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Comparison of Damage to Kidneys and Liver Caused by Lethal *Babesia rodhaini* Infection and Non-lethal *Babesia microti* Infection in Mice

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ABSTRACT

Two mouse babesioses, lethal *Babesia rodhaini* and non-lethal *Babesia microti* infections, were examined to determine if damage to kidneys and liver is correlated with the prognosis of these infections. All *B. rodhaini*-infected mice died after a sudden increase of parasitemia, severe hemolysis, and excretion of reddish hemoglobinuria. All *B. microti*-infected mice survived after a temporary moderate increase of parasitemia, moderate hemolysis, and excretion of greenish urine. *B. rodhaini*-infected mice showed immune complex-induced mesangiopathic glomerulonephropathy, moderate renal tubular necrosis, and extensive liver necrosis. In the glomerulonephropathy, electron microscopy showed electron-dense deposits in the mesangial matrix and along the glomerular basement membrane. Biochemical analysis of blood and urine from these mice confirmed renal damage in terms of increased BUN and of proteinuria that contained albumin and protein of more than 200kDa molecular weight, and hepatic damage in terms of an increase in serum direct bilirubin. *B. microti*-infected mice had relatively mild immune complex-induced mesangiopathic glomerulonephropathy, mild renal tubular necrosis, and focal liver necrosis. BUN and serum direct bilirubin showed no increase, and proteinuria contained no detectable proteins of more than 200kDa. These data suggest that the severity of damage in the kidneys and liver is correlated with the prognosis of the two *Babesia* infections.

INTRODUCTION

Babesia is a species of hemoprotozoan parasite that induces babesiosis in domestic and experimental animals. *Babesia* infection induces hemolytic anemia, hemoglobinemia, and subsequent disorder of kidneys and liver. Damage of kidneys and liver is the most conspicuous pathological change in *Babesia* infections. In the kidneys, pathological changes include renal tubular necrosis induced by hemoglobinemia (Habela et al. 1991, Hussein 1977b, Liddel et al. 1980) and/or glomerulonephritis (Maegraith et al. 1957, Rogers 1971, Hussein 1977b) induced by immune

complexes(Annable and Ward 1974). Pathological changes in the liver encompass focal, mid-zonal or centrolobular necrosis of the parenchyma (Habela et al.1991, Liddel et al. 1980, Hussein 1977a, Maegraith et al. 1957, Rogers 1971, Paget et al. 1962). In mouse babesiosis, *B. rodhaini* and *B. microti* infections show different prognoses. *B. rodhaini* is lethal to mice whereas *B. microti* generally is not lethal (Clark and Allison 1974). We compared these two mouse babesioses that have different clinical outcomes with regard to pathological changes occurring in kidneys and liver, and with regard to analysis of blood and urine that reflect the functional conditions of these organs.

MATERIALS AND METHODS

Animals and Parasites: Male and Female BALB/c mice, 6-7 weeks old, were used in these experiments. *Babesia rodhaini* (Australia strain) and *Babesia microti* (Munich strain) were provided by the Kyushu Branch of National Institute of Animal Health (Ministry of Agriculture, Forestry and Fishery, Kagoshima, Japan) and by Prof. O.A. Heydorn (Free University of Berlin, Berlin, Germany) respectively, and maintained by blood passage in BALB/c mice. Mice were inoculated intraperitoneally (i.p.) with $1x10^7$ parasitized erythrocytes (PRBC). The percentage of parasitemia was determined by counting the number of PRBC in tail blood smears stained with Giemsa. Hematocrit of the blood, which was obtained from the infraorbital venous plexus of mice, was measured by the microhematocrit method. Blood serum and urine samples were collected daily and stored at -20℃ until use.

Biochemical analysis of blood serum: Blood sera were obtained from centrifuged blood collected from mouse hearts under ether anesthesia, and examined for serum bilirubin and blood urea nitrogen (BUN). Serum bilirubin concentration was measured with the alkaline-azobilirubin method (Bilirubin B II-test Wako, Wako Pure Chemical Industries,Ltd., Tokyo, Japan). BUN was measured with a commercial kit (Unikitrate BUN, Kanto Chemical, Tokyo, Japan).

Urinary protein analysis: Urine was obtained from mice by forced urination. Urinary protein concentration was measured with the Bradford method with bovine serum albumin as standard. For detection of hemoglobin in the urine, urine and hemoglobin solutions were examined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), loading samples on a 12% gel concentration with a Mini-PROTEAN II cell (Bio-Rad Laboratories, CA, U.S.A.). The hemoglobin solution was obtained from BALB/c mouse blood. The blood was washed three times with physiological saline, frozen and thawed twice, diluted with double-distilled water, and centrifuged 13000xg for 20 min. The supernatant was used as a hemoglobin solution against which urine samples were compared. After electrophoresis, the gels were stained with o-dianisidine solution for hemoglobin detection. To estimate the molecular weight of protein in the urine, samples were loaded on a 7.5% gel concentration and proteins were stained with 0.1% Coomassie brilliant blue R-250 after electrophoresis.

