Evidence of Classical Complement Pathway Involvement in a Subset of Patients with Warm Autoimmune Hemolytic Anemia

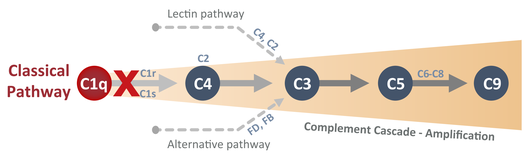
Jeffrey Teigler[1], Julian Low[1], Shawn Rose[1], Ellen Cahir-McFarland[1], Ted Yednock[1], Henk-André Kroon[1], Sanjay Keswani[1], Ronald S. Go[2], and Wilma Barcellini[3]

[1]Annexon Biosciences, South San Francisco, CA, USA; [2]Division of Hematology, Mayo Clinic, Rochester, Minnesota, USA; [3]UO Ematologia, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

INTRODUCTION

- Autoimmune hemolytic anemia (AIHA) is a constellation of diseases caused by autoantibodies targeting red blood cells (RBCs) with or without complement activation[1]
- Two main types of AIHA are cold agglutinin disease (CAD) and warm autoimmune hemolytic anemia (wAIHA), and while the role of the complement cascade is firmly established in CAD, its role in wAIHA remains unclear[1,2]
- Activation of the classical complement pathway and autoantibody activity against RBCs in AIHA can be triggered by C1q binding to autoantibodies (**Figure 1**)[1,3]

Figure 1. The Classical Complement Pathway



- Diagnosis of AIHA is completed with the direct antiglobulin test (DAT), which measures IgG and/or complement C3 on the surface of RBCs in patient blood samples and can provide evidence of antibody-mediated complement activity[1]
  - A negative DAT test could be misleading, as the assay is insensitive to detect IgM and only provides information on cells that have not been eliminated from circulation[1,3]
- Therefore, we explored additional measures which could suggest evidence of complement activation in AIHA patients
  - In contrast to assays that measure in vivo complement deposition on the surface of endogenous RBC from AIHA patients directly, these additional measures allow detection of complement-depositing autoantibodies with no impact from selective destruction of complement-coated RBC in vivo

OBJECTIVE

- