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Experimental transfusion-induced *Babesia microti* infection: Dynamics of parasitemia and immune responses in a rhesus macaque model

Sanjeev Gumber^{1,3,*}, Fernanda S Nascimento⁴, Kenneth A Rogers², Henry S Bishop⁴, Hilda N Rivera⁴, Maniphet V Xayavong⁴, Sushil G Devare⁵, Gerald Schochetman⁵, Praveen K Amancha², Yvonne Qvarnstrom⁴, Patricia P Wilkins⁴, and Francois Villinger^{2,3}

¹Division of Pathology, Yerkes National Primate Research Center, Atlanta, GA, USA

²Division of Microbiology and Immunology, Yerkes National Primate Research Center, Atlanta, GA, USA

³Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, USA

⁴Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control & Prevention, Atlanta, GA, USA

⁵Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL, USA

Abstract

BACKGROUND—Babesiosis is an emerging tick-borne infection in humans. The increasing numbers of reported cases of transfusion-associated babesiosis (TAB), primarily caused by *Babesia microti*, represents a concern for the safety of the U.S. blood supply.

STUDY DESIGN AND METHODS—This study investigated kinetics of parasitemia, innate immune responses and dynamics of antibody responses during *B. microti* infection in rhesus macaques using blood smears, quantitative PCR (qPCR), flow cytometry, and indirect fluorescent antibody testing. A total of 6 monkeys were transfused with either hamster or monkey-passaged *B. microti* infected erythrocytes (2 and 4 monkeys, respectively) simulating TAB.

RESULTS—The prepatent period in monkeys inoculated with hamster-passaged *B. microti* was 35 days compared with 4 days in monkeys transfused with monkey-passaged *B. microti*; the latter monkeys also had markedly higher parasitemia levels. The duration of the window period from the first detected parasitemia by qPCR analysis to the first detected antibody response ranged from 10–17 days. Antibody responses fluctuated during the course of the infection. Innate responses assessed by the frequencies of monocytes and activated B cells correlated with the kinetics and magnitude of parasitemia. On day 14, additional activation peaks were noted for CD14⁺CD16⁺ and CD14⁻CD16⁺ monocytes and for CD11c⁺ myeloid dendritic cells, but only in animals

*Corresponding author: Sanjeev Gumber, Division of Pathology, Yerkes National Primate Research Center, Emory University, 954 Gatewood Road NE, Atlanta, Georgia 30329, USA. Tel.: +1 404 727 7022; Fax: +1 404 727 4531; sgumber@emory.edu.

Conflict-of-interest disclosure

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transfused with monkey-passaged *B. microti*. Parasitemia persisted in these immunocompetent animals, similar to human infection.

CONCLUSION—The results suggest that transfusion-associated transmission of *B. microti* leads to rapid onset of parasitemia (day 4) in rhesus macaques, detectable antibody response 14 days later and persistent parasitemia.

Keywords

Antibody; Babesia; Immune; Parasitemia; PCR; Rhesus; Transfusion

INTRODUCTION

Babesiosis is a tick-borne disease caused by intraerythrocytic parasites, including *Babesia microti* and other *Babesia* species.^{1,2} *Babesia* infection can range from asymptomatic to severe.^{1,3,4} In severe or persistent cases who require treatment, clindamycin and quinine⁵ or an alternative regimen using atovaquone and azithromycin have been recommended.⁶ *B. microti* infection is not usually clinically silent,^{7,8} complications of *B. microti* include hemolytic anemia, acute respiratory distress syndrome, disseminated intravascular coagulopathy, congestive heart failure, hepatic failure, splenic rupture and renal failure.^{9–12} In particular, persons who are asplenic, elderly, or otherwise immunocompromised are at increased risk for clinical manifestations and life-threatening infection.^{1,3,4}

Transfusion-associated babesiosis (TAB) is a growing public health concern for the safety of the US blood supply since it may lead to severe morbidity and mortality. Babesiosis became a nationally notifiable disease in January 2011.¹³ *B. microti* has been implicated in the majority of US tick-borne and TAB cases, including 159 of the 162 cases of TAB that were identified during the period of 1979–2009; the other 3 cases were caused by *B. duncani*.¹³ These figures likely underestimate the actual number of TAB cases, as infections are not always diagnosed nor recognized as transfusion-associated and investigated.¹³ In the United States, *B. microti* is endemic in parts of the Northeast and upper Midwest, whereas sporadic cases of infection with *B. duncani* have been identified in several western states.¹³ Acute, symptomatic cases of babesiosis typically are diagnosed by light-microscopic identification of organisms on Giemsa-stained thin blood smears.^{9,14} *B. microti* elicits both IgM and IgG antibody responses in humans, and serologic testing is commonly used as evidence for transmission in retrospective investigations of donors,^{15–17} although recent attempts to detect *Babesia* nucleic acids in blood products have been made to confirm transmission.^{17–19} In addition, investigational high throughput antibody screening assays including Enzyme immunoassay (EIA) and arrayed fluorescence immunoassay (AFIA) showed promising results for donor screening.^{17,20,21} FDA has closely monitored the results of an investigational protocol evaluating *B. microti* serology and PCR and recently solicited formal input from an advisory committee on appropriate screening methodology and donor deferral, but has yet to define a temporary deferral period for donors who have tested positive for *B. microti*.²¹

Nonhuman primates—particularly, rhesus macaques (RMs) are the most widely used animal models of human disease because of their close genetic, physiologic, and metabolic

similarity to humans.²² RMs have been used successfully to study the kinetics of *B. microti* parasitemia.^{23–25} However, these studies did not document the acute innate immune responses during *B. microti* parasitemia, as well as before and after seroconversion. Further, immune responses to *B. microti* infection have been evaluated in a limited number of studies.^{26,27} Such issues are central to developing effective strategies to prevent TAB, including an automated high-throughput assay for *B. microti* donor screening. Therefore, we conducted a formal, comprehensive investigation using the RM animal model to fill key gaps and model human TAB with *B. microti*.