Tissue processing and staining: Tissue samples were collected when infected mice had high parasitemia and excreted discolored urine. Small pieces of the kidneys and liver specimens were processed for histological, immunohistological, and electron microscopic studies. For histology, tissues were fixed in 10% phosphate-buffered formalin, dehydrated in alcohol and xylene, embedded in paraffin, and sectioned at 4μm. Sections were stained with hematoxylin and eosin (H&E), periodic-acid-Schiff

(PAS), and methenamine silver. For electron microscopy, renal tissues were fixed in 2.5% glutaraldehyde in 0.05M phosphate buffer (pH 7.4) and 4% sucrose for 2 hr, postfixed in 1% osmium tetroxide for 2 hr, dehydrated in increasing concentrations of ethyl alcohol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 100 CX electron microscope. For immunofluorescent study, small blocks of kidney tissue were embedded in O.C.T. compound (Tissue-Tek, Division of Miles Laboratories Inc., Naperville, U.S.A.), frozen in dry ice-acetone, cut with a cryostat (Leitz 1720 digital, Wetzlar, Germany), dried, and stained with immune sera.

Antibodies: Antibodies to *B. rodhaini* and *B. microti* were obtained from rats immunized with mouse erythrocytes infected with the respective parasites. Rats were inoculated i.p. with $1x10^8$ PRBC, and after 3 weeks rats were inoculated i.p. with a booster of same dose of PRBC. Four weeks after booster inoculation, sera were taken from the rats. Indirect fluorescent antibody test showed that the titer of both anti-*B. rodhaini* and anti-*B. microti* was 1:4096 against *B.rodhaini*-infected and *B. microti*-infected RBC respectively. These sera were used as primary antibodies for detection of *Babesia* antigens in kidneys. FITC-conjugated IgG fraction of goat anti-rat IgG (Organon Teknika Corp., West Chester, U.S.A.) was used secondary antibody. FITC-conjugated (Fab') goat anti-mouse IgG or anti-mouse IgM and FITC-conjugated goat IgG fraction anti-mouse C3 were obtained from TAGO Inc. (CA, U.S.A.) and Organon Teknika Corp. (West Chester, U.S.A.), respectively.

Immunostaining: Deposition in glomeruli of IgG, IgM, C3, and Babesia antigens was examined by direct or indirect fluorescent antibody staining.

Direct Immunofluorescent antibody staining: IgG, IgM, and C3 in glomeruli were examined by direct immunofluorescent antibody staining. Kidney sections were treated with blocking solution (2% skimmed milk- 5% normal goat serum in PBS) at 37℃ for 30 min and incubated with FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-mouse IgM, or FITC-conjugated goat IgG fraction to mouse C3 at 37℃ for 1 hr, washed in PBS, and examined with a fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan).

Indirect Immunofluorescent antibody staining: Kidney sections were treated with blocking solution at 37℃ for 30 min and incubated with either rat anti-*B. rodhaini* or *B. microti* serum or normal rat serum as control at 37℃ for 30 min. These sections were washed in PBS, incubated FITC-conjugated IgG fraction of goat anti-rat IgG at 37℃ for 30 min, washed in PBS, and examined with a fluorescence microscope.

RESULTS

1) *B. rodhaini* infection in mice.

a)Clinical data

Parasitemia appeared on the 2nd day of infection and progressively increased until the 7th day when all mice died (Fig. 1). The hematocrit decreased after the infection and fell 30% (normal value 48-49%) (Fig. 2). By the 4th day of infection, all mice excreted greenish urine, which at days 5-7 became reddish. The appearance of discolored urine corresponded to a high parasitemia. SDS-PAGE analysis confirmed the reddish urine was hemoglobinuria (Fig. 3). *B. rodhaini* infection induced severe hemolysis in the mice before death.

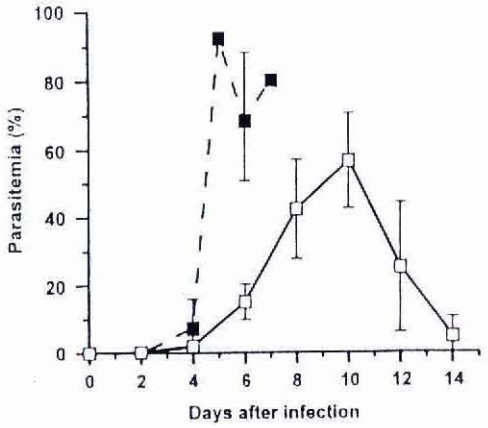


Fig. 1. Changes of parasitemias in *Babesia*-infected mice. *B. rodhaini*-infected mice (-■-) showed sudden increase of parasitemia until the 7th day when all mice died. *B. microti*-infected mice (-□-).

