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A novel laboratory technique demonstrating the influences of *RHD* zygosity and the RhCcEe phenotype on erythrocyte D antigen expression

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Abstract

D antigen is the most immunogenic and clinically relevant antigen within the complex Rh blood group system. Variability of D antigen expression was first described decades ago but has rarely been investigated quantitatively, particularly in the context of *RHD* zygosity along with RhCcEe serological phenotype. With IRB approval, 107 deidentified blood samples were analyzed. Rh phenotypes were determined serologically by saline technique using monoclonal antibodies against D, C, c, E, and e antigens. *RHD* zygosity was determined using both PCR-restriction fragment length polymorphisms and quantitative real-time PCR techniques. A novel and robust method was developed for quantitation of erythrocyte D antigen sites using calibrated microspheres and flow cytometry, allowing correlation of D antigen density with *RHD* zygosity and expression of Rh CcEe antigens. Subjects homozygous for *RHD* expressed nearly twice the number of D antigen sites compared with *RHD* hemizygotes (33,560 ± 8,222 for DD versus 17,720 ± 4,471 for Dd, $P < 0.0001$). Expression of c or E antigens was associated with significantly increased erythrocyte D antigen expression, whereas presence of C or e antigens reduced expression. These data and this novel quantitation method will be important for future studies investigating the clinical relevance of D antigen variability.

Introduction

The Rh blood group is a complex system of blood antigens found on human erythrocytes and is second in clinical importance only to the ABO blood group in the field of transfusion medicine [1,2]. Erythrocyte D antigen (derived from *RHD* and located on the RhD protein) is the most immunogenic of the over 50 Rh blood group antigens that have been identified to date; D antigen expression has important clinical implications in the diagnosis and management of hemolytic disease of the newborn (HDN) [3,4], autoimmune hemolytic anemia [5], and alloimmunization [6,7]. Polyclonal preparations of RhD immune globulin are administered prophylactically to pregnant women for the prevention of HDN [8] and therapeutically for the management of children and adults with immune thrombocytopenia (ITP) [9,10].

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Wide variability in erythrocyte D antigen expression was first described over 50 years ago in quantitative studies measuring red blood cell (RBC) uptake of ^{131}I-labeled polyclonal anti-D, using high titer anti-D serum from a "housewife who had been immunized to the RhD antigen by multiple pregnancies [11–15]." For example, a series of elegant experiments by Rochna and Hughes-Jones in 1961 demonstrated a variability in D antigen expression ranging from 9,900–33,000 binding sites per RBC among 23 donors tested [13]. In 1965, Silber et al. first noted the influence of the RhCcEe phenotype on D antigen expression. The expression of C antigen, and to a lesser extent e antigen, was associated with reduced D expression, and a suppressive effect was postulated [16,17]. These studies documented substantial differences in erythrocyte D antigen expression and the authors speculated presciently that this variability might have clinical implications.

Variability of erythrocyte D antigen expression is therefore documented based on older techniques, but D antigen variability has not been quantitated using a combination of modern flow cytometry and molecular biology techniques in the context of *RHD* zygosity and RhCcEe phenotype. Recent reports of D antigen quantitation have focused mostly on D variants such as weak D and partial D, relying on a "standard RBC" to determine the number of D antigen sites per RBC [18,19]. This method was determined to be the most reliable method of D antigen quantitation by the 4th International Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens [18], but the antibodies and the critical standard RBC used for the Workshop are reagents that are no longer commercially available.

In this report, we describe a novel, robust, and reproducible method of erythrocyte D antigen quantitation using a commercially available anti-D antibody and calibrated microspheres, which demonstrates marked antigenic variation among D-positive individuals. Further analysis revealed strong associations between D antigen expression and *RHD* zygosity, which were also influenced by the presence of other clinically relevant Rh antigens (RhCcEe). On the basis of these findings, we hypothesize that variable D antigen expression may have important clinical implications in the management and treatment of HDN and ITP. Our new laboratory techniques using commercially available reagents may become important tools for these future investigations of hematological disorders where D antigen expression may play a critical role.

Methods

Patients

With IRB approval, deidentified peripheral blood samples (only age and ethnicity were recorded) from patients at St. Jude Children's Research Hospital were analyzed, using discarded blood collected for routine blood counts. Patients with either primary hematological or oncological diagnoses were included, but samples were excluded from analysis if the child had known erythrocyte abnormalities, had been transfused within 120 days, had received chemotherapy or radiation therapy within 5 years, or had ever undergone stem cell transplantation. The vast majority of these samples were obtained from patients receiving care in the After Completion of Therapy or the Hemostasis/Thrombosis clinics. Within 24 hr of blood collection, an aliquot was tested for D antigen expression by

quantitative flow cytometry, another aliquot was used for standard serological RhCcDEe typing, and the remainder was used for isolation of genomic DNA for *RHD* zygosity testing.