MATERIALS AND METHODS

Ethics statement

All RMs were born at Yerkes National Primate Research Center (YNPRC) and were maintained at the YNPRC of Emory University in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals, 8th edition. YNPRC has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International since 1985. The experiments were approved by the Emory Institutional Animal Care and Use Committee as well as biosafety review board. The animals were fed monkey diet (Purina) supplemented daily with fresh fruits or vegetables and water ad libitum. Additional social enrichment, including the delivery of appropriate safe toys, was provided and overseen by the Yerkes enrichment staff, and animal health was monitored daily and recorded by the animal care staff and veterinary personnel, available 24 hours, 7 days a week. Monkeys were caged in socially compatible same-sex pairs to facilitate social enhancement and well-being. Male Golden Syrian Hamsters (*Mesocricetus auratus*) were used to propagate *B. microti* Gray strain parasites in accordance with the rules and regulations within CDC animal use protocol “Production of antigen in hamsters and gerbils for the serodiagnosis of babesiosis”.

Study design and experimental infections

The study was conducted in three different phases as illustrated in Figure 1. A total of 6 adult (7–13 years), female, non-splenectomized, Indian RMs were assigned to this study, specifically 2 monkeys for phase 1 (RF19, RGc8), 3 monkeys in phase 2 (RLk10, RZz9, RCq10), and 1 animal for phase 3 (RVf12). All the animals were prescreened to rule out prior malaria and/or *Babesia* infection using blood smears, PCR and IFA test. The animals were pretreated with diphenhydramine prior to exposure to *B. microti* to prevent potential anaphylaxis. Since *B. microti* cannot be grown *in vitro*, freshly collected blood from parasitemic hamsters was utilized as the source of primary inoculation for phase 1. Each of the 2 RMs was inoculated with 2.8 mL of heparinized hamster blood intravenously containing approximately 6×10^9 hamster-passaged *B. microti* Gray strain. After inoculation, blood samples were collected aseptically from the femoral vein in EDTA on days 1, 4, 7, 14 and weekly thereafter (Figure 1), for PCR, IFA, flow cytometry, Giemsa-stained thin/thick blood smears, complete blood count and chemistry analyses. Blood smears were also prepared from blood obtained by ear prick procedure to determine the parasitemia at various time points (Figure 1). On day 56, 10 mL blood from each phase 1 monkey was harvested and erythrocytes from each animal were washed in 7.5 mL normal saline and mixed together

to a total volume of 15 mL for transfusion into three phase 2 monkeys (Figure 1). Blood smears from pooled blood sample were also prepared to estimate parasitemia. Each phase 2 animal was inoculated intravenously with 5 mL of infected erythrocytes and saline mixture corresponding to approximately 1.5×10^7 organisms of monkey-passaged *B. microti* obtained from phase 1. On day 63 of phase 2, blood was collected from RM RCq10, in which *B. microti* had become undetectable in both thin and thick blood smears but remained positive by IFA and PCR, to test whether transfusion of such blood had the potential to transmit *Babesia*. Five mL of washed erythrocytes corresponding to a total dose of 25,000 monkey-passaged *B. microti* (based on qPCR) from phase 2 were prepared as described above and transfused intravenously to RM RVf12 (phase 3). In phase 3, additional blood samples were collected on days 10, 12, and 18. All animals in this study were treated for 10 days with atovaquone (Amneal Pharmaceuticals) @ 20 mg/kg orally twice a day and azithromycin (Greenstone Limited) @ 12 mg/kg orally once a day starting on day 122. The animals were monitored thereafter by blood collections at various time points to document that no parasites were found and to conduct additional analyses as described above. Phase 1 and 3 animals (Figure 1) needed a second course of treatment starting on days 193 and 200, respectively, to achieve parasitic clearance.

Estimation of parasitemia by light microscopy

Peripheral blood thin and thick smears were prepared from EDTA whole blood, stained with Wright-Giemsa stain. Thin blood smears were used for parasite identification and thick blood smears for quantification of organisms detected as described previously.²⁸ Parasitemia was expressed as parasites/ μ L of blood in a thick blood film by counting parasites until 200 sequential WBCs were enumerated and using the simple mathematical formula:

$$\text{Parasites}/\mu\text{L} = \frac{\text{Total number of WBC}}{200} \times \text{Number of parasites counted}$$

Nested PCR (nPCR) and Quantitative PCR (qPCR)

DNA isolation was performed on 200 μ L of EDTA whole-blood using QIAamp DNA Blood Mini Kit using the QIAcube automated system (QIAGEN, CA). Detection of *B. microti* DNA was performed in a nPCR assay as described elsewhere,²⁹ with some modifications. For qPCR, a standard curve was created by serially diluting three blood samples from experimentally infected hamsters with known parasitemia levels, corresponding to 220,000, 935,000 and 1,280,000 parasites/ μ L, respectively (Figure S1). qPCR was performed using a published TaqMan real-time PCR assay targeting the 18S rRNA gene.³⁰ Each sample was run in triplicate with appropriate negative and positive controls. The Ct-values were compared to the standard curve to derive numbers of parasites present in the RM samples. Parasitemia was expressed as parasites/ μ L of blood.