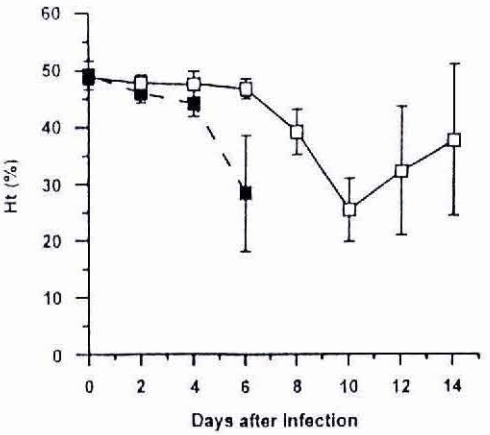


Fig. 2. Decrease of hematocrit in Babesia-infected mice. *B. rodhaini* infection (-■-). *B. microti* infection (-□-).

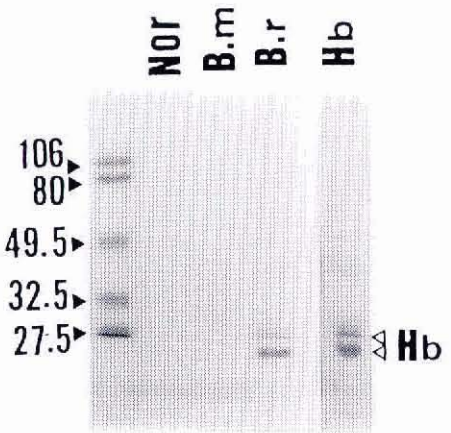


Fig. 3. Hemoglobin-stained SDS-PAGE (12% acrylamide) of mouse urine samples and mouse hemoglobin solution: Normal mice urine (lane Nor); *B. microti*-infected mice urine (lane B.m); *B. rodhaini*-infected mice urine (lane B.r); Hemoglobin solution (lane Hb). Standard urinary proteins of known molecular weight are shown (left lane). Arrow heads on the right margin indicate the position of the hemoglobin. Sizes on the left margin are in kilodaltons.

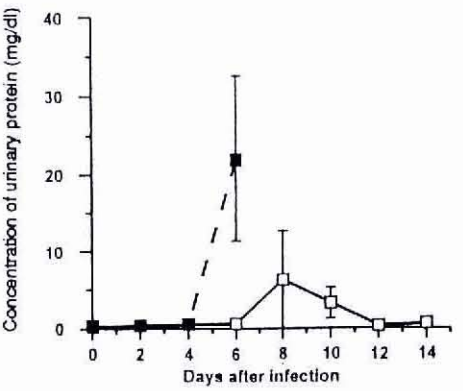


Fig. 4. Changes of proteinuria of mice infected with *B. rodhaini* (-■-) and *B. microti* (-□-).

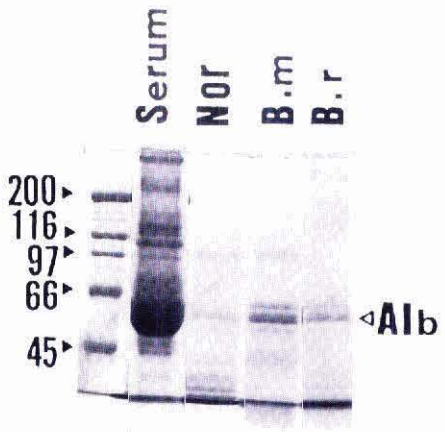


Fig. 5. SDS-PAGE (7.5% acrylamide) of mouse urine samples stained with Coomassie brilliant blue R-250. Lanes Nor, B.m, and B.r are same as in Fig. 3. Normal mouse serum (lane Serum) and standard serum proteins of known molecular weight (left lane) are shown. An arrow head on the right margin indicates the position of the albumin. Sizes on the left margin are in kilodaltons.

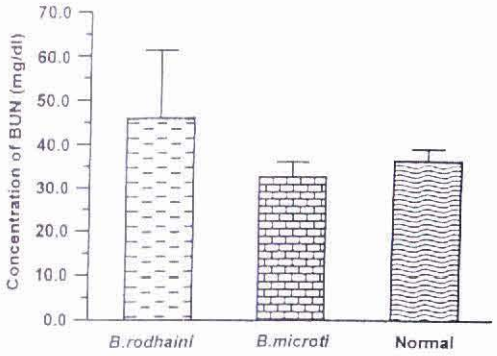


Fig. 6. BUN concentration in *Babesia*-infected mice. Significant increases are seen in *B. rodhaini*-infected mice ($p<0.05$).

