



Iran . J . Immunol
ISSN 1735-1383

Iran. J. Immunol. December 2008, 5 (4), 189-200

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Article Type: Review

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Index Medicus and Pubmed

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REVIEW ARTICLE

Immunologic Basis and Immunoprophylaxis of RhD Induced Hemolytic Disease of the Newborn (HDN)

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ABSTRACT

RhD antigen is the most immunogenic and clinically significant antigen of red blood cells after ABO system. It has historically been associated with hemolytic disease of the newborn (HDN) which is now routinely prevented by the administration of polyclonal anti-D immunoglobulin. This management of HDN has proven to be one of the most successful cases of prophylactic treatment based on antibody mediated immune suppression (AMIS). Despite the increasing efficiency of treatment, the mechanism of action of anti-D is not completely defined. There is a widespread interest in obtaining a reliable therapeutic monoclonal anti-D, due to difficulty of maintaining a pool of high titer volunteer donors for plasma collection and also increasing demand for antenatal prophylaxis and safety issues with plasma derived products. Candidate monoclonal anti-D preparations should demonstrate appropriate functionality in both in vitro and in vivo assays comparable to polyclonal anti-D immunoglobulin. These criteria are reviewed in addition to the factors regulating development of D specific immune response in D negative individuals and its suppression in HDN prophylaxis.

Keywords: Anti-D immunoglobulin, D antigen, Hemolytic Disease of the Newborn (HDN), Monoclonal anti-D, Rh Blood Group System

INTRODUCTION

The D antigen is an extremely potent immunogen of the Rh blood group system present on the surface of human red blood cells (RBCs). It is a non-glycosylated integral membrane protein of approximately 30 kDa, encoded by the RHD gene in the RH locus of D

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positive individuals (1). Most D negative individuals have complete deletion of this gene and the frequency of this status is estimated to be 15-17% among Caucasians (2). These individuals could develop specific antibody against the D antigen (anti-D) if transfused with D positive blood. Similarly, D negative women carrying a D positive fetus may become immunized by fetal D⁺ RBC at parturition. Anti-D immunoglobulin G (IgG) developed by mother can be transported across the placenta in subsequent pregnancies and lead to fetal RBC hemolysis creating a pathological condition known as hemolytic disease of the newborn (HDN) (3). Since the introduction of anti-D immunoglobulin prepared from the plasma of immunized donors 40 years ago, and its routine administration after parturition to RhD negative women delivering an RhD positive infant, rate of maternal alloimmunization has reduced dramatically (4). However, alloimmunization still occurs in some cases mainly due to exposure to fetal RBC during pregnancy (5). To prevent this, anti-D should be given antenatally; nevertheless, due to insufficient supply of anti-D, this treatment is not routinely performed universally. Monoclonal anti-D is an attractive substitute as it will provide unlimited supplies of a standardized and safe product. A number of monoclonal candidates are currently being evaluated in clinical studies for their potential to substitute polyclonal anti-D immunoglobulin. Other immunotherapeutic modalities are also being studied which need further investigation.

RH BLOOD GROUP SYSTEM

Rh blood group system is the most immunogenic and the most polymorphic blood group system in man and currently 48 serologically defined antigens have been identified which belong to this system (2, 6). It is also the most clinically significant blood group system after ABO in transfusion medicine (7). The most important components of the Rh system are D and CcEe proteins encoded by two highly related genes RHD and RHCE, respectively. These proteins are believed to form tetrameric structures known as Rh complexes in the red cell membrane which may contain one D subunit, one CE subunit and two subunits of another molecule called Rh-associated glycoprotein (RhAG) (8). Although recently it has been proposed that Rh protein oligomeric complexes may indeed be trimolecular (9). Physiologically, it is suggested that Rh proteins could play a role in maintaining the cell membrane stability and the regulation of red cell shape (10), based on the observations that individuals lacking all the Rh proteins (Rh null phenotype) suffer from clinical syndromes characterized by a chronic hemolytic anemia of varying severity, an increased osmotic fragility and abnormalities of red cell morphology (known as spherostomatocyte), cation transport and membrane phospholipids organization (11, 12).

RHD and RHCE genes (96% identity) are located in the RH locus on chromosome 1p34-p36 in tandem organization and opposite orientation interspersed by a third gene, SMP1, whose function is currently not defined (6). The most common Rh polymorphism (RhD positive/RhD negative) is associated in most cases with the presence or absence of the RHD gene caused by deletion (13). With respect to Rh CcEe proteins, it has been proposed that codominant expression of Cc and Ee polypeptides is regulated by the single RHCE gene through alternative splicing events giving rise to multiple Rh isoforms (14), though distinct transcript for each polypeptide has also been isolated (15). Both RhD and RhCcEe proteins are composed of 417 amino acids and their structural

models are predicted to consist of 6 extracellular loops, 12 transmembrane and 7 intracellular protein segments with both C and N terminal protein ends residing in the cell (16, 17). D antigen differs from CcEe proteins in 32-35 amino acids, 9-10 of which are located on extracellular loops and the rest are present in the transmembrane and cytoplasmic regions but can still affect the topology of the protein in the membrane (17, 18). There is far more homogeneity between CcEe proteins such that C and c polypeptides differ in 4 (19) and E and e polypeptides only in one amino acid residue (20). The close proximity of RHD and RHCE genes on the same chromosome and the fact that they have mainly identical regions allows for numerous exchange events between them mostly by a process called gene conversion resulting in hybrid proteins accounting for "D variant" or "partial D" categories defined as individuals who lack parts of the normal D antigen. In addition, there are other RhD polymorphisms such as D_{el} and weak D (D^u) phenotypes characterized by reduced D antigen level on the surface of RBCs resulting from various mutations in the RHD gene. A review by Westhoff (21) presents an overview of the molecular complexity of the Rh D protein and its diverse phenotypes.

