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Protective Effects of Passively Transferred Merozoite-Specific Antibodies against *Theileria equi* in Horses with Severe Combined Immunodeficiency

Robert H. Mealey,[a] Lowell S. Kappmeyer,[b] Massaro W. Ueti,[b] Bettina Wagner,[c] and Donald P. Knowles[a,b]

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA[a]; Animal Disease Research Unit, USDA Agricultural Research Service, Pullman, Washington, USA[b]; and Department of Population Medicine and Diagnostic Science, Cornell University, Ithaca, New York, USA[c]

Theileria equi **immune plasma was infused into young horses (foals) with severe combined immunodeficiency. Although all foals became infected following intravenous challenge with homologous *T. equi* merozoite stabilate, delayed time to peak parasitemia occurred. Protective effects were associated with a predominance of passively transferred merozoite-specific IgG3.**

Theileria equi is a tick-transmitted apicomplexan hemoprotozoan parasite of horses worldwide and is one of the etiologic agents of equine piroplasmosis (EP). The United States had been considered free of EP prior to the *T. equi* outbreaks in Florida and Missouri in 2008 and 2009 (1) and the current *T. equi* outbreak in Texas (14). Acute *T. equi* infections are characterized by hemolytic disease of varying severity, and horses that survive acute infection remain infected for life, serving as reservoirs for transmission to naïve horses.

In contrast to immunocompetent foals, foals with severe combined immunodeficiency (SCID) rapidly develop high levels of parasitemia and severe clinical disease following inoculation with *T. equi*-parasitized erythrocytes, indicating that adaptive immune responses are required for control (3). Equine SCID is caused by a frameshift mutation in the gene encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (15, 24), resulting in a complete lack of functional B and T lymphocytes (8). Because innate immunity is intact in SCID foals (3, 7, 12, 13), the above results also emphasize the inability of innate immune responses to control *T. equi* parasitemia.

Although the precise mechanisms of adaptive immune control of *T. equi* are not known, both humoral and cell-mediated responses are likely involved. In immunocompetent horses, the development of merozoite-specific IgGa and IgGb during acute *T. equi* infection correlates with control of parasitemia, while merozoite-specific IgG(T) appears only after resolution of parasitemia (1a). Equine IgG subclasses have been reassigned such that IgGa corresponds to IgG1, IgGb corresponds to IgG4 and IgG7, and IgG(T) primarily corresponds to IgG5 and, to a lesser extent, IgG3 (20–22). Since IgG1, IgG3, IgG4, and IgG7 all bind complement and interact with Fc receptors (6), complement activation and opsonization by *T. equi* merozoite-specific antibodies likely play important roles in resolution of acute parasitemia and maintenance of long-term control (1a). In addition, vaccination with a killed merozoite immunogen results in reduced parasitemia and clinical disease in donkeys undergoing lethal *T. equi* challenge (5). Protective effects are associated with high titers of whole merozoite antigen-specific antibodies and merozoite antigen-specific lymphocyte proliferative responses (5), suggesting that both antibody and cell-mediated responses contribute to *T. equi* immune control. However, studies dissecting the relative roles of antibodies and T lymphocytes in protection against *T. equi* infection have not been done.

The current study was designed to test the hypothesis that humoral immune responses would independently control *T. equi* replication. Because SCID foals lack functional B and T cells, they provide a powerful and unique opportunity to finely dissect the protective effects of immune interventions against *T. equi* in the complete absence of other *de novo* adaptive immune responses. The SCID foals used in this study were obtained by selective breeding of Arabian horses heterozygous for the SCID trait (3, 10, 11, 16). Foals were approximately 1 month of age and included six experimental animals and three control animals. The six experimental SCID foals (E1-S, E2-S, E3-S, E4-T, E5-T, and E6-B) received intravenous (i.v.) infusions of immune plasma prior to and after *T. equi* challenge. Two SCID foals (C1 and C2) were inoculated with *T. equi* (but received no plasma infusions) as part of a previous study (3) and served as historical controls for the *T. equi* merozoite-parasitized erythrocyte stabilate inoculum. A third control SCID foal (C3) received nonimmune normal horse plasma prior to and after *T. equi* challenge. Five immunocompetent horses persistently infected with the same *T. equi* Florida isolate (4) were used as immune plasma donors in this study. Horses H024 and H026 had been inoculated i.v. with 1×10^9 parasitized erythrocytes 1 year before immune plasma was obtained. Horses H059, H072, and H076 were infected by *Rhipicephalus microplus* tick transmission (18), also 1 year before immune plasma was obtained. All experiments using horses and foals were approved by the Institutional Animal Care and Use Committee.

