Microbiology and Pathology, Umeå University, Umeå, Sweden

The African relapsing fever spirochete *Borrelia crocidurae* forms aggregates with erythrocytes, resulting in a delayed immune response. Mice were infected with *B. crocidurae* and monitored during 50 days after infection. Spirochetes were observed extravascularly at day 2 after infection. Two days later, inflammatory responses, cell death, and tissue damage were evident. The pathologic responses in lungs and kidneys were similar, whereas the symptoms in the brains were delayed, with a less pronounced inflammatory response. Microemboli were found in the blood vessels, possibly a result of the erythrocyte aggregation. The *B. crocidurae* infection emerged more rapidly than has been described for Lyme disease–causing *Borrelia* species. In addition to erythrocyte rosetting, the presence of extravascular *B. crocidurae* indicates a novel route for these bacteria to propagate and cause damage in the mammalian host. The histopathologic findings in this study may explain the clinical manifestations of human relapsing fever.

Species of *Borrelia* pathogenic for humans cause two kinds of diseases, Lyme borreliosis and relapsing fever [1, 2]. Unlike most other pathogenic spirochetes, borreliae are arthropod-borne and are distributed worldwide. Lyme disease is caused by *Borrelia burgdorferi* sensu lato, which is transmitted by hard-bodied ticks of the family Ixodidae, whereas relapsing fever is caused by several *Borrelia* species and is mainly transmitted by soft-shelled ticks of the family Argasidae.

Relapsing fever can be epidemic or endemic. The epidemic relapsing fever, caused by the louseborne species *B. recurrentis*, has plagued Asia and Europe during the last century and caused a mortality rate of up to 40% in areas in which it is epidemic [3]. The most recent extensive epidemic occurred during World War II and its aftermath in southern Europe, the Mediterranean region, the Middle East, and the northern region of Africa [4]. During the past decade, there have been several reports of epidemic relapsing fever in Africa. The endemic form of relapsing fever has been reported in different regions of the world, including Europe, North America, South America, Africa, and Asia [5–7].

Patients with relapsing fever characteristically experience one or more cycles of spirochemia. Each cycle consists of a febrile period with microscopically visible spirochemia lasting for 3–7 days, followed by nonfebrile periods of increasing lengths [8, 9]. The relapsing nature of this infection depends on the spirochetes’ ability to undergo antigenic variation, which has been extensively studied in the North American relapsing fever species *B. hermsii*. The antigenic variation results in the exchange of a surface lipoprotein called the variable major protein (Vmp), switching at a frequency of $10^{-4}$ to $10^{-3}$ per cell per generation [10–12].

The pathogenesis of relapsing fever is diverse, depending on the infecting *Borrelia* species and the host species. Several clinical reports have shown that the borreliae can influence the blood coagulation system and cause myocardial damage [13–16]. Relapsing fever can also cause meningitis, focal deficits, hemiplegia, paraplegia, epilepsy, paresthesias, pains, pupillary abnormalities, peripheral and cranial neuritis, and myelitis [17–22]. A recently discovered relapsing fever species from a patient in Spain has been shown to cause meningitis in laboratory mice [23–25].

Infection with *B. crocidurae* is severe and often fatal in rats but is usually less severe in humans [6]. It has been reported that natives in areas of endemcity experience a milder form of the disease than do visitors [7]. This observation may be explained by a partial immunity in the local inhabitants, which is lacked by visitors and laboratory animals that have not previously been exposed to the pathogen. In some regions of Africa in which relapsing fever is endemic, the relationship between the disease and the tick vector is well known by the inhabitants. When people move to new homes, they bring ticks from their old house to maintain immunity, which possibly protects them against local relapsing fever strains [7].