Erythrocyte D antigen quantitation

Quantitation of erythrocyte D antigen sites was determined by flow cytometry, using calibrated microspheres (Quantum Simply Cellular, Bangs Laboratories, Fishers, IN) with known antibody binding capacities to create a calibration curve used for quantitation. The four populations of microspheres are coated with increasing levels of IgG specific for the Fc portion of human IgG. Calibration is performed by the manufacturer prior to distribution; using a precise number of surface-labeled microspheres, fluorescence intensity is assigned in molecules of equivalent soluble fluorochrome (MESF) units through direct comparison with fluorescence measurements from solutions of the same pure fluorochrome. Given the known 1:1 antibody: fluorophore ratio of the quality control antibody, the MESF value of each antibody-saturated microsphere represents the antibodybinding capacity. Microsphere fluorescence was determined using the same antibody and the same flow cytometry settings as subject samples. Each subject sample was run in triplicate, and freshly drawn blood from the same Caucasian male human volunteer was included with each set of analyses to ensure consistency of results (the coefficient of variation for these control samples was <5%).

The RBC count (number of erythrocytes per μL) was used to standardize the antibody incubation phase; 2×10^5 RBCs were diluted in 1 mL of a 0.5% bovine serum albumin in phosphate-buffered saline solution. Cells were centrifuged at 1,500 rpm × 5 min, and supernatant was poured off. After repeating this washing step three times, 50 μL monoclonal FITC-conjugated anti-D (LDG76) antibody (Quant-Rho, Quotient Biodiagnostics, Newtown, PA) was added, and the solution was mixed thoroughly by vortexing. This antibody is an IgG1κ heterohybrid (human-murine) monoclonal antibody specific for epD3 in the nine epitope model and epD5 in the thirty-six epitope model of D antigen and has been shown to have a high affinity for D antigen, achieving sufficiently high antibody-to-cell ratio [20]. The volume of antibody was determined in pilot experiments to ensure antibody saturation (data not shown). Reactions were incubated in the dark at room temperature for 30 min. Simultaneously, one drop of each of four Quantum Simply Cellular microspheres were added to 100 μL PBS-BSA with 100 μL anti-D-FITC (volume also determined in pilot experiments to ensure saturation) prior to 30-min incubation in the dark at room temperature. After incubation, 1 mL PBS-BSA was added to all tubes for an additional three washing steps. Cells were then resuspended in 100 μL PBS-BSA and analyzed by flow cytometry. FITC-conjugated Mouse IgG1 isotype control (BD Biosciences, San Jose, CA) was included with each sample and an additional “blank” microsphere population with no specific antibody binding capacity was analyzed by flow cytometry to determine background fluorescence.

Forward scatter versus side scatter with a logarithmic scale was used to appropriately gate RBC and microsphere populations. At least 10,000 events were collected at a rate of about 500 events per second using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). A calibration curve was created for each batch analysis, by plotting the known antibody binding capacity (ABC) of each microsphere (*y*-axis) versus the geometric mean

fluorescence value (FL-1) of each microsphere (*x*-axis). The ABC of each sample was calculated and the number of antigen sites per RBC was determined by subtracting the ABC of the blank population (i.e., the background fluorescence).

DNA isolation

Genomic DNA was isolated from ~1 mL of peripheral blood collected in EDTA, using a modified salting-out precipitation method [21] and Gentra PureGene Blood kit (Qiagen, Valencia, CA). Purified DNA was resuspended in double distilled water to a concentration of 50 ng/μL for PCR-RFLP and 5 ng/μL for real-time PCR analysis of *RHD* zygosity.

RHD zygosity determination by PCR-RFLP and RQ-PCR

PCR with restriction fragment length polymorphism (PCR-RFLP) was used initially to identify the hybrid rhesus box as previously described [22]. The most common genetic mechanism for the RhD negative phenotype is deletion of *RHD*, identified by the presence of a hybrid Rhesus box that reflects newly juxtaposed DNA sequences (Fig. 1). In Caucasians, it is estimated that 98–99% of D-negative alleles are due to this gene deletion and can be identified by isolation of the hybrid rhesus box [23,24]. Among non-Caucasian populations, there is a higher frequency of inactivating mutations in *RHD*, particularly in the upstream and downstream rhesus boxes that are not identified by PCR-RFLP [25]. Published data have demonstrated that among persons of African descent, *RHD* deletion is responsible for 75–78% of D-negative alleles [26,27].

