FOLIA PARASITOLOGICA 50: 237–239, 2003

RESEARCH NOTE

BABESIA MICROTI: PARASITAEMIA AND ANTIBODY RESPONSES TO PRIMARY AND CHALLENGE INFECTIONS IN BALB/c MICE

Katarzyna Pastusiak[1], **Ewa Konopka**[2], **Maria Doligalska**[3] and **Edward Siński**[3]

[1]Witold Stefański Institute of Parasitology, Polish Academy of Sciences, Twarda 51/55, 00-818 Warszawa, Poland;

[2]National Institute of Hygiene, Department of Medical Parasitology, Chocimska 24, 00-791 Warszawa, Poland;

[3]Department of Parasitology, Institute of Zoology, Faculty of Biology, Warsaw University, Miecznikowa 1, 02-096 Warszawa, Poland

Babesia microti (Franca, 1909), an intraerythrocytic, tick-transmitted haemoprotozoan parasite is a common pathogen of free-living small mammals, particularly rodents. Babesiosis caused by this species of *Babesia* is gaining increasing interest as emerging zoonosis in humans (Parry M.F., Fox M., Burka S.A., Richar W.J. 1977: J. Am. Vet. Med. Assoc. 238: 1282–1283; Homer M.J., Aguilar-Delfini, Telford III, S.R., Krause P.J., Persing D.H. 2000: Clin. Microbiol. Rev. 13: 451–469). This is especially the case in North America, where human babesiosis is caused predominantly by *B. microti* and occasionally by a newly recognized species called WA1 piroplasm (Dammin G.J., Spielman A., Benach J.L., Piesman J. 1981: Hum. Pathol. 12: 398–400; Persing D.H., Herwaldt B.L., Glaser C., Lane R.S., Thomford J.W., Mathiesen D., Krause P.J., Phillip D.F., Conrad P.A. 1995: New Engl. J. Med. 332: 298–303). However, in Europe, human babesiosis is rather unique but more lethal, and mostly caused by *Babesia divergens* (Mac Fadyean et Stockman, 1911). Human infections occur always or nearly always in persons who lack a spleen (Brasseur P., Gorenflot A. 1992: Mem. Inst. Oswaldo Cruz 87, Suppl. 3: 131–132). Also, quite recently a new pathogenic *B. microti*-like species from dogs was described in Germany (Zahler M., Rinder H., Schein E., Gothe R. 2000: Vet. Parasitol. 89: 241–248).

In natural conditions, *B. microti* is transmitted by *Ixodes ricinus* complex ticks, in Europe, predominantly by nymphs of *I. ricinus*. Free-living small rodents (*Clethrionomys glareolus*, *Apodemus flavicollis*, *Microtus arvalis*) are important reservoir hosts. Although in enzootic regions these animals generally are parasitaemic, chronic infection (less than 0.5% infected erythrocytes) evidently persists for few weeks (Karbowiak G., Siński E. 1996: Acta Parasitol. 41: 50–51; Bajer A., Pawełczyk A., Behnke J.M., Gilbert F.S., Siński E. 2001: Parasitology 122: 43–54).

Babesia microti is widely used as an experimental model for the study of mechanisms of protective immunity induced by both natural and controlled infections (Cox F.E.G., Young A.S. 1969: Parasitology 59: 257–268; Hu R., Yeh M.T., Hyland K.E., Mather T.N. 1996: J. Parasitol. 82: 728–732). Protection to subsequent infection is afforded partly by antibodies against surface and E/S antigens of free merozoites or infected erythrocytes (Winger C.M., Canning E.U., Culverhouse J.D. 1989: Parasitology 3: 341–348). In the acute phase of infection IgM antibodies are produced as a first response against the parasite (Chen D., Copeman D.B., Burnell J., Hutchinson G.W. 2000: Parasite Immunol. 22: 81–88). Also, specific IgG antibodies are responsible for reducing the number of parasites in the blood (Hu et al. 1996, op. cit.). However, the role of humoral immunity to *B. microti* infection in rodents is not fully clarified (Clawson M.L., Paciorkowski N., Rajan T.V., La Vake C., Pope C., La Vake M., Wikel S.K., Krause P.J., Radolf J.D. 2002: Infect. Immun. 70: 5304–5306).