IMMUNE RESPONSE TO RHD ANTIGEN

Alloimmunity to D antigen in D negative individuals develops in the context of exposure to D⁺ blood, which occurs either through incompatible transfusion or pregnancy. D antigen, being a protein, elicits a T cell dependent immune response (22, 23) characterized by the uptake of the antigen into antigen presenting cells (APCs) of the spleen and lymph nodes, followed by its processing into short peptides in the phagolysosome and the presentation of these peptides on the surface of APCs in association with the major histocompatibility complex (MHC) class II molecules, where they can interact with the corresponding T cell receptors on the surface of CD4⁺ helper T cells. This interaction and further costimulation by the APCs will lead to proliferation and generation of B cell stimulatory cytokines that induce activation and proliferation of B cell clones expressing the antigen specific surface immunoglobulin and their differentiation into antibody secreting plasma cells. Upon first exposure to D antigen, the immune response is relatively slow and may not be detectable for at least 4 weeks. It might result in formation of immunoglobulin M (IgM) alloantibodies. It also produces memory B and T cells that are long-lived and upon subsequent exposure create a rapid response by production of high affinity IgG antibodies that are detectable as early as 48 h post exposure. The high immunogenicity of D antigen is due to the number of antigen specific peptides that can be generated in vivo in D negative individuals expressing only CcEe proteins that as mentioned earlier differ from D antigen in 32-35 amino acid residues. Whereas, C and c which differ by four amino acids and E and e which differ by one amino acid are potentially less immunogenic as fewer specific peptides will be generated when C/c, E/e incompatible transfusions are performed. Using synthetic linear peptides derived from the known amino acid sequence of D protein, it was shown that the level of anti-D response in D negative individuals was directly proportional to the number of peptides that could stimulate antigen specific memory T cells of these individuals to proliferate in vitro (24). It was further demonstrated in this study that these T cells were restricted primarily to human leukocyte antigen (HLA) class II-DR15. A high anti-D titer, and more severe HDN, has also been associated with HLA-DRQB*0201 (25). Similarly, a separate study showed significant overrepresentation of HLA-DRB1*1501 allele in RhD negative donors who had produced anti-D antibodies in response to exposure to RhD posi-

tive RBCs (26). Interestingly, it was demonstrated that expression of human HLA-DR15 transgene in mice which normally do not react to D antigen confers on them the ability to respond to immunization with purified RhD protein and make specific IgG antibodies (27). On the other hand, there are contrasting reports defying the existence of any meaningful association between a specific HLA allele and induction of anti-D response (28, 29). Possibility of HLA restriction in T cell response to D antigen might partly explain why some D negative individuals (5-15%) remain "non-responders" to D antigen despite repeated exposures. These individuals are usually poor responders to other red cell antigens as well (23). However, those who believe the response to RhD is not HLA restricted consider the status of "non-responder" as being not absolute as it depends strongly on the volume and genotype of transfused RBC, ABO antigens incompatibility between fetus and mother, the number of injections and any history of previous administration of passive anti-D (30). Using the technique of EBV transformation in combination with limiting dilution assay (LDA) and Poisson statistical analysis, we have already demonstrated that the variation in degree of "responsiveness" to D antigen in a group of naturally immunized Rh negative women partly reflects the differences in the frequency of D specific B-lymphocyte precursors in each donor (31).

HEMOLYTIC DISEASE OF THE NEWBORN

Hemolytic disease of the fetus and newborn (HDFN) results from maternal IgG antibodies against fetal RBC antigens that cross the placenta to the fetal circulation during gestation and cause RBC destruction and complications before birth (HDF), or anemia and hyperbilirubinemia after birth (HDN) or both. In most severe cases, it causes hydrops fetalis characterized by total body edema, hepatosplenomegaly, and heart failure which usually lead to intrauterine death (32). D antigen being highly immunogenic is the most important cause of HDN (85%) followed by Kell (10%) and c (3.5%) antigens (33). Rarely, cases of HDN have been documented to be associated with immunization against other red cell antigens for instance Rh C, E/e and G antigens (34-37) and antigens of other blood group systems (38, 39). In some HDN cases a combination of antibodies to D, C and G have been identified, accurate determination of which forms an essential aspect of the management of such affected cases (40). There are also numerous reports on the contribution of each of anti-D IgG subclasses, specifically IgG1 and IgG3 antibodies to the severity of HDN (40-43). Some have suggested that HDN occurs most often when IgG1 anti-D and IgG3 anti-D are both present in the maternal serum (40, 41); whereas, others (42, 43) have shown that IgG1 anti-D alone may cause severe HDN. This isotype restriction reflects the efficiency of interaction between IgG1 and IgG3 antibodies and cell surface receptors belonging to Fc γ receptor I and III classes present on effector cells especially macrophages engaged in immune destruction of antibody sensitized RBCs. Studying the sera and clonal B cell lines obtained from a small group of women alloimmunized through pregnancy, we demonstrated that all the subjects had produced specific antibody of IgG1 subclass, while IgG3 and IgM antibodies were also generated in some donors. Production of IgG2 was identified to a lesser extent while none of the donors were shown to have produced specific antibody of IgG4 subclass. All the donors' fetuses were affected with HDN; some were saved by exchange transfusion while some either gave still birth or their fetuses were fatally affected with hydrops fetalis and were lost. However, number of donors in our study was not suffi-

cient enough to illustrate a relationship between the isotype of specific antibodies and severity of HDN (44).