Forward primer rez7 and reverse primer rnb31 (specific for the downstream Rhesus box) [22] were used for PCR amplification using the Qiagen Long Range PCR Kit (Valencia, CA). Annealing occurred at 66°C and extension at 68°C for 5 min. In order to identify the hybrid Rhesus box, PCR amplicons were then digested with *PstI* restriction enzyme (Promega Corporation, Madison, WI) for 3 hr at 37°C, and fragments were resolved using a 2.5% agarose gel. The hybrid Rhesus box (representing *RHD* deletion) has three *PstI* sites in the PCR amplicon resulting in four fragments of 1,888, 567, 397, and 179 bp (Fig. 1). The intact downstream Rhesus box of D+ haplotypes lack 1 *PstI* site and, therefore, produces three fragments of 1,888, 746, and 397 bp (Fig. 1). Dd hemizygotes have the presence of both haplotypes and produce five fragments of 1,888, 746, 567, 397, and 179 bp. (Fig. 1).

To confirm *RHD* zygosity assignment, real-time quantitative PCR (RQ-PCR) was then performed as previously described [23]. *RHD* exon 10 was amplified and quantified in relation to a reference gene, RNase P. RQ-PCR reactions were set up in a reaction volume of 12 μL. All components, including TaqMan probes, were supplied by Applied Biosystems. Reaction mixtures consisted of Amplitaq Gold DNA polymerase (0.12 μL), GeneAmp 10× PCR Gold Buffer (1.2 μL), uracil-N-glycosylase (0.048 μL) and 25 mM $MgCl_2$ (1.2 μL), dNTPs (1.2 μL), 3.6 μL H_2O, 20 ng DNA and either *RHD* (Hs07226363_cn, Applied Biosystems) or RNase P probe (0.6 μL). Reactions were carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems) and consisted of 2-min incubation at 50°C, followed by a 10-min denaturation step at 95°C, 40 cycles of 95°C for 15 sec and 60°C for 1 min. RQ-PCR data were analyzed, and relative gene expression was calculated using StepOne™ Software, v2.0 (Applied Biosystems).

Statistical analyses

Flow cytometry data were analyzed using FlowJo Flow Cytometry Statistical Software (Tree Star, Ashland, OR). Statistical analyses were performed using GraphPad Prism 4 graphical and statistical software (GraphPad Software, La Jolla, CA). Fisher exact tests, *t*-tests, and ANOVA analyses with *P* values less than 0.05 were considered statistically significant.

Results

Rh antigen frequency

A total of 107 samples were collected and analyzed. By serological phenotyping and PCR testing for *RHD* zygosity, a total of 90 samples were D antigen positive (84%) and 17 were D antigen negative (16%). Of the 107 samples, 103 had concordant *RHD* zygosity as determined by PCR-RFLP and RQ-PCR. There were four subjects (three Caucasian, one Hispanic) with discordant *RHD* zygosity results. One subject serotyped as D-negative but both PCR-RFLP and RQ-PCR identified *RHD* hemizygosity. In this case, serotype results were respected, and the subject was assigned dd status. The remaining three discordant results were initially identified as D-positive by serology, DD by PCR-RFLP, but Dd by RQ-PCR. Given reports of mutations affecting upstream and downstream rhesus boxes [25], hemizygosity was assigned based on the RQ-PCR results. When compared with African-Americans, Caucasians were more likely to lack D antigen (100% of African-Americans were D-positive versus 78.5% of Caucasians, $P = 0.005$). The D antigen positive cohort comprised 50 DD (50/107 = 47%) and 40 Dd (40/107 = 37%) individuals. Table I illustrates the frequency of Rh antigens by ethnicity.

The RhC antigen was present in 64 (60%) subjects, all of whom were also D-positive (33 DD, 31 Dd). Of the 43 C-negative samples, 17 (40%) were D-negative and 26 (60%) were D-positive (17 DD, 9 Dd). When compared with African-Americans, Caucasians were more likely to express C antigen (63.3% versus 38.1%, $P < 0.05$ using Fisher's exact test, Table I). The majority of subjects (84.1%) expressed c antigen; of the 90 c-positive samples, 73 (81%) were D-positive (35 DD, 38 Dd) and 17 (19%) were D-negative. All 17 c-negative subjects were D-positive (15 DD, 2 Dd). The remaining seven subjects were of American Indian, Asian, or Hispanic descent.

E antigen was present in 25 (23%) of subjects, almost all of whom were also D-positive (21 DD, 3 Dd). Of the E-negative subjects, 66 (80.5%) were D-positive (29 DD, 37 Dd) and 16 (19.5%) were D-negative. One hundred and four subjects (97%) were e-positive.

