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EPITOPE SPECIFICITY AND ISOTYPE OF MONOCLONAL ANTI-D ANTIBODIES DICTATE THEIR ABILITY TO INHIBIT PHAGOCYTOSIS OF OPSONIZED PLATELETS.[a]

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Running title: Monoclonal anti-D inhibits platelet phagocytosis.

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Rh immune globulin (WinRho®SDF) is an effective treatment for autoimmune thrombocytopenic purpura (AITP), however, maintaining a sustained supply for its use in AITP and its primary indication, hemolytic disease of the newborn, makes the development of alternative reagents desirable. We compared WinRho®SDF and six 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 WinRho®SDF significantly ($p<0.0001$) reduced phagocytosis of fluroescently-labeled opsonized platelets in an Fc-dependent manner. Of the MoAnti-D that shared specificity but differed in isotype, only IgG3 antibodies could significantly ($p<0.0001$) inhibit platelet phagocytosis. In contrast, two MoAnti-D shared isotype and differed in specificity, however, only one could significantly ($p<0.0001$) inhibit platelet phagocytosis. The results suggest that MoAnti-D epitope specificity and isotype are critical requirements for optimal inhibition of opsonized platelet phagocytosis.

Introduction

Autoimmune thrombocytopenic purpura (AITP) is a bleeding disorder where platelets are opsonized by autoantibodies and destroyed by Fc receptor (R)-mediated phagocytosis.[1,2] Treatment options can include corticosteroids or splenectomy, and if these fail to raise platelet counts, more potent compounds may be used.[1,2] Rh immune globulin (WinRho® SDF) also increases platelet counts in AITP,[3-5] however, the competition for supplies of WinRho® SDF for it's primary indication, hemolytic disease of the newborn (HDN), has limited its use in AITP. A potential alternative to WinRho®SDF is the use of MoAnti-D.

The D antigen is a 32 kD erythrocyte (RBC) protein encoded by the RHD gene which has several allelic variants that is able to generate different epitopes allowing the production of many MoAnti-D.[6-13] Hundreds of MoAnti-D have been produced for phenotypic studies and many have been characterized for their ability to inhibit physiological processes in models of HDN.[9-13] With respect to AITP, however, only one study attempted to treat AITP patients with MoAnti-D, but platelet counts were not increased.[14] It was suggested that the failure was due to the monoclonal preparation missing factors present in polyclonal anti-D.[15] Subsequently, few studies attempted to understand why MoAnti-D preparations are not effective in AITP. This study examined the ability of MoAnti-D to inhibit opsonized platelet phagocytosis in vitro and results demonstrate that both epitope specificity and isotype need to be considered for production of a MoAnti-D preparation that mimics the effects of polyclonal WinRho® SDF.

Material and Methods

Antibodies: Serum prepared from a patient with AITP previously screened for high-titred IgG anti-platelet autoantibodies was obtained from Dr. John Freedman (St. Michael's Hospital, Toronto, ON, Canada). Murine IgG2a monoclonal anti-human MHC (W6/32) was produced from hybridoma HB-95 (ATCC#HB-95). WinRho®SDF was obtained from the Cangene Corporation (Winnipeg, MB,

Canada). Human MoAnti-D BRAD3 (IgG3; clone 1A3-3) and BRAD5 (IgG1; clone 1A11) that share D specificity (Loop 6/7) were obtained from the International Blood Group Reference Laboratory (Bristol, UK).[10] Human MoAnti-D IgG1r9B8 (IgG1; clone rRh9B8-14-1-M5) and IgG3r9B8 (IgG3; clone rRh9B8-C12-1) share identical V-regions (Loop3/Loop6 M169L/M170R.D350H) but differ in isotypes whereas IgG1Rh113 (IgG1; clone rRh113) and IgG1Rh178 (IgG1; clone rRh4-29-178) differ in D specificity (Loop6 D350H and Loop 4/6 F223V,D350H,G353/A354N respectively) but share isotypes and were produced as previously described.[11] Because efficacy of MoAnti-D is proportional to the amount on RBC,[13] the anti-D antibodies were titrated on RBC and bound with a FITC-labeled goat anti-human IgG (H+L chain specific, #GTX72737, GeneTex, San Antonio, TX). The anti-D amounts that showed equivalent fluorescence (binding to RBC) were used in the phagocytosis assay; WinRho®SDF(1.9 ug), IgG1Rh113 (4.0 ug), IgG1Rh178 (1.1 ug), IgG1r9B8 (3.0 ug), IgG3r9B8 (3.3ug), BRAD3 (0.7ug) and BRAD5 (1.1ug). $F(ab')_2$ fragments of WinRho® SDF were prepared as previously described;[16] purity by HPLC analysis was >96%.