IMMUNOPROPHYLAXIS OF HDN

HDN due to D immunization is prevented in the vast majority of cases by passive administration of anti-D immunoglobulin, prepared from plasma of D negative hyperimmunized donors, to RhD negative women within 72 hours of each delivery of an RhD positive fetus. A dose of 300 µg is commonly used in North America, 100 µg in the UK and 200-250 µg in Europe and elsewhere which will protect against 10-15 ml of fetal RhD positive red cells (45). In Iran a dose of 300 µg is routinely used for both postnatal and antenatal intervals. Fetomaternal haemorrhage (FMH) at parturition in 99.3% of women leads to transfer of less than 4 ml fetal red cells which will be effectively removed from maternal circulation by one injection of anti-D immunoglobulin; however, 0.3% of women have more than 15 ml FMH at delivery hence should be given an additional dose of anti-D (46). In some countries where FMH is monitored, patients with large FMH are followed up for 1 to 2 days after injection to check whether fetal red cells have been cleared and if not, additional anti-D is given (47). A high titer intravenous anti-D preparations (1500 IU) are also employed to prevent sensitization of RhD negative mothers receiving large volumes of fetal RhD+ blood. Anti-D is prescribed to all RhD negative and D variant women following a potentially sensitizing event such as delivery of an RhD positive infant. The incidence of D alloimmunization in pregnancy has decreased from 14% to between 1 and 2% following the introduction of postnatal prophylaxis with passive anti-D. The addition of routine antenatal anti-D prophylaxis at weeks 28 and 34 of gestation in some countries has reduced the immunization cases to 0.1% (32). Nevertheless, antenatal prophylaxis is not universally applied due to a variety of factors one of which is availability and cost of anti-D. Many of women who do receive antenatal anti-D treatment might eventually give birth to a D negative fetus; therefore, identification of fetal blood group status is important in order to be able to restrict antenatal anti-D immunoglobulin to mothers carrying an RhD positive fetus. Although genotyping can be performed on fetal DNA obtained through chorionic villus sampling or from amniotic fluid, current research is focused on identifying fetal genotype from maternal plasma as a source of fetal DNA as a far less invasive procedure (45). This technique is being developed as a routine diagnostic test.

IMMUNOLOGIC BASIS OF ANTI-D PROPHYLAXIS

Despite the highly effective clinical use of anti-D in preventing HDN, the mechanism of treatment remains poorly understood. It is proposed to be mainly dependent on the rapid clearance of anti-D coated D⁺ RBC from maternal circulation by the spleen following the interaction of IgG anti-D with macrophages expressing three classes of IgG receptors, FcγRI, FcγRII and FcγRIIIa. This will lead to early destruction of D antigen before it is recognized by the immune system (48). Nevertheless, in some cases when more than 10 ml FMH occurs following delivery, anti-D coated D⁺ RBC are not cleared from the circulation at the same rate and yet passive anti-D still protects against a specific immune response (49). This observation suggests that antibody clearance theory cannot be the sole mechanism in suppressing the humoral immune response against D antigen. Further support has come from studies of sheep red blood cell clearance in a mice

model of AMIS (antibody mediated immune suppression), showing that T cell priming was not prevented after transfusion of IgG-opsonized RBCs, indicating that the antigen was visible to the immune system. Moreover, delivery of IgG-opsonized RBCs to phagocytic cells was insufficient to attenuate the B-cell response to RBCs. Finally, it was demonstrated that upon challenge with a mixture of opsonized and untreated RBCs, there was a dose dependent reduction of the antibody response. The authors suggest that the attenuation of the antibody response by anti-RBC IgG is not due to active immune suppression or clonal deletion at T-cell or B-cell level, but rather it is the result of B cell unresponsiveness to IgG-opsonized RBCs (50, 51). Another hypothesis is dependent on the role of immunomodulatory cytokines in explaining anti-D mediated immune suppression as demonstrated by Branch et al. (52) showing that the plasma levels of transforming growth factor β (TGF- β) and prostaglandin E2 were significantly increased 48 hours after administration of antenatal anti-D immunoglobulin. TGF- β may bind to aggregated IgG on coated red cells and be localized to antigen-specific B cells and exert its inhibitory effect. In addition to B cells, TGF- β has a profound long-lasting inhibitory effect on T cell priming (53).

Concurrent engagement of the immunoglobulin molecules on surface membrane of antigen-specific B-cells and the Fc γ RIIb is known to induce inhibitory signals leading to suppression of antibody production to the corresponding antigen (54). This mechanism has been proposed to be effective in anti-D immunoprophylaxis (55, 56). Infusion of intravenous immunoglobulin (IVIg) collected from plasma of normal individuals with no previous history of RhD immunization has also been shown to downregulate the immune response in some clinical conditions such as recurrent spontaneous abortion and autoimmune thrombocytopenic purpura (57). These immunological effects have been attributed to signaling through both activating Fc γ R (Fc γ RI and Fc γ RIII) on dendritic cells and the inhibitory Fc γ RIIb on B-cells (58).

MONOCLONAL ANTI-D FOR PROPHYLACTIC TREATMENT

With increasing success of passive anti-D prophylactic program in treatment of HDN, very few women are now immunized and able to donate plasma. Immunization of D negative male volunteers hence serves to make up for the shortage. On the other hand, despite stringent safety guidelines, there are concerns over the inherent risk of transmission of agents such as variant-Creutzfeldt-Jakob disease (vCJD) through administration of human plasma derived products (59). An effective monoclonal anti-D antibody would eliminate the need for preparation and use of plasma derived anti-D.

Over the years, several monoclonal anti-D antibodies have been produced in different laboratories through Epstein-Barr virus (EBV) immortalization and generation of B-lymphoblastoid cell lines (LCLs) derived from immunized D negative donors (60, 61). Since mice do not recognize the D antigen, thus no murine monoclonal anti-D antibodies have ever been produced. In order to enrich for D specific LCLs, some laboratories have utilized rosetting of D-specific B-LCLs with D⁺ RBC before cloning (60, 62). In an attempt to increase the antibody production of these cell lines, some have been fused with mouse myeloma cells to produce heterohybridomas (62-64). Monoclonal anti-D antibodies of IgM isotype derived by similar techniques in our laboratory (65) and by other workers (66, 67) serve as efficient D typing serological reagents; however, in order to be effective as a replacement for plasma derived anti-D immunoglobulin, the monoclonal anti-D should be of IgG isotype.