The relapsing fever spirochete *B. crocidurae* was first isolated from the blood of a musk shrew in Senegal and was later iden-
and fentanyl citrate (0.315 mg/mL) plus flunisone (10 mg/mL; Janssen, Sanderton, High Wycombe, UK; ratio of 1 : 1 : 2, midazolam–fentanyl citrate/flunisone–sterile water) and killed by cardiac exsanguination. Citrate-buffered blood was transferred to Eppendorf tubes and centrifuged for 5 s at 8000 g. The blood was washed repeatedly with 500 µL of PBS (pH 7.4) and the supernatants were collected. After each collection step, the numbers of spirochetes were estimated by microscopy. Borreliae were cultured at 34°C in BSK II medium (Sigma, St. Louis) complemented with 10% rabbit serum and 0.7% gelatin and incubated [31].

Isolation of clonal populations of B. crocidurae. A clonal population of B. crocidurae spirochetes was obtained by the limiting dilution technique described elsewhere [10]. By inoculating groups of mice with decreasing concentrations of spirochetes, the probability of infecting a mouse with only 1 Borrelia organism increases.

The numbers of B. crocidurae in freshly isolated samples from infected mice were determined by counting the bacteria in a Petroff-Hauser chamber with use of phase-contrast microscopy. The spirochetes were diluted in PBS, and dilutions theoretically containing 1, 10, and 100 bacteria/mL were used to infect healthy mice. Ten mice were injected with 0.1 mL of each dilution, the objective being to obtain a group of 10 mice in which only one mouse developed spirochetemia. The spirochtemic mouse was killed and exsanguinated by cardiac puncture, and the clonal population was isolated as described above.

Isolation of new serotypes. A clonal population of the original B. crocidurae isolate was used to infect a healthy BALB/c mouse. The number of spirochetes in the blood of the mouse was monitored microscopically. One sample was taken during the first relapse of spirochetemia, and a second sample was taken during the next relapse. The samples were amplified by passage through healthy mice before dilution and injection into healthy mice, to obtain clonal populations as described above.

Histology, immersion, and perfusion fixation. Either the animals were perfusion fixed or the tissues were immersion fixed in a 4% formaldehyde solution after dissection. The vascular system was rinsed via the right carotid artery with Ringer’s solution (0.15 M NaCl, 2.7 mM KCl, 18 mM CaCl2, and 2.4 mM NaHCO3), followed by perfusion with 4% formaldehyde. Tissues were postfixed in the same solution. After fixation, the tissues were dehydrated and embedded in paraffin or methacrylate plastic (Histo-Resin; Janssen, Sanderton, High Wycombe, UK; ratio of 1 : 1 : 2, midazolam–fentanyl citrate/flunisone–sterile water).
**Figure 3.** Lung tissues of mice infected with *Borrelia crocidurae*. A, Cross-section of blood vessel in lung with erythrocyte aggregates and polymorphonuclear cell infiltrations at day 7 after infection. B, Leukocytes (arrows) and erythrocytes (arrowheads) infiltrated area at day 8 after infection. C, Higher magnification of same area, depicting extravascular erythrocytes and fragmented extravascular neutrophilic leukocytes. Hematoxylin-eosin stain; bars, 20 µm.

LKB, Stockholm), cut into 2- to 5-µm sections, and stained with hematoxylin-eosin for examination by light microscopy.

To identify the microglia cells in the brain sections, a rat anti-mouse F4/80 antigen (Serotec, Oxford, UK) was used. The sections were processed as described elsewhere [25, 32]. Briefly, after incubation with the antibody to F4/80, sections were incubated with anti-rat IgG. The streptavidin–horseradish peroxidase–biotin complex was developed by diaminobenzidine and H₂O₂.

**Treatment of tissues for cryosectioning.** The tissues were fixed by immersion in 10 vol of paraformaldehyde, saturated with 30% sucrose, and embedded in OCT (Tissue Tek; Miles Laboratories, Elkhart, IN) compound medium for frozen tissue specimens. The embedded tissues were sectioned in a Cryostat (Microm, model HM505E; Laborgeräte, Walldorf, Germany) and stained with hematoxylin-eosin or with fluorescent antibodies.

