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Research Article

THE RH ENIGMA PROSPECTS AND PITFALLS

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ABSTRACT
In transfusion medicine, after the ABO blood groups, the D^u antigen is the most significant. A high proportion of people whose red cells lack D^u will make anti-D if exposed to the D antigen by pregnancy or transfusion. Accordingly, all D negative patients, especially females of child bearing ages should be transfused with D negative red cells. A retrospective analysis for a period of 1 year was done. Blood samples that were negative for RhD by immediate spin tube method were tested for weak D antigen. Of a total of 15,666 blood samples13,800 were found (88.08%) to be Rh positive. Of the remaining 2000 blood samples (12.76%) were Rh negative. There were 25 blood samples (0.15%) with D antigen expression. Although uncommon, all health care professionals should be aware of this entity to avoid anti D alloimmunisation.

Key Words: *Anti D typing; Rh Blood Group, D^u Antigen*

INTRODUCTION
The Rh blood group system is one of the most complex blood systems, including 49 different antigens. The D antigen itself consists of more than 30 distinct epitopes, more than 100 known haplotypes and similar phenotypes of different alleles (Srikrishna *et al.,* 2001).

Initially weak D formerly, called D^u was characterized by a reduction of antigen D expression on red cells. However, known variants of the D antigen, also-called partial D phenotypes, can have a weak D expression, such as the D^{va} and D^{vi} phenotypes. Individuals whose RBCs carried a partial D phenotype could be immunized to D epitopes lacking in their RBCs and were at risk. Therefore, it is necessary to distinguish weak D from partial D. Individuals with weak D phenotypes do not produce antibodies in response to pregnancy or transfusion, however, these RBCs can provoke anti D immunization in D negative recipients (Srikrishna *et al.,* 2001).

MATERIALS AND METHODS
Ours is the licenciated 900 bedded, tertiary care, teaching hospital based blood bank situated in Kolar, southeastern Karnataka, with the facilities for blood and blood components collection, preparation, storage & distribution. In addition to the routine hospital demand, our blood bank caters to the demand of the neighboring district of Chikkaballapura, Chittor districts of Andhra Pradesh and also Hosur and Krishnagiri districts of Tamil Nadu.

Rh blood group typing of all donor blood samples in our institute were analyzed for a period from January 2012 to December 2012. There were a total of 15,666 subjects. Routine Rh typing was done using the (IS) method. Blood samples, which were negative for agglutination by (IS), were further tested. Samples showing agglutination after incubation or after addition of Anti Human Globulin (AHG) serum were considered to be weak D. Appropriate controls were used. Equal volumes each of anti D serum and 2-5% washed red cell suspension were placed in a clean glass test tube. They were mixed and incubated at $37^{\circ}C^{in}$ a water bath for 45-60 minutes as per manufacturer's instructions.

Alternatively, in an emergency situation, we incubated for 10 minutes and then centrifuged at 1000 rpm for one minute to obtain a rapid result. However, standard AHG techniques were always performed for confirmation. The tube was gently re–suspended and the cell button observed for agglutination. If the test cells were agglutinated (except in negative control tube) the test was recorded as positive. If the test cells control tube was not agglutinated or the results were doubtful, the cells were washed three to four times

with large volumes of normal saline. After the final wash, the saline was decanted and one to two drops of antiglobulin serum was added accordingly to manufactures instructions.
Later, the contents of the test tube were mixed and the tube was centrifuged at 1000rpm for 30 seconds. The cell button was then gently re-suspended and examined for agglutination. All negative results were confirmed under the microscope.

RESULTS

The results are depicted in Table1.All samples that were negative for Rh antigen by the IS method. The weak D antigen was subjected to further tests. Appropriate controls were used. Of a total of 15,666 blood samples, were 13,800 found (88.08%) to be Rh positive. Of 2000 samples (12.76%) were Rh negative. There were 25 blood samples (0.15%) with D antigen expression.