Aside from traditional techniques of human monoclonal antibody production, more recently human RhD specific monoclonal antibodies have also been generated by various approaches using recombinant DNA technology. These include production of anti-D recombinant antibodies by panning phage display libraries of Fab fragments from a hyperimmune donor and the expression of a complete immunoglobulin construct in baculovirus-insect cell expression system (68) or Chinese hamster ovary (CHO) cells (69) and by antibody engineering technique from the DNA coding for anti-D in lymphoblastoid cell lines and heterohybridomas and its expression in rodent myeloma cell lines (55).

In order to be considered for prophylactic treatment, the monoclonal anti-D preparations must fulfill certain criteria and their performance evaluated in a number of *in vitro* and *in vivo* functional assays. For the rest of this review, we will try to present an overview of these assays in relation to their use in estimating the efficacy of a number of monoclonal anti-Ds in quest for a suitable and efficient substitute for polyclonal anti-D.

Primarily, the antibodies should be serologically determined to be specific for the D antigen and bind only to D positive but not D negative red cells. They should also demonstrate a high affinity constant for their antigen comparable to polyclonal anti-D preparations for which the functional affinity constants varied between 3.1×10^8 to 4.2×10^8 mol/l, as determined by an enzyme linked immunosorbent assay (ELISA) of solubilized anti-D bound to sensitized RBCs (70). Monoclonal anti-Ds with lower affinity constants show greater dissociation from the fetal red cells, a condition not exactly desirable for generation of monoclonal substitutes with prophylactic value. Epitope specificity of the candidate monoclonal anti-D antibodies is also a determining factor in their selection for immunoprophylaxis. The RhD antigen is composed of different epitopes and a polyclonal anti-D preparation contains a mixture of immunoglobulins directed against these various epitopes. Individuals known as "D variants" or "partial D" lack parts of the normal D antigen and if challenged with normal D positive blood can produce antibody to those epitopes of the antigen which they lack (71). The last international workshop on Rh serology summarized the agglutination pattern of 142 Rh specific monoclonal antibodies with D variant red cells and concluded that there are 30 different reaction patterns or epitopes for D antigen (72). No single anti-D monoclonal antibody is capable of reacting with all the partial D types implication of which is that in order to ensure that a monoclonal anti-D for immunoprophylaxis will be efficient in clearing fetal cells with partial D antigens that could potentially induce an anti-D response in a D negative mother, it might be necessary to blend a mixture of monoclonal anti-D antibodies with different specificities into a functional cocktail (73). Despite the apparently diverse epitope specificity, anti-D antibodies seem to recognize limited antigenic determinants of the RhD molecule. This is supported by the fact that of the 35 D-specific amino acids only seven residues are located in the extracellular region of the D molecule. Thus, only these residues are available for binding of anti-D on RhD+ RBC (23, 74). Restricted epitope specificity of the anti-D antibodies is also indicated by the restricted immunoglobulin variable region heavy (VH) and light (VL) chain genes repertoire. More than 70% of the VH genes rearranged in anti-D antibodies are selected from the VH3.33 superspecies genes (75, 76) and almost 10% of the anti-D VH genes belong to the VH4.34 member of the VH4 gene family (75). Our recently established IgM anti-D monoclonal antibody (MG-1G7) was also found to express the VH4.34 germline gene (unpublished observation). Altogether, these findings imply involvement of a focused antibody response to the RhD antigen.

In addition to the above factors which can be grouped as serological criteria, selection of monoclonal anti-D for therapeutic use depends on its functional ability in interacting with effector cells mainly through binding of the Fc region of anti-D to the receptors Fc γ RI, Fc γ RIIa and Fc γ RIII. These receptors are present on splenic macrophages; however, normally these particular cells are not available for experimental purposes. Hence, a number of in vitro assays have developed that use Fc γ R⁺ effector cells from peripheral blood. Monocytes express Fc γ RI and Fc γ RIIa and natural killer (NK) cells have Fc γ RIIIa (30). Adherence and formation of rosettes, and phagocytosis as measured by chemiluminescence due to oxidative burst caused by this process are in vitro measures of interaction with Fc γ RI. Antibody dependent cellular cytotoxicity (ADCC) as measured by radiolabeled chromium release from red cells in presence of NK cells is an indication of the interaction with Fc γ RIIIa (73). In general IgG1 anti-D promotes greater monocyte phagocytosis than IgG3; whereas IgG3 anti-D promotes more adherence and extracellular lysis by monocytes of sensitized red cells than IgG1 anti-D (77). With regards to anti-D mediated haemolysis via Fc γ RIIIa on NK cells, only some monoclonal IgG1 anti-Ds have proven effective and IgG3 anti-Ds are generally inactive in this assay (78). Two monoclonal anti-D antibodies (BRAD-3 & BRAD-5) produced by Epstein-Barr virus-transformed B-LCLs (62) are two examples of antibodies with in vitro effector functions almost comparable to polyclonal anti-D. BRAD-5 (IgG1) was found to mediate high extracellular hemolysis of antibody coated red cells by NK cells in ADCC assay; whereas BRAD-3 (IgG3) was shown to adhere to monocytes and induce Fc γ RI-mediated phagocytosis, chemiluminescence and extracellular lysis of antibody coated red cells more efficiently than BRAD-5 (79). Both antibodies recognize an immunodominant epitope (ep D6/7) on the RhD antigen (62) and have been adapted to large scale culture in hollow-fibre bioreactors with a similar glycosylation pattern to that of human serum IgG (80). They have both been used in clinical studies as the next step in generation of therapeutic monoclonal anti-D.

Basically a number of criteria are considered important in determining the in vivo efficacy of a monoclonal anti-D. The anti-D must demonstrate a long plasma half-life which is desirable for antenatal prophylaxis to ensure anti-D levels are enough to prevent D-immunization at the time of FMH; it must be able to efficiently clear RBCs from the circulation and must prevent D immunization. A recent insightful review by Kumpel presents an excellent overview of the use of different available monoclonal anti-Ds in clinical trials (81). To determine the plasma half life of anti-D, it is injected into D negative subjects in the absence of D positive red cells and kinetics of antibody survival is then calculated by quantitating anti-D concentrations in serum samples taken at intervals (81). BRAD-3 and BRAD-5 were determined to have plasma half lives of 22.2 and 10.2 days, respectively upon intramuscular injections of 300 μ g BRAD-5 and 1500 μ g BRAD-3. The plasma half life of polyclonal anti-D (100 μ g) in the same study was determined to be 15.6 days (82). There are variations between such studies because of different dose of anti-D, route of injection, timing of samples and method of calculation; however in general some monoclonal anti-Ds and recombinant anti-Ds are rapidly eliminated compared to polyclonal anti-D (81).