**In situ detection of borreliae by use of immunofluorescence.** Formalin-fixed and paraffin-embedded tissues were cut into 6-µm sections and affixed to microscopy glass slides. Histologic samples were deparaffinized in 100% xylene for 3 min, followed by 6 min in 100% ethanol and 6 min in 95% ethanol, and were rehydrated for 3 min in 70% ethanol. The sections were immersed in methanol and acetone (1 : 1) at 20°C for 20 min, followed by a 15-min incubation in 0.25% Triton X-100 in HEPES-buffered saline (HBS; 10 mM HEPES and 150 mM NaCl at pH 7.4). The tissue sections were then rinsed 3 times for 5 min in HBS. The reconditioned sections were incubated in blocking buffer (5% goat serum in HBS) for 45 min. To let the antibody bind to borreliae in the tissue sections, the glass around the tissue was cleaned and the tissue section was encircled with wax (PAP-Pen; Research Products International, Mt. Prospect, IL) before addition of the primary antibody. The tissue sections were never allowed to dry during the overlay procedures. The sections were incubated with the *Borrelia*-specific anti-flagellin antibody H9724 (diluted 1 : 20 in 0.5% goat serum and 0.1% Triton X-100 in HBS) overnight at 4°C, followed by 3 rinses in HBS for 5 min each. The sections were incubated with the secondary antibody, Cy3-F(ab)₂ fragment anti-mouse IgG heavy and light chain (Jackson Immunoresearch Laboratories, West Grove, PA), for 1 h in the dark. The samples were washed 3 times for 5 min each with HBS before mounting by means of fluorescent mounting medium (Dako, Århus, Sweden). The distributions of bacteria in the samples were studied by fluorescence microscopy.

**Silver staining.** Paraffin-embedded sections of tissue samples were deparaffinized as described above and washed with ethanol, followed by immersion in 1% uranyl nitrate hexahydrate (Fluka, Buchs, Switzerland) in 70% ethanol to reduce the background, and incubated at 55°C for 30 min. The sections were washed once in deionized water, rinsed in 95% ethanol, and immersed in 10% gum mastic (Sigma) in absolute alcohol for 30 s. The samples were then immersed in 95% ethanol and washed in water. The sections were soaked in 1% silver nitrate solution for 6 h at 55°C in the dark, followed by washing in distilled water. The samples were placed in developing solution (1.7% hydroquinone, 0.3% sodium sulfite, 4% formalin, 11% acetone, 11% pyridine) for 10 min. The sections were then briefly washed with distilled water, followed by 95% ethanol, and were finally immersed in acetone. The samples were washed once in 100% xylene and mounted by use of mounting medium.

**SDS-PAGE.** Bacteria were harvested from 15 mL of an exponentially growing in vitro culture and washed 3 times with PBS. After centrifugation, the pellet was resuspended in 100 µL of PBS, and a whole cell lysate was obtained by boiling the bacteria for 10 min in sample buffer (Laemmli buffer+50 mM β-mercaptoethanol).

Bacterial proteins were separated on a 12.5% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250 (Sigma).

**Hematologic status.** For the study of effect on hematologic
status, 20 mice were divided randomly into 4 equal groups: 1 group contained uninfected control animals, and the other 3 groups were infected with *B. crocidurae* and were killed at days 2, 5, and 15, respectively. After cardiac exsanguination, blood was collected. Blood hemoglobin content, erythrocyte particle concentration, erythrocyte indices of mean corpuscular volume, erythrocyte volume fraction (hematocrit), thrombocyte particle concentration, and leukocyte particle concentration with differential count of neutrophils, lymphocytes, and monocytes were determined by use of an automated Sysmex SE-9000 hematology analyzer (Toa Medical Electronics, Kobe, Japan).

The animal model. In an initial experiment, 10 BALB/c mice were infected with $10^6$ cells of *B. crocidurae* serotype 1 and serotype 2 spirochetes, and 1 mouse was killed each day during the first 10 days after infection. The kidneys, lungs, brain, liver, and spleen from each mouse were macroscopically examined and were cryosectioned for histopathologic study.