The present study was designed to test the protective effect of a *B. microti* strain maintained by syringe passage under laboratory conditions since 1991 to challenge the infection of BALB/c mice. We have measured and compared the parasitaemia of primary and challenge infections as well as IgM, IgG1 and IgG2a antibody response, against specific *B. microti* antigens.

Babesia microti strain (King's 67 strain), originally obtained in 1991 from Dr. S. Randolph (Oxford University) has been maintained by weekly syringe passage using both sexes of BALB/c mice. Fifty two male BALB/c mice aged 8–12 weeks, used in this study, were divided in two groups (26 animals each). Animals were kept in clean labelled plastic cages and provided with food and water *ad libitum*. The first group was injected once with 5×10^7 parasitized mouse erythrocytes by the intraperitoneal route. The second group was infected twice; the first infection on day 0 (as in group 1) and the second infection on day 38 after the first injection. Parasitaemia was examined on days 2, 4, 6, 7, 8, 9, 10, 11, 13, 14, 18 and 24 post primary infection (DPPI) and on 4, 8, 14, 18 and 24 days post challenge infection (DPCHI).

The presence of *B. microti* was detected by microscopic examination of blood smears stained with Giemsa. Two smears were obtained from the tail tip of each mouse on these days. One hundred erythrocytes were examined and the level of parasitaemia was expressed as the percentage of parasitized erythrocytes.

Heparinized blood was collected from the mice when the level of parasitaemia was about 75% of infected erythrocytes. The blood samples were centrifuged at 1000 *g* for 5 min and the parasites were isolated according to Machado et al. (Machado R.Z., Valadao C.A.A., Melo W.R., Alessi A.C. 1994: Braz. J. Med. Biol. Res. 27: 2591–2598).

Purified piroplasms were homogenized in 0.1 M PBS, pH 7.4, containing protease inhibitors (PMSF, TPCC), sonicated

Address for correspondence: E. Siński, Department of Parasitology, Institute of Zoology, Faculty of Biology, Warsaw University, Miecznikowa 1, 02-096 Warszawa, Poland. Phone: ++22 554 1113; Fax: ++22 554 1203; E-mail: esinski@biol.uw.edu.pl

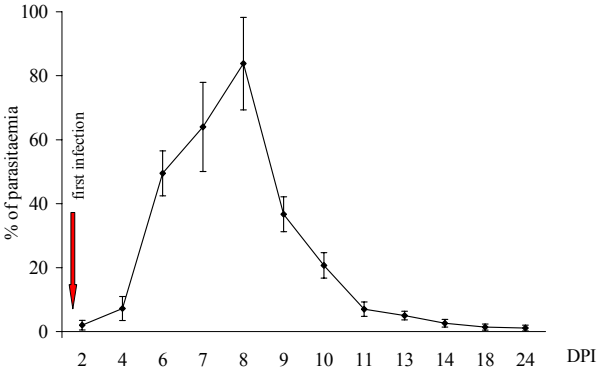

Fig. 1. Mean *Babesia microti* parasitaemia ± SD in mice. The arrow indicates the point at which primary dose was given.