Clearance studies are usually performed by two different approaches: 1) clearance of ex vivo labelled autologous RBCs presensitized with anti-D antibodies, 2) clearance of labelled allogeneic D positive RBCs in male D negative volunteers after intravascular or intramuscular administration of anti-D. The second approach is usually followed by multiple samplings over a period of at least 6 months to document any immunization.

Additionally the donors are later challenged unprotected with D positive RBC to assess the nature of immune response (primary as opposed to secondary response) (83). BRAD-3 showed rapid red cell clearance of autologous D positive red cells at almost similar rates to polyclonal anti-D (84) and both BRAD-3 and BRAD-5 were efficient in removing more than 90% of allogeneic red cells from the circulation of D negative volunteers with BRAD-5 being similar to polyclonal anti-D (85); however, both antibodies were used at doses three times that of polyclonal anti-D. Challenge with D positive RBC indicated that all the responders had been protected from immunization by passive BRAD-3 or BRAD-5. Overall, the anti-D antibodies (both monoclonal and recombinant) used in clinical studies have produced variable results in terms of clearance efficiency, protection against immunization, induction of pro-inflammatory events and half lives, details of which are described elsewhere (81).

Recently a recombinant monoclonal anti-D (IgG1), R297, was tested in a clinical phase I study to assess its ability to induce the clearance of autologous antibody coated red cells in male D negative volunteers (86). It demonstrated a more rapid RBC elimination compared to that of polyclonal anti-D at similar coating levels and induced no clinical, biological or immunologic adverse effects in the subjects studied. Authors believe the high efficiency of R297 in clearance studies to be due to its higher Fc γ RIIIa binding and ADCC activity which is the result of its optimum glycosylation profile (55). In summary, many monoclonal anti-Ds have been developed and studied in laboratories around the world for their potential as prophylactic reagent and all of these studies have contributed a great deal to our understanding of interaction of antibodies with the effector cells of the immune system and the mechanism of anti-D mediated immune suppression.

FUTURE WORK AND RESEARCH

Basically, three different alternative strategies to prevent HDN which are independent of the classical anti-D immunoglobulin administration are currently under investigation:

1. Development of recombinant mutant IgG anti-D deficient in hemolytic activity in order to block the hemolytic activity of maternal anti-D by binding to D⁺ fetal RBC (87, 88). These engineered antibodies have been generated either by deletion of the hinge region of the heavy chain and a further mutation to link the light chains by a disulfide bond for added stability, which efficiently removed the Fc γ receptor mediated hemolysis and phagocytosis of RBC (87), or by designing an IgG1 hybrid Fc region which was inactive in binding to Fc γ receptors and complement (88).
2. Investigation on the effect of regulatory CD25⁺ T cells (Treg1) which inhibit allogeneic reactions through the production of IL-10 and serve to suppress the antibody response in vivo (89).
3. Use of immunodominant RhD peptides to switch off (tolerize) the immune response to D antigen by administration through nasal route. Proof of concept for this strategy has already been demonstrated in humanized HLA-DR15 transgenic mice in which administration of each of these peptides to the nasal mucosa before immunization with purified RhD protein led to inhibition of T-cell priming and blocked specific antibody response (27). Clinical trials for this novel, noninvasive protocol of suppressing anti-D production in women are currently under consideration (90).

ACKNOWLEDGEMENTS

Some of our results presented in this article were obtained from studies supported by grants from the Food and Drug Administration of the Ministry of Health and Medical Education of Iran, The Medical Biotechnology Network of Iran, Pasteur Institute of Iran and Tehran University of Medical Sciences.