In the next experiment, 58 BALB/c mice, divided into 2 equal groups, were infected with $10^6$ *B. crocidurae* serotype 1 and serotype 2 spirochetes. During the first 10 days after infection, 2 mice infected with each serotype were killed each day. The kidneys, lungs, and brain from 1 mouse were fixed by perfusion, and the same organs from the other were fixed by immersion. At days 12, 15, 22, 29, 36, and 50 after infection, 1 mouse from each group was killed, and the tissues were dissected after perfusion fixation. The advantage of using perfusion fixation is depletion of the capillary system of blood cells and retention of the natural morphology of the tissue. Additionally, by use of this technique, areas containing microemboli can more readily be observed.

As a negative control, mice were infected with the nonerythrocyte-aggregating North American relapsing fever spirochete *B. hermsii*. Seventeen BALB/c mice were infected with $10^6$ cells of *B. hermsii* (serotype 7). One mouse was killed and perfusion fixed each day on days 1–10, 12, 15, 22, 29, 36, and 50, and the organs were immersion fixed with a 4% formaldehyde solution. *B. crocidurae* serotype 1 and 2 and *B. hermsii* serotype 7 were passaged once in BALB/c mice before infection of experimental mice.

To investigate how kidney lesions are correlated with the spirochetal progress in mice infected with *B. crocidurae*, kidneys were sectioned. One kidney was dissected each experimental day and was cut into 2-μm sections, separated by 20 μm. Ten randomly selected visual fields per section were examined by light microscopy. The sections were scored blindly for the presence of foci with inflammation and dead cells.

Blood samples from the tail veins of the mice were microscopically examined each day for development of spirochetemia. The mice infected with *B. crocidurae* were also randomly examined for presence of head tilt, spinning in the air when lifted by the tail, or swimming inability.

Statistics. Statistical analyses of hematologic data were done by use of SPSS 8.0 for Windows (SPSS, Chicago). The Kruskal-Wallis one-way analysis of variance by ranks test was used. Post hoc comparisons between pairs of means were made by use of the Mann-Whitney U test, with a downward adjustment of the level to compensate for multiple comparisons. $P \leq .052$ was considered significant.

Results

Infection and symptoms in mice. We have shown elsewhere that the African relapsing fever spirochete *B. crocidurae* forms aggregates with erythrocytes both in vivo and in vitro [30]. The present study aimed to develop a model for the pathologic effects of erythrocyte rosetting in mice. Previously, different strains of mice, BALB/c, B6, 57BL/6, and C3H/HeN, were infected with clonal populations of *B. crocidurae*. All strains developed spirochetemia and several concomitant relapses [30].

The immunocompetent BALB/c mice infected with, in theory, a single spirochete of *B. crocidurae* serotype 1 or 2 developed relapsing fever. The two different serotypes of *B. crocidurae* used in this model system were serotype 1, expressing a Vmp of 37 kDa, and serotype 2, expressing a 21.5-kDa Vmp.

At least two relapses were observed during the experimental infections. Borreliae were detected in mouse blood from day 1 or 2 after infection (figure 1). The courses of spirochetemia in mice infected with *B. crocidurae* serotype 1 and serotype 2 were similar to each other. However, mice infected with *B. crocidurae* serotype 2 developed several symptoms, such as tilted head, tendency to spin when lifted by the tail, and swimming inability, during the same period. On average, these symptoms lasted up to day 22 after infection. Mice infected with the etiologic agent of North American relapsing fever, *B. hermsii*, did not show any of the symptoms described above.

The study of the hematologic status of mice infected with *B. crocidurae* revealed a neutrophilic and lymphocytic leukocytosis at the peak of spirochetemia at day 5 after infection (table 1). After the spirochetal peaks, at day 15 after infection, neutrophils returned to a normal level, whereas lymphocytosis was still detectable. Another interesting finding was thrombocytopenia at day 2 after infection. In addition, there was a decreased level of hemoglobin at day 5 after infection, which was completely restored at day 15 after infection. However, the thrombocyte particle concentration remained at a low level during the whole experiment.

In dissecting mice infected with *B. crocidurae*, we observed macroscopic hemorrhages on the surface of the liver, lungs, and kidneys at day 4 after infection. Enlargement of the spleen was also observed.

