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TRANSFUSION UPDATE

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Sugar Free Antibodies: A New Treatment for Idiopathic Thrombocytopenic Purpura and Autoimmune Hemolytic Anemia

3

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INTRODUCTION

Autoimmune destruction of self-tissue is the consequential result of a convergence of several factors, both genetic and environmental, that effectively dislocates the immune system's ability to tolerate self-antigens but simultaneously retains its focus on those perceived as foreign. Although many of these autoimmune targeted self-antigens are known, immune system triggers can be more elusive. Nevertheless, once autoimmunity has been initiated, immune complexes (IC) play as a significant driving force in maintaining chronic inflammation and subsequently pathology in many of these conditions. ICs are antibody/antigen assemblies, usually IgG antibody subtype, sometimes found systemically but primarily at the site of autoimmune self-antigen recognition.[1] Sugar free antibodies are deglycosylated IgG having a dominant suppressive effect on inflammation and points to a unique class of therapeutic immunoglobulins for the treatment of autoimmunity.

The enzyme EndoS from *Streptococcus pyogenes* is an immunomodulatory molecule hydrolyzing the conserved glycans in the effector part of immunoglobulin G (IgG). EndoS is remarkably specific for IgG, and hydrolysis having profound effects on IgG effector functions. EndoS pre-treatment of IgG, or direct administration to animals with experimental antibody-mediated autoimmune diseases, inhibits development of disease or cures animals from established disease. The properties of EndoS make it a unique experimental tool and an attractive alternative to current therapies of conditions involving pathogenic antibodies.[2]

SUGAR FREE ANTIBODIES IN IMMUNE THROMBOCYTOPENIC PURPURA

Collin et al.[3] adopt a novel experimental approach to this challenge: enzymatic removal of carbohydrates from IgG Fc *in vivo*. The enzyme chosen, EndoS, is isolated from a common human pathogen *Streptococcus pyogenes* and specifically hydrolyzes the Fc glycans between the two core GlcNAc residues. This modification of Fc abolishes the binding of IgG to Fc receptors and reduces complement activation. In their study, Collin et al. first showed that EndoS, at low concentration, efficiently hydrolyzes the glycan on IgG in the complex environment of human blood *in vitro*. They then went on to show that the hydrolysis could be achieved efficiently in the circulation of a live animal by injecting rabbits with EndoS and monitoring total IgG. Low concentrations of EndoS were effective without perturbing the total IgG concentration or disrupting other serum glycoproteins. Furthermore, the IgG glycan hydrolysis effect was observed even in the face of a host antienzyme response. To investigate whether the glycan modification might have utility in modifying IgG activity, they chose a mouse model of a human autoimmune disease, immune thrombocytopenic purpura (ITP), in which platelets are targeted by autoantibodies. In the model, polyclonal antiplatelet IgG is injected into the mice to cause disease. Collin et al. showed that pretreatment of the antiplatelet IgG with EndoS or treatment of the mice with EndoS after initiation of disease prevented lethal thrombocytopenia in the majority of animals. Most impressively, even if disease was allowed to proceed to symptoms, as might be the case for humans presenting with ITP, EndoS treatment was able to provide considerable benefit.[3]

How does EndoS Exercise a Protective Function?

One plausible mechanism is that "activating" signals from C1q, Fc_Rs, and MBL binding are all reduced or abolished, whereas the interaction with the "inhibitory" Fc_RIIB receptor is preserved. This mechanism may reflect the natural immunosuppressive role of this enzyme. *S. pyogenes* has evolved several mechanisms to evade and diminish the adaptive immuneresponse, one of these appears to effectively switch off the effector functions of IgG.

SUGAR FREE ANTIBODIES IN AUTOIMMUNE HEMOLYTIC ANEMIA

The presence and pathogenicity of autoantibodies directed against red blood cells (RBCs) have been extensively

investigated and implicated in a number of autoimmune diseases, such as autoimmune hemolytic anemia (AIHA) and systemic lupus erythematosus.[4] The RBC surface contains many epitopes recognizable by alloantibodies and autoantibodies, including more than 300 blood group antigens located on glycoproteins and glycolipids but also nonpolymorphic/nonblood group protein structures.[5] The most common targets of pathogenic RBC autoantibodies are epitopes on the Rh protein, encoded by 2 homologous genes on chromosome 1, *RHD* and *RHCE*.[6] More than half of all autoantibody specificities in patients with AIHA of the common so-called warm type include immunoglobulin G (IgG) antibodies against these Rh epitopes, which are consequently often used to model AIHA experimentally.