REFERENCES

- 1 Bloy C, Blanchard D, Lambin P, Goossens D, Rouger P, Salmon C et al. Human monoclonal antibody against Rh(D) antigen: partial characterization of the Rh(D) polypeptide from human erythrocytes. *Blood*. 1987; 69: 1491-7.
- 2 Wright MS. The Rh blood group system. In: Quinley ED, editor. *Immunohematology: Principles and practice*. Lippincott Company, Philadelphia: 1993. p.106-16.
- 3 Zupanska B. Assays to predict the clinical significance of blood group antibodies. *Curr Opin Hematol*. 1998; 5:412-6.
- 4 Moise KJ. Management of rhesus alloimmunization in pregnancy. *Obstet Gynecol*. 2002; 100: 600-11.
- 5 Urbaniak SJ. The scientific basis of antenatal prophylaxis. *Br J Obstet Gynaecol*. 1998; 105 (suppl.18):11-8.
- 6 Suto Y, Ishikawa Y, Hyodo H, Uchikawa M, Juji T. Gene organization and rearrangements at the human Rhesus blood group locus revealed by fiber-FISH analysis. *Hum Genet*. 2000; 106:164-71.
- 7 Avent ND, Reid ME. The Rh blood group system: a review. *Blood*. 2000; 95:375-87.
- 8 Heitman J, Agre P. A new face of the Rhesus antigen. *Nat Genet*. 2000; 26:258-9.
- 9 Conroy MJ, Bullough PA, Merrick M, Avent ND. Modelling the human rhesus proteins: Implications for structure and function. *Br J Haematol*. 2005; 131:543-51.
- 10 Le Van Kim C, Colin Y, Cartron JP. Rh proteins: Key structural and functional components of the red cell membrane. *Blood Rev*. 2006; 20:93-110.
- 11 Sturgeon P. Hematological observations on the anemia associated with blood type Rhnull. *Blood*. 1970; 36:310-20.
- 12 Nash R, Shojania AM. Hematological aspect of Rh deficiency syndrome: a case report and review of the literature. *Am J Hematol*. 1987; 24:267-75.
- 13 Colin Y, Cherif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood*. 1991; 78:2747-52.
- 14 Le Van Kim C, Cherif-Zahar B, Raynal V, Mouro I, Lopez M, Cartron JP et al. Multiple Rh mRNAs isoforms are produced by alternative splicing. *Blood*. 1992; 80:1074-8.
- 15 Simsek S, de Jong CA, Cuijpers HT, Bleeker PM, Westers TM, Overbeeke MA et al. Sequence analysis of cDNA derived from reticulocyte mRNAs coding for Rh polypeptides and demonstration of E/e and C/c polymorphisms. *Vox Sang*. 1994;67:203-9.
- 16 Le Van Kim C, Mouro I, Cherif-Zahar B, Raynal V, Cherrier C, Cartron JP et al. Molecular cloning and primary structure of the human blood group Rh D polypeptide. *Proc Natl Acad Sci USA*. 1992; 89: 10925-9.
- 17 Eyers SA, Ridgwell K, Mawby WJ, Tanner MJ. Topology and organization of human Rh (rhesus) blood group-related polypeptides. *J Biol Chem*. 1994; 269:6417-23.
- 18 Arce MA, Thompson ES, Wagner S, Coyne KE, Ferdman BA, Lublin DM.. Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood*. 1993; 82:651-5.
- 19 Avent ND, Daniels GL, Martin PG, Green CA, Finning KM, Warner KM. Molecular investigation of the Rh C/c polymorphisms. *Transfus Med*. 1997; 7(suppl. 1):18.
- 20 Issitt PD. An invited review: The Rh antigen e, its variants, and some closely related serological observations. *Immunohematology*.1991; 7:29-36.
- 21 Westhoff CM. Review: the Rh blood group D antigen...dominant, diverse, and difficult. *Immunohematology*. 2005; 21:155-63.
- 22 Ouwehand WH, Wallington TB. Adaptive immunity and transfusion. *Vox Sang*. 2004; 87(suppl. 1):35-8.
- 23 Urbaniak SJ. Alloimmunity to RhD in humans. *Transfus Clin Biol*. 2006; 13:19-22.
- 24 Scott LM, Barker RN, Urbaniak SJ. Identification of alloreactive T-cell epitopes on the Rhesus D protein. *Blood*. 2000; 96:4011-9.
- 25 Hilden JO, Gottvall T, Lindblom B. HLA phenotypes and severe Rh(D) immunization. *Tissue Antigens*. 1995; 46:313-5.
- 26 Urbaniak SJ, Scott LM, Hall AM, Acim LS, Barker RN. Peptides derived from the RhD protein generate regulatory cytokines in vitro and induce mucosal tolerance to RhD in HLA-DR15 transgenic mice [abstract]. *Blood*. 2002; 100:25.
- 27 Hall AM, Cairn LS, Altmann DM, Barker RN, Urbaniak SJ. Immune responses and tolerance to the RhD blood group protein in HLA-transgenic mice. *Blood*. 2005; 105:2175-9.
- 28 Smith NA, Ala FA, Lee D, Love EM, Makar YF, Pamphilon DH et al. A multi-centre trial of monoclonal anti-D in the prevention of Rh-immunization of RhD⁻ male volunteers by RhD⁺ red cells. *Transfus Med*. 2000;10 (suppl. 1): 8.
- 29 Kruskall MS, Yunis EJ, Watson A, Awdeh Z, Alper CA. Major histocompatibility complex markers and red cell antibodies to the Rh(D) antigen. Absence of association. *Transfusion*. 1990; 30:15-9.
- 30 Kumpel BM. Monoclonal anti-D development programme. *Transpl Immunol*. 2002; 10:199-204.
- 31 Pasha RP, Shokrgozar MA, Bahrami ZS, Shokri F. Frequency analysis of B lymphocytes specific for Rh antigens in naturally immunized Rh-negative women. *Vox Sang*. 2004; 86:62-70.
- 32 Eder AF. Update on HDFN: new information on long-lasting controversies. *Immunohematology*. 2006; 22:188-95.
- 33 Van Kamp IL, Klumper FJ, Oepkes D, Meerman RH, Scherjon SA, Vandenbussche FP et al. Complications of intrauterine intravascular transfusion for fetal anemia due to maternal red cell alloimmunization. *Am J Obstet Gynecol*. 2005; 192:171-7.
- 34 Huber AR, Leonard GT, Driggers RW, Learn SB, Gilstad CW. Case report: moderate hemolytic disease of the newborn