Histopathologic changes in the lungs of mice infected with *B. crocidurae*. The first sign of histopathology was seen as macroscopic lesions at day 4 after infection in the lungs of mice infected with *B. crocidurae* (figure 2). After whole-body perfusion with PBS, the lungs from mice infected with *B. hermsii* (figure 2A) and control mice injected with PBS were cleared of blood. However, blood was retained in the lungs of mice infected with *B. crocidurae*, and the lesions were seen at the macroscopic level as hemorrhages (figure 2B). Microscopically, erythrocyte aggregates were found in blood vessels in the lungs of mice infected with *B. crocidurae* (figure 3A) but not in mice infected with *B. hermsii*. Lung sections from mice infected with *B. crocidurae* had several areas with microscopic hemorrhages...
Lesions in kidneys of infected mice. *B. crocidurae* and *B. hermsii* were found vascularly (figure 4A, 4B) and extravascularly (figure 4C) in the kidneys at day 3 after infection. The kidney sections from mice infected with *B. crocidurae* had hemorrhages in the medulla from day 4 to day 8 after infection (figure 4D). During the same period, we observed retention of blood cells and erythrocyte aggregates in the dilated capillaries and leukocyte accumulations in and around the microvessels (figure 3B, 3C).

Tissue sections from mice infected with *B. crocidurae* were stained with Cy3-labeled monoclonal anti-flagellin antibody or with a modified silver staining (Dieterle’s). From day 2 after infection, *Borrelia* spirochetes were found extravascularly in the lungs of mice infected with both serotypes of *B. crocidurae*.
in sections from perfusion-fixed mice (Figure 4E). The kidney sections initially exhibited inflammatory lesions seen as polymorphonuclear leukocyte accumulations in and around microvessels (Figure 4E), and after day 6 after infection, the foci of inflammation had no apparent association with blood vessels. During the same period, we could observe small foci of degenerating parenchyma (Figure 4F) and polymorphonuclear cells. The investigated tissues from mice infected with *B. hermsii* did not show any of these pathologic lesions.

To determine the possible association between inflammation and cell death, and the time required for development of the observed damages, the pathologic lesions were quantified. Multiple sections were cut through the kidneys, and each section was searched for the presence of foci with inflammation and degenerated cells (Figure 5). As described above, inflammation and cell death were detected from day 4 after infection. The lesions in mice infected with *B. crocidurae* peaked at day 5 after infection (with an average of 9.5 inflammatory areas/organ) and at day 12 after infection (8 inflammatory areas/organ). The largest number of areas with dead cells (21.5 areas/organ) was seen at day 8 after infection.

The occurrence of spirochetemia, rosette formation, neurologic symptoms, and histopathologic findings in the kidneys of mice during the course of infection with *B. crocidurae* are summarized in Figure 6.

**Induction of meningoencephalitis by relapsing fever Borrelia.** *B. crocidurae* spirochetes were detected in erythrocyte aggregates in the brain vessels of infected mice (Figure 7A) and were found extravascularly in the brain at day 4 after infection (Figure 7B), although they were observed earlier in the lungs and kidneys. Spirochetes were found in the parenchyma, leptomeninges, and plexus choroiides, as well as intravascularly in the brain. In the vessels, spirochetes were associated with either the endothelium (Figure 7C) or erythrocytes (Figure 7D). The parenchymatous spirochetes were mainly found in the basal diencephalon, the brain stem, the cerebellum, and the plexus choroiides (Figure 7E). In the cerebellum, *B. crocidurae* were found in the granular layers, adjacent to the Purkinje’s cells (Figure 7F).

Brain sections of mice infected with *B. crocidurae* had sparse foci of inflammatory cells seen as microglial cells (Figure 7G), as well as small assemblies of lymphocytes and granulocytes. Cells in the brain section reacted with a monoclonal antibody against mature mouse macrophage-microglia cells (data not shown). These inflammatory foci were observed in both gray and white matter and had no close association with blood vessels. A more pronounced inflammatory reaction was found in the choroid plexus and in the leptomeninges of infected mice (Figure 7H). This was seen as assemblies of lymphocytes and granulocytes.

