



Mechanisms of anti-D action in the prevention of hemolytic disease of the fetus and newborn

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Anti-D is routinely and effectively used to prevent hemolytic disease of the fetus and newborn (HDFN) caused by the antibody response to the D antigen on fetal RBCs. Anti-D is a polyclonal IgG product purified from the plasma of D-alloimmunized individuals. The mechanism of anti-D has not been fully elucidated. Antigenic epitopes are not fully masked by anti-D and are available for immune system recognition. However, a correlation has frequently been observed between anti-D-mediated RBC clearance and prevention of the antibody response, suggesting that anti-D may be able to destroy RBCs without triggering the adaptive immune response. Anti-D-opsonized RBCs may also elicit inhibitory FcγRIIB signaling in B cells and prevent B cell activation. The ability of antigen-specific IgG to inhibit antibody responses has also been observed in a variety of animal models immunized with a vast array of different antigens, such as sheep RBCs (SRBC). This effect has been referred to as antibody-mediated immune suppression (AMIS). In animal models, IgG inhibits the antibody response, but the T-cell response and memory may still be intact. IgG does not mask all epitopes, and IgG-mediated RBC clearance or FcγRIIB-mediated B-cell inhibition do not appear to mediate the AMIS effect. Instead, IgG appears to selectively disrupt B cell priming, although the exact mechanism remains obscure. While the applicability of animal models of AMIS to understanding the true mechanism of anti-D remains uncertain, the models have nevertheless provided us with insights into the possible IgG effects on the immune response.

The D polypeptide is a highly immunogenic antigen and can cause clinically significant antibody responses in D-negative individuals.¹ The decision to use anti-D to prevent D alloimmunization was originally based upon two different observations.^{2,3} Firstly, the incidence of hemolytic disease of the fetus and newborn (HDFN) was reduced in cases of ABO incompatibility between the mother and the child, which was thought to occur because anti-A or anti-B IgM antibodies destroyed the incompatible fetal RBCs before immune system recognition.² Secondly, the inhibitory effect of antibodies on RBC immunization has been observed in different animal models⁴ and termed antibody-mediated immune suppression (AMIS).⁵ It was therefore thought that anti-D may prevent D immunization by one of these mechanisms.^{2,3}

Anti-D has been in use for several decades; however several problems are associated with its use. Current anti-D preparations are prepared from the plasma of human donors immunized to the D antigen and as such exist in a limited supply. While the risk for transmission of known infectious

diseases with these preparations is thought to be minimal, there have been reports of transmission of the Hepatitis C virus in the past.¹ There is also a theoretical concern regarding the transmission of variant Creutzfeldt-Jacob disease,¹ as well as concern for the entry of new unknown pathogens into a vulnerable population.

As part of the efforts to replace polyclonal anti-D, several monoclonal or recombinant anti-D antibodies have been developed (reviewed in Beliard⁶ and Kumpel⁷). However, monoclonal/recombinant anti-Ds have had a variable effect, and some anti-Ds have unexpectedly enhanced the anti-D response.⁷

The ability of anti-D to prevent the antibody response to D-positive RBCs is not an isolated phenomenon. It has long been known that antigen-specific IgG administered with an antigen can inhibit antigen-specific antibody responses in various animal models.^{4,8} The findings on the mechanism of action in animal models of AMIS may be relevant for the understanding and design of monoclonal/recombinant anti-

D antibodies and the selection of an optimal monoclonal product able to prevent the immune response to the D antigen.

The Antibody Response to RBCs

The antibody response to RBCs likely involves the activation of antigen-specific B cells, which is elicited by RBC-mediated ligation of the B-cell receptor (BCR) on the B-cell surface and cognate interaction between RBC-specific T and B cells.⁹ Various models have been proposed for naïve B-cell encounter with an antigen.¹⁰ B cells may recognize the antigen directly or the antigen presented on the surface of follicular dendritic cells, migrating dendritic cells, or subcapsular sinus macrophages.¹⁰ B cells can acquire antigens from the particulate surface without internalizing the entire particle.¹⁰ It has also been shown that membrane-bound antigens can be acquired in a membrane-bound form following receptor-ligand interaction, leading to incorporation of membrane patches into B and T cells, a process termed “troglodytosis.”¹¹ However, the precise mechanism of B-cell recognition of D-positive RBCs is not fully understood.