Control SCID foal C3 received five one-liter infusions of pooled nonimmune preinfection plasma obtained from horses H024 and H026. Experimental SCID foals E1-S, E2-S, and E3-S received five one-liter infusions of pooled immune plasma from stabilate-inoculated horses H024 and H026, while experimental SCID foals E4-T and E5-T received eight one-liter infusions of pooled immune plasma from tick transmission-infected horses H059, H072, and H076. Finally, experimental SCID foal

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Address correspondence to Robert H. Mealey, rhm@vetmed.wsu.edu.

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TABLE 1 SCID foals, plasma donor horses, and plasma infusion schedules

SCID foal	Immune plasma donors[a]	Days plasma was infused (dpi)[b]
Controls		
C1	None	None
C2	None	None
C3	None	−4, −2, 0, 2, 5
Experimental animals		
E1-S	H024, H026	−4, −2, 0, 2, 5
E2-S	H024, H026	−4, −2, 0, 2, 5
E3-S	H024, H026	−4, −2, 0, 2, 5
E4-T	H059, H072, H076	−4, −2, 0, 2, 5, 7, 9, 11
E5-T	H059, H072, H076	−4, −2, 0, 2, 5, 7, 9, 11
E6-B	H024, H026, H059	−4, −2, 0, 2, 5, 7, 9, 11, 13

[a] Control SCID foal C3 received nonimmune plasma obtained from horses H024 and H026 before they were infected with *T. equi.* Immunocompetent horses H024 and H026 were infected with *T. equi* by i.v. inoculation of parasitized erythrocytes, 1 year before plasma was obtained. Immunocompetent horses H059, H072, and H076 were infected with *T. equi* by tick transmission 1 year before plasma was obtained.
[b] On each infusion day, donor plasma was pooled and a total of one liter was infused i.v. dpi, days post-*T. equi* inoculation.

E6-B received nine one-liter infusions of pooled immune plasma from stabilate-inoculated horses H024 and H026 and tick transmission-infected horse H059 (Table 1). Four hours after the third plasma infusion, all SCID foals were inoculated i.v. with the same Florida *T. equi* strain (4) used as described above to infect the plasma donors, using 2 ml blood stabilate containing 49% merozoite-parasitized erythrocytes. This was the same stabilate used to inoculate the two historical SCID controls C1 and C2 (3).

Body temperature and overall clinical status were monitored daily in all foals, as were packed cell volume (PCV) and percent parasitized erythrocytes (PPE) determined by microscopic examination of blood smears (1, 3). Previous work confirmed that during the rise in *T. equi* parasitemia, between 9 and 15 days postinoculation (dpi), quantitation of parasites in the blood by microscopic examination closely agrees with real-time PCR (17). The study endpoint for all foals was humane euthanasia, which was performed when the following occurred: PCV of $\leq$15 with concurrent PPE of $>$20 and/or advanced progressive clinical disease. All statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) and a significance level (α) of 0.05.

Passively transferred *T. equi* merozoite antigen-specific antibody titers in SCID foal sera, including identification of merozoite-specific IgGa, IgGb, and IgG(T) subclasses, were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (1, 4). The same was done for the donor-derived immune plasma used for infusion into each SCID foal recipient. Murine monoclonal antibodies (MAbs) CVS48, CVS39, and CVS38 (AbD Serotec, Raleigh, NC) were used to identify IgGa, IgGb, and IgG(T), respectively. Anti-IgGa CVS48 recognizes recombinant equine IgG1 exclusively, anti-IgGb CVS39 recognizes recombinant IgG4 and IgG7, while anti-IgG(T) CVS38 strongly recognizes recombinant IgG5 but also displays some weak recognition of recombinant IgG3 (6). In addition, MAbs 159-4 and 416-2 were used to identify IgG3 and IgG5, respectively (2, 23). Sera (or plasma) were diluted 1:500 for all merozoite-specific IgG subclass ELISAs.

