Monoclonal antibodies that mimic the action of anti-D in the amelioration of murine ITP act by a mechanism distinct from that of IVIg

Seng Song, Andrew R. Crow, Vinayakumar Siragam, John Freedman, and Alan H. Lazarus

The mechanism of action of intravenous immunoglobulin (IVIg) and polyclonal anti-D–mediated reversal of immune thrombocytopenia (ITP) is still unclear. However, in a murine model of ITP, the therapeutic effect of IVIg appears to be wholly dependent upon the expression of the inhibitory Fc receptor, FcγRIIB. We previously demonstrated that, similar to anti-D in humans, 2 erythrocyte-reactive monoclonal antibodies (TER119 and M1/69) ameliorated murine ITP and inhibited reticuloendothelial system (RES) function at doses that protected against thrombocytopenia. The current study evaluated the involvement of the inhibitory and activating Fc receptors, FcγRIIB and FcγRIIA, respectively, in the TER119 and M1/69-mediated inhibition of thrombocytopenia. In contrast to IVIg, in FcγRIIB-deficient mice, both monoclonal antibodies ameliorated ITP and both significantly down-regulated the level of expression of the activating FcγRIIA in splenic macrophages. These results indicate that anti-erythrocyte antibodies that ameliorate ITP act independently of FcγRIIB expression but are dependent upon the activating FcγRIIA.

Study design

Mice

FcγRIIB−/− mice (B6;129S4-FcγR2Iβtm1Rav/J) and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the St Michael’s Hospital Research Vivarium. Induction and reversal of ITP

Thrombocytopenia was induced by daily intraperitoneal injection of 2 μg rat anti–mouse integrin αIIb antibody (PharMingen, Mississauga, ON, Canada) as previously described.16 On day 2, mice were injected intravenously with 50 μg monoclonal antibody TER119 or M1/69 (PharMingen) or intraperitoneally with 2 g/kg IVIg (Gamimune, 10%; Bayer, Elkhart, IN). Whole blood was collected daily via the saphenous vein into capillary tubes preloaded with 5 μL 1% EDTA (ethylene-diaminetetraacetic acid) in phosphate-buffered saline (PBS); pH 7.2; 50 μL blood was diluted in 1200 μL 1% EDTA in PBS (1:2.5), and centrifuged at room temperature at 170g for 2 minutes to isolate platelet-rich plasma (PRP). Fifty microliters of PRP was diluted into 9.95-mL Isoton II diluent (Couler Corporation, Miami, FL) and platelet count determined using a Beckman Z2 Coulter Counter (Couler Corporation).

Induction and prevention of erythrocyte clearance

To block erythrocyte clearance, mice were injected intravenously with 50 μg 2.4G2 (an FcγRIIB/FcγRIIA blocking antibody)19,20 or rat IgG as negative control, or intraperitoneally with 2 g/kg IVIg. After 2 hours, erythrocyte clearance was induced with the intravenous administration of 50 μg TER119. After a further 24 hours, the blood was collected and erythrocytes enumerated as previously reported.16

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Preparation of splenocytes and flow cytometric analysis

Twenty-four hours after treatment with TER119, M1/69, or 30-F1 (Phar-Mingen) or IVlg as indicated, the spleen was removed, mechanically disrupted in 5 mL PBS containing 0.5% bovine serum albumin (BSA), and filtered through 70-μm nylon mesh strainer.21 Erythrocytes were lysed using 0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2 EDTA (ACK) lysis buffer22 and washed in PBS/BSA. One million cells in 50 μL were incubated with a murine macrophage marker (PE-CY5–anti-F4/80; Cedarlane Laboratories, Hornby, ON, Canada) and fluorescein isothiocyanate (FITC)–2.4G2 antibody (PharMingen) for 30 minutes at 4°C with constant shaking. The cells were washed and acquired on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA).