Hyperaemia of the meninges and petechial hemorrhages in the brain parenchyma were also found. Mice infected with *B. hermsii* had no detectable histopathology in the brains during the study.

**Discussion**

Erythrocyte rosetting has been observed during infection of humans and animals with the West African *B. crocidurae*, the East African *B. duttoni* [29, 30], and North African *B. hispanica* (unpublished data). Significantly, this is the first description of the histopathology during erythrocyte-rosetting relapsing fever in an animal model. The pathologic effect of the louseborne relapsing fever–causing species, *B. recurrentis*, has been thoroughly described in histologic terms. Histiocytic myocarditis, multiple microabscesses replacing the nodular white pulp of the spleen, and hepatitis with foci of midzonal necrosis have been described in Grivet monkeys [33] and in investigations of a number of fatal cases of human louseborne relapsing fever [34].

In the present study, we found that the clonal populations of *B. crocidurae* serotypes 1 and 2 had equal ability to establish infection in immunocompetent mice. The distributions of serotype 1 and 2 spirochetes in different organs, such as lungs and kidneys, were indistinguishable, except for brains. Infection with *B. crocidurae* serotype 2 caused more severe neurologic symptoms than did infection with *B. crocidurae* serotype 1. Thus, *B. crocidurae* serotype 2 may have evolved a tissue tropism that is directed to the brain. These neurologic symptoms are similar to those described by Cadavid et al. [35] for the neurotropic North American relapsing fever species, *B. turicatae*. Different serotypes of *B. turicatae* have different abilities to cause symptoms in SCID mice. In that study, serotype A invaded the central nervous system, whereas serotype B caused arthritis in mice. In addition, the neurotropic relapsing fever–causing *Borrelia* species *B. turicatae* and *B. duttoni*, as well as the less neurotropic *B. hermsii* [36, 37], are found in the brains of infected mice.

After infecting mice with *B. crocidurae*, we observed an inflammatory response in the brains occurring during the same period as the neurologic symptoms. On the basis of the distribution of parenchymatous spirochetes and inflammatory foci in the brain parenchyma, we conclude that not all spirochetes...
Figure 6. Summary of time course of histopathology in kidney and various symptoms in mice infected with *Borrelia crocidurae*

- Neurologic symptoms
- Cell death
- Inflammatory foci
- Vasculitis
- Extravascular spirochetes
- Rosettes
- Spirochetemia

Days after infection

give rise to inflammation. In contrast to the relatively mild parenchymatous inflammatory response, there was a more pronounced reaction in the leptomeninges and the choroidal plexus. We suggest that the blood-brain barrier is incomplete in hampering spirochetal invasion of the brain parenchyma. Because no such barrier exists in the leptomeninges, these will probably show an inflammatory reaction similar to that seen in other internal organs. Both the parenchymal lesions and the leptomeningeal changes might contribute to the neurologic symptoms observed. It is also possible that the brain injury could partly be explained by the observed ability of relapsing fever–causing *Borrelia* species and *B. burgdorferi* to adhere to neural cells [38–40]. Our histopathologic findings for brain tissue may explain the neurologic symptoms seen in this study, which correlate well with the symptoms described for humans [17, 19, 21].

The histopathology of lungs and kidneys were similar in mice infected with *B. crocidurae*. In areas with macroscopic hemorrhages, inflammation with leukocyte infiltrates and extravasated erythrocytes were also observed. The absence of these lesions in the blood vessels in mice infected with the non-erythrocyte-aggregating *B. hermsii*, and the fact that spirochetes can be found extravascularly in tissues during infection in mice [37], led us to propose that the observed lesions are the result of aggregation of erythrocytes by *B. crocidurae*, which forms emboli in the blood-capillary system. Despite an average diameter of 7.2 μm, erythrocytes are able to pass through the smallest capillaries of only 3–4 μm in diameter in mammals, because of the plasticity of these cells. We believe that erythrocytes bound to *B. crocidurae* may be unable to pass through capillaries and will hence cause microemboli, which is the likely cause of lesions in infected animals. Presumably, microemboli reduce the blood flow and thereby damage the surrounding tissues, which could explain some of the clinical symptoms seen in patients with relapsing fever caused by *B. crocidurae* [26, 27].