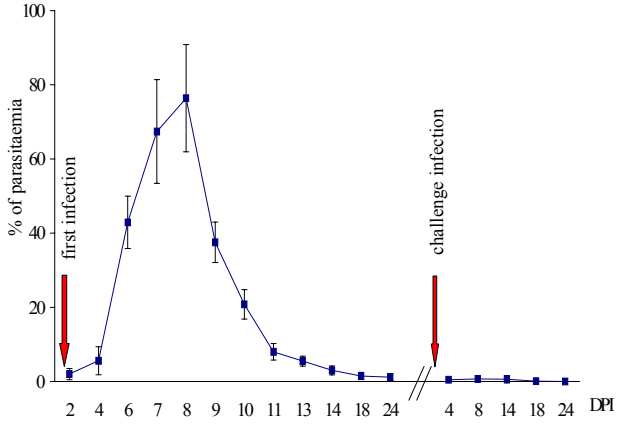

Fig. 2. Mean *Babesia microti* parasitaemia ± SD in mice. The arrows indicate the points at which primary and challenge doses were given.

10 times for 30 s and filtered with 0.22µm pore membrane. The homogenate was mixed overnight before centrifugation. The supernatant was dialyzed in PBS with inhibitors and condensed with Centriplus Model 30 (Amicon) concentrators. All preparations were carried out on ice. The protein content was estimated by Folin method. The antigen was used at 1.75 mg/ml concentration.

Blood samples were taken on 8, 14 and 24 DPPI and on the same days post challenge infection (DPCHI). Sera from uninfected mice were used as a control. Sera samples were stored at –20°C until use.

The antigens of *B. microti* were electrophoresed on 5% stacking gel and 15% acrylamide slab gels using Minigel System (Bio-Rad Laboratories, Hercules, California). Western blotting was performed with sera diluted 1:20, incubated overnight at 4°C, then incubated 1 h at 37°C with biotinylated monoclonal anti-mouse IgG1 and IgG2a antibodies (The Binding Site, UK) diluted 1:100 and monoclonal horseradish peroxidase-conjugated antibody anti-IgM (Sigma-Aldrich, Steinheim, Germany) diluted 1:500. Nitrocellulose membrane with anti-IgG1 and anti-IgG2a antibodies were then incubated 1 h at 37°C in streptavidin conjugated with horseradish peroxidase (Sigma-Aldrich, Steinheim, Germany) diluted 1:1000. The bound antibodies were visualised by adding 0.02% (w/v) 3,3´-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Steinheim, Germany) and 0.05% hydrogen peroxide. The reaction was stopped by washing in distilled water. Gels were stained with Coomasie brilliant blue and silver-stained with $AgNO_3$.

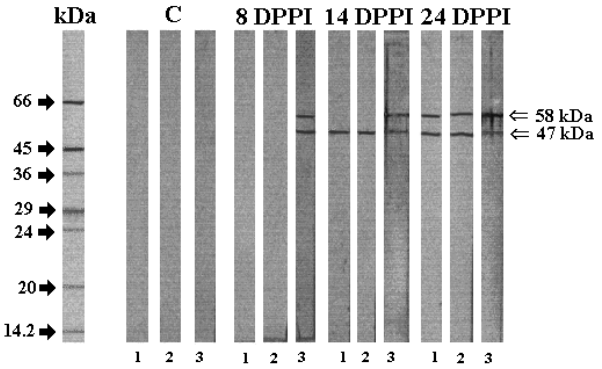

Fig. 3. Western blot of *B. microti* merozoite crude antigen with IgG1 (1), IgG2a (2) and IgM (3) serum antibody. The gel was a 15% discontinuous polyacrylamide gradient. C control serum obtained from non-infected mice; 8, 14, 24 DPPI serum obtained from infected mice after primary infection.

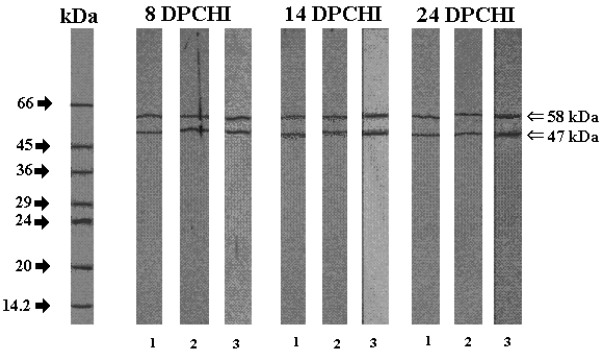

Fig. 4. Western blot of *B. microti* merozoite crude antigen with IgG1 (1), IgG2a (2) and IgM (3) serum antibody. 8, 14, 24 DPCHI serum obtained from mice after challenge infection.

