Development of Specific Immunoglobulin Ga (IgGa) and IgGb Antibodies Correlates with Control of Parasitemia in *Babesia equi* Infection

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Received 8 September 2005/Returned for modification 13 October 2005/Accepted 12 December 2005

In this study, the kinetics of specific immunoglobulin G (IgG) isotypes were characterized in *Babesia equi* (*Theileria equi*)-infected horses. IgGa and IgGb developed during acute infection, whereas IgG(T) was detected only after resolution of acute parasitemia. The same IgG isotype profile induced during acute infection was obtained by equi merozoite antigen 1/saponin immunization.

*Babesia equi*, also described as *Theileria equi* (18), is a tick-transmitted protozoan that infects horses. The parasite replicates within erythrocytes and causes anemia, which characterizes the acute stage of infection. Following resolution of acute parasitemia, horses remain life-long carriers but develop a long-term protective immunity against clinical disease (20, 23). Acquired immune responses are necessary to control parasitemia, although lysis of erythrocytes does not require adaptive immunity (11). Thus, following lysis of infected erythrocytes, parasites are accessible to antibody and associated mechanisms of antibody-mediated killing. Protective mechanisms associated with antibodies include neutralization of extracellular parasites by blocking of cell entry or opsonization of infected erythrocytes and complement lysis of either organisms or infected cells (6). Although high titers of antibody alone are not able to protect against *Babesia* infection, they are correlated with reduction of replication and clearance of the parasite (5, 7, 8).

Seven genes for immunoglobulin G (IgG) heavy chain constant in horses have recently been described (24); however, only four IgG antibody isotypes have been characterized in equine serum, IgGa, IgGb, IgGc, and IgG(T). IgGa and IgGb antibodies are cytotoxic and capable of both complement activation and opsonization, while IgG(T) and IgGc are noncytotoxic and may block the protective effects of cytotoxic antibodies by competitive binding to antigen (15, 17). In this study, the kinetics of the most prevalent IgG isotypes in horse serum, the IgGa, IgGb, and IgG(T) antibodies (22), were characterized during acute and chronic *B. equi* infection; the potential use of these isotypes as immunological markers for infection control and vaccine development is discussed.

Mixed-breed ponies, between 2.5 and 4 years of age and negative for *B. equi* as tested by competitive enzyme-linked immunosorbent assay (ELISA) (12) and nested PCR (23), were experimentally infected with *B. equi* by two methods: parenteral inoculations and tick transmission. The use of horses in this study complied with all relevant national guidelines and Washington State University policies. A stabilate of a *B. equi* isolate obtained in Florida (13) was used to infect horses and also to prepare the antigen for ELISA. For the parenteral inoculation group, four ponies were inoculated three times at 21-day intervals. The first injection was intravenous and contained 10^9 infected red blood cells; the second and third injections were intravenous and subcutaneous, respectively, and each contained 10^7 infected red blood cells. Two horses were infected with *B. equi* by experimental *Boophilus microplus* tick transmission as described elsewhere (12). During the experiments, the horses were regularly monitored for clinical signs and percentages of parasitized erythrocytes (PPE). Following *B. equi* infection, all horses, infected by either method, had transitory parasitemia (Fig. 1) and developed acute signs of infection, including fever and a decrease in packed cell volume (data not shown).

The titers of IgG antibodies in sera were determined by ELISA, using *B. equi* merozoite extract as the antigen (13) and monoclonal antibodies anti-IgGa (CVS48), anti-IgGb (CVS39), and anti-IgG(T) (CVS40) (15, 21). To validate the ELISA, both the optimal antigen amount and the monoclonal antibody dilutions that showed the maximum difference in absorbance values between the reference positive and negative sera were determined by checkerboard titration with reference sera diluted 1:10. The ELISA was adapted from a protocol described previously (13), with the following modifications: several 10-fold dilutions (from 1:10 to 1:100,000) of each horse
After washing, 0.5 μg/ml of each anti-equine isotype monoclonal antibody was used to detect the horse IgG isotype bound to the antigen. Six normal equine control serum samples were run in duplicate on each plate and used to calculate the threshold, which resulted in an absorbance value equal to the threshold, was calculated from the best fit line of the absorbancies versus the reciprocal of dilutions for each serum sample.

The statistical analyses in this study were performed using NCSS 2001 software (NCSS, Kaysville, UT), and a P value of \( p \leq 0.05 \) was considered significant.

As summarized in Table 1, horses infected by parenteral inoculation developed IgGa antibodies at 1 week postinfection (WPI), followed by IgGb antibodies at 3 WPI. Both IgGa and IgGb antibodies were detected at 3 WPI in the experimental tick transmission group. IgG(T) antibodies were detected only after resolution of acute parasitemia, at 4 WPI in the horses infected by parenteral injection and between 9 and 14 WPI in those infected by tick transmission. The profiles of IgG isotype antibody responses from 0 to 14 WPI in both groups were significantly different (\( P < 0.01 \)), as analyzed by multivariate analysis of variance (MANOVA). By analyzing each isotype antibody individually (Hotelling-Lawley test), it was determined that this significance resulted only from titers of IgGa antibody (\( P < 0.01 \)). As illustrated in Fig. 1, the delayed appearance of IgGa in the horses infected by tick feeding was probably related to the longer prepatent period (13 days) presented by the group, which was significantly longer than that in the group infected by parenteral injection (4.7 days), as determined by ANOVA (\( P < 0.01 \)). After primary infection, the horses infected parenterally resolved the parasitemia in a mean of 11 days postinfection, and after the second and third B. equi injections, the PPE was significantly lower than that following primary infection (a period of 4 days prior to and 4 days after the parasitemia peak was considered for analysis; \( P < 0.01 \)). These data indicate that the development of the cytophilic antibodies, IgGa and IgGb, correlate with parasitemia control in B. equi infection.