Platelets and RBC: CPDA blood was drawn from laboratory volunteers under a St. Michael's Hospital REB-approved protocol. Informed consent was provided in accordance with the Declaration of Helsinki. PRP was prepared, platelets were counted and labeled with 20uM CellTracker Green CMFDA (CM-G; Invitrogen), washed and resuspended in PBS. Where indicated, CM-G-labeled platelets were opsonized with either 5 ug W6/32 or a 1:2 dilution of AITP serum for 30 minutes, washed and used in the phagocytosis assay. After removal of PRP, the buffy coat was removed and the RBC pellet was washed six times in PBS. RBC were adjusted to $4x10^8$ 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 10^7 cells/ml were activated with 50ng/ml

phorbol 12-myristate 13-acetate. The reaction was started by incubating $5x10^6$ THP-1 cells with $250x10^6$ platelets in 0.1 ml duplicate tubes for 2h on ice or at 37°C. Extracellular fluorescence was quenched by addition of 0.1% trypan blue. Tubes were centrifuged at 200xg for 10 min at 4°C, the supernatant discarded and 200 ul of 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 (PI) was calculated by the formula: median FL1 fluorescence at 37°C / median FL1 fluorescence at 0°C. Where 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 and B).[17] When non-opsonized RBC were added, there was no effect on W6/32-opsonized or autoantibody-opsonized platelet phagocytosis at any RBC:THP-1 ratio (Figures 1C and 1D). RBC opsonized with WinRho® SDF, however, mediated a significant ($p<0.0001$) Fc-dependent inhibition of both W6/32- and autoantibody-mediated platelet phagocytosis (Figures 1C and 1D). Wiener et al[18] determined that anti-D opsonized RBC phagocytosis was mediated by high affinity FcγRI on monocytes whereas Miescher et al[19] demonstrated MoAnti-D mediated RBC clearance was associated with FcγRIIA and FcγRIIIA polymorphisms. Furthermore, Coopamah et al[20] 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 ($p<0.0001$) inhibited opsonized platelet phagocytosis (Figures 2A and 2B). 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 WinRho®SDF 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 two MoAnti-D sharing IgG1 isotypes but differing in specificity (IgG1Rh113/IgG1Rh178) were used in the assay, only one could significantly ($p<0.01$) reduce platelet phagocytosis (Figure 2C). The mechanism of how the MoAnti-D specificity affects opsonized platelet phagocytosis is unknown but may relate to the molecules orientation on the RBC surface. For example, Christiaansen et al[25] demonstrated that orientation of monoclonal antibodies on target cells was critical for determining whether they mediated their biological 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 due to differing affinities, however, in control experiments with RBC alone, there was no reduction in anti-D fluorescence (not shown). In an attempt to address these issues, we mixed the 6 MoAb expecting that the 3 non-inhibitory antibodies would confer at least some of their lack of inhibition, however, the mixture significantly inhibited platelet phagocytosis as well as WinRho®SDF (Figure 2D). This paradoxical finding may be related to studies demonstrating that blending different IgG1 and IgG3

isotypes of 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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Authors contributions: Kjaersgaard, performed research, analyzed data, wrote paper; Aslam, designed research, performed research, analyzed data; Kim, Designed research, performed research; Speck, designed research, performed research, Freedman; Designed research, wrote paper; Stewart and Wiersma, constructed monoclonal antibodies, designed research, wrote paper; Semple, designed research, wrote paper.

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Figure Legends.

Figure 1. Representative flow cytometric histogram fluorescence of **(A)** control THP-1 phagocytosis of W6/32-opsonized platelets at 0°C (·····) and 37°C (——) and **(B)** THP-1 phagocytosis of W6/32-opsonized platelets at 37°C in the presence of non-opsonized RBC (10:1 RBC:THP-1 ratio, ——), WinRho®SDF-opsonized RBC (1:1 RBC:THP-1 ratio, ------) or WinRho®SDF-opsonized RBC (10:1 RBC:THP-1 ratio, ·····). The median values of the THP-1 fluorescence for W6/32-opsonized platelets at 0°C was 4.2±1.2 and at 37°C was 79.6+28.4 (N=91). These values were used to calculate the percent inhibition of **(C)** W6/32 opsonized platelet phagocytosis by non-opsonized RBC (□), RBC opsonized with WinRho®SDF (●) or RBC bound with WinRho®SDF $F(ab')_2$ fragments (○) and **(D)** IgG autoantibody opsonized platelet phagocytosis by non-opsonized RBC (□), RBC opsonized with WinRho®SDF (●) or RBC bound with WinRho®SDF $F(ab')_2$ fragments (○). The data in panel C and D are presented as the mean percent inhibition (±SD) at the indicated RBC:THP-1 ratios (N=20 for panel C and N=8 for panel D) and were calculated by the formula: 1 - PI (added RBC) / PI (platelets alone) x 100. Statistical significance (p values) by an unpaired t test is shown: all solid symbols were compared with the corresponding non-opsonized RBC inhibition, ★; $p<0.0001$.