Indirect fluorescent antibody test

An IFA assay for IgG antibodies to *B. microti* was used as described earlier.³¹ Briefly, sera were diluted fourfold starting at 1:4 to 1:4096. 20 μ L of sera was added to the appropriate well on the coated *B. microti* slides and incubated for 30 min at 37°C. Slides were washed for 5 min with PBS and dried followed by addition of 20 μ L goat anti-human immunoglobulin cross reacting with rhesus immunoglobulin labeled with fluorescein (FITC,

1 mg/mL) at 1:50 in PBS with 0.1% Evans blue and were incubated for 30 min at 37°C. The fluorescence was determined with an Olympus BX60 microscope equipped with a X-cite Series 120-Q fluorescence illumination source at 400× magnification. A titer of 1:64 with *B. microti* antigen was considered a positive reaction.

Flow cytometry

Freshly isolated peripheral blood mononuclear cells (PBMC) ($\sim 1 \times 10^6$) were stained with three fluorescent antibody panels with predetermined concentrations of antibodies against CD3 (SP34-2), CD4 (L200), CD8 (RPA-T8), CD20 (L27), CD28 (CD28.2), CD95 (DX2), NKG2A (Z199), HLADR (Tu36), CD38 (AT-1), CD14 (M5E2), CD16 (3G8), CD11c (3.9), CD123 (7G3), CD21 (B-Ly4), CD27 (MT271), CD127 (R34.34), CD25 (4E3), CD86 (IT2.2), CD25 (1HT44H32) (Table S2). Cells were incubated with the antibody cocktails for 30 min at 4°C, washed with PBS containing 2% FBS, permeabilized with perm/fix buffer (BD) washed with 2 mL of perm wash buffer. The cells were then additionally stained with anti-Ki67 (B56) or for Foxp3 (206D). Cells were finally fixed in 1% paraformaldehyde, acquired on LSR-II flow cytometer driven by FACS DiVa software. Analysis of the acquired data was performed using FlowJo software (version 9.4.11; TreeStar).

Statistical analyses

Flow cytometry data were analyzed using a 2-tailed paired t-test to compare activation patterns in phase 1 and 2 animals.

RESULTS

Level and duration of parasitemia during *B. microti* infection

To model TAB in monkeys, we utilized a 3 tiered experimental protocol: The initial phase was to generate monkey-adapted *B. microti* for direct transfusion of “high acute” parasitemia (phase 2) and low chronic parasitemia (phase 3). For the xenogeneic transfusion (phase 1), patent infection developed in both monkeys inoculated with 6×10^9 hamster-passaged *B. microti* (Gray strain) after 35 and 49 days in the 2 monkeys (RF19, RGc8) based on qPCR and blood smear data, respectively (Figures 2 and S2). The parasitemia peaked on day 56 (RGc8) and 63 (RF19) after experimental infection, and the peak parasitemia detected with qPCR and blood smears showed good temporal correlation. Peak parasitemia ranged from 1227 to 1679 organisms/ μ L on thick blood smears or 11,319 to 15,676 organisms/ μ L blood by qPCR. After peak parasitemia, both animals showed intermittent low level parasitemia (< 50 organisms/ μ L) prior to initiating drug treatment on day 122. This low level parasitemia persisted in RF19 based on nPCR/qPCR analysis leading to a second round of therapy on day 193. Erythrocytes from both phase 1 monkeys were collected on day 56 and transfused to phase 2 monkeys (corresponding to 1.5×10^7 organisms). Even though the parasite dose was > 100 fold lower than the dose administered to phase 1 monkeys, the prepatent period was markedly reduced (4 days). The peak parasitemia identified (qPCR and blood smears) on day 14 or day 21 post infection was also markedly higher in 2 of the 3 phase 2 monkeys compared to phase 1 monkeys (Figure 3). Phase 2 RM RCq10 surprisingly exhibited markedly lower peak parasitemia, suggesting individual differences in susceptibility. Beyond the acute parasitemia, these monkeys also exhibited intermittently

detectable low level chronic parasitemias (<50 organisms/ μ L) prior to atovaquone/azithromycin treatment. The intermittent chronic low level parasitemia was detected predominantly by nPCR, the most sensitive technique, though confirmed by qPCR followed by measurable transient rebounds in IFA titers (Figures 2–4 and Table S1), while parasites could not be detected in blood smears beyond the acute infection.