- due to anti-G. *Immunohematology*. 2006; 22:166-70.
- 35 Trevett TN Jr, Moise KJ Jr. Twin pregnancy complicated by severe hemolytic disease of the fetus and newborn due to anti-g and anti-C. *Obstet Gynecol*. 2005; 106:1178-80.
 - 36 McAdams RM, Dotzler SA, Winter LW, Kerecman JD. Severe hemolytic disease of the newborn from anti-e. *J Perinatol*. 2008; 28:230-2.
 - 37 Thakral B, Agrawal SK, Dhawan HK, Saluja K, Dutta S, Marwaha N. First report from India of hemolytic disease of newborn by anti-c and anti-E in Rh(D) positive mothers. *Hematology*. 2007; 12:377-80.
 - 38 Ishihara Y, Miyata S, Chiba Y, Kawai T. Successful treatment of extremely severe fetal anemia due to anti-Jra alloimmunization. *Fetal Diagn Ther*. 2006; 21:269-71.
 - 39 Kim WD, Lee YH. A fetal case of severe hemolytic disease of newborn associated with anti-Jk(b). *J Korean Med Sci*. 2006; 21:151-4.
 - 40 Iyer YS, Kulkarni SV, Gupte SC. Distribution of IgG subtypes in maternal anti-D sera and their prognostic value in Rh hemolytic disease of the newborn. *Acta Haematol*. 1992; 88:78-81.
 - 41 Downing I, Bromilow IM, Templeton JG, Fraser RH. A retrospective study of red cell maternal antibodies by chemiluminescence. *Vox Sang*. 1996; 71:226-32.
 - 42 Nance SJ, Arndt PA, Garratty G, Nelson JM. Correlation of IgG subclass with the severity of hemolytic disease of the newborn. *Transfusion*. 1990; 30:381-2.
 - 43 Lubenko A, Contreras M, Rodeck CH, Nicolini U, Savage J, Chana H. Transplacental IgG subclass concentrations in pregnancies at risk of hemolytic disease of the newborn. *Vox Sang*. 1994; 67:291-8.
 - 44 Pasha PKR, Bahrami ZS, Niroomanesh S, Ramzi F, Razavi AR, Shokri F. Specificity and isotype of Rh specific antibodies produced by human B-cell lines established from allo-immunized Rh negative women. *Transfusion and Apheresis Science*. 2005; 33:119-27.
 - 45 Urbaniak SJ, Greiss MA. RhD hemolytic disease of the fetus and the newborn. *Blood Rev*. 2000; 14:44-61.
 - 46 Urbaniak SJ. Royal College of Physicians of Edinburgh/Royal College of Obstetricians and Gynecologists consensus conference on anti-D prophylaxis. *Transfus Med*. 1997; 7:143-4.
 - 47 Bowman J. Rh-immunoglobulin: Rh prophylaxis. *Best Pract Res Clin Haematol*. 2006; 19:27-34.
 - 48 Kumpel BM. On the immunologic basis of Rh immune globulin (anti-D) prophylaxis. *Transfusion*. 2006; 46:1652-5.
 - 49 Lubenko A, Williams M, Johnson A, Pluck J, Armstrong D, MacLennan S. Monitoring the clearance of fetal RhD-positive red cells in FMH following RhD immunoglobulin administration. *Transfus Med*. 1999; 9:331-5.
 - 50 Brinc D, Le-Tien H, Crow AR, Freedman J, Lazarus AH. IgG-mediated immunosuppression is not dependent on erythrocyte clearance or immunological evasion: implications for the mechanism of action of anti-D in the prevention of hemolytic disease of the newborn? *Br J Haematol*. 2007; 139:275-9.
 - 51 Brinc D, Le-Tien H, Crow AR, Siragam V, Freedman J et al. Immunoglobulin G-mediated regulation of the murine immune response to transfused red blood cells occurs in the absence of active immune suppression: implications for the mechanism of action of anti-D in the prevention of haemolytic disease of the fetus and newborn? *Immunology*. 2008; 124:141-6.
 - 52 Branch DR, Shabani F, Lund N, Denomme GA. Antenatal administration of Rh-immune globulin causes significant increases in the immunomodulatory cytokines transforming growth factor- β and prostaglandin E2. *Transfusion*. 2006; 46:1316-22.
 - 53 Kumpel BM. In vivo studies of monoclonal anti-D and the mechanism of immune suppression. *Transfus Clin Biol*. 2002; 9:9-14.
 - 54 Amigorena S, Bonnerot C, Drake JR, Choquet D, Hunziker W, Guillet JG et al. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science*. 1992; 256:1808-12.
 - 55 Siberil S, de Romeuf C, Bihoreau N, Fernandez H, Meterreau JL, Regenman A et al. Selection of a human anti-RhD monoclonal antibody for therapeutic use: impact of IgG glycosylation on activating and inhibitory Fc gamma R functions. *Clin Immunol*. 2006; 118:170-9.
 - 56 Kumpel BM, Elson CJ. Mechanism of anti-D-mediated immune suppression--a paradox awaiting resolution? *Trends Immunol*. 2001; 22:26-31.
 - 57 Jolles S, Sewell WA, Misbah SA. Clinical uses of intravenous immunoglobulin. *Clin Exp Immunol*. 2005; 142:1-11.
 - 58 Siragam V, Crow AR, Brinc D, Song S, Freedman J, Lazarus AH. Intravenous immunoglobulin ameliorates ITP via activating Fc gamma receptors on dendritic cells. *Nat Med*. 2006; 12:688-92.
 - 59 Burnouf T, Padilla A. Current strategies to prevent transmission of prions by human plasma derivatives. *Transfus Clin Biol*. 2006; 13:320-8.
 - 60 Crawford DH, Barlow MJ, Harrison JF, Winger L, Huehns ER. Production of human monoclonal antibody to rhesus D antigen. *Lancet*. 1983; 1:386-8.
 - 61 Goosens D, Champomier F, Rouger P, Salmon C. Human monoclonal antibodies against blood group antigen. Preparation of a series of stable EBV immortalized B clones producing high levels of antibody of different isotypes and specificities. *J Immunol Methods*. 1987; 101:193-200.
 - 62 Kumpel BM, Poole GD, Bradley BA. Human monoclonal anti-D antibodies. I. Their production, serology, quantitation and potential use as blood grouping reagents. *Br J Haematol*. 1989; 71:125-9.
 - 63 Thompson KM, Hough DW, Maddison PJ, Melamed MD, Hughes Jones NC. The efficient production of stable, human monoclonal antibody secreting hybridomas from EBV-transformed lymphocytes using the mouse myeloma X63-Ag8.653 as a fusion partner. *J Immunol Methods*. 1986; 94:7-12.
 - 64 Rapaille A, Francois-Gerard C, Donnay D, Sondag-Thull D. Production of stable human-mouse hybridomas secreting monoclonal antibodies against Rh D and c antigens. *Vox Sang*. 1993; 64:161-6.
 - 65 Pasha RP, Roohi A, Shokri F. Establishment of human heterohybridoma and lymphoblastoid cell lines specific for the Rh D and C antigens. *Transfus Med*. 2003; 13:83-92.
 - 66 Thompson KM, Melamed MD, Eagle K, Gorick BD, Gibson T, Holburn AM et al. Production of human monoclonal IgG and IgM antibodies with anti-D (rhesus) specificity using heterohybridomas. *Immunology*. 1986; 58:157-63.
 - 67 Raache R, Rapaille A, Sondag-Thull D, Abbadi MC. Production of monoclonal antibodies specific for the ABO blood group and rhesus D antigens. *Arch Inst Pasteur Alger*. 1998; 62:118-37.
 - 68 Edelman L, Margaritte C, Chaabihi H, Monchatre E, Blanchard D, Cardona A et al. Obtaining a functional recombinant anti-rhesus (D) antibody using the baculovirus-insect cell expression system. *Immunology*. 1997; 91:13-9.