T cells are activated by antigen-presenting cells (APCs), including macrophages, dendritic cells, as well as B cells, although mature dendritic cells are often the major APCs that activate T cells. It has been shown that transfusion of RBCs under inflammatory conditions targets the RBCs into splenic dendritic cells, induces dendritic cell maturation, and promotes the anti-RBC T-cell response in mice.¹² Antigen presented by B cells may also be sufficient for CD4⁺ T cell activation.¹³ However, the role of B cells in the D-specific T-cell response is not known. Recent studies have also indicated novel pathways that can regulate B-cell activation, such as activation induced by Toll-like receptors (TLRs),^{14,15} interleukin (IL)-4 signals derived from CD11b⁺Gr-1⁺ cells,¹⁶ and potential inhibition mediated by T-regulatory cells.¹⁷

Animal Models with Potential Relevance to the Anti-D Effect

Over the course of a century, the inhibitory AMIS effect has been consistently observed in a variety of different models. These early studies of AMIS first established that the antigen-specific IgG antibody can suppress the antibody response to particulate antigens, such as RBCs, more than 50 years before the discovery of the anti-D effect. Initially, it was shown that antiserum prevented the antibody response to cattle RBCs in rabbits.¹⁸ Subsequently, similar antibody effects were observed in rats and mice challenged with blood cells, including SRBCs,¹⁹ rabbit RBCs,²⁰ horse RBCs,²¹ platelets^{22,23} and leukocytes.²⁴ The inhibitory IgG effect has also been observed using soluble antigens, such as γ -globulins, administered in adjuvants.⁴ IgG-mediated

prevention of the antibody response was also achieved using bacterial and viral products, including flagellum and bacteriophage Φ X174.⁴ The same effect of maternal IgG has been observed using several different vaccines, such as measles, polio, varicella-zoster, influenza, rotavirus, tetanus, diphtheria, and pertussis.²⁵

Anti-D has been primarily studied in humans, particularly in D-negative male volunteers transfused with D-positive RBCs.¹ Two strategies have been used to study anti-D responses in mice. In one approach, SCID mice were reconstituted with peripheral blood lymphocytes from a human donor immunized to the D antigen. Following challenge with D-positive RBCs, these mice responded with a secondary response to the D-positive RBCs.²⁶ In a different and more recent approach, mice expressing the human MHC class II molecule capable of D-antigen presentation (HLA-DR15) were generated. These mice presented the D peptides to T cells, and T-cell responsiveness has also been detected following challenge with purified D protein or peptides.²⁷ However, anti-D has not yet been tested for the ability to prevent the immune response in these models.

An animal model for HDFN has also been developed, based on the blood group system in rabbits containing allotypes expressed from the Hg locus (Hg^A, Hg^D, Hg^F). In this model, female rabbits are first immunized against allogeneic RBCs and then mated with a male rabbit homozygous for the incompatible allotype.^{28,29} The maternal anti-RBC response was observed during pregnancy and this mediated RBC lysis.

There are important differences between the immune responses suppressed by AMIS or anti-D. In animal models of AMIS, the immune response is designed to be reproducible and rapid, and occurs with a high frequency of responders. Furthermore, these responses are not associated with clinical conditions, such as HDFN. As a result, the animal models of AMIS have been criticized for relevance to anti-D.⁵

Potential Mechanisms of Anti-D and AMIS Action

Several major hypotheses were proposed about the mechanism of AMIS^{4,5,8} and some have been considered as potential explanations of the anti-D effect;³⁰ it was hypothesized that IgG may achieve its effect by mediating rapid antigen clearance, by inhibiting T cells or B cells, or by sterically blocking B-cell epitopes on the antigen.

Antigen Clearance Hypothesis

According to this hypothesis, IgG prevents the antibody response by accelerating the phagocytosis and removal of RBCs from circulation by the mononuclear phagocytic

system, particularly by macrophages, before their recognition by the immune system.³¹ IgG-opsonized RBCs may interact with activating IgG receptors (FcγRs) on effector cells and elicit phagocytosis.³² IgG may also accelerate RBC clearance by stimulating complement activation on the RBC surface, followed by RBC lysis or complement receptor-mediated phagocytosis. However, anti-D itself does not activate complement,³³ suggesting that FcγR-mediated phagocytosis may be the dominant mechanism of anti-D-mediated RBC clearance.