To determine the clinical relevance of low chronic parasitemia, blood collected from RM RCq10 at day 63 post transfusion (blood smears with no parasites found and barely positive qPCR signal) was transfused to phase 3 monkey (RVf12), amounting to about 25,000 monkey-passaged *B. microti* organisms, based on qPCR. However, this low dose exposure also resulted in patent infection. The prepatent period was 4 days based on qPCR/nPCR and blood smears detected *B. microti* on day 21. Peak parasitemia was detected on day 35 (Figure 4, 528 organisms/ μ L by blood smears and 10,631 organisms/ μ L by qPCR). RVf12 also showed intermittently detectable low level parasitemia (<50 organisms/ μ L) on blood smears until day 70 followed by nPCR/qPCR detection prior to starting treatment on day 122. This animal, similar to phase 1 RF19 required a 2nd course of treatment to eliminate detectable parasite.

The kinetics and detection of parasitemia have been summarized in Table 1 and Figures 2–4, suggesting clear differences in prepatency depending on the source of the TAB, while peak parasitemia in animals transfused with monkey-passaged *B. microti* appear to reflect the infectious dose, though phase 3 only comprised 1 animal. There was fluctuation of parasitemia after the acute peak with no pattern in the timing of recurrences of detectable parasitemia during chronic infection.

Of note, clinical symptoms, e.g. flu-like illness, and deviations in hematological parameters (hemoglobin, hematocrit, WBC or differential counts) were not observed in these animals.

Antibody responses during and after *B. microti* infection

The kinetics of parasitemia and antibody titers are summarized in Table 1 and Figures 2–4. During phase 1, the first antibody IFA responses (1:64) were detected on days 35 and 49, on the day of, or 2 weeks after the first qPCR detection for RGc8 and RF19, respectively. The peak antibody titers (1:4096) in both animals were detected on day 56 coinciding with peak parasitemia. The antibody titers gradually declined to 1:1024 on day 84, and lower levels (1:64) were still observed for RGc8 on day 207. Conversely, RF19's titer fluctuated, and a titer of 1:1024 was observed on day 207 despite the absence of detectable parasitemia. In phase 2, the first positive antibody titers (1:256) were detected on day 14 i.e. 10 days after the first detectable parasitemia. Peak antibody titers were identified on day 21 again, coinciding with the peak parasitemia. Of note, peak antibody titers in 2 of the 3 phase 2 RMs and the phase 3 RM were markedly lower than in phase 1 monkeys. The first antibody detection in the phase 3 monkey was on day 21, likely attributable to the comparatively small inoculum transfused. With the exception of RCq10, the peak antibody titers rapidly declined in all animals, though fluctuations in antibody titers were noted for the monkeys transfused with monkey-passaged *Babesia* but not with hamster-passaged parasites, suggesting occasional parasite replication which is not necessarily detectable in blood.

Innate immune responses to *B. microti* infection

We hypothesized that *B. microti* infection invokes innate responses *in vivo*. Therefore, we monitored PBMCs at periodic intervals over the course of the study to address changes and activation of various cell lineages. Evidence of immune activation was most clearly observed in B cells, T cells, monocytes, and myeloid dendritic cells, whereas changes in NK cells (NKG2a+) or T regulatory cells (CD25+/FoxP3+/CD127-) were not observed in blood. As expected for parasite driven responses, the kinetics for immune activation paralleled those of parasite burden. B cell activation/proliferation—assessed by the frequency and density of Ki67 expression on CD20+ cells—showed a biphasic increase on days 7 and 49 in both phase 1 animals (Figure 5). In the phase 2 monkeys, B cell activation peaked sharply on day 7 and decreased thereafter towards baseline values by day 21 or 35. Activation of B cells in the phase 3 animal was weaker, which may reflect the comparatively low inoculum. For all 6 monkeys, the peak activation was essentially reflective of tissue memory (CD21-/CD27-, $p=0.0441$ on day 7) and of memory (CD21-/CD27+; $p=0.0198$ on day 7 and $p=0.0012$ on day 14 relative to baseline) B cell subsets, whereas the naïve B cell subsets (CD21+/CD27-) did not show such change (not shown).

Activation of T cells was predominantly seen in the CD8+ central memory T cells (Tcm) (CD28+CD95+) on day 7 post transfusion for animals infected with monkey-passaged *B. microti*, as measured by the percentages of Ki67+ and HLA-DR^{hi} cells (Figure S3), whereas such increases in percentages were much more modest in phase 1 animals. In contrast to CD8+ cells, CD4+ T cells showed little evidence of activation, except perhaps in a phase 1 monkey (RGc8; data not shown).

For monocytes and myeloid dendritic cells, activation measured by increases in Ki67 and CD86 mean fluorescence intensity demonstrated low and highly variable levels of activation without a clear trend in phase 1 monkeys (data not shown). However, in phase 2 monkeys, acute activation was clearly seen on day 14 for activated CD14+/CD16+ and atypical CD14-/CD16+ monocytes, as well as for myeloid dendritic cells (Figure S4). This activation was highest for RCq10, followed by RLk10 and RZz9, suggesting that activation of monocytic lineages was not proportional to the magnitude of parasitemia. Activation of these subsets was minimal in the phase 3 monkey (data not shown).