Regardless of the method of infection, all three isotypes were present during chronic infection. Fifty serum samples from carrier field horses, previously known to be positive for B. equi by a complement fixation test and competitive ELISA (9, 13), were also tested to characterize the IgG isotype response during chronic B. equi infection. The average titers (plus or minus standard deviations) of IgGa, IgGb, and IgG(T) antibodies in these naturally infected horses and in the horses experimentally infected in this study, tested at 22 WPI (parenteral inoculation) and 39 WPI (tick transmission), were 5,947 (±1,300), 12,028 (±4,959), and 6,954 (±3,474), respectively. The IgG isotype profiles were similar in all groups, as tested by MANOVA (\( P > 0.05 \)), indicating that the isotypes produced

### Table 1. ELISA titers of IgGa, IgGb, and IgG(T) antibodies in horses experimentally infected with B. equi

<table>
<thead>
<tr>
<th>WPI</th>
<th>Parenteral injection</th>
<th>Tick transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgGa (SD)</td>
<td>IgGb (SD)</td>
</tr>
<tr>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1</td>
<td>336 (497)*</td>
<td>25 (33)</td>
</tr>
<tr>
<td>3–4</td>
<td>885 (236)*</td>
<td>507 (834)*</td>
</tr>
<tr>
<td>9</td>
<td>6,422 (1,993)*</td>
<td>22,226 (19,505)*</td>
</tr>
<tr>
<td>14</td>
<td>5,427 (1,707)*</td>
<td>23,805 (20,182)*</td>
</tr>
<tr>
<td>18</td>
<td>6,359 (3,349)*</td>
<td>17,284 (11,688)*</td>
</tr>
<tr>
<td>22</td>
<td>7,316 (2,111)*</td>
<td>24,332 (23,556)*</td>
</tr>
</tbody>
</table>

\( * \), significant difference compared with the preinfection titer (0 WPI), as determined by ANOVA (\( P < 0.05 \)).

\( b \) Data obtained at 4 weeks postinfection.
during the chronic stage of _B. equi_ infection are not affected by the mode of transmission of the parasite.

The importance of cytophilic antibodies in controlling pathogen replication in hemoparasitic, viral, and bacterial infections has been reported previously (1, 3–5, 7, 14, 16, 19). Since the induction of cytophilic antibodies is dependent on the production of gamma interferon by T cells (2, 14), the IgG isotype profile may reflect the overall type of immune response (Th1 or Th2), as recently demonstrated for _Rhodococcus equi_ infection (14). Herein, we showed that the _B. equi_-specific cytophilic antibodies, IgGa and IgGb, are correlated with parasitemia control, suggesting that the IgG isotype profile might also be useful as a potential immunological marker for _B. equi_ infection control and vaccine development. To test this hypothesis, we showed that the IgG antibody isotype profile induced by infection can be produced by a subunit antigen, recombinant equi merozoite antigen 1 (rEMA-1) in saponin was tested as an immunization model. EMA-1 is an immunodominant surface protein of _B. equi_ which induces the first measurable antibody response in horses during control of acute parasitemia (11, 12). rEMA-1 was obtained and purified as previously reported (10, 12), and four horses (mixed-breed ponies, 3 to 6 years old) were immunized eight times with 80 g of the protein (54 g in the last immunization) in phosphate-buffered saline containing 6 mg of Quil A saponin during a period of 40 months. Specific _B. equi_ IgGa and IgGb antibodies were induced by rEMA-1/saponin immunization, with no measurable IgG(T) (Fig. 2). After the first three immunizations, the titers of IgGa and IgGb antibodies were significantly higher than the preimmunization titers (_P_ < 0.001). These results indicate that the IgG isotype profile associated with control of acute infection can be induced by subunit immunization.

Since the horses did not produce IgG(T) antibodies following immunization, their ability to produce IgG(T) was tested by infecting them with 10⁹ live _B. equi_ erythrocyte-stage parasites by intravenous injection at 10 days after the last immunization. All horses developed IgG(T) antibodies following _B. equi_ infection, with a mean titer of 3,058 at 2 WPI. Although rEMA-1 immunization induced high titers of IgGa and IgGb (Fig. 2), the horses developed clinical disease following _B. equi_ inoculation (data not shown). Several reasons related to the antigen used may explain this outcome. For instance, it is not known whether rEMA-1 is a replica of native EMA-1 for induction of immunity or whether native or recombinant EMA-1 is capable of inducing the necessary repertoire of protective immunity. These results reinforce the potential use of IgG isotypes as immunological markers for infection control and vaccine development.

In conclusion, this study demonstrated the kinetics of IgG isotypes in _B. equi_ infection and the correlation of IgGa and IgGb antibodies with control of parasitemia. It was also shown that the same IgG isotype profile developed during acute infection can be induced by a subunit immunization using saponin as an adjuvant.

We thank Ralph Horn and Will Harwood for the excellent technical assistance.

This work was supported by USDA-ARS CRIS 5348-32000-020-00D. C. W. Cunha was supported by the CAPES Foundation through the Brazilian government.