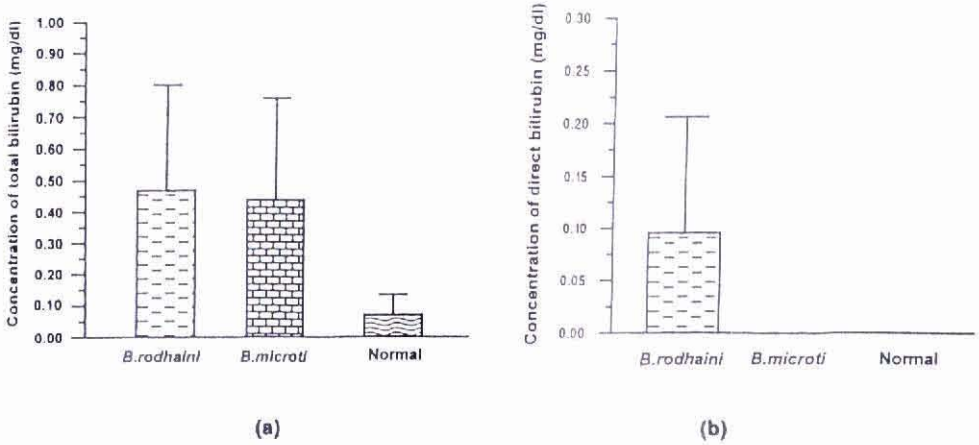


Fig. 7. Serum bilirubin concentration in *Babesia*-infected mice. (a) Both *B. rodhaini* and *B. microti*-infected mice showed increase of total bilirubin. Values are significantly different between *B. rodhaini*-infected mice and normal mice ($p<0.05$). (b) Significant increase of direct bilirubin in *B. rodhaini*-infected mice ($p<0.05$).

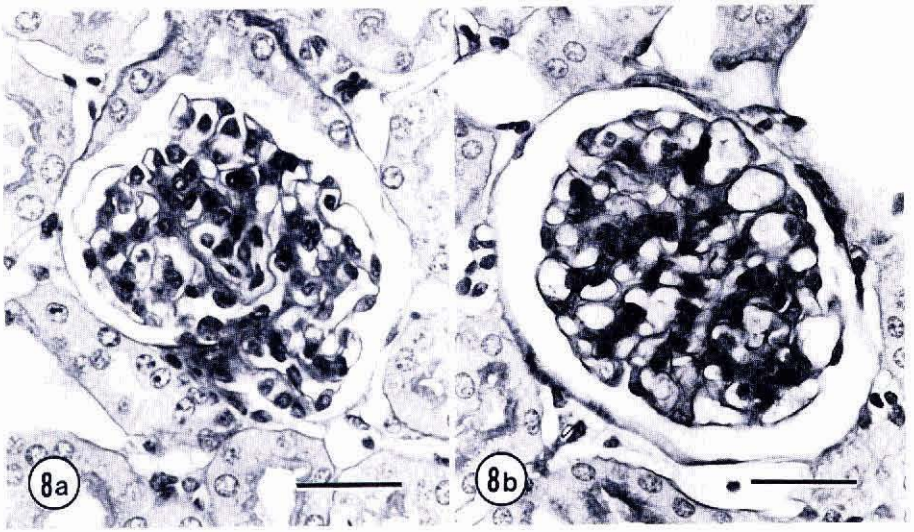


Fig. 8. PAS stained sections showing an increase in number of mesangial cells and mesangial matrix. **a**, *B. rodhaini*-infected mouse kidney. Bar= 10 μ m. **b**, B. *microti*-infected mouse kidney. Bar=10 μ m.

Biochemical analysis of the urine indicated functional damage to the kidneys. Proteinuria was detected by the 2nd day after infection and markedly increased to 24.6 $\pm$9.5mg/ml immediately before death (Fig. 4). SDS-PAGE analysis of the urine (Boesken et al. 1973) showed not only a prominent band of albumin but also several conspicuous bands of larger proteins that were more than 200 kDa molecular weight (Fig. 5). Biochemical tests of the blood serum showed an increase in BUN (Fig. 6) and bilirubin concentration (Fig. 7a and b) before the mice died. BUN, which is a useful serum marker to estimate renal function, reached 46.1$\pm$15.3mg/dl (control: 36.4$\pm$2.7mg/dl). Total bilirubin concentration was 0.47$\pm$0.33mg/dl (control: 0.07$\pm$0.06mg/dl), and direct bilirubin concentration was 0.10$\pm$0.11mg/dl (control: 0.01$\pm$0.03mg/dl). Total bilirubin dominant bilirubinemia corresponded to severe hemolysis in *B. rodhaini*-infected mice. The increase of direct bilirubin suggests functional damage of liver tissue.

b) Pathology of the kidneys and liver

When the mice had the highest parasitemia and were excreting reddish urine, we examined the microscopic appearance of kidneys and livers. Mesangiopathic glomerulonephropathy was the main histopathological change in the kidneys and was characterized by endocapillary proliferation, increase of mesangial matrix, and mild thickening of the basement membrane (Fig. 8a). Additionally, tubular necrosis sometimes was observed (not shown).