It is likely that penetration of the endothelium by *B. crocidurae* occurs in two steps. During the beginning of spirochetemia (days 1–2), *B. crocidurae* spirochetes reach the adjacent tissues. When the spirochete titer is higher (days 4–7), larger aggregates and emboli are formed in the blood vessels, which may cause vascular and tissue damage, resulting in hemorrhages and inflammation. The blood cells will now leak into the tissues together with the spirochetes. This hypothesis is in concordance with the finding of *B. crocidurae* in tissues without extravasated erythrocytes during the early stage of infection and in association with erythrocytes, adjacent to areas of inflammation, in later stages. The dissemination of *B. crocidurae* and the emergence of a systemic infection resemble that seen in infection with *Treponema pallidum*, the spirochete agent of syphilis. Thus, syphilis also result in an inflammatory reaction in and around blood vessels [41].

The hemorrhages in lungs and kidneys of mice infected with *B. crocidurae* may also explain the tendency toward decreased hemoglobin at day 5 after infection. The increase in leukocyte particle concentration with neutrophilia and lymphocytophilia at day 5 after infection is transformed to a state with only lymphocytophilia at day 15 after infection. A possible explanation of the neutrophilia could be that the hemorrhage in the inflamed tissues stimulates not only the erythropoiesis but also the generation of neutrophils and thrombocytes. The most pronounced change in blood cell count is the severe thrombocytopenia, especially during the early stage of infection. *B. crocidurae* can bind thrombocytes efficiently in vivo (unpublished data), which has also been shown for *B. burgdorferi*, the agent
Figure 7. Infection with relapsing fever–causing *Borrelia* species in brains of mice. 

A. Aggregates of *Borrelia crocidurae* spirochetes and red blood cells at day 5 after infection.  
B. Two spirochetes in cerebral parenchyma at day 4 after infection. Note large pyramidal neuron at upper center.  
C. Spirochete in intraparenchymatous cerebral blood vessel at day 4 after infection. Spirochete is partly associated with endothelium (arrow).  
D. Aggregate of spirochete and several red blood cells in intraparenchymatous cerebral blood vessel at day 6 after infection.  
E. Spirochetes (arrows) close to blood vessel in plexus choroideus at day 4 after infection.  
F. *Borreli* spirochete (arrow) in parenchyma of upper part of granular layer of cerebellum at day 4 after infection.  
G. Neurons surrounded by enlarged astrocytes and microglial cell (arrow), indicating reactive gliosis, at day 15 after infection.  
H. Lymphocytic infiltration in immediate vicinity of choroid plexus of lateral ventricle at day 5 after infection.  

Stains: A–F, modified Dieterle’s silver impregnation; G, H, hematoxylin-eosin; bars, 10 μm.
of Lyme disease [42–44], and for another relapsing fever–causing Borrelia species [25]. Such binding may partly explain the decrease in the number of thrombocytes in the blood. Endothelial injury during the infection may also result in adherence and consumption of thrombocytes [45].

Surprisingly, some polymorphonuclear leukocytes and parenchymal cells of the kidneys (figure 4F) and lungs (data not shown) of mice infected with B. crocidurae appeared to undergo cell death. Although it is well known that polymorphonuclear leukocytes have a short half-life and many extravasated neutrophils die during the inflammatory response, the mechanism behind the parenchyma cell death during B. crocidurae infection is unknown. Cell death could be induced directly by factors released from or by contact with the spirochete or indirectly by microemboli causing hypoxia [46, 47]. Furthermore, the tissue-invading spirochetes may induce an inflammatory response, but inflammation may also be a secondary effect to hypoxic tissue damage and cell death. In the kidney, two separate inflammatory peaks were observed, one at day 5 after infection and the other at day 12 after infection. The early reaction may be caused by an acute inflammatory response against the spirochetes in the tissues, and the second peak could be triggered by tissue damage.