Erythrocyte D antigen expression

An example of the quantitative flow cytometric technique is illustrated in Fig. 2. Erythrocytes expressing D antigen were easily distinguished from D-negative samples, although substantial variation in expression was noted among positive samples (Table II). Overall, the average D antigen expression measured in the 90 D-positive individuals was 26,686 ± 10,292 antibody-binding sites, with a range of 6,192–56,508 sites. When analyzed according to the *RHD* zygosity, subjects homozygous for *RHD* expressed nearly double the number of D antigen sites than hemizygous individuals (33,560 ± 8,222 for DD versus

17,720 ± 4,471 for Dd, $P < 0.0001$, Fig. 3). This effect was upheld in all ethnic groups tested (Table III). Within each of these cohorts, however, there was still substantial variability observed (coefficient of variation 24.5% for DD and 25.2% for Dd).

Influence of the RhCcEe phenotype

Because earlier studies on D antigen expression suggested an influence from additional Rh antigens, we next analyzed our results according to the expression of Cc and Ee antigens. Among all D-positive subjects, the expression of C antigen was associated with decreased D antigen expression (24,840 ± 9,384 with C versus 30,350 ± 11.820 without C, $P = 0.02$). This trend was observed in both DD homozygotes and Dd hemizygotes (Table II). In contrast, the expression of c antigen expression had no effect on D antigen expression for the entire D-positive cohort, but among DD homozygotes, the expression of c antigen was associated with a significant increase in D antigen expression (36,170 ± 7,913 with c versus 27,480 ± 5,280 without c, $P = 0.0002$, Table II).

Among all D-positive individuals, the expression of E antigen was associated with significantly increased D antigen expression (34,510 ± 8,748 with E antigen versus 23,620 ± 9,458 without E antigen, $P < 0.0001$). This effect was most easily observed among DD individuals, where E expression had a significant effect on D antigen expression (Table II). Given that there were only three hemizygous Dd individuals who coexpressed E antigen, the effect of E antigen expression in this small cohort was impossible to ascertain, particularly considering the confounding in trans effect of C antigen. The influence of e antigen expression was evident among DD individuals, where the presence of e antigen significantly reduced D antigen expression (32,760 ± 7,749 with e antigen versus 46,150 ± 4,997 without e antigen, $P = 0.01$) Although there were only three subjects who lacked e antigen, all three were DD individuals with marked increases in D antigen expression, among the highest observed in all subjects (Table I).

Discussion

The Rh gene locus is located on chromosome 1 and contains two large highly homologous and closely linked genes (*RHD* and *RHCE*) that each encodes a highly hydrophobic channel protein with 12 transmembrane domains [28,29]. *RHD* encodes the RhD protein; D antigen is represented by more than 30 epitopes along the extracellular portion of the RhD protein [2]. In contrast, *RHCE* encodes the RhCE protein that carries the Cc and Ee antigens in different combinations [2,30,31]. Given their proximity and homology, these genes are highly susceptible to genetic exchange.

Of the more than 50 Rh antigens that have been identified to date, the most common and clinically significant Rh antigens are D, C, c, E, and e [1]. Cc and Ee represent four discrete antigens, distinct from each other due to polymorphisms of the *RHCE*, so it is possible to express both C and c, as well as E and e antigens. However, in the absence of a distinct genetic polymorphism or protein, the term “d” actually represents the lack of D antigen, typically due to a genetic deletion within the *RHD* locus. The d phenotype (absence of D antigen expression) is significantly more prevalent among Caucasians (17%) than individuals of African (7%) or Asian (2%) descent [32]. In Caucasians, it has been

established that most D-negative phenotypes are due to complete deletion of the *RHD* [21,33].

Although wide variability of erythrocyte D antigen expression was recognized 50 years ago using radiolabeled antibodies [15], accurate quantitation of D antigen sites on RBC has not been commonly reported using flow cytometry and has not been analyzed according to *RHD* zygosity. To date, flow techniques have been used primarily to describe antigen density of weak D or D variant individuals, but these quantitative reports relied on fluorescent intensity of a frozen CcDEe "RBC standard." Although the original report describes a RBC standard (DCcEe) with a D antigen density of 27,500 D antigen sites per cell [18], other reports using a DCcEe standard report varying antigen density as low as 21,500 [9,34–37]. Comparatively, in our report, there were 11 DCcEe samples with an average D antigen expression of 34,640 ± 5,585 antigen sites per RBC. This reference sample was developed by the investigators themselves and is not commercially available, although the R1R2 (DCcEe) phenotype has been recommended as a reference when quantitating D antigen.

Commercially available quantitative flow cytometry kits rely on calibrated microspheres/beads with a known number of bound antibodies, a known number of microsphere-bound fluorochromes, or microspheres with calibrated and clearly defined antibody-binding capacity. Our initial attempts at developing a reliable and reproducible method for D antigen quantitation were challenging due to lack of reliable fluorochrome-conjugated anti-D antibody reagents, technical problems with various commercial quantitative systems, RBC agglutination following secondary antibody incubation, and difficulties with interpreting the calibration curve with apparent results orders of magnitude higher than would be expected. After trialing different commercial antibodies and quantitation methods that were unsuccessful mostly due to lack of reliable and reproducible data with different reagents, we were able to develop a reproducible and robust method described here, using reagents that are all commercially available.