Electron microscopy showed an increase in mesangial cells and matrix, as well as electron-dense deposits, in the mesangial matrix and along the glomerular basement membrane (Fig. 9a and c). The basement membrane was somewhat thickened and

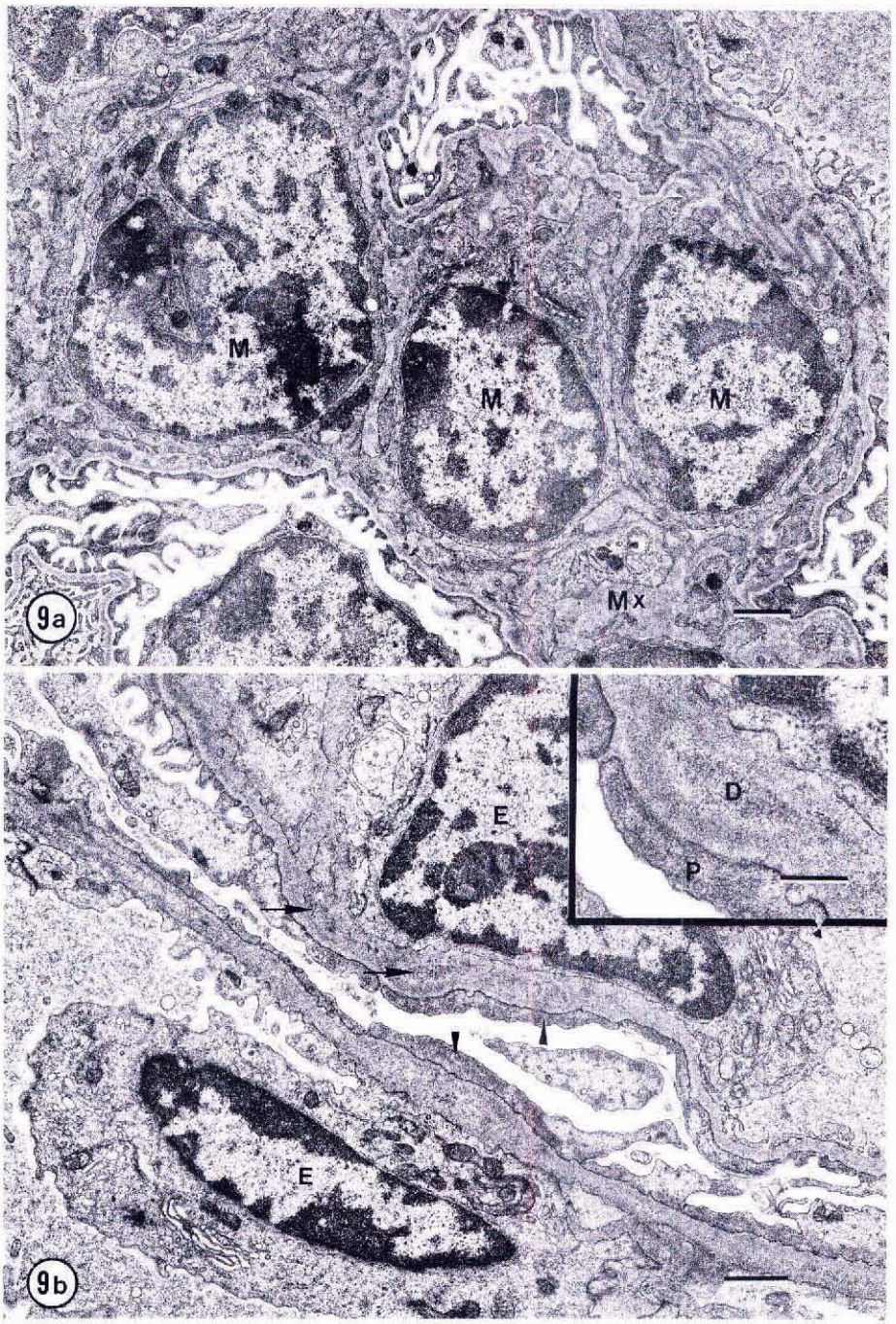
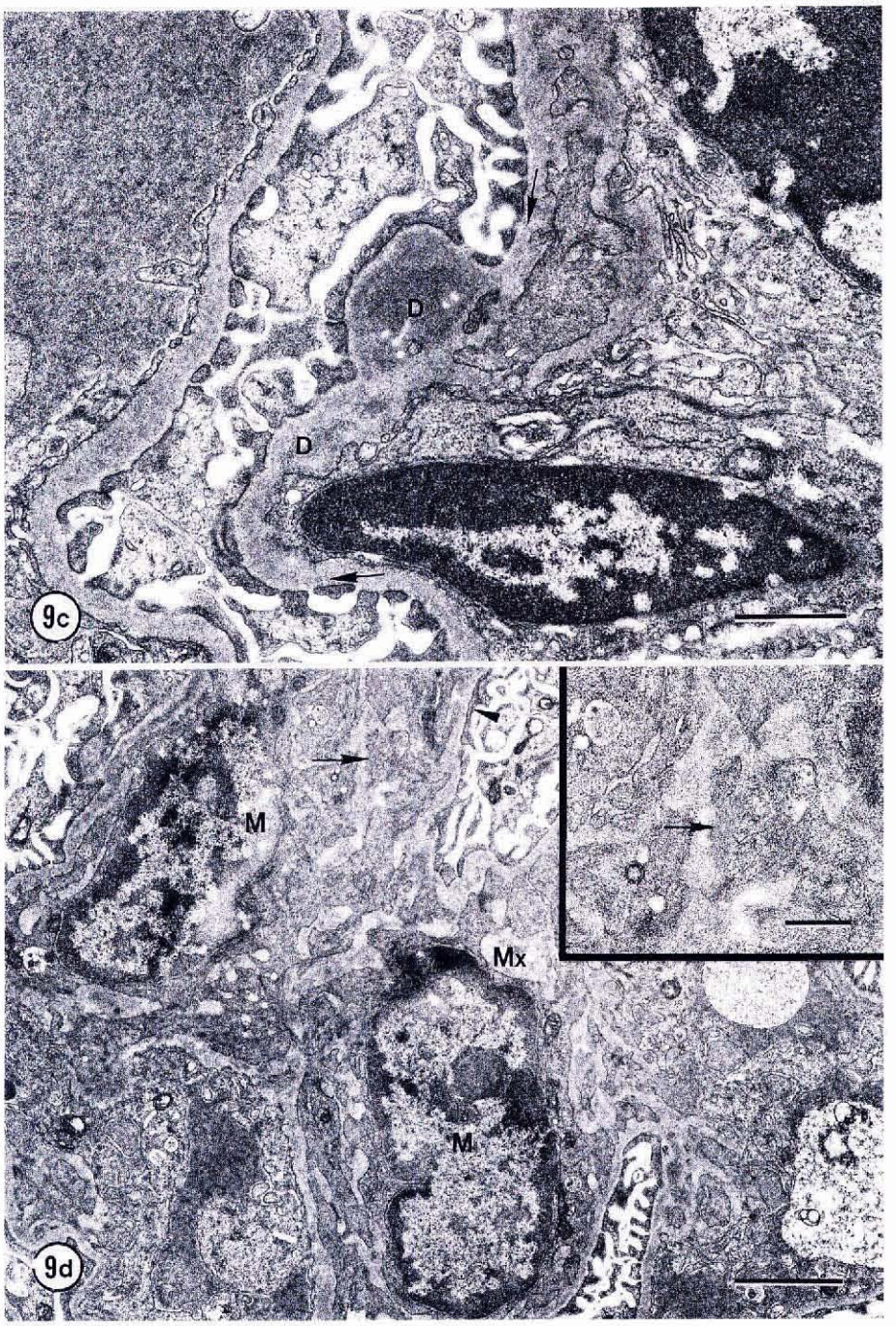


Fig.9. Electron micrograph of glomerulus in *B. rodhaini* (**a**,**b**,**c**) and *B. microti* infection (**d**). **a**, Mesangial expansion with an increase of cells (M) and matrix (Mx). Bar=1 μ m. **b**, Fusion of foot processes (arrow heads) and electron-dense deposits (arrows) along the basement membrane. Endothelial cells (E). Bar=1 μ m. Inset: High magnification of electron-dense deposits (D) and fused foot



processes (P).Bar=0.5 μ m. **c**, Electron-dense deposits (D) along the basement membrane (arrows). Bar=1 μ m. **d**, Mesangial expansion with increase in cells (M) and matrix (Mx). Electron-dense deposits (arrow). Fusion of foot processes (arrow head). Bar=2 μ m. Inset: High magnification of electron-dense deposits in the mesangial matrix (arrow). Bar=0.5 μ m.

foot processes of podocytes were focally fused (Fig. 9b). Immunohistochemistry revealed deposits of IgG (Fig. 10a), IgM, C3 and *Babesia* antigens in the mesangium regions and along the basement membrane.

Light microscopy of the liver showed extensive mid-zonal and centrolobular necrosis, with leukocyte infiltration around the necrotic areas (Fig. 11a). Many leukocytes including lymphocytes, monocytes, and macrophages were in the sinusoids and dilated central veins and interlobular veins.

2) *B. microti* infection in mice

a) Clinical data

Parasitemia appeared on the 2nd day after infection, reached its peak (51.8 ± 4.9%) by the 10th day, and gradually decreased by the 14th day when mice recovered from the infection(Fig. 1). Their hematocrit decreased after infection and fell to 36.8 ± 9.0% at the 10th day (Fig. 2). Greenish urine appeared by the 8-10th day and gradually disappeared as mice recovered from the infection. Proteinuria was found at the time high parasitemia. SDS-PAGE analysis of the urine showed a band which corresponded to albumin (Fig. 5). Hemoglobin and proteins larger than 200kDa were not detected in the greenish urine. Biochemical tests of the blood serum showed an increase in bilirubin concentration (Fig. 7). Total bilirubin concentration was 0.44 ± 0.32 mg/ dl; however, direct bilirubin concentration was within normal limits. The increase of indirect bilirubin suggested that greenish urine was bilirubinuria. The BUN concentration was not increased (Fig. 6).

b) Pathology of the kidneys and liver

Kidneys showed mesangiopathic glomerulonephropathy with an increase in mesangial cells and mesangial matrix (Fig. 8b). Immunohistochemical observations showed deposits of IgG, IgM, C3 and *B. microti* antigens in the mesangial regions and along the basement membrane (Fig. 10b). Electron microscopy showed electron-dense deposits in the mesangial matrix and along the basement membrane, and fusion of

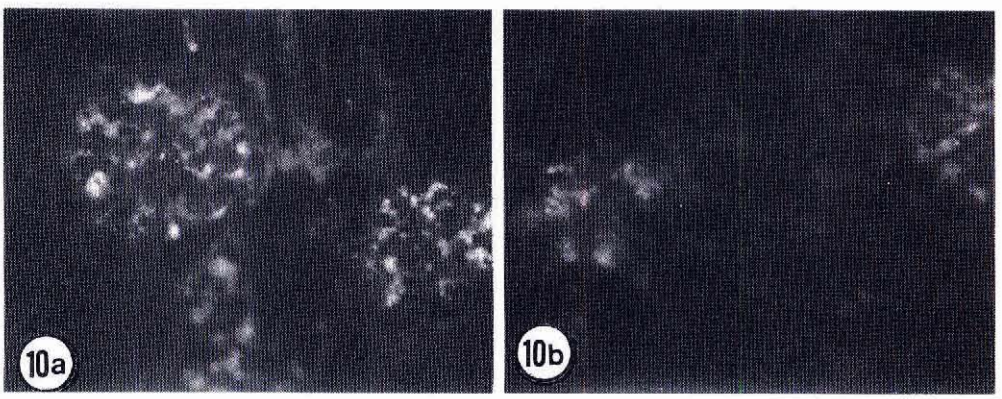


Fig.10. **a**, Granular deposition of IgG in the mesangium of *B. rodhaini*-infected mice kidney. **b**, Granular deposition of IgM in the mesangium of *B. microti*-infected mice kidney.

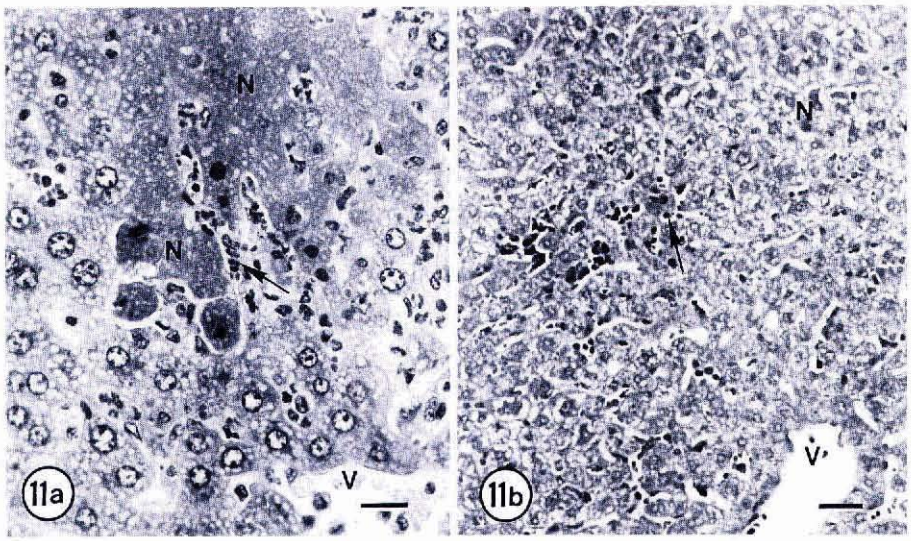


Fig.11. H&E stained sections. **a**, Mid-zonal necrosis(N) of the liver parenchyma in *B. rodhaini* infection. Leukocyte infiltration (arrow) around the necrotic area. Central vein(V). Bar=10 μ m. **b**, Focal necrosis (N) of the liver parenchyma in *B. microti* infection. Leukocyte infiltration (arrow). Central vein(V).Bar=10 μ m.

podocytes foot processes (Fig. 9d). However, these ultrastructural changes were relatively mild in *B. microti* -infected mice compared to *B. rodhaini*-infected mice. Renal tubular damage was mild (not shown).

Liver tissue showed focal necrosis and some leukocyte infiltration in the mid-zone of liver lobules (Fig. 11b). These necrotic changes were less severe in *B. microti*-infected mice than in *B. rodhaini*-infected mice.

DISCUSSION

B. rodhaini infection was different from *B. microti* infection in its clinical course. All *B. rodhaini*-infected mice died after the sudden increase of parasitemia and excretion of reddish hemoglobinuria. In contrast, all *B. microti*-infected mice survived after a temporary moderate increase in parasitemia and in excretion of greenish urine. The decrease of hematocrit and the increase of indirect bilirubin confirmed intravascular hemolysis, which caused discolored urine in both *Babesia* infections. The excretion of hemoglobinuria suggested that serum hemoglobin concentration exceeded serum haptoglobins, which capture and bind free hemoglobin molecules and prevent their excretion. These date indicate that the intravascular hemolysis was more severe in *B. rodhaini*-infected mice than *B. microti*-infected mice.

Biochemical analysis of blood and urine showed functional conditions of kidneys in these *Babesia* infections. Both types of the infected mice excreted proteinuria , which contained albumin. However, the specific data (such as the increase of BUN and

proteinuria containing large molecular weight proteins that were more than 200kDa) suggested that *B. rodhaini*-infected mice had more severe functional damage of kidneys than did *B. microti*-infected mice. BUN is a useful parameter of diagnosis of the renal involvement in *Babesia* infections (Hussein 1977b, Maegraith et a.l 1957, Rogers 1971, Wright 1972). The excretion of large proteins indicates severe disorder of the filter function of kidneys.

Glomerulonephropathy was a characteristic pathological change in both babesioses. The deposition of IgG, IgM, C3 and Babesia antigens in glomerulus strongly suggested that babesioses caused the immune complex-induced mesangiopathic glomerulonephropathy. Similar immune complex-induced glomerulonephropathy was reported in rat babesiosis (Annable and Ward 1974) and in *Plasmodium* infections (Aikawa et al. 1988, Allison et al. 1969, Dixon 1966, Ward and Kibukamusoke 1969, Hartenbower et al. 1972, Bhamarapravati and Boonpucknavig 1973, Boonpucknavig and Sitprija 1979). In babesiosis, the deposition of electron-dense materials was first manifested in the mesangial area and along basement membranes; these densities may correspond to the immune complex deposits. It should be noted that although such deposits were immediately obvious by electron microscopy, they were not discernible by conventional light microscopy. With electron microscopy, these deposits were ubiquitous in the glomerular basement membranes of *B. rodhaini*-infected mice, but sporadic in the *B. microti*-infected animals. These ultrastructural changes in *B. rodhaini*-infected kidneys corresponded to the relatively severe functional damage incurred by these organs.

The kidneys of the infected mice also had mild tubular damage. It has been postulated that excessive hemoglobin in concert with tissue hypoxia is toxic to renal tubules (Braun et al. 1970). This is consistent with the fact that renal tubular necrosis was more severe in *B. rodhaini*-infected mice with relatively severe hemolysis and low hematocrit than in *B. microti*-infected mice.

The extensive liver necrosis and increase of direct bilirubin in *B. rodhaini*-infected mice showed that liver damage was relatively severe in *B. rodhaini* infection. Babesiosis induces the characteristic focal, mid-zonal, and/or centrolobular liver necrosis in various animals (Habela et al. 1991, Liddel et al. 1981, Hussein 1977a, Maegraith et al. 1954, Rogers 1971). Clark et al. (1987) suggested an excessive release of tumor necrotic factor could account for mid-zonal liver damage in *Plasmodium* infection. However, the mechanism of the liver necrosis has not yet been fully elucidated.

We showed that severity of the damage to kidneys and liver under the conditions of high parasitemia and discolored urine excretion is correlated with the prognosis of these two *Babesia* infections. The damage to kidneys and liver, as well as severe hemolytic anemia, in *B. rodhaini* infection seemed to cause failing of the mice, eventually leading to their death.

ACKNOWLEDGMENTS

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