Reduced red blood cell destruction by antibody fragments

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Antibodies to blood group antigens can cause immune RBC destruction directly (extravascular destruction) or indirectly through subsequent complement activation (intravascular hemolysis). The Fc portion of the IgG antibody is responsible for the effector functions of immune RBC destruction. We hypothesized that sensitization of RBCs with blood group antigen–specific IgG antibodies lacking their Fc portion would escape from the recipient's immune system, allowing for a longer survival period of the RBCs in the circulation. Direct injection of mouse RBC-specific Ter-119 monoclonal antibody into mice resulted in a more severe anemia compared with that in mice injected with the Ter-119 F(ab´)$_2$ fragment. We found that mouse RBCs coated in vitro with the Ter-119 F(ab´)$_2$ fragment, when transfused into mice, survived longer in circulation compared with RBCs coated with whole Ter-119 IgG molecule. The data support the conclusion that antibodies can be rendered less pathogenic through removal of their Fc portion. ***Immunohematology* 2006;22:11–14.**

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Alloantibodies and autoantibodies to blood group antigens can cause immune RBC destruction, resulting in transfusion reactions and autoimmune hemolytic diseases.[1] Antibodies to human glycophorin A (GPA)–associated antigens can cause hemolytic transfusion reactions and HDN.[2] Ter-119, a rat IgG2b monoclonal antibody, is a mouse erythroid-specific antibody that recognizes GPA3 and causes autoimmune hemolytic anemia after injection into mice.[4] This indicates that the Ter-119 antibody pathogenicity is analogous to that of human GPA antibodies. Because the Fc portion of the antibody molecule mediates its effector functions, including binding to Fc receptors and complement components,[5] we hypothesized that blood group–specific antibodies would be rendered nonpathogenic by removal of their Fc domains. We chose to use the F(ab´)$_2$ fragment as compared with the monovalent Fab fragment because of its higher binding affinity (avidity) for the target antigens. Although the use of antibody fragments directed against a variety of antigens has been described, there have been only two published studies that addressed their in vivo application for antibodies directed against RBC antigens. Specifically, in two previous reports, the in vivo effects of F(ab´)$_2$ prepared from anti-D were compared to whole IgG but conflicting results had been obtained.[6,7] In one report, the antibody fragment preparation resulted in complete clearance of D+ RBCs,[7] whereas in the other report, only about one-third of the RBCs were destroyed.[6] The explanation given for these unexpected results was that the antibody fragment preparations used for the studies had different degrees of contamination with intact IgG molecules, which were responsible for RBC destruction.

In this study, we compared the in vivo effects of intact Ter-119 antibody with those of a highly purified preparation of its F(ab´)$_2$ fragment. We found that direct injection of Ter-119 antibody into mice resulted in a more severe anemia compared with that observed in mice injected with the F(ab´)$_2$ fragment. In addition, mouse RBCs sensitized in vitro with the Ter-119 F(ab´)$_2$ fragment exhibited prolonged survival in vivo as compared with RBCs coated with the whole IgG molecule.

Materials and Methods

Injection of antibodies into mice

Ter-119 whole IgG and its F(ab´)$_2$ fragment were purchased (BD Pharmingen, San Diego, CA). Ter-119 F(ab´)$_2$ fragment had approximately 1 percent IgG contamination as determined by SDS-PAGE and HPLC analysis (data not shown). Eight to 10-week-old C57BL/6 mice were injected intraperitoneally with nonagglutinating doses of Ter-119 whole IgG molecule or its F(ab´)$_2$ fragment. Blood samples (25 µl) were obtained by retro-orbital sinus bleeding at the time points indicated after antibody injection and whole blood count was performed on the samples using Advia 120 Hematology System (Bayer, Tarrytown, NY).

Transfusion of antibody-sensitized RBCs into mice

Mouse RBCs were opsonized in vitro with Ter-119 or its F(ab´)$_2$ fragment by incubating 1 × 10^9/mL RBCs with various nonagglutinating concentrations of antibody for 30 minutes at 37°C (data not shown). The sensitized RBCs were checked by flow cytometry using a FITC-conjugated goat anti-rat IgG (Vector Laboratories, Burlingame, CA) to ensure uniform coating of all RBCs. In addition, the percentage of antigen sites that were occupied by the antibodies was measured by flow cytometry using a PE-conjugated Ter-119 antibody (BD Pharmingen). After they were labeled with PKH-26 (Sigma, St. Louis, MO) according to manufacturer's instructions, the antibody-coated RBCs were injected by the tail vein into 8- to 10-week-old C57BL/6 female mice. Blood samples were obtained by retro-orbital sinus bleeding at the time points indicated after transfusion and the clearance of fluorescent RBCs was measured by flow cytometry as previously described.[8] Ter-119 sensitization levels on the transfused RBCs were determined by two-color flow cytometry using FITC-conjugated rabbit anti-rat IgG heavy and light chains (Vector Laboratories) adsorbed to prevent any cross-reactivity with mouse immunoglobulins. We also checked the transfused RBCs for possible sensitization with mouse C3, IgG, or IgM components by flow cytometry but none were detected (data not shown).