Anti-RBC IgG can clear D-positive RBCs from the circulation.³⁴⁻³⁶ The correlation between antigen clearance and anti-D protection has favored the antigen clearance hypothesis as an explanation for AMIS.^{7,35} As a result, the antigen clearance hypothesis has also been the focus for the testing of alternative anti-D products. Newly developed monoclonal and recombinant anti-D antibodies have been tested using both in vivo and in vitro assays based on the ability of IgG to interact with FcγRs on effector cells.³⁷ Similarly, in vivo assays have measured the ability of various anti-D preparations to clear RBCs from circulation.^{35,38} Based on these assays, several monoclonal/recombinant anti-D antibodies have been developed, albeit with limited success.^{6,7} Importantly, the correlation between antigen clearance and the protective anti-D effect has not always been observed for all monoclonal/recombinant anti-D products.^{6,7} Monoclonal anti-D preparations vary in their ability to prevent the antibody response and can sometimes cause an enhanced antibody response to the D antigen under certain conditions, despite efficient antigen clearance.^{6,7}

In a further development of the antigen clearance hypothesis, it has been proposed that IgG can mediate immunosuppressive clearance of RBCs, while clearance in the absence of IgG would trigger inflammation and promote the immune response.³¹ The ability of some monoclonal anti-D to enhance D immunization may occur as a result of “inflammatory” antigen clearance.^{7,31} However, the mechanisms behind RBC clearance that would lead either to activation or inhibition of the immune response have not been elucidated.

In the animal models of AMIS, there is little support for the antigen clearance hypothesis. In some animal models of AMIS, rapid clearance of RBCs was detected independently of anti-RBC IgG.^{39,40} Furthermore, the AMIS effect correlates with the number of IgG molecules attached to SRBCs, rather than the ability of the IgG to fix complement or bind FcγRs.⁴¹ The inhibitory IgG effect was also found to be independent of complement.⁴² Lastly, it has been shown that the FcRγ chain, which associates with FcγRs and is absolutely required for FcγR-mediated phagocytosis, is not at all necessary for the AMIS effect in a murine model using

SRBCs as foreign RBCs.⁴³

The antigen clearance hypothesis assumes that antigen removal by macrophages is sufficient to prevent the antibody response. However, antigen could also be “cleared” by dendritic cells, which should then promote an immune response by presenting antigens to B and T cells.^{44,45} To test the likelihood of mononuclear phagocytic system-mediated RBC presentation to B cells, we recently examined whether the transfusion of IgG-opsonized SRBCs pre-internalized or pre-bound by adherent mononuclear cells (enriched in phagocytic cells and dendritic cells) inhibited the immune response. Rather than inhibiting the response, as would be expected based upon the antigen clearance model, transfusion of the mononuclear cells actually elicited a potent antibody response upon infusion into naïve recipient mice.⁴⁰

The Role of T Cells

T cells are necessary for the antibody response to RBCs.⁴⁶ As a result, the prevention of HDFN may occur following prevention of the D-specific T-cell response. T cells specific to the D polypeptide have recently been identified and are likely necessary for the response to D-positive RBCs.^{47,48} However, whether anti-D has any effect on the T-cell response to D-positive RBCs has not yet been determined.

T-cell activation has been directly tested under AMIS conditions in several animal models and was shown to be normal.^{40,43,49} Maternal IgG antibodies, while inhibiting the antibody responses in infants following vaccination, do not affect T-cell priming nor change the type of cytokines produced by these T cells.²⁵ Immunological memory was likewise also not prevented under AMIS conditions.^{50,51} Unfortunately, AMIS models may not be relevant tests of T-cell function following anti-D administration. B cells, instead of other APCs, may elicit T-cell activation in D alloimmunization.

FcγRIIB-mediated B-cell Inhibition Hypothesis

It was hypothesized that RBCs and IgG form a complex that may deliver a negative signal to inactivate antigen-specific B cells.^{52,53} However, in the SRBC model of AMIS, using mice genetically deficient in FcγRIIB (FcγRIIB^{-/-} mice), it has been formally shown that FcγRIIB is not necessary for the AMIS effect.^{43,54,55}

Several novel FcR-like molecules (FCRL)⁵⁶ (previously referred to as FcR homologs or FcRH) have been identified in mice and humans.⁵⁷ Although, it has not yet been shown that these FCRLs can bind monovalent or aggregated IgG, we speculate that some of these FCRLs may mediate the B-cell inhibition instead of the classical FcγRIIB.