- 69 Miescher S, Zahn-Zabal M, de Jesus M, Moudry R, Fisch I, Vogel M et al. CHO expression of a novel human recombinant IgG1 anti-RhD antibody isolated by phage display. *Br J Haematol.* 2000; 111:157-66.
- 70 Debbia M, Braossard Y, Lambin P. Measurement of the affinity of anti-D in the serum of immunized mothers and in immunoglobulin preparations with unlabeled antibodies. *Transfusion.* 2005; 45:975-83.
- 71 Avent ND, Reid ME. The Rh blood group system: a review. *Blood.* 2000; 95:375-87.
- 72 Scott M. Section 1A: Rh serology. Coordinator's report. *Transfus Clin Biol.* 2002; 9:23-9.
- 73 Scott ML. Monoclonal anti-D for immunoprophylaxis. *Vox Sang.* 2001; 81:213-8.
- 74 Liu W, Avent ND, Jones JW, Scott ML, Voak D. Molecular configuration of Rh D epitopes as defined by site-directed mutagenesis and expression of mutant Rh constructs in K562 erythroleukemia cells. *Blood.* 1999;94:3986-96.
- 75 Andersen PS, Haahr-Hansen M, Coljee VW, Hinnerfeldt FR, Varming K, Bregenholt S et al. Extensive restrictions in the VH sequence usage of the human antibody response against the Rhesus D antigen. *Mol Immunol.* 2007;44:412-22.
- 76 Dohmen SE, Mulder A, Verhagen OJ, Eijssink C, Franke-van Dijk ME, van der Schoot CE. Production of recombinant Ig molecules from antigen-selected single B cells and restricted usage of Ig-gene segments by anti-D antibodies. *J Immunol Methods.* 2005;298:9-20.
- 77 Hadley AG, Kumpel BM, Leader K, Merry AH, Brojer E, Zupanska B. An in vitro assessment of the functional activity of monoclonal anti-D. *Clin Lab Haematol.* 1989; 11:47-54.
- 78 Kumpel BM, Beliard R, Brossard Y, Edelman L, de Haas M, Jackson DJ et al. Section 1C: Assessment of the functional activity and IgG Fc receptor utilization of 64 IgG Rh monoclonal antibodies. Coordinator's report. *Transfus Clin Biol.* 2002; 9:45-53.
- 79 Kumpel BM. In vitro functional activity of IgG1 and IgG3 polyclonal and monoclonal anti-D. *Vox Sang.* 1997; 72:45-51.
- 80 Kumpel BM, Rademacher TW, Rook, GA, Williams PJ, Wilson IB. Galactosylation of human IgG monoclonal anti-D produced by EBV-transformed B-lymphoblastoid cell lines is dependent on culture method and affects Fc receptor-mediated functional activity. *Hum Antibodies Hybridomas.* 1994; 5:143-51.
- 81 Kumpel BM. Efficacy of RhD monoclonal antibodies in clinical trials as replacement therapy for prophylactic anti-D immunoglobulin: more questions than answers. *Vox Sang.* 2007; 93:99-111.
- 82 Goodrick J, Kumpel B, Pamphilon D, Fraser I, Chapman G, Dawes B et al. Plasma half lives and bioavailability of human monoclonal Rh D antibodies BRAD-3 and BRAD-5 following intramuscular injection into Rh D-negative volunteers. *Clin Exp Immunol.* 1994; 98:17-20.
- 83 Beliard R. Monoclonal anti-D antibodies to prevent alloimmunization: lessons from clinical trials. *Transfus Clin Biol.* 2006; 13:58-64.
- 84 Thomson A, Contreras M, Gorick B, Kumpel B, Chapman GE, Lane RS et al. Clearance of Rh D-positive red cells with monoclonal anti-D. *Lancet.* 1990; 336:1147-50.
- 85 Kumpel BM, Goodrick MJ, Pamphilon DH, Fraser ID, Poole GD, Morse C et al. Human Rh D monoclonal antibodies (BRAD-3 and BRAD-5) cause accelerated clearance of Rh D+ red blood cells and suppression of Rh D immunization in Rh D- volunteers. *Blood.* 1995; 86:1701-9.
- 86 Beliard R, Waegemans T, Notelet D, Massad L, Dhainaut F, Romeuf C et al. A human anti-D monoclonal antibody selected for enhanced FcγRIII engagement clears RhD⁺ autologous red cells in human volunteers as efficiently as polyclonal anti-D antibodies. *Br J Haematol.* 2008; 141:109-19.
- 87 Nielsen LK, Green TH, Sandlie I, Michaelson TE, Dzeigiel MH. In vitro assessment of recombinant, mutant immunoglobulin G anti-D devoid of hemolytic activity for treatment of ongoing hemolytic disease of the fetus and newborn. *Transfusion.* 2008; 48:12-9.
- 88 Armour KL, Parry-Jones DR, Beharry N, Ballinger JR, Mushens R, Williams RK et al. Intravascular survival of red cells coated with a mutated human anti-D antibody engineered to lack destructive activity. *Blood.* 2006; 107:2619-26.
- 89 Roncarolo M, Levings MK. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol.* 2000; 12:676-83.
- 90 Urbaniak SJ. Noninvasive approaches to the management of RhD hemolytic disease of the fetus and newborn. *Transfusion.* 2008; 48:2-5.