DISCUSSION

In the US, almost 5 million patients are recipients of red blood cells transfusion annually, the safety of which remains a constant source of concern.³² Babesiosis is now the most commonly reported transfusion-associated parasitic disease.^{33,34} Clinical features of TAB cases are usually considered similar or more severe than those observed after arthropod vectored transmission, primarily due to underlying health-related issues and lowered immune competence in blood recipients, promoting the development of clinically manifest babesiosis following blood transfusion with a *Babesia*-contaminated unit.⁸

While there is increased interest in developing a high-throughput assay for *B. microti* blood donor screening, further research is also needed to understand the kinetics of parasitemia and antibody responses. Various experimental animal models have been used to investigate

the underlying pathophysiology of *Babesia* infection, and hamsters are commonly used as a model to study babesiosis and explore drug therapies.^{35,36} Mouse models are usually less representative because they develop low grade parasitemia.³⁷ Despite the presence of existing rodent models, the pathophysiology of human babesiosis is not completely understood. Therefore, in the present study we investigated clinically relevant aspects of transfusion-associated transmission of *B. microti* in a primate model that is physiologically closer to man.

Data on duration of *B. microti* parasitemia or persistence of infection in humans are limited.^{38,39} In this study, the prepatent period in monkeys inoculated with hamster-passaged Gray stain cells ranged from 35 to 49 days, and the peak parasitemia on blood smears ranged from 1227 to 1679 organisms/ μ L. Morphologically, the parasites resembled those seen in the hamsters, humans and NHP. In the late 1970s and early 1980s, RMs have been used successfully to study the kinetics of *B. microti* parasitemia.^{23–25} These studies reported a prepatent period of 15 to 46 days in monkeys infected with the hamster-passaged Briard strain of *B. microti*, and peak parasitemia ranged from 496 to 3906 organisms/ μ L. Parasitemia persisted for at least 90 days in all the animals and parasites were detected 559 days after inoculation in one animal,²³ suggesting variability in the ability of *B. microti* strains to replicate *in vivo*. In addition, recurrences of parasitemia were seen following splenectomy in RMs infected intravenously or via *B. microti* infected nymphal ticks.^{24,25} The phase 2 and 3 animals in this study revealed a far shorter prepatent period (4 days) than the phase 1 monkeys suggesting a possible host adaptability of *B. microti*, and a far more representative model of TAB in humans. The basis for such variability in parasite dynamics between hamster-passaged and monkey-passaged *B. microti* strains may be secondary to parasite genome mutations, or the acquisition of species specific host markers that somehow facilitate replication *in vivo*. It has been reported that comparative studies of parasite genomes can reveal genetic adaptations to parasitism.⁴⁰ However, the extent to which the combination of host environment and genetic variation accounts for variability in pathogenicity associated with *B. microti* strains remains to be investigated.

The possibility of asymptomatic persistence of *B. microti* in blood donors is supported by numerous TAB cases.^{13,41,42} A recent study documented that seropositive donors can have protracted low level parasitemia,³⁸ which was corroborated in the present study up to the time that the monkeys received antimicrobial therapy. The possible explanation of recurrent parasitemia in this study could be due to combination of premunition (concomitant immunity) and/or process of “antigenic variation” which has been reported to occur in *Babesia* spp. including *B. microti* and is thought to aid *Babesia* spp. survival by evasion of host adaptive immune responses.^{43–45}

One major challenge posed by this mostly clinically silent infection is the possibility of persistent parasitemia that is below the level of detection of currently available tests. As seen here, detection of parasites in blood smears, the current reference for patient diagnosis in acutely ill patients, including transfusion recipients, is relatively insensitive, and absence of *Babesia* detection by this method does not exclude the possibility of parasite transmission to transfusion recipients (Figures 3, 4). Although the number of animals available to us did not allow for extensive testing of transmission during chronic *Babesia* infection, the

transmission of *B. microti* from a phase 2 monkey to the phase 3 monkey readily occurred even though qPCR values and nPCR signals at the time of collection were very low (5 organisms/ μ L of blood), providing proof of concept that such transmission does occur. Further, antibody titers measured by IFA were quite variable post infection, and often barely positive, despite low (intermittently detectable) levels of *Babesia* nPCR/qPCR signals, suggesting that single nucleic acid based test or IFA assay available to date may not be adequate by itself for adequate donor screening to prevent *Babesia* transmission, particularly after the acute phase of *Babesia* infection. This is not secondary to the sensitivity of individual assays but more to the size of the representative samples tested. The findings in the current study underscore that the protracted low levels of parasitemia pose a risk for transmission. Of note, the transfusion used in our study differs from human blood transfusion by the absence of blood storage as in human blood banking conditions. In addition, a single course of prescribed atovaquone and azithromycin failed to clear the parasites in 3 animals suggesting additional courses of treatment are required for complete clearance of *B. microti*, which requires further investigation. Similar finding has been reported in few *B. microti* infected humans where the subjects were retreated with atovaquone and azithromycin due to persistent positive *B. microti* PCR.³⁸

Little is known also regarding the minimal infectious dose of *B. microti* which can cause infection in naïve hosts. The monkey in phase 3 of this study became infected with a dose of 25,000 *B. microti* organisms obtained from 5 mL of erythrocytes harvested during the chronic phase of infection even though no parasites could be detected by blood smear. Considering the average volume of a human blood transfusion for adults, this dose represents approximately 1.5×10^6 *B. microti* in a blood unit assuming 300 mL of packed RBC/unit. Of note, while the low dose transfused resulted in a delayed peak of parasitemia and lower magnitude measured by blood smear, the qPCR parasitemia loads were similar to those of the other monkeys transfused with higher *Babesia* doses.