Using mice as an animal model, we have shown that the African relapsing fever agent B. crocidurae causes severe histopathology, which correlates with the clinical disease manifestations described elsewhere [14, 33, 35, 48–50]. Several animal models have been developed to investigate the neurologic manifestations of Lyme disease and relapsing fever borreliosis [24, 35, 51–53]. These studies have shown that the symptoms in mice, which mimic the clinical presentation in humans with systemic Lyme disease, are due to the neurologic lesions [52]. However, our model applies not only to studies of symptoms similar to those of neuroborreliosis but also to symptoms caused by the erythrocyte rosetting. Thus, this model can be extended to investigate lesions caused by other microorganisms with the ability to bind to and aggregate cells in the vascular system, such as Plasmodium species, the causative agent of malaria. The similarities between the erythrocyte rosettes caused by B. crocidurae and Plasmodium species are striking; both parasites may use the rosettes as a transport system to a possible brain reservoir [54]. We are currently investigating the hematologic effect of the erythrocyte rosetting in this animal model. Moreover, characterization of the tissue tropism and the molecules involved in rosette formation may be a key to understanding the pathology of relapsing fever. These interactions are also under investigation.

### Acknowledgments

We thank Alan G. Barbour, Staffan Normark, Hans Wolf-Watz, Jana Jass, and Dominic McCafferty for critically reading the manuscript; Kjell Granqvist for evaluating the hematological results; and Elisabeth Dahlberg, Birgitta Ekblom, and Sigrid Kiltzer for their skilful technical assistance.

### References


### Table 1. Hematologic data from mice infected with Borrelia crocidurae and from healthy uninfected mice.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Day 2</th>
<th>Day 5</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPC, ×10⁹/L</td>
<td>1.84 ± 0.55</td>
<td>1.46 ± 0.42</td>
<td>3.96 ± 0.54*</td>
<td>2.82 ± 1.44</td>
</tr>
<tr>
<td>EPC, ×10⁹/L</td>
<td>9.36 ± 0.35</td>
<td>8.82 ± 0.48</td>
<td>7.92 ± 1.39</td>
<td>10.66 ± 1.63</td>
</tr>
<tr>
<td>B-Hb, g/L</td>
<td>141.2 ± 4.53</td>
<td>125.8 ± 9.04</td>
<td>119.6 ± 10.09</td>
<td>161.6 ± 19.80</td>
</tr>
<tr>
<td>EVF, %</td>
<td>47.2 ± 1.36</td>
<td>42.8 ± 2.04</td>
<td>41 ± 6.48</td>
<td>50.2 ± 8.01</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>49.6 ± 0.80</td>
<td>48.8 ± 1.12</td>
<td>51.2 ± 2.14</td>
<td>46.8 ± 0.75</td>
</tr>
<tr>
<td>TPC, ×10⁹/L</td>
<td>967.8 ± 42</td>
<td>327 ± 32.13a</td>
<td>119 ± 22a</td>
<td>293.8 ± 39a</td>
</tr>
<tr>
<td>Neutrophils, ×10⁹/L</td>
<td>0.9 ± 0.11</td>
<td>0.62 ± 0.15</td>
<td>2.14 ± 0.19b</td>
<td>0.5 ± 0.11</td>
</tr>
<tr>
<td>Lymphocytes, ×10⁹/L</td>
<td>0.6 ± 0.08</td>
<td>0.74 ± 0.12</td>
<td>1.74 ± 0.30a</td>
<td>2.14 ± 0.55</td>
</tr>
<tr>
<td>Monocytes, ×10⁹/L</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.12 ± 0.06</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± SD for 5 mice/group. LPC, leukocyte particle concentration; EPC, erythrocyte particle concentration; B-Hb, blood hemoglobin; EVF, erythrocyte volume fraction (hematocrit); MCV, mean corpuscular volume (erythrocyte indices); TPC, thrombocyte particle concentration.

*P < .032.