With the exception of E6-B, all foals became parasitemic within 7 to 12 dpi, with the peak PPE ranging between 20 to 49% and occurring between 9 and 15 dpi (Fig. 1a). Ascending parasitemia was associated with fever (rectal temperature $>$ 101.5°F) and a corresponding decrease in PCV in all foals (data not shown). Although all experimental foals eventually developed parasitemia and clinical disease, infusions of immune plasma resulted in a significantly delayed time to peak PPE (Fig. 1b) compared to that of controls. Compared to control foals, time to peak PPE was delayed in foals infused with immune plasma from stabilate-infected donors but not in foals infused with immune plasma from tick transmission-infected donors (Fig. 1c). Interestingly, the time to peak PPE in foal E6-B (infused with immune plasma from both stabilate- and tick transmission-infected donors) exceeded the 95% upper confidence interval limit for the other groups by 9 days (Fig. 1c), which was considered significant. In this foal, low-level parasitemia was detectable by dpi 12, but ascending parasitemia did not begin until 21 dpi, with a peak PPE of 26% occurring on dpi 27 (Fig. 1a).

Immune plasma infusions resulted in passively transferred merozoite-specific serum antibodies that were detected with similar rates of peak acquisition and subsequent decay (Fig. 1d). Peak titers occurred after 5 to 8 infusions and ranged from 5,300 to 10,000. The exceptions were foals E4-T and E5-T, which received immune plasma derived from the tick transmission-infected donors. Merozoite-specific serum antibody titers in these two foals remained below 2,300. Of the experimental foals, these two foals had the shortest times to peak parasitemia (which were not significantly different from the control values; see above) and among the highest PPE levels (Fig. 1a). Interestingly, IgG(T)/IgG5 comprised the predominant passively transferred merozoite-specific serum IgG subclass in these two foals (Fig. 2d and e), while IgG3 was the predominant subclass in the other four experimental foals (Fig. 2a to c and f). Compared to the other experimental foals, foals E4-T and E5-T had the lowest relative levels of passively transferred merozoite-specific serum IgG3, IgGa (IgG1), and IgGb (IgG4 and IgG7) and the highest relative levels of merozoite-specific serum IgG(T)/IgG5. There were no clear differences in merozoite-specific serum antibody titers or IgG subclasses among the other four experimental foals, which included the most significantly protected foal, E6-B. The passively transferred merozoite-specific antibody titers and relative levels of the different merozoite-specific IgG subclasses in each foal correlated with those in the corresponding infused immune plasma (data not shown). No merozoite-specific serum antibodies were detected in any of the three control foals (data not shown).

Our results indicated that in SCID foals completely lacking the ability to mount adaptive immune responses, passive transfer of immune plasma containing *T. equi* merozoite-specific antibodies significantly delayed the onset of peak parasitemia and clinical disease following homologous *T. equi* merozoite stabilate challenge. These protective effects were most evident in foal E6-B, which was infused with immune plasma derived from both stabilate-inoculated and tick transmission-infected donor horses. Although the mechanisms contributing to the greater protective effects observed in E6-B are not precisely known, the combination of antibodies elicited by the two different modes of infection in the plasma donor horses may have resulted in enhanced targeting of