Our data demonstrate a near twofold increase in the expression of D antigen in subjects homozygous for *RHD* when compared with *RHD* hemizygotes (Fig. 3). This copy number "dose effect" of *RHD* was perhaps predictable but not previously documented does not fully explain expression variability since considerable variation in D antigen expression was still noted within both the DD and the Dd cohorts (Fig. 3). The potential impact of minor Rh antigen expression (Cc and Ee) on D antigen expression was suggested by previous reports [16,17]. We not only confirmed that the presence of C antigen or e antigen was associated with reduced D antigen expression but also suggest that the presence of c or E antigen may be associated with increased D antigen expression (Table II). When both C and c were present, D antigen expression remained high suggesting the positive effects of c antigen appear to outweigh the negative or potentially suppressive effects of C antigen expression. Although significant advances have been made in the understanding of Rh protein structure and function [1,38,39], the interactions between D antigen and C/c or E/e antigens that could alter membrane expression of D antigen are not clear. As tandem duplicated genes, it is possible that *RHCE* expression has a direct suppressive effect on *RHD* transcription. Alternatively, *RHCE* expression could influence *RHD* mRNA translation, or be involved with post-translational modifications. Finally, given the tight trimeric structure of the Rh

protein superfamily [38], it is possible that coexpression of D and C antigens results in more steric hindrance on the RBC membrane, thereby reducing D antigen expression.

There are several potential limitations of this report. First, our samples came from pediatric patients rather than normal adult controls; this was primarily for convenience, and our data should be applicable to all patient groups. Second, our sample size was relatively small, yet 107 samples provided us with enough data to identify clear differences in D antigen expression by flow cytometry and also significant influences from the RhCcEe phenotype. Larger studies should be able to validate and extend our findings and allow better evaluation of the effects of E antigen expression. Third, an incorrect assignment of *RHD* zygosity status is possible for subjects with a silenced but not deleted *RHD*, particularly in non-Caucasian subjects [25]. Despite our attempts at confirming *RHD* zygosity by using two complementary assays, it is possible with an inactivating mutation as described in the literature [25,27] are misassigned DD status due to inability to identify these mutations by PCR-RFLP or RQ-PCR [25,27]. In our report, there are two DD samples (both African American) shown as outliers in Fig. 3 with <20,000 D antigen sites, who were perhaps erroneously assigned DD status. Full DNA sequencing of *RHD* (and perhaps *RHCE*) would be necessary to exclude a point mutation that affected D antigen expression. A fourth potential limitation was the monoclonal anti-D antibody itself; our reagent is approved by the FDA and used clinically to identify fetomaternal hemorrhage and is reported to recognize all clinically significant partial D antigens. As over 30 RhD epitopes have been identified [40,41], it is possible that some epitopes are missed. Although comparable studies have used several antibodies when quantifying antigen expression [18], the lack of availability of reliable anti-D monoclonal antibodies limited our selection. A final potential limitation is that we did not specifically investigate the presence of *RHD* variants, for example, weak D, partial D, or Del; however, our technique should prove to be a useful adjunct for these future investigations.

In summary, this report describes a novel and reliable flow cytometric method for the quantitation of erythrocyte D antigen expression. Using this method, we demonstrated *RHD* dosage effect and the contribution of minor Rh antigens to D antigen expression. Routine Rh serology, frequently performed in the clinical laboratory, can help to stratify subjects with higher (presence of c or E) and lower D antigen (presence of C or e) expression. However, these quantitative methods are necessary to understand more fully the variability of D antigen expression. This method will be useful for future studies that investigate the relationships between D antigen expression and the variable responses and toxicities of anti-D therapy. Anti-D is administered therapeutically for ITP only to individuals who type as D-positive, yet standard treatment with 50–75 μg/kg leads to highly variable and unpredictable platelet responses, as well as variable and sometimes excessive declines in hemoglobin concentration [10,42–44]. There are no data at this point to suggest that *RHD* zygosity or Rh phenotype has an effect on the efficacy or toxicity of anti-D therapy for patients with ITP, but as anti-D therapy involves binding of antibody to RBC D antigens for immune blockade [45], the presence of high D antigen expression as identified with these techniques may help to understand treatment responses and toxicities, both clinically relevant goals.

