Rh immune globulin (WinRho SDF; Cangene, Mississauga, ON, Canada) is an effective treatment for autoimmune thrombocytopenic purpura; however, maintaining a sustained supply for its use in autoimmune thrombocytopenic purpura and its primary indication, hemolytic disease of the newborn, makes the development of alternative reagents desirable. We compared Rh immune globulin and 6 human monoclonal anti-D antibodies (MoAnti-D) with differing isotypes and specificities for their ability to opsonize erythrocytes and inhibit platelet phagocytosis in an in vitro assay. Results demonstrated that opsonization of erythrocytes with Rh immune globulin significantly (P < .001) reduced phagocytosis of fluorescently labeled opsonized platelets in an FC-dependent manner. Of the MoAnti-D that shared specificity but differed in isotype, only IgG3 antibodies could significantly (P < .001) inhibit platelet phagocytosis. In contrast, 2 MoAnti-D shared isotypes and differed in specificity; however, only one could significantly (P < .001) inhibit platelet phagocytosis. The results suggest that MoAnti-D epitope specificity and isotypes are critical requirements for optimal inhibition of opsonized platelet phagocytosis. (Blood. 2007;110:1359-1361)
Platelets and red blood cells

CPDA blood was drawn from laboratory volunteers under a St. Michael’s Hospital Research Ethics Board-approved protocol. Informed consent was obtained in accordance with the Declaration of Helsinki. Platelet-rich plasma was prepared, platelets were counted and labeled with 20 μM CellTracker Green CMFDA (CM-G; Invitrogen, Carlsbad, CA), washed, and resuspended in phosphate-buffered saline. When indicated, CM-G–labeled platelets were opsonized with either 5 μg W6/32 or a 1:2 dilution of AITP serum for 30 minutes, washed, and used in the phagocytosis assay. After removal of platelet-rich plasma, the buffy coat was removed and the RBC pellet was washed 6 times in phosphate-buffered saline. RBC were adjusted to 4 × 10^6 in 100 μL and incubated with the indicated amounts of anti-D for 40 minutes and used in the phagocytosis assay.

Phagocytosis assay

Phagocytosis of platelets was performed by a method previously described.17 Human THP-1 cells (ATCC® TIB-202) were counted and 10^5 cells/mL were activated with 50 ng/mL phorbol 12-myristate 13-acetate. The reaction was started by incubating 5 × 10^6 THP-1 cells with 250 × 10^6 platelets in 0.1-mL duplicate tubes for 2 hours on ice or at 37°C. Extracellular fluorescence was quenched by addition of 0.1% trypan blue. Tubes were centrifuged at 200g for 10 minutes at 4°C, the supernatant discarded, and 200 μL LDS DNA stain (Invitrogen) added. Flow cytometry was performed using a FACSort flow cytometer; cells were acquired through an FL3 gate and intracellular FL1 fluorescence was determined. Phagocytic index was calculated by the formula: median FL1 fluorescence at 37°C/median FL1 fluorescence at 0°C. When indicated, titrations of RBC were added to the assay.

Statistical analysis

An unpaired t test for comparison between means was used.

Results and discussion

When labeled platelets were opsonized with W6/32, a significant increase in THP-1 intracellular fluorescence was observed, as previously described (Figure 1A,B).17 When nonopsonized RBC were added, there was no effect on W6/32-opsonized or autoantibody-opsonized platelet phagocytosis at any RBC:THP-1 ratio (Figure 1C,D). RBC opsonized with WinRho SDF, however, mediated a significant (P < .0001) Fc-dependent inhibition of both W6/32- and autoantibody-mediated platelet phagocytosis (Figure 1C,D). Wiener et al18 determined that anti-D-opsonized RBC phagocytosis was mediated by high-affinity FcγR l on monocytes, whereas Miescher et al19 demonstrated MoAnti-D-mediated RBC clearance was associated with FcγRIIA and FcγRIIB polymorphisms. Furthermore, Coopamah et al20 reported that anti-D-mediated erythrophagocytosis was associated with a monocytic Fc-dependent oxidative burst. Perhaps anti-D-mediated inhibition of platelet phagocytosis is related to these observations and we are currently studying this.

Of the MoAnti-D (BRAD-3/BRAD-5 and IgG1r9B8/IgG3r9B8) that shared epitope specificity but differed in IgG isotype, only IgG3 antibodies significantly (P < .001) inhibited opsonized platelet phagocytosis (Figure 2A,B). These results are consistent with studies demonstrating that some IgG3 isotypes of MoAnti-D are superior to their IgG1 counterparts in mediating responses related to HDN prevention.13,21,23 They may be related to the observation that certain FcγRIIA and IIIA alleles display differential binding to human antibody isotypes.24 Of interest, one IgG3 MoAnti-D (IgG3r9B8) achieved similar levels of platelet phagocytosis inhibition as Rh immune globulin, suggesting even single MoAnti-D products can mimic polyclonal anti-D. Nonetheless, these results may explain why an IgG1 MoAnti-D failed to raise platelet counts in patients with AITP.14

When 2 MoAnti-D sharing IgG1 isotypes but differing in specificity (IgG1R113/IgG1R178) were used in the assay, only one could significantly (P < .01) reduce platelet phagocytosis (Figure 2C). The mechanism of how the MoAnti-D specificity affects opsonized platelet phagocytosis is unknown but may relate to the molecule’s orientation on the RBC surface. For example, Christiaansen et al25 demonstrated that orientation of monoclonal antibodies on target cells was critical for determining whether they mediated their biologic effect. Perhaps MoAnti-D with a particular specificity binds D in a manner that does not allow interaction with Fc receptors. In addition, although we equilibrated anti-D binding, some may have been lost from the RBC surface as a result of FcγR engagement.26 Of interest, one IgG3 MoAnti-D (IgG3r9B8) achieved similar levels of platelet phagocytosis inhibition as Rh immune globulin, suggesting
MoAnti-D can synergistically inhibit erythrophagocytosis in HDN and respiratory burst in monocytes.21,23

In conclusion, MoAnti-D inhibition of opsonized platelet phagocytosis is dependent on isotype and epitope specificity suggesting that these preparations can be produced to mimic polyclonal anti-D and perhaps be therapeutically effective in AITP.

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Authorship

Contribution: M. Kjaersgaard performed the research, analyzed data, and wrote the paper; R.A. designed the research, performed the research, analyzed the data; M. Kim designed and performed the research; E.R.S. designed and performed the research; J.F. designed the research and wrote the paper; D.I.H.S. AND E.J.W. constructed monoclonal antibodies, designed the research, and wrote the paper; and J.W.S. designed the research and wrote the paper.

Conflict-of-interest disclosure: D.I.H.S. and E.J.W. are employed by Cangene, whose product (WinRho SDF) was studied in the present work. All other authors declare no competing financial interests.

Correspondence: John Semple, St. Michael’s Hospital, 30 Bond St., Toronto, ON, Canada, M5B 1W8; e-mail: simplesj@smh.toronto.on.ca.
Epitope specificity and isotype of monoclonal anti-D antibodies dictate their ability to inhibit phagocytosis of opsonized platelets

Mimi Kjaersgaard, Rukhsana Aslam, Michael Kim, Edwin R. Speck, John Freedman, Donald I. H. Stewart, Erik J. Wiersma and John W. Semple