Human antibodies against RhD (anti-D) or rabbit antibodies against human RBC (anti-RBC) can result in accelerated phagocytosis and removal of RBC from the circulation by monocytes/macrophages in the spleen and liver.[7,8] Fcγ receptor (FcγR)-mediated erythrophagocytosis, in addition to other IgG effector functions, such as activated complement, oxidative burst, and cytokine production by FcγR expressing effectors cells, play an essential role in shortened RBC survival.[9-13] The ability of normal human monocytes to bind and phagocyte IgG sensitized RBCs via FcγR and mediate immune RBC destruction is highly related to the glycosylation status of the Fc domain on the anti-RBC antibodies.[14-19]

A study was undertaken to establish the effects of EndoS on antibody-mediated destruction of RBCs with a future therapeutic application in mind. Here it was shown, using monocytes from blood and the THP-1 monocytic cell line, that pretreatment of anti-RBC with EndoS abrogates erythrophagocytosis and subsequent activation of monocytes. The complement-dependent hemolytic effects of anti-RBC added to whole human blood were greatly decreased by pretreatment of this antibody with EndoS. Finally and most importantly, EndoS showed inhibitory effects on a model of AIHA in mice. These results suggest that anti-RBC antibodies can be rendered less pathogenic by treatment with EndoS, which may be regarded as a possible future inhibitor of antibodymediated destruction of RBCs and AIHA.

Mechanism of Action

Treatment of Anti-D with EndoS does not Affect the Binding of Anti-D to RBCs

EndoS inhibits binding of IgG to soluble and monocyte-bound FcγR by hydrolyzing the N-linked glycan on the heavy chain of IgG.[20] In a study conducted by Allhorn M, the results showed that the treatment of anti-D with EndoS did not affect the antigen recognition by this antibody and the amount of RBC-bound IgG was not influenced by EndoS treatment.

EndoS Inhibits Phagocytosis and Adherence of Sensitized RBCs by Monocytes

The effect(s) of EndoS on phagocytosis of anti-D sensitized RBCs by monocytes was analyzed using monocyte monolayer assay. Monolayers of monocytes were cultured on slides, and RBCs presensitized with anti-D were added to the monolayers. The degree of phagocytosis was determined using light microscopy and expressed as the number of monocytes with adherent/phagocytosed RBCs by analyzing 100 monocytes. As expected, the phagocytosis of anti-D–sensitized RBCs was significantly greater than nonsensitized RBCs and was efficiently eliminated by pretreatment of anti-D with EndoS ($p < 0.001$). This clearly shows that the treatment of anti-D with EndoS before sensitization of RBCs abrogates the phagocytosis-enhancing activity of anti-D and decreases the accumulation of intracellular hemoglobin in monocytes.

EndoS Inhibits Oxygen Metabolite Production by Monocytes Induced by Sensitized RBCs

In an attempt to further elucidate the effects of EndoS on monocyte-driven cell cytotoxicity to sensitized RBCs, we measured the release of oxygen metabolites from monocytes using a chemiluminescence test. RBCs were opsonized with immune plasma with high titers of anti-D from 3 persons. Monocyte responses to sensitized RBCs were compared with their response to unsensitized cells and expressed as a ratio (opsonic index). RBCs incubated with immune plasma gave positive results in chemiluminescence test with opsonic index 2.5. In contrast, monocyte responses to RBCs incubated with immune plasma pretreated with EndoS were significantly decreased (plasma 1, $p < 0.07$; plasma 2, $p < 0.02$; plasma 3, $p < 0.015$) and in some cases even below the control plasma. These results suggest that treatment of immune plasma with EndoS abrogates *in vitro* metabolic oxidative responses by monocytes.

EndoS Inhibits IL-8 Secretion from Monocytes Induced by Sensitized RBCs

It has been reported that IL-8, besides being produced by monocytes in response to lipopolysaccharide, tumor necrosis factor-α, or IL-1, is implicated as an indicator of RBC incompatibility and mediator of the pathologic events in hemolysis in, for example, hemolytic transfusion reactions.[21-23] It has been shown that monocytes exposed to sensitized RBC's release IL-8, which was measured by ELISA method, as expected a significant rise in levels of IL-8 were observed. Noticeably, RBCs sensitized with anti-D pretreated with EndoS showed significantly decreased secretion of IL-8 in THP-1 cells ($p < 0.001$) and bloodmonocytes ($p < 0.003$), which was comparable with the secretion of IL-8

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by THP-1 cells and monocytes because of stimulation with nonsensitized RBCs ($p < 0.001$ and $p < 0.002$, respectively. These results demonstrate that EndoS does not have any direct cytotoxic effects on monocytes.