DISCUSSION

The term D^u was coined in the year 1946, (Stratton *et al.*, 2005). Later, in the year 1948 (Race *et al.*, 2005) and in the year 1950 (Stratton *et al.*, 2005) studied this antigen further and showed that it was an inherited characteristic. They found that D^u red cells were not agglutinated directly by anti-Rh (D) serum, but required subsequently antiglobulin addition to show the presence of this antigen.
"High grade D^u are those RBCs directly agglutinate with selected high-protein potentiated incomplete anti-D reagents whereas "Low-grades" D^u are those which have a strict requirement for antiglobulin. The D^u antigen is unique among blood groups because it expresses atleast 30 epitopes distributed along the extracellular portion. Thus a change, or changes, in the amino acid sequences of RhD may not ablate the entire D antigen but can cause epitopes loss, giving rise to variant forms of D antigens known as partial D^u. Partial D" RBCs, a phenomenon less common than weak D, usually contain normal number of RhD protein, although the protein is mutated in an exofacial loop, eliminating at least one D-specific epitope on the RhD protein.
Weak D is phenotype with either qualitative or quantitative difference in the Rh D^u moiety resulting in a weakened expression of the antigen. The problems that arise from weak D^u antigen are due to its low immunogenicity which gives rise to conflicting laboratory reports, as to whether an individual is Rh D positive or negative. Weak D^u demonstrates reduced quantities of the D antigen because of mutations in the protein's transmembrane domains. As the name implies, these RBCs tend to demonstrate either weak or no hemagglutination at IS phase.
In earlier years, blood banks used polyclonal antisera and these contained low titres as compared to monoclonal antibodies. The blood group reporting was affected by the titre of anti D^u antibodies in the test reagent. An individual with weak D^u antigen was labeled Rh D^u positive by one laboratory and Rh D^u negative by another. Such conflicting reports lead to inadvertent transfusion of Rh D^u positive blood to Rh D^u negative recipients.
The DEL phenotype is a third group of D variants. DEL cannot be detected using routine serological reagents or the weak D test. It is, however easily detected by genetic analysis (Wagner *et al.*, 2000). DEL RBCs contain an extraordinarily low number of D antigens but, despite this paucity, can cause primary and secondary (Stratton *et al.*, 2005) and (Race H, *et al.*, 2005) immune responses against the D antigen in D negative recipients. Fortunately its incidence is low and it is found predominately amongst the Japanese and Chinese population (Stratton *et al.*, 2005).
As molecular genotyping of the RHD genesis complicated by its size, propensity for rearrangements with the related RHCE gene, and significant variability between ethnic groups, it is best performed in a dedicated academic laboratory with expertise in interpreting the gene's many alleles. There may be fewer than 100 such dedicated laboratories in the world (Bhatia *et al.*, 1997).
The Canadian Society of Obstetricians and Gynecologists in its guidelines on the prevention of D allo-immunization, mandated the use of the weak D test in pregnant women (Srikrishna A *et al.*, 2001).This policy classifies those women who demonstrate hem-agglutination with anti-D reagents in the weak D test

as D^u positive, which however, might unnecessarily increase the number of sensitization. This adverse outcome could be easily prevented if RHD genotyping is used in resolving weak D and partial D individuals because serological tests cannot discriminate between these weak D types and those that are susceptible to allo-immunization; only a molecular analysis of the RHD gene can distinguish between weak D types (Williams *et al.*, 2000) .

The D^u antigen is highly immunogenic, and if a Rh D positive blood is transfused to an Rh D negative recipient, the recipient is likely to develop anti D^u allow antibodies and thereafter cannot be transfused with Rh D^u positive blood. Furthermore, if sensitized Rh negative women conceived a RhD positive foetus, the passage of anti-D^u antibodies across the placenta to the unborn baby resulted in hemolytic disease of the newborn. The clinical significance of weak D is the transfusion of such red cell to Rh D^u immunized subject can result in a haemolytic transfusion reaction. If weak-D red cells are transfused to a Rh negative subject it may lead to alloimmunisation to the Rh D antigen. Subsequent transfusion of blood from these donors to a sensitized individual may result in accelerated destruction of donor RBC.

The low frequency of Rh negativity in our set up is accentuated by low donor drive and suboptimal blood transfusion services, which virtually relegated the daunting responsibilities of donor recruitment and blood collections to individual hospital blood banks (Makroo *et al.*, 2010) Consequently, there is severe scarcity of RhD negative blood in rural Indian blood banks, and therefore, we should scale down the number of requests for RhD negative blood by subjecting all the negative patients who require transfusion to indirect antiglobulin testing (IAT) in order to minimize unnecessary transfusions (Bhatia HM *et al.*, 1997).

Identification of D^u positive patients has numerous advantages where RhD negative bloods are scarce. Firstly, the major advantages is that such patients can be transfused with RhD positive donor blood without any risk of sensitization since they do possess the D^u antigen, which they expressed at lower levels on their red cells. Secondly, valuable and scare RhD negative blood would be conserved to be used for patients that are genuine RhD negative. This is particularly important since donor blood is always in short supply and inadequate to meet the clinical requirements (Makroo *et al.*, 2010). Thirdly, female patients that are identified as D^u positive will be subjected to unnecessary and costly anti-D immunoglobulin infections if they deliver RhD positive babies. This is important in our setup because of the poor socio-economic status of the rural population (Srikrishna *et al.*, 2001).