Steric Hindrance Hypothesis

The steric hindrance hypothesis suggests that IgG binds the antigen and prevents the BCR from recognizing the corresponding epitopes.⁵⁸ In the case of anti-D, it is estimated that most of the D epitopes are not blocked by polyclonal anti-D and that only 5% of D-antigen sites are bound under these conditions.⁵⁹ Free D epitopes can be detected following administration of anti-D.⁶⁰ Monoclonal anti-Ds were also able to prevent antibody responses by binding only 10% to 15% of D epitopes.^{35,61} However, the interaction between B cells and D-positive RBC has not been studied in detail. For example, IgG binding a fraction of D epitopes may be sufficient to prevent putative synapse formation between the RBC and B cell and prevent full B-cell activation.

In animal models of AMIS, the ability of IgG to prevent the antibody response correlates with the IgG affinity for the antigen and the amount of IgG bound to the antigen, which was interpreted as support for the steric hindrance hypothesis of AMIS.⁴¹ Nevertheless, it has been observed that it is not necessary to block all epitopes on the antigen to prevent the antibody response. For example, the amount of IgG that prevents the antibody response does not saturate all the epitopes on SRBCs⁶² or platelets.²³ This indicates that free antigens are available under AMIS conditions. Furthermore, we have recently shown that an immunogenic dose of non-sensitized SRBCs given concurrently with a desensitizing dose of IgG-opsonized SRBCs results in a proportionally reduced anti-SRBC response.⁵¹ According to the steric hindrance hypothesis, a normal antibody response should have been mounted against the unopsonized SRBCs.

According to both the antigen clearance and the B-cell inhibition hypotheses, the AMIS effect should be dependent on the Fc portion of the IgG. In contrast, according to the steric hindrance hypothesis, the IgG effect only requires the F(ab')₂ portion of the IgG. Consequently, a series of studies have been initiated to determine whether the Fc portion of the IgG is necessary for its effect on the antibody response. However, both Fc-independent^{43,63} and Fc-dependent^{64,65} suppression of the antibody response have been reported, and this issue has remained largely unresolved.^{8,53}

Recently, we tested the interaction between B cells and IgG-opsonized RBCs in vitro using syngeneic mouse RBCs or SRBCs coupled with an immunologically well characterized antigen, lysozyme, and transgenic B cells specific for lysozyme (MD4 B cells). Both RBCs and IgG-opsonized RBCs interacted with B cells and elicited early B-cell activation (manuscript in preparation). Thus, simple steric hindrance does not appear a likely explanation of AMIS. Instead, IgG appears to inhibit late stages of B-cell activa-

tion. It may be speculated that antigen-specific B cells internalize antigen together with the antigen-specific IgG. Following successful acquisition and internalization of the antigen + IgG complex, antigen processing/presentation may be altered by the IgG. It has been shown that antigen processing by B cells and other APCs can be altered when the antigen forms an immune complex with IgG. Distinct peptides can be processed from such complexed antigens compared to processing of antigen alone. In this way, IgG can prevent or enhance the presentation of specific peptides and the proliferation of epitope-specific T cells, although the effect on the antibody response has not been determined.^{66,67} Furthermore, the IgG internalized together with its target antigen can itself be processed and possibly presented to T cells.⁶⁸ IgG Fc-derived peptides presented by MHC class II may recruit IgG-specific CD4⁺CD25⁺Foxp3⁺ natural regulatory T cells. Regulatory T cells may be able to downregulate antibody production by B cells,¹⁷ and regulatory T cells specific for IgG-derived peptides have been detected.⁶⁹

Conclusions

The ability of IgG to prevent the antibody response to a co-administered antigen has been observed in a variety of different animal models using vastly different antigens. Despite a century of efforts, the mechanism of this IgG effect has not yet been clearly established. While the anti-D effect tends to correlate with antigen clearance, antigen clearance may not be a sufficient mechanism to explain the prevention of D alloimmunization. Animal models of AMIS suggest that T cells and APCs need not be inhibited for AMIS to occur and that AMIS may in fact be a B-cell-centric phenomenon. We speculate that the IgG alters antigen processing and presentation leading to reduced T-cell help and B-cell activation and decreased humoral immunity against the original antigen. Future studies need to examine B-cell activation and antigen presentation under AMIS conditions at the level of individual antigen-specific B cells. Finally, the role of B cells in the T-cell response to RBCs, as well as the role of T-cell inhibition in the protection from HDFN should be examined.

Disclosures

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