Results and discussion

Salama et al23 initially postulated that the success of IVlg in treating ITP was due to reduced macrophage binding of sensitized platelets by competitive blockade from immunoglobulin-coated erythrocytes. This was supported by observations that infusion of IVlg in patients with ITP prolonged the clearance of radiolabeled anti–Rh (D)–sensitized erythrocytes in vivo.1 Recently, a clear erythrocytes. This was supported by observations that infusion of platelets by competitive blockade from immunoglobulin-coated treating ITP was due to reduced macrophage binding of sensitized antibodies may functionally block and/or down-regulate the expression of FcyRIIIA, resulting in the decreased platelet phagocytosis. To define the possible modulating effect of these anti-erythrocyte antibodies on FcγRIIIA expression, we analyzed the expression of FcγRIIIA on splenocytes in mice (Figure 1A, E). In contrast, the antibody-coated erythrocytes engage the Fc region of IgG. Thus, selective blockade of FcγRIIIA, using 2.4G2 in FcγRIIB−/− mice, would prevent the phagocytosis of antibody-coated erythrocytes by macrophages. As shown in Figure 2B, pretreatment of FcγRIIB−/− mice with 2.4G2 significantly inhibited the erythrocyte destruction by subsequent administration of TER119; in contrast, control IgG had no effect on erythrocyte destruction induced by TER119. These data support the contention that anti-erythrocyte antibodies that ameliorate ITP may do so by engaging FcγRIIIA and blocking and/or down-regulating the receptor. To establish a stronger link between the observed effects on erythrocyte destruction and the correction of platelet counts, we have investigated these phenomena in the same animals and observed the expected relationship (Figure S1, available on the Blood website; see the Supplemental Figures link at the top of the online article).

It should be noted that the model of ITP used here is a simple model of immune thrombocytopenia without all of the complex attributes of a full-blown autoimmune disease such as human ITP. Thus, while this model provides a selective and powerful approach to understanding specific pathophysiologic mechanisms of IVlg action in ITP, the model may also be limited by virtue of this simplicity.

In this report, we have used several monoclonal anti-erythrocyte–specific antibodies to mimic the therapeutic effects of anti-D in ITP. The TER119 antigen is a molecule associated with cell-surface glycoporphin A, and the monoclonal antibody TER119 specifically recognizes murine erythrocytes.20 Our previous study has demonstrated that TER119 mimics the action of anti-D to effectively

![Figure 1. Anti-erythrocyte antibodies (TER119 and M1/69) do not require the inhibitory Fc receptor FcγRIIB in the amelioration of murine ITP. (A) FcγRIIB−/− (C57BL/6) and (B) FcγRIIB−/− mice were injected with 2 μg antiplatelet antibody on days 0 to 3 ( ). All mice received an injection of 200 μL PBS ( ) or 2 g/kg IVlg ( ) or 50 μg TER119 ( ) or M1/69 ( ) on day 2 ( ). The y-axis denotes the days, the y-axis denotes the platelet count; n = 7 for each group. Data are expressed as mean ± SEM.](image-url)
ameliorate murine thrombocytopenia. A small prospective study to test a single human monoclonal anti-D in 7 D-positive patients with chronic ITP was unsuccessful, and this is in general agreement with our previous work, which has demonstrated that not all monoclonal anti-erythrocyte antibodies can ameliorate murine ITP. The monoclonal antibodies that effectively ameliorated the thrombocytopenia did react with the murine red cells and bound more strongly than did IVIg (Figure S2). In contrast, the antibody less reactive (equivalent to the batch of IVIg (lot #26N1LL1) used throughout these experiments with murine red cells did not ameliorate ITP. Therefore, antibodies that bind poorly to red cells (eg, 30-F1 and IVIg) may, in contrast to those that bind strongly to red cells (eg, TER119 and M1/69), not mediate “anti-D–like” effects. However, the mechanisms of action of IVIg therapy may be more complex in humans than in mice, and it is difficult to rule out the possibility that IVIg does not have anti-D–like effects in humans.

In summary, the anti-D–like antibodies TER119 and M1/69 require activating FcγRIIIA but not the inhibitory FcγRIIB in suppressing murine ITP. Since there appears to be different mechanisms of action involved with IVIg and anti-erythrocyte antibodies in ITP, we speculate that patients who do not benefit from one treatment may benefit from the other, or there may be an additive effect, although this remains to be demonstrated.

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