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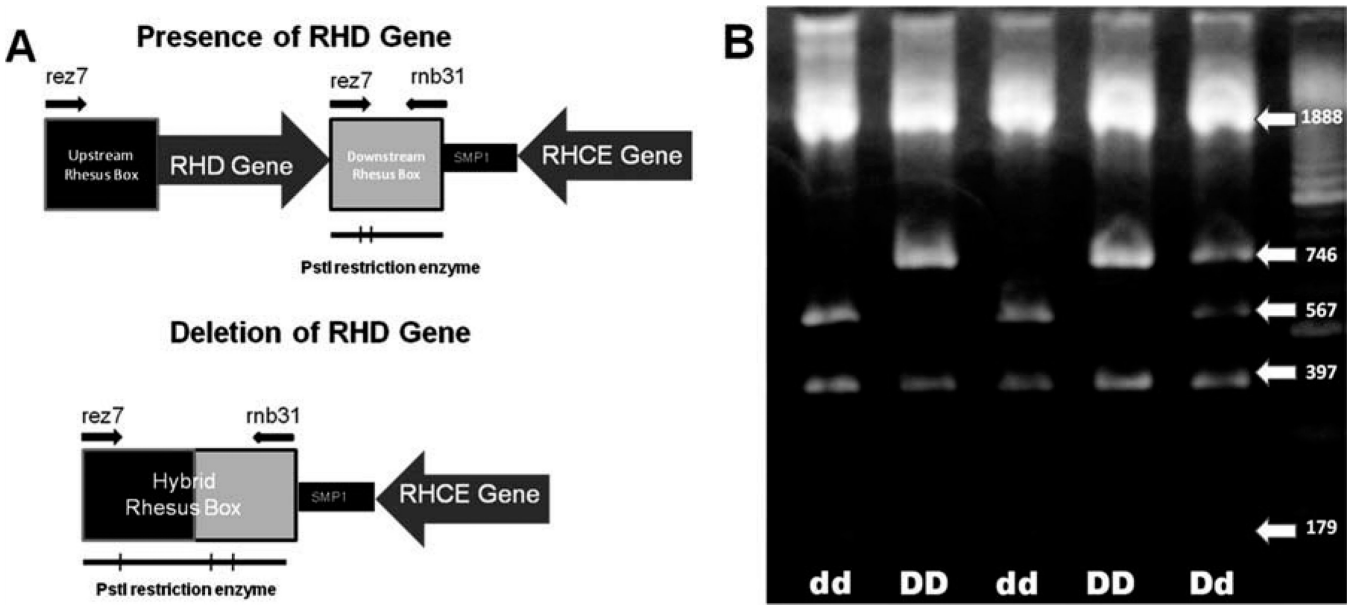

Figure 1.

Identification of *RHD* gene deletion. Panel A (adapted from Ref. 27) illustrates the *RHD* gene locus on chromosome 1, its proximity to the *RHCE* gene, and the presence of flanking upstream and downstream rhesus boxes. Most D-negative haplotypes (particularly among Caucasians) are due to a deletion of the *RHD* gene, leading to the formation of the "hybrid rhesus box [22]." Panel B illustrates the distinct banding pattern obtained with PCR amplification of the upstream and hybrid boxes (using primers rez7 and rnb31) and subsequent digestion with the PstI restriction enzyme. Amplification of the downstream Rhesus box alone (indicating DD homozygosity) results in three DNA fragments of 1,888, 746, and 397 bp. The hybrid Rhesus box (present in D-negative individuals) has an additional PstI site and results in fragments of 1,888, 567, 397, and 179 bp. Heterozygous Dd individuals have one allele expressing the *RHD* gene and one allele lacking the *RHD* gene (and hence expressing the hybrid Rhesus box) and thereby has fragments of 1,888, 746, 567, 397, and 179 bp.