Statistical analysis

The significance of differences between groups of mice was calculated using ANOVA; only p values less than 0.05 were considered significant.

Results

In a previous study by Jordan et al., it was shown that nonagglutinating doses of Ter-119 antibody caused anemia when injected into mice and that RBC destruction was mediated by erythrophagocytosis by splenic macrophages.[4] To compare the ability of RBC-specific Ter-119 antibody and its F(ab´)$_2$ fragment to cause anemia, C57BL/6 mice were directly injected with either whole IgG molecule or its F(ab´)$_2$ fragment. After a single injection of the antibodies at a nonagglutinating concentration (80 µg) in both groups of mice, all circulating RBCs of the mice were sensitized with the injected antibodies (Fig. 1A). In mice injected with the Ter-119 F(ab´)$_2$ fragment, there was a slight decrease in the Hb levels of all treated mice (n = 4) over a 24-hour period (Fig. 1B). In contrast, after

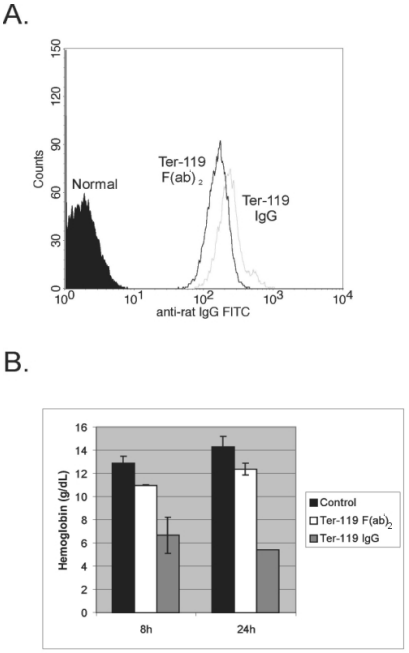

Fig. 1. Direct injection of Ter-119 whole IgG and its F(ab´)$_2$ fragment into mice. (**A**) A representative flow cytometric analysis of RBCs bled from C57BL/6 mice that had been injected with 80 µg Ter-119 whole IgG or F(ab´)$_2$ fragment at 8 hours postinjection using a goat anti-rat secondary antibody, demonstrating that all circulating RBCs were coated with the antibodies. RBCs from mice not injected with antibody ("Normal") were used as a control. (**B**) Mice were bled 8 and 24 hours after injection of 80 µg of the Ter-119 antibody (whole IgG or F[ab´]$_2$ fragment) in order to determine blood Hb concentration. Control mice (n = 4) were not injected with antibody (where n is the number of animals per group). One of four mice injected with Ter-119 whole IgG died by 8 hours postinjection and another two were dead by 24 hours. None of the mice (n = 4) injected with Ter-119 F(ab´)$_2$ fragment died. Data are presented as means ± standard errors.

injection of an equivalent amount of whole IgG Ter-119 antibody, one of four mice was dead by 8 hours postinjection and the other three had developed anemia. By 24 hours postinjection, only one mouse was alive and it had severe anemia (Fig. 1B). When we injected a lower concentration of the whole IgG Ter-119 (50 µg), all treated mice (n = 4) survived but developed anemia (Hb levels of 8 ± 1.2 g/dL at 24 h postinjection). These data indicate that the F(ab´)$_2$ fragment of Ter-119 is less pathogenic than the whole antibody molecule and that anemia caused by the RBC-specific Ter-119 antibody can be reduced by removal of its Fc portion.

We next opsonized equivalent numbers of mouse RBCs in vitro with different nonagglutinating concentrations (7 µg/mL, 5 µg/mL, or 3 µg/mL) of either Ter-119 or its F(ab´)$_2$ fragment and injected them into C57BL/6 mice. RBCs opsonized in vitro with 7 µg/mL or 5 µg/mL of the whole IgG Ter-119 antibody