Innate responses such as monocyte activation has been reported upon infection of monkeys with *B. microti*.⁴⁶ Another study reported higher frequency of IL-10 producing B cells (Bregs) and CD4⁺CD25⁺FoxP3⁺ T cells during the course of *B. microti* infection in mice.¹⁹ Our study confirmed an early activation of monocyte subsets and myeloid dendritic cells corresponding to the peak parasitemia, suggesting a role for these cell lineages in the early control of parasitemia. The sharp decline in activation following this time point coincides with a drop in parasitemia and it has been demonstrated that in mice, monocyte/macrophages are important for parasite control.⁴⁷ Although this study did not examine other tissues, it is possible that these monocytes are migrating to tissues such as the spleen where they develop into macrophages and assist in the removal of infected erythrocytes. Conversely, memory B cells activation preceded the peak of parasitemia, suggesting the induction of adaptive responses precedes the peak of parasitemia. Anecdotally, the animal with the lowest peak parasitemia (RCq10, phase 2) exhibited the highest level of immune activation overall, suggesting that perhaps these responses are indeed involved in the control of parasite replication. These responses were transient only, even though low levels of parasitemia persisted in most monkeys, suggesting that perhaps *Babesia* may have developed strategies to limit anti-parasitic defenses allowing for its survival at low levels.

Taken together, the study has enhanced our understanding of *B. microti* host invasion and suggested possible evolutionary adaptation of *B. microti* strains to a specific host and illustrated the complexity of persistence of *B. microti*. Overall, the antibody response was consistently detected 14 days after the first detection of parasitemia. qPCR detected infections several days before IFA upon homologous monkey infections while results from both techniques were matched for the detection of the hamster derived infections. However, the onset of parasitemia was far more rapid during the homologous transfusion. In addition, the data presented here provide a potential model to study the complexity of host-parasite interaction and the mechanisms of parasite persistence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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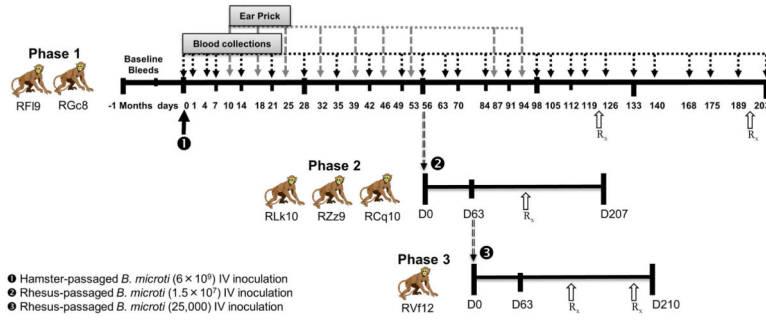


Figure 1. Study design. Phase 1: On day 0, two rhesus macaques were inoculated with erythrocytes containing 6×10^9 hamster-passaged *B. microti*. In phase 2, three monkeys were transfused with 1.5×10^7 monkey-passaged *B. microti* obtained on day 56 of phase 1. One monkey in phase 3 was transfused with 25,000 monkey-passaged *B. microti* acquired from a phase 2 monkey. All animals in this study were treated with atovaquone and azithromycin for 10 days starting on day 122. Phase 1 and 3 animals received a second course of treatment starting on days 193 and 200, respectively. Blood samples for PCR, IFA, flow cytometry and ear prick for blood smear examination were collected at multiple time points during the three phases of the study.