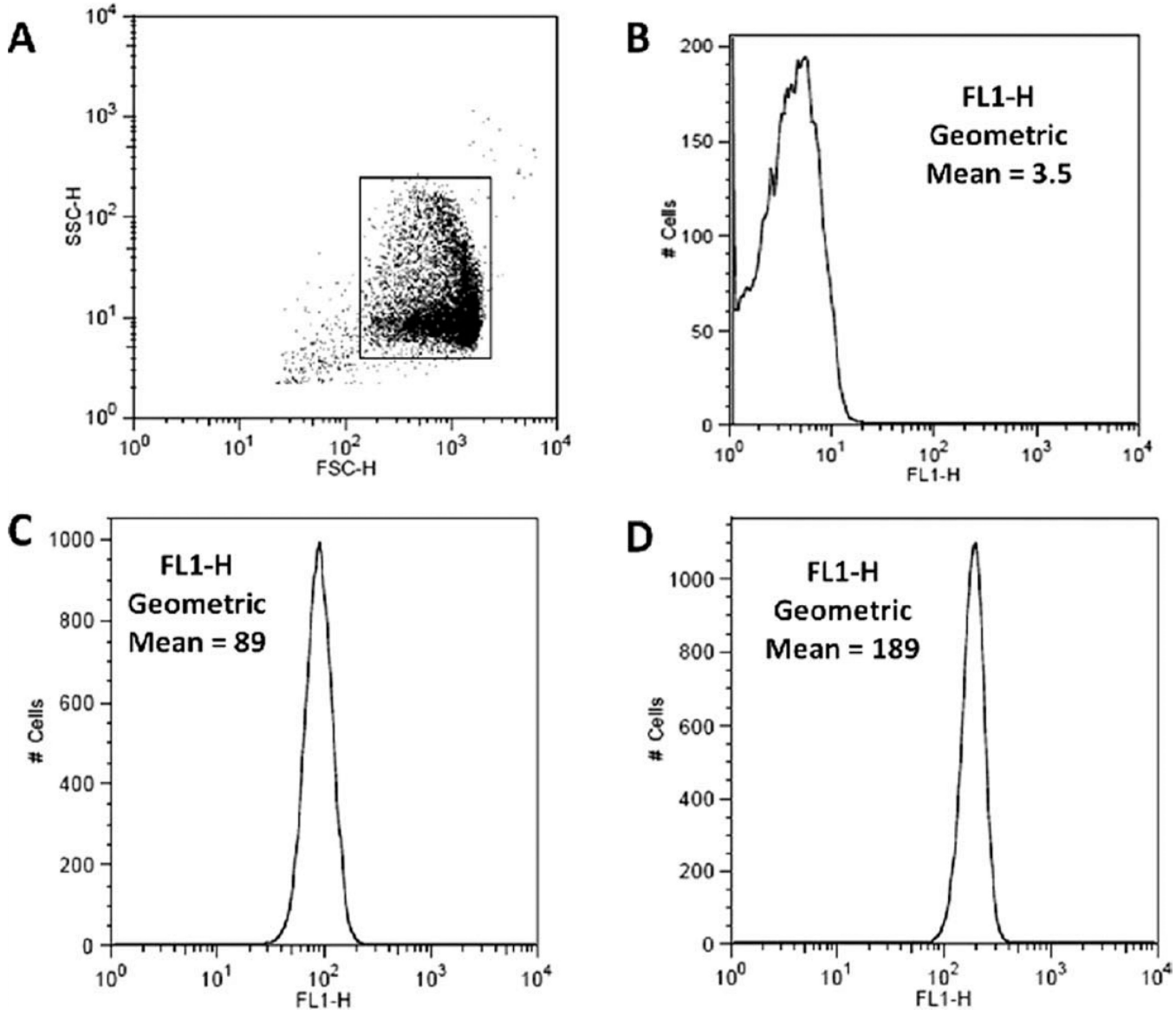

Figure 2.

Quantitation of erythrocyte D antigen expression by flow cytometry. Panel A demonstrates clustering of RBC, allowing for easy gating. The subsequent panels demonstrate clearly distinguishable fluorescence intensities of typical dd (panel B), Dd (Panel C), and DD (Panel D) individuals. FL-1 geometric mean fluorescence was used in conjunction with the calibration curve to calculate the number of D antigen sites per RBC, as described in METHODS.