All mice after primary infection were readily infected. The patterns of parasitaemia after primary and challenge infection in mice are shown in Fig. 1 and Fig. 2. In the first group of animals (Fig. 1) the percentage of parasitaemia progressively increased and peaked synchronously in all mice at 8 DPPI (mean value, 80% erythrocytes). The parasitaemia subsequently declined to 10% of infected erythrocytes at 10 DPPI and to 3% of infected erythrocytes at 14 DPPI. At the end of experiment at 24 DPPI, the parasitaemia was 0.1%, practically non-detectable. After challenge infection, *B. microti* was detected very rarely with a maximum of less than 1% of infected erythrocytes (Fig. 2).

SDS-PAGE electrophoresis of the crude *B. microti* merozoite antigen revealed high heterogeneity of protein bands ranging between 6.5 and 205 kDa of molecular weight (data not shown). However, only two of the protein bands (47 kDa and 58 kDa) revealed in electrophoresis reacted with specific IgM, IgG1 and IgG2a antibodies. After primary dose,

the IgM response was detected at 8 DPPI against both protein bands. IgG antibodies reacted at 14 DPPI only with one 47-kDa protein and later at 24 DPPI also with 58-kDa protein band (Fig. 3). After challenge dose, IgM and IgG antibodies recognized both 47-kDa and 58-kDa protein bands from 8 DPCHI to the end of experiment (Fig. 4).

The piroplasms of rodents are apparently distributed all over the world (Šebek Z., Rosický B., Sixl W. 1977: Folia Parasitol. 24: 211–220; Homer et al. 2000, op. cit.). In contrast to the USA, in European countries the health problem of zoonosis caused by *B. microti* is entirely unknown. However, not more than 20 cases of human babesiosis have been reported in Europe since 1957, most of them due to *B. divergens*. Various studies have demonstrated that mice are susceptible to *B. microti* infection (Shortt H.E., Blackie E.J. 1965: J. Trop. Med. Hyg. 68: 37–42; Cox and Young 1969, op. cit.). However, many strains of *B. microti* cause only a transient parasitaemia and the infected mouse spontaneously recovers from the acute infection (Igarashi I., Hosomi T., Kaidoh T., Omatta Y., Saito A., Suzuki N., Aikawa M. 1993: J. Protozool. Res. 3: 144–155). Thus, in order to study the immunity to *B. microti*, male BALB/c mice infected with 5×10^7 parasitized mouse erythrocytes and challenged with the same dose of parasites were used. All of the animals after primary infection developed detectable parasitaemia ranging from 69% to 97% at its peak, which generally was reached on day 8 post infection.

The presented results indicate that the parasite self-limiting starts and parasitaemia levels begin to decline approximately 10 days after infection. However, at the time of the third and fourth weeks post infection, the same animals maintain chronic infection, when less than 0.3% of erythrocytes are infected. The level of parasitaemia observed in this study was higher than the results obtained by other authors. Mzembe et al. (Mzembe S.A.T., Lloyd S., Soulsby E.J.L. 1984: Z. Parasitenkd. 70: 753–761), using the same strain of *Babesia*, revealed that, in NIH mice infected with 1×10^8 infected erythrocytes, the peak of parasitaemia appeared 10 days after infection, and also the level of parasitaemia was much lower, with a maximum of 55% of infected erythrocytes.