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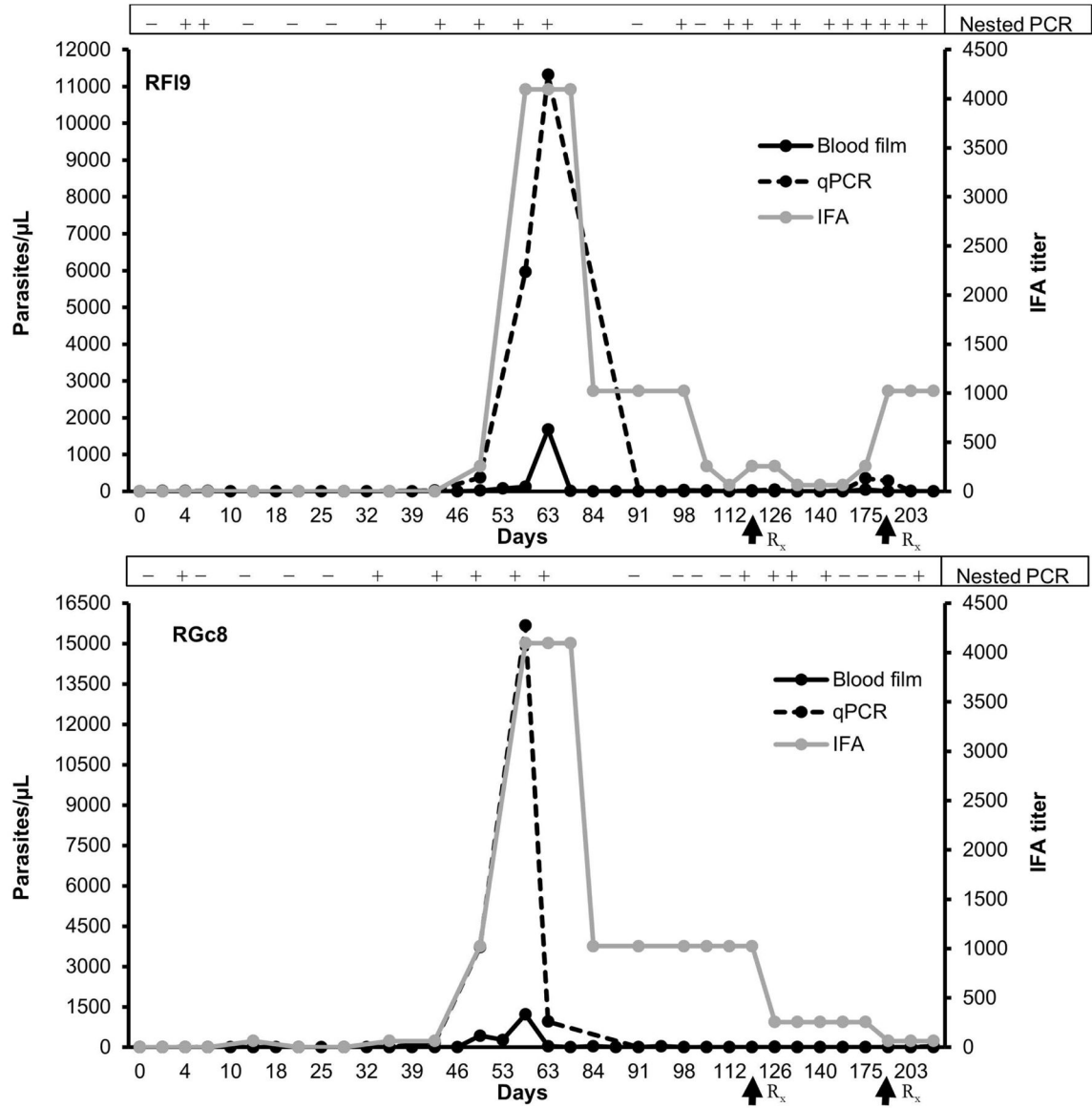


Figure 2. Course of parasitemia in the two phase 1 rhesus macaques (RF19, RGc8) transfused with hamster-passaged *B. microti*. The parasitemia values (left y axis) were compared with an IFA test titer (right y axis) and nested PCR. A titer of 1:64 with *B. microti* antigen was considered a positive reaction. Parasitemia levels determined on thick blood smears and qPCR were expressed as parasites/μL. Nested PCR results are reported as positive or negative. Arrows indicate initiation of atovaquone/azithromycin treatment.

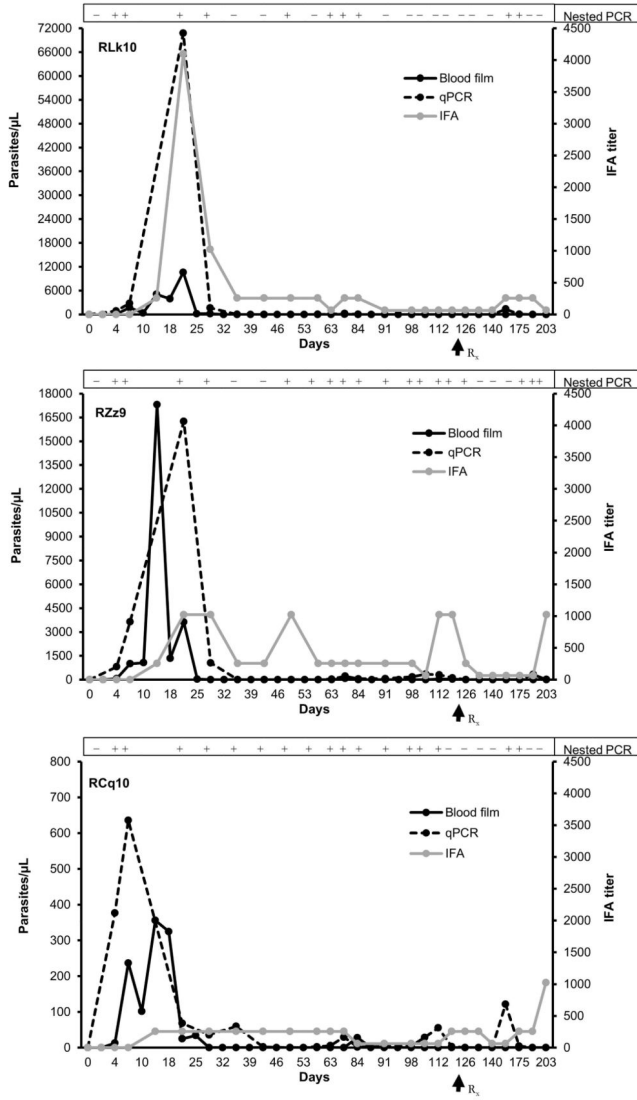


Figure 3. Parasitemia in the three phase 2 rhesus macaques (RLk10, RZz9, and RCq10) transfused with monkey-passaged *B. microti*. The parasitemia values (left y axis) were compared with an IFA test titer (right y axis) and nested PCR. A titer of 1:64 with *B. microti* antigen was considered positive. Parasitemia levels determined on thick blood smears and qPCR were expressed as parasites/μL. Nested PCR results are reported as positive or negative. Arrows indicate initiation of atovaquone/azithromycin treatment.