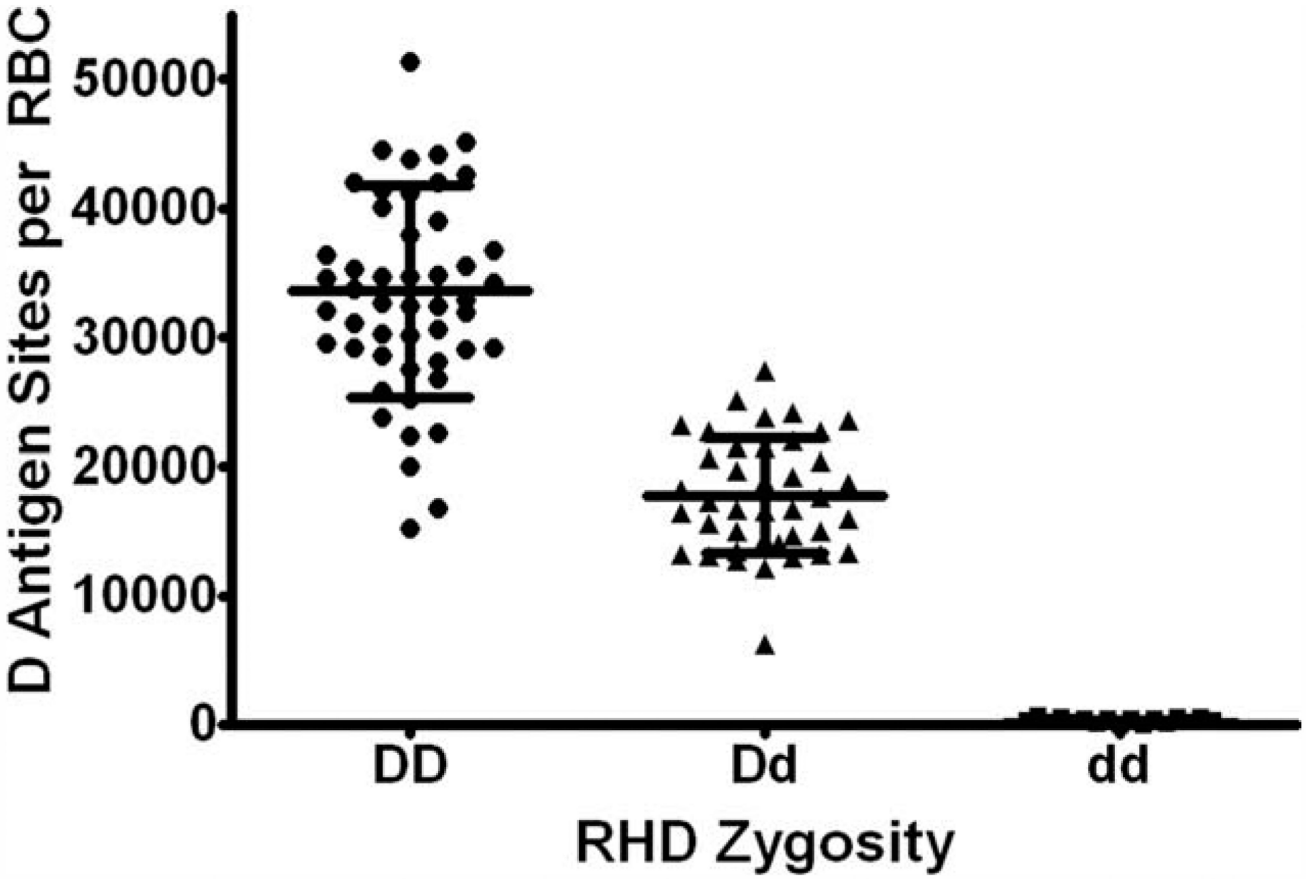

Figure 3.

Erythrocyte D antigen expression and *RHD* Zygosity. The Y-axis represents number of D antigen sites per RBC as determined by quantitative flow cytometry, as described in METHODS. On average, homozygous DD individuals expressed nearly double the number of D antigen sites than heterozygous Dd individuals (DD mean antigen sites 5 33,560 ± 8,222, median 5 32,720; Dd mean antigen sites 5 17,720 ± 4,471, median 5 16,970).

TABLE I

Distribution (%) of Rh Antigens by Ethnicity

Ethnicity	*N*	D	C	c	E	e
Caucasian	79	78.5	63.3	81.0	24.1	96.2
African American	21	100.0	38.1	95.2	23.8	100.0
Other	7	100.0	85.7	85.7	14.3	100.0

Distribution of Rh antigens by ethnicity, as determined by serological techniques described in Methods.

TABLE II

Influence of Minor Rh Antigens on D Antigen Expression

	N	D Antigen Sites	*P*-value
DD + C	33	32,080 ± 6,873	0.08
DD − C	17	36,440 ± 9,956	
DD + c	35	36,170 ± 7,913	0.0002
DD − c	15	27,480 ± 5,280	
DD + E	21	36,270 ± 7,830	0.02
DD − E	29	31,600 ± 8,062	
DD + e	47	32,760 ± 7,747	0.01
DD − e	3	46,150 ± 4,797	
Dd + C	31	17,150 ± 3,976	0.05
Dd − C	9	19,710 ± 5,699	
Dd + c	38	17,840 ± 4,532	0.48
Dd − c	2	15,490 ± 3,048	
Dd + E	3	22,210 ± 2,726	0.06
Dd − E	37	17,360 ± 4,408	
Dd + e	40	17,720 ± 4,471	NA
Dd − e	0	NA	

The presence of c and E antigen are positively associated with D antigen expression, particularly in DD individuals. Although e antigen was lacking in only three subjects, all three had markedly increased D antigen expression. D antigen sites are reported as mean number of sites per RBC ± 1 standard deviation.

TABLE III

D Antigen Expression by Ethnicity among RhD Positive Individuals

	N	DD	Dd
All D-positive Samples	90	33,560 ± 8,222	17,720 ± 4,471
Caucasians	62	32,572 ± 7,225	17,889 ± 4,120
African-Americans	21	34,058 ± 10,358	17,431 ± 7,518
Other	7	38,992 ± 3,544	14,882 ± 1,624

The effect of *RHD* zygosity was upheld across all ethnicities with DD individuals expressing nearly double the number of D antigen sites when compared to Dd individuals.