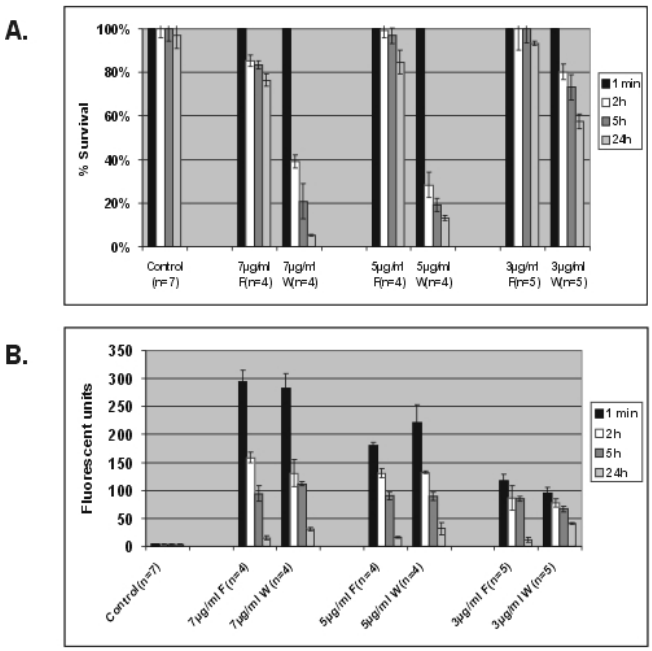

Fig. 2. Survival of transfused RBCs, opsonized in vitro with Ter-119 whole IgG or its F(ab´)$_2$ fragment, in mice. RBCs were opsonized in vitro with different concentrations of Ter-119 or its F(ab´)$_2$ fragment. Although all the RBCs were uniformly coated with all antibody concentrations tested, these antibody concentrations were nonsaturating and at 5 µg/mL antibody dose used to coat RBCs, about 50 percent of the target antigenic sites were still reactive with a fluorescently labeled Ter-119 antibody (data not shown). Following labeling with fluorescent dye PKH-26, the RBCs were injected intravenously at a final Hct of 25% in 200 µL into C57BL/6 mice. At times indicated, venous blood was sampled and analyzed by flow cytometry for the fraction of fluorescent RBCs. To show the clearance kinetics, injected RBCs at 1 minute posttransfusion were taken as 100 percent and the remaining RBCs were calculated at different time points as the average for each group of mice. Error bars depict the standard error of the mean (SEM). Survival of transfused RBCs opsonized in vitro with (**A**) 7 µg/mL, 5 µg/mL, and 3 µg/mL of Ter-119 whole IgG or Ter-119 F(ab´)$_2$ fragment in mice. Control (n = 7) mice were injected with an identical number of dye-labeled, but not opsonized, RBCs. Ter-119 whole IgG coated RBCs had lower survival rates at all concentrations tested over a 24-hour period ($p < 0.05$ as compared to control). The level of antibody coating of the transfused RBCs opsonized in vitro with (**B**) 7 µg/mL, 5 µg/mL, and 3 µg/mL of Ter-119 whole IgG or Ter-119 F(ab´)$_2$ fragment was measured by flow cytometry using a FITC-conjugated anti-rat IgG at indicated times posttransfusion and is presented in fluorescence intensity units on the y-axis as the average for each group of mice (error bars depict the SEM). As expected, the secondary antibody did not react with control, nonopsonized, transfused RBCs.

were found to be rapidly cleared from the circulation so that by 5 hours posttransfusion only about 20 percent of these RBCs remained in circulation (Fig. 2A and 2B). In contrast, RBCs that were opsonized with similar concentrations of the F(ab´)$_2$ fragment were only slightly reduced in numbers compared with control, nonopsonized RBCs (Fig. 2A and 2B). Even at lower (3 µg/mL) antibody concentrations, the Ter-119 coated RBCs were cleared more rapidly than those opsonized with similar concentrations of the F(ab´)$_2$ fragment (Fig. 2A). To ensure that the improved in vivo survival of transfused F(ab´)$_2$ -coated RBCs was not due to differences in the levels of antibody sensitization, we measured relative antibody levels on the transfused RBCs and found comparable levels of antibody coating on the circulating RBCs of both groups of mice (Fig. 2B). Thus, RBCs opsonized with the Ter-119 F(ab´)$_2$ fragment have a higher survival in vivo than whole IgG–sensitized RBCs.

Discussion

We have shown that RBC destruction by the RBC-specific Ter-119 antibody can be reduced by removal of its Fc portion. In addition, we demonstrated that RBCs opsonized with the F(ab´)$_2$ fragment of the Ter-119 antibody have a higher survival in vivo than RBCs opsonized with similar concentrations of whole IgG molecule. Although the protective effects of antibody fragments in vivo for RBC survival have been previously investigated, the results obtained were confounded by the contamination of their antibody fragment preparation with intact IgG.[6,7] Our experiments were performed with an F(ab´)$_2$ fragment preparation that was about 99 percent pure. Altogether, the data are consistent with the concept that the Fc portion of the antibody fragment mediates RBC destruction.

The data suggest that antibody fragments lacking the Fc portion may be used to escape recognition from the recipient immune system, although future studies are needed to assess the survival of F(ab´)$_2$-coated RBCs in mice that have preexisting antibodies. There is precedent for masking target antigens using F(ab´)$_2$ fragment. For example, direct injection of F(ab´)$_2$ fragment with specificity against human platelet antigens to patients with certain thromboembolic disorders induces dose-related inhibition of platelet aggregation by blocking the target antigen.[9] Although it remains to be tested, the data suggest that antibody fragments may have potential for masking blood group antigens to prevent antibody-mediated RBC destruction. In the future, using the transfusion protocols described here, we hope to test this possibility by directly injecting F(ab´)$_2$ fragments or Ter-119 F(ab´)$_2$-coated RBCs into mice with preexisting Ter-119 antibodies and measuring RBC survival.

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