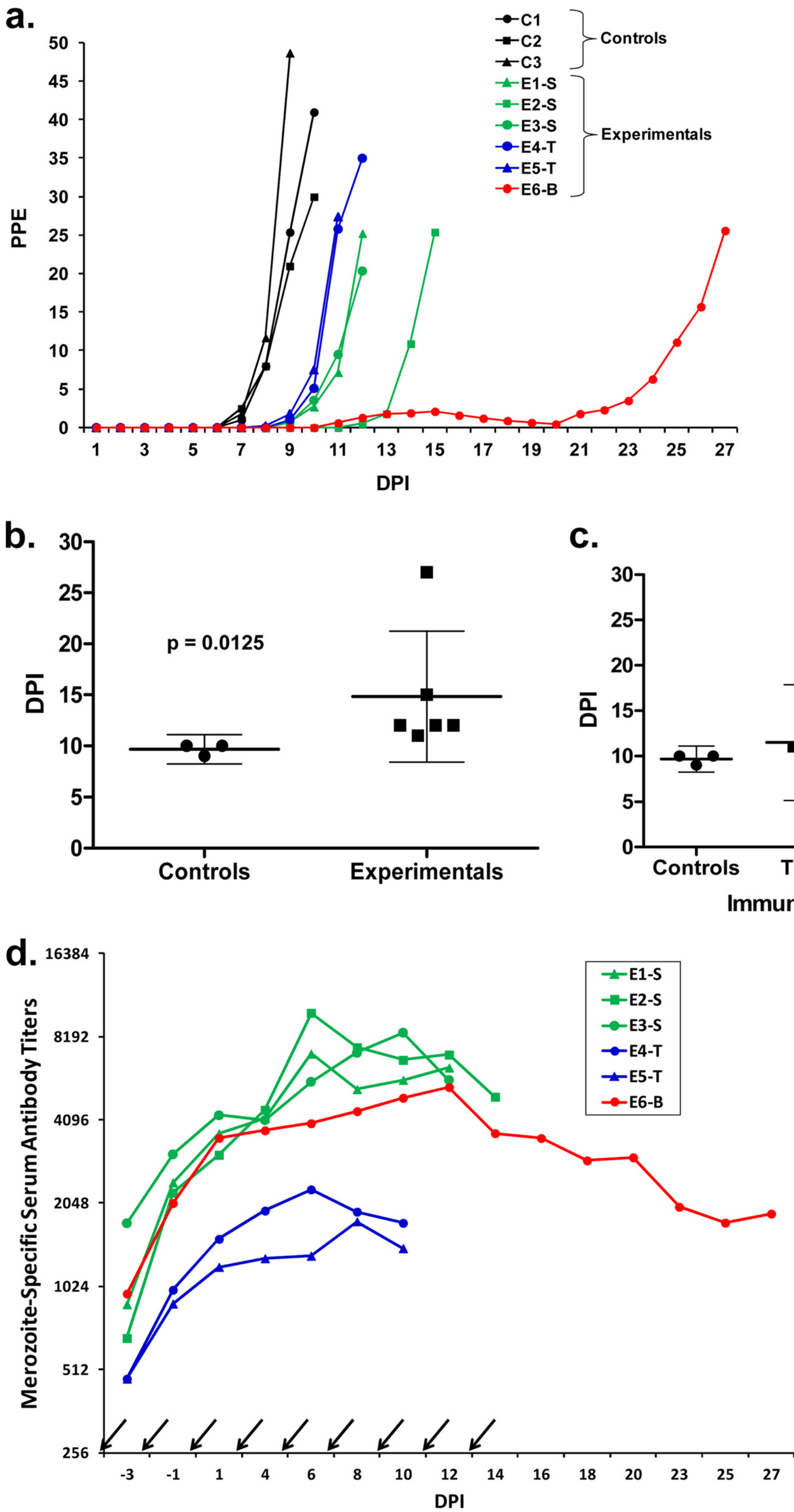

FIG 1 (a) Levels of parasitemia in SCID foals following i.v. inoculation of *T. equi* blood stabilate. PPE, percent parasitized erythrocytes as determined by microscopic evaluation of blood smears stained with Giemsa stain; dpi, days post-*T. equi* inoculation. (b) Mann-Whitney comparison of times to peak parasitemia (in days) in control and experimental foals (one-tailed *P* value). Horizontal lines are means, and error bars are 95% confidence intervals (CI). (c) One-way analysis of variance (ANOVA) (with Tukey's test for multiple comparisons) of times to peak parasitemia (in days) between groups based on the donor source of the immune plasma. Error bars are 95% CI. The asterisk indicates a *P* value of <0.05 in comparison with the control group. (d) Passively transferred *T. equi* merozoite-specific serum antibody titers in experimental SCID foals as measured by ELISA. Arrows at the *x* axis indicate days that immune plasma was infused.