EndoS Inhibits Hemolysis of Sensitized RBCs and Prevents Binding of C1q to IgG-sensitized RBCs

Human anti-D antibodies of IgG isotype do not generally activate the complement pathway, and RBCs coated with these antibodies are preferentially cleared by mononuclear phagocytic cells via FcγRs. It was clearly shown that pretreatment of antibody by EndoS is not a prerequisite for inhibition of hemolysis and that a direct addition of EndoS to blood was sufficient to exclude the hemolytic effect of anti-RBC ($p < 0.001$). EndoS by itself did not have any effect on hemolysis ($p < 0.001$). Furthermore, addition of increasing concentrations of anti-RBC IgG to whole blood caused a strong agglutination reaction as expected. Remarkably, addition of EndoS-treated antibody abrogated this effect. In conclusion, these data indicate that EndoS possesses antihemolytic activities as demonstrated by pretreatment of anti-RBC with enzyme or by a direct addition of EndoS to anti-RBC-containing blood.

It has been reported that C1q, the recognition subunit of the first component of the classic complement pathway, binds to IgG-sensitized RBCs.[24] Because the binding of C1q to IgG is dependent on the glycosylation state of IgG, we decided to explore the effect(s) of EndoS on deposition of C1q onto IgG-sensitized RBCs and with this further elucidate the mechanism for the antihemolytic effects of EndoS. There was significantly decreased fluorescence intensity in the samples containing RBCs sensitized with EndoS-treated anti-RBC ($p < 0.001$) compared with RBCs treated with native antibody. These results indicated an inability of C1q to bind to these RBCs, providing further evidence for the complement-inhibitory/antihemolytic properties of EndoS.

EndoS Inhibits AIHA In Vivo

The deposition of C1q and C3 on RBCs was analyzed by flowcytometry 24 hours after injection of EndoS. This revealed a significant decrease in C1q and C3 deposition on RBCs in mice treated with EndoS compared with PBS-treated mice (mean fluorescence intensity: C1q, 28.4 ± 6.5 vs 42.6 ± 7.8, $p < 0.02$; C3, 13.1 ± 4.8 vs 29.6 ± 9.7, $p < 0.01$), suggesting a reduced activation ofcomplement. The results showed that there was a substantial reduction of iron deposits in livers from mice treated with EndoS, compared with mice without treatment of EndoS. In addition, the effect of EndoS on 34-3C IgG1 and IgG2a class-switch variants was also determined. We found protective effect of EndoS on IgG1 induced anemia but not on IgG2a-induced anemia. When measuring the extent of C1q and C3 deposits in mice injected with complement-activating 34-3C IgG2a, no significant differences were visible compared with mice without EndoS treatment. Data indicate that EndoS partially inhibits development of anemia mediated by anti-RBC reactive antibodies in an IgG subclass-dependent manner in mice.

SUGAR FREE ANTIBODIES IN OTHER AUTOIMMUNE DISEASES

Nandakumar et al. used the mouse anticollagen type II (anti-CII) mediated-arthritis model to show that deglycosylated IgG, regardless of Fab antigen specificity, reduces inflammation in a dose-dependent manner.[1] This finding suggests that deglycosylated IgG has a dominant suppressive effect on inflammation and points to a unique class of therapeutic immunoglobulins for the treatment of autoimmunity.[1]

FUTURE OF THIS THERAPEUTIC MODALITY

The idea that antibody-mediated autoimmunity could be therapeutically controlled by a single enzyme is certainly an attractive one, but will Fc deglycosylation become a therapeutic reality?

Several questions remain. EndoS is a bacterial protein and is readily targeted for removal by antibodies, which would normally limit the half-life of the protein in the blood. However, Collin et al. report that, although antibodies to EndoS were observed in their study, the enzyme nonetheless exhibited reasonable pharmacokinetics. A possible explanation for this observation is that the normal Fc-mediated mechanisms for clearing foreign proteins are reduced, because the IgGs bound to the enzyme are themselves deglycosylated by the enzyme. Another potential obstacle might be that EndoS would cleave glycans other than those on IgG with unknown consequences. Encouragingly, however, EndoS appears unusually specific for the glycans on IgG, probably a consequence of its intrinsic affinity for Fc.

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