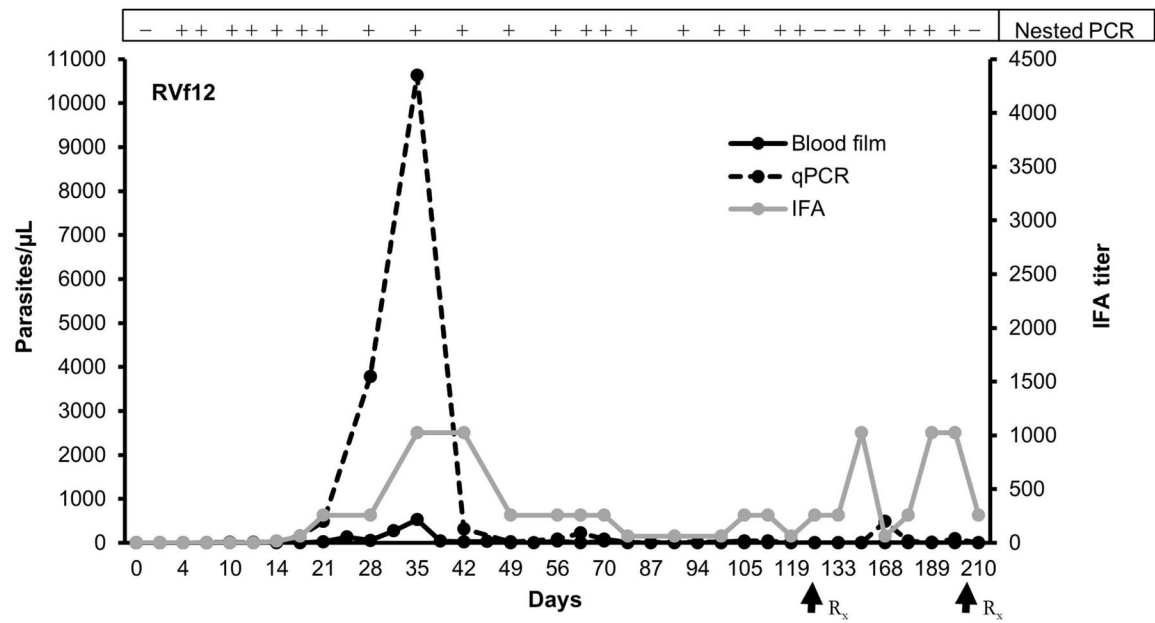


Figure 4. Parasitemia in the one phase 3 rhesus macaque (RVf12) transfused with monkey-passaged *B. microti*. The parasitemia values (left y axis) were compared with an IFA test titer (right y axis) and nested PCR. A titer of 1:64 with *B. microti* antigen was considered positive. Parasitemia levels determined on thick blood smears and qPCR were expressed as parasites/ μL . Nested PCR results are reported as positive or negative. Arrows indicate initiation of atovaquone/azithromycin treatment.

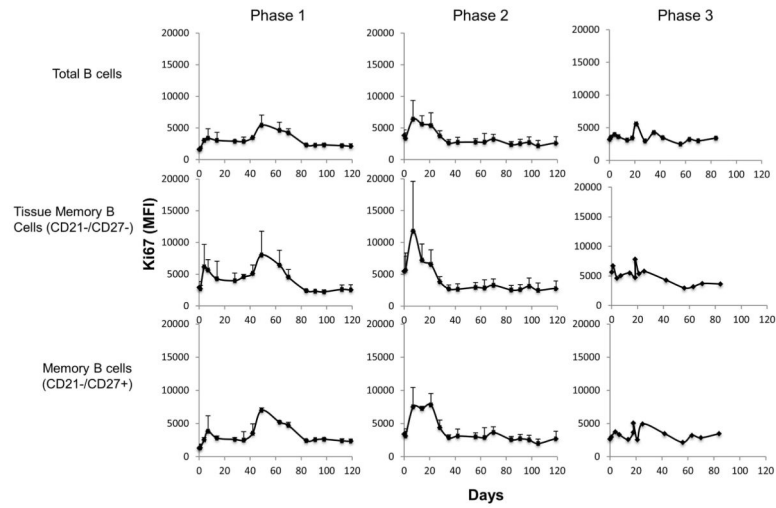


Figure 5. Activation of total and subpopulations of B cells in rhesus macaques during the course of the three phases of the study of *B. microti* infection. Lines represent the average Ki67 MFI for each phase with the error bars indicating standard deviation. MFI: mean fluorescence intensity.

Table 1

Kinetics of parasitemia and antibody titers in the rhesus macaques infected with *B. microti* during the three phases of the study

Screening Test	<u>Hamster-passaged <i>B. microti</i></u>	<u>Monkey-passaged <i>B. microti</i></u>	
	Phase 1	Phase 2	Phase 3
qPCR parasitemia *			
First detection	35	4	4
Peak detection	56–63	14–21	35
Blood smear parasitemia *			
First detection	49	4	21
Peak detection	56–63	14–21	35
IFA titer *			
First response	35–49	14	21
Peak response	56	21	35

* Data correspond to respective study day(s) in the particular phase (Phase 1: monkeys RF19 and RGc8; Phase 2: monkeys RLk10, RZz9, and RCq10; Phase 3: monkey RVf12)

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