Figure 2. Inhibition of IgG autoantibody-opsonized platelet phagocytosis by RBC opsonized with **(A)** MoAnti-D IgG3r9B8 (●) or IgG1r9B8 (○), **(B)** MoAnti-D BRAD3 (●) or BRAD5 (○), **(C)** MoAnti-D IgG1Rh113 (●) or IgG1Rh178 (○) and **(D)** RBC opsonized with a mixture of all 6 MoAnti-D (●). The data is presented as the mean percent inhibition (±SD) at the indicated RBC:THP-1 ratios (N=6) and was calculated as in Figure 1. Statistical significance (p values) by an unpaired t test is shown: all solid symbols were compared with the corresponding non-opsonized RBC inhibition in panel 1B, ★; $p<0.0001$.

Figure 1

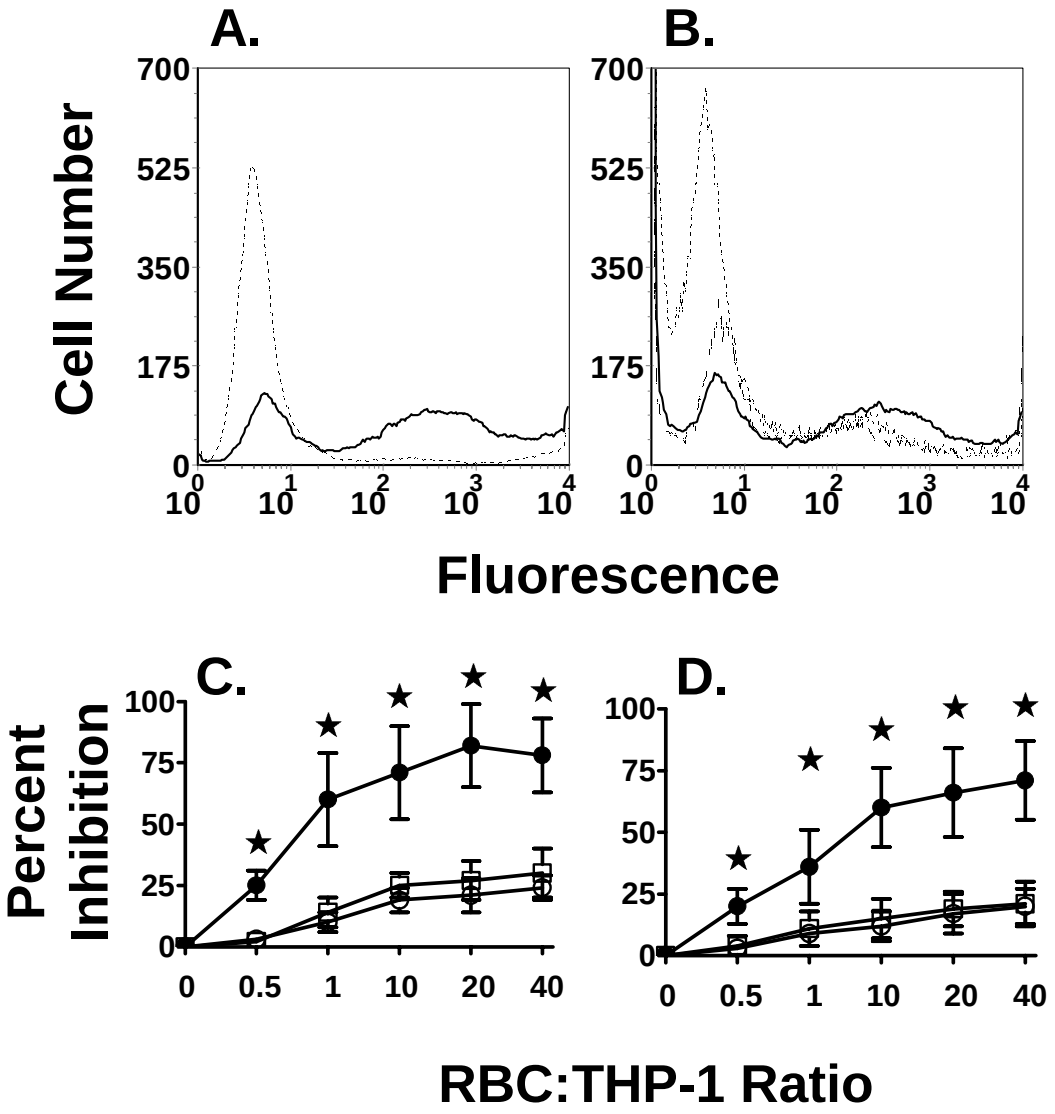
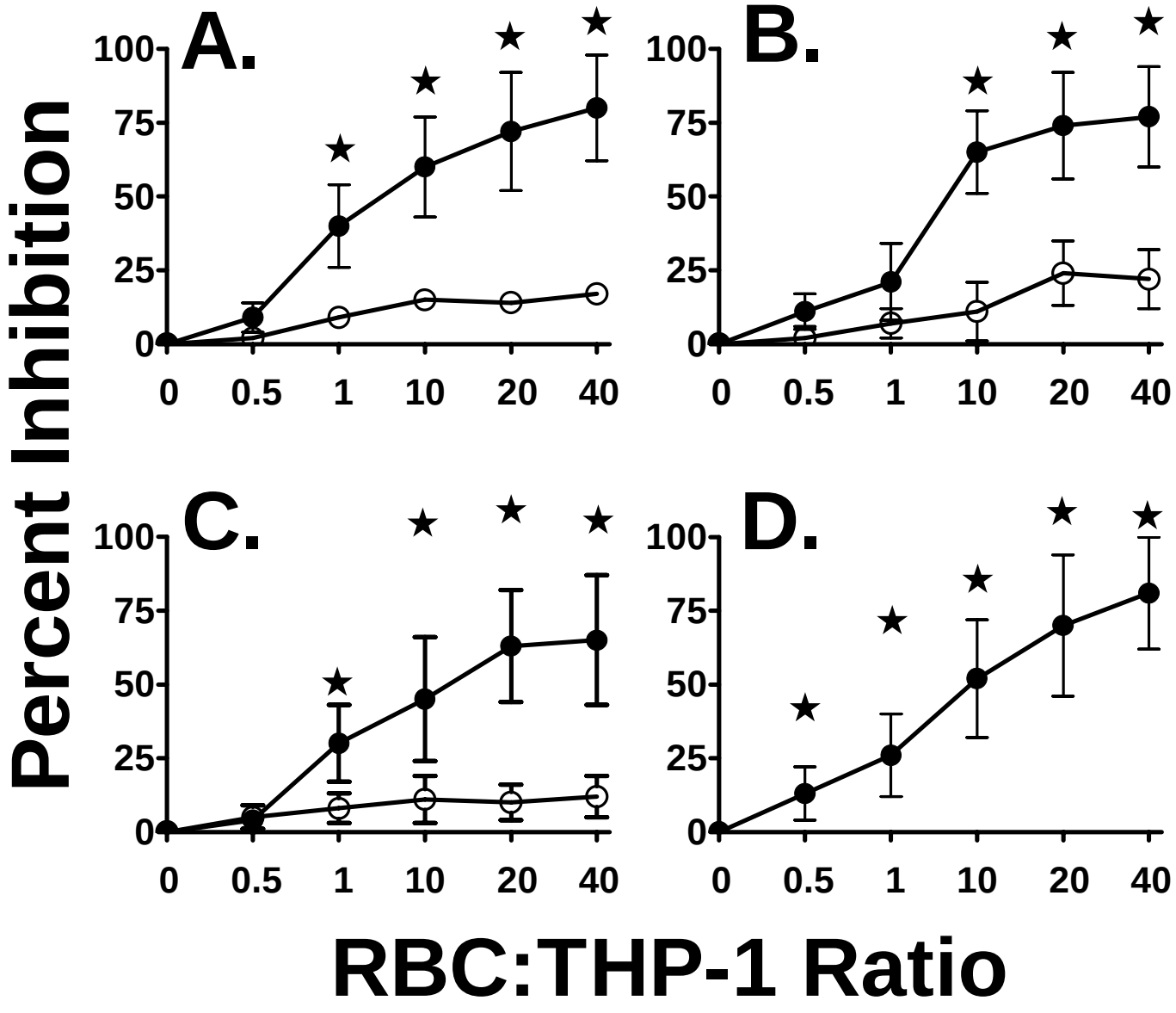

A.
B.
Cell Number
700
525
350
175
0
10^0
10^1
10^2
10^3
10^4
Fluorescence
C.
D.
Percent Inhibition
100
75
50
25
0
0
0.5
1
10
20
40
RBC:THP-1 Ratio

Figure 2


A.
B.
C.
D.
Percent Inhibition
RBC:THP-1 Ratio
100
75
50
25
0
0
0.5
1
10
20
40