It is not necessary test infants or cord blood samples for weak D as the immunogenicity of D^u is low and the immunizing dose by a feto-maternal hemorrhage will never be large. The risk of material sensitization is extremely low and the area where most uncertainties remain is whether or not donors need to be tested for D^u.

The use of IAT for RhD (D^u) (Srikrishna *et al.*, 2001) typing can be dangerous as patients with have been falsely recorded as D^u positive when controls have been omitted or wrongly interpreted. If a mistyped Rh-negative female patient was then transfused with RhD positive blood, the consequences due to the serious risk of alloimmunization would be more serious than if the test had not be performed.

Likewise, it is unnecessary to perform a D^u test on samples from pregnant women, the administration of anti-D immunoglobuline either ante or post –natally to a woman with a weak D phenotype will cause no harm and could be of benefit to those whose RBCs contain a D^u category antigen.

Two genes, the RHD and RHCE encode the antigens of Rh blood group. It is generally believed that weak D phenotype could arise from three different genetic mechanisms- (a) A person may inherit a RHD gene that encodes for a weak expression. (b)The 'D' gene may interact with the 'C' gene of the corresponding chromosome in the genotype Cde / Cde or cDe / Cde and for unknown reasons; the expression of the D antigen is depressed. (c) The RHD gene may not encode for all the epitopes that make up the complete D antigen (Tippet P *et al.*, 1998).

(Wagner *et al.*, 2000) identified the phenomena of the weak D genotype in D negative samples using molecular techniques. Therefore, it seems that the main problem with these variants is not their characterization as false D negative, but the potential that they can be D^u positive. In pregnancy, this

means that immunoglobulin will be administered, and in transfusion medicine, D^u negative units will be selected, in most of the cases without justification.

The prevalence of Rh negativity in our donor population has been estimated to be 12.76% and that of weak D antigen 0.15% respectively whereas (Makroo *et al.*, 2010) in their study regarding weak D prevalence among Indian blood donors observed a prevalence of 7.19% of Rh negativity and that of weak D antigen as 0.01%. (Bhatia *et al.*, 1997) [placed] the incidence of weak D in India at 0.3-0.5%. A study by (Sri Krishna *et al.*, 2001) noted the incidence to be 0.15% (Srikrishna *et al.*, 2001). It is possible that the use of potent monoclonal anti D reagents may account for the slightly higher incidence of weak D in our study.

However, a study analyzing repeat antibody screens of serologically D negative patients with weak D alleles who have been exposed to D positive red cells is needed to quantify the absolute risk of sensitization.

Hence, strategies in blood banks regarding the weak D types and the immunization should be established, to reliably type these individuals as RhD positive, not prone to anti-D immunization. The application of methods based on molecular biology could allow fast and economic large scale detection in a near future.

Table: 1 Distribution of Rh antigen & D^u positivity among the donors

Total samples	RhD Positive (+)		RhD Negative (-)		Du Positive	
	n	%	n	%	n	%
15,666	13,800	88.08%	2,000	12.76%	25	0.15

The D antigen is unique among blood groups because it expresses 30 epitopes distributed along the extracellular portions of the RhD protein (Contreras *et al.*, 1989). Changes, in the amino acid sequence of RhD may not ablate the entire D antigen but can cause epitopes loss, giving rise to variant forms known as partial D (Urbuniak SJ, *et al.*,1981).

New discoveries relating to the RhD gene, and an appreciation of its variant phenotypes such as weak D and partial D, have challenged the way that D status is assigned to both blood donors and blood product recipients (Srikrishna A *et al.*, 2001). The Rh antigen D positive is highly immunogenic and clinically significant in transfusion and pregnancy. As many as 80% of D negative recipients of a unit if D positive red cells will form anti-D. Ideally D negative patients should be transfused with D negative components (Makroo *et al.*, 2010).

Conclusion

Weak D Rh antigen presents a peculiar clinical situation. The subject may face problems in Rh blood group typing and prevent a potential risk of alloantibody formation when transfused with Rh positive blood. Allo-immunisation of females with weak-D while in the child-bearing age, is disastrous and results in haemolytic disease of the newborn. It would be prudent to consider individuals with a weak D antigen as Rh D positive when presenting as a donor and Rh D negative when confronted as a recipient.

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