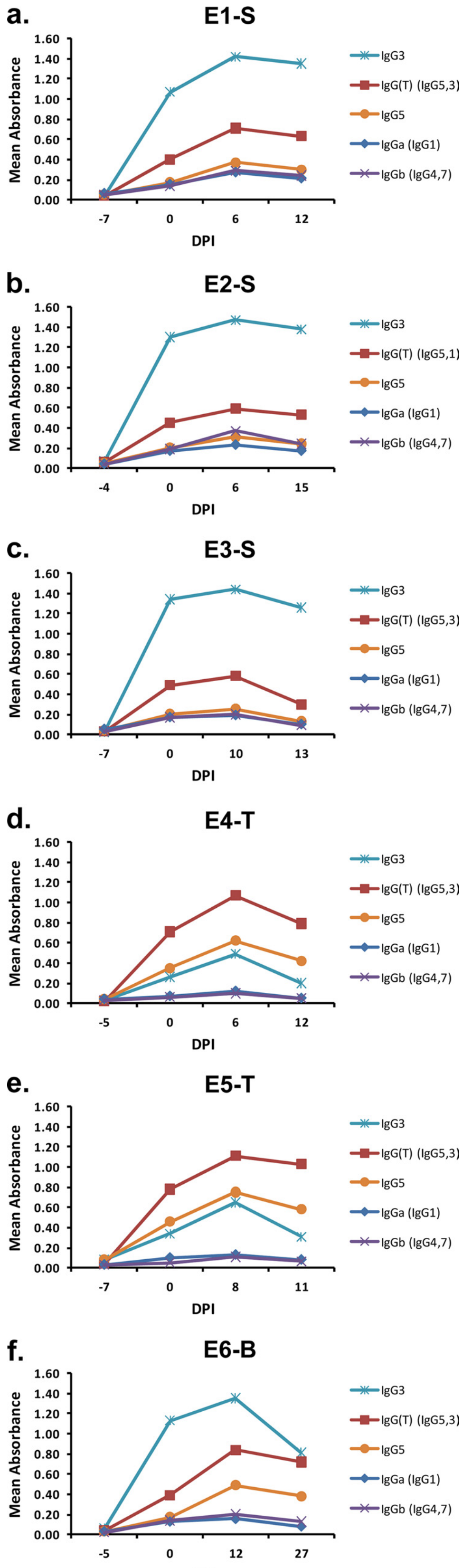

FIG 2 (a to f) Mean absorbance at 620 nm as measured by ELISA for passively transferred *T. equi* merozoite-specific IgG subclasses in sera from each experimental SCID foal collected at various time points. Sera were diluted 1:500.

protective epitopes. Merozoite-specific IgG3 was the predominant passively transferred IgG subclass in sera from experimental foals with the highest level of protection, suggesting that this subclass is important for control of parasitemia. This observation is not surprising, given that IgG3 is capable of triggering a strong respiratory burst via Fc receptor binding and that it is the most potent activator of complement of all the equine IgG subclasses (6). It is also not surprising that the two foals with the lowest levels of protection had the lowest serum levels of IgG3, IgG1, IgG4, and IgG7 (all capable of Fc binding and complement activation) and the highest relative levels of IgG(T)/IgG5, neither of which activates complement (6, 9). Assuming these mechanisms are important for *T. equi* merozoite clearance, it is possible that more profound and consistent protective effects would have been observed had the immune plasma infusions resulted in higher levels of IgG3, IgG1, IgG4, and IgG7. Based on our previous work, immune plasma harvested from infected donors shortly after resolution of acute parasitemia (as opposed to a year afterward, as was done here) would likely contain the highest levels of these IgG subclasses (1a) and could result in enhanced protective effects. Although the present results are encouraging, it is probable that the most robust protection against natural tick-borne *T. equi* infection requires both antibody and T cell responses directed not only against erythrocyte merozoites but also against the earlier sporozoite stages.

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