The higher level of parasitaemia could be caused by the higher virulence of *B. microti*. The parasites were maintained under constant laboratory conditions and were passaged in BALB/c mice for at least 10 years. It has been known that virulence of this parasite in laboratory mice increased and could lead even to death (Cox and Young 1969, op. cit.). The pattern of parasitaemia revealed in this study confirmed the results obtained previously by Konopka (unpublished). Infection of BALB/c mice by this strain of parasite appears to follow a predictable course. In contrast to the primary infection, the challenge dose resulted in a very low level of parasitaemia (0–3%) during the time of the study. It means that mice recovered from primary infection are fully resistant to the challenge infection. This clearing of the parasite is due to both humoral and cellular immune responses (Chen et al. 2000, op. cit.). However, the mechanisms of host recovery following *B. microti* infection after challenge, and the maintenance of a chronic infection, are still unknown.

The next step of our study was to characterise the protein bands of the crude *B. microti* merozoite antigen and, in addition, determine which of these bands reacted with specific IgM and IgG anti-parasite antibodies.

SDS-PAGE electrophoresis revealed a high heterogeneity of protein bands varying from 6.5 to 205 kDa. Using immunoblotting, only two of the detected protein bands (47 kDa and 58 kDa) reacted positively with IgM, IgG1 and IgG2a antibodies. After primary infection, the IgM antibody detected both protein bands 8 DPPI. IgG1 and IgG2a antibodies only detected the 47-kDa protein band 14 DPI. Later, at 24 DPPI, the IgG1 and IgG2a antibodies recognized both protein bands. It seems that both immunoglobulin isotypes IgG1 and IgG2a play an important defence role against these intraerythrocytic protozoan parasites and the time of their appearance strongly correlated with rapid parasite clearing. The immune system effectively "primed" with the first inoculation of parasites stimulated specific IgG antibodies that can prevent secondary infection by binding and neutralizing sporozoites before they succeed in invading erythrocytes.

Some observations suggest that in mice after primary infection with *B. microti* T lymphocytes, a subpopulation of $CD4^+$ cells and gamma interferon are especially involved in the regulation of the immune response (Igarashi I., Suzuki R., Waki S., Tagawa Y., Seng S., Tum S., Omatta Y., Hosomi T., Saito A., Nagasawa H., Iwakura Y., Suzuki N., Mikami T., Toyoda Y. 1999: Infect. Immun. 67: 4143–4148; Chen et al. 2000, op. cit.), and that different isotypes of antibody (IgM and IgG) take part in the protective immunity to this infection (Chen et al. 2000, op. cit.). However, some recent data indicate that cellular immunity is rather critical for the clearance of *B. microti* in BALB/c mice (Clawson et al. 2002, op. cit.).

After challenge infection, IgM, IgG1 and IgG2a antibodies in sera of infected mice reacted more strongly with the two protein bands of merozoite antigen. During this time, the parasitaemia achieved a very low level. These results indicated that appearance of specific IgG1 and IgG2a antibodies correlated well with the recovery and the final clearance of babesiosis from infected BALB/c mice. It seems most likely that these antibodies inhibit erythrocyte invasion by, and promote opsonisation of, merozoite and infected erythrocytes by activated cellular mechanisms (Taylor-Robinson A.W. 1995: Parasitol. Today 11: 334–342). Also, there is evidence from experimentally infected golden hamsters that appearance of a detectable anti-*B. microti* IgG response corresponds to clearing of parasites from the blood (Hu et al. 1996, op. cit.). On the other hand, it seems that two proteins of crude merozoite antigen (47 kDa and 58 kDa) are able to induce an effective, protective immunity against challenge infection.

In the light of our recently published data (Karbowiak et al. 1996, op. cit.; Bajer et al. 2001, op. cit.), which revealed that less than 1% of naturally infected bank voles (*Clethrionomys glareolus*) had chronic infection with less than 0.5% of the erythrocytes infected, this study provides additional information on the possible role of chronic infections in forming the zoonotic reservoir of *B. microti* infection as newly emergent tick-borne zoonosis.

Received 10 March 2003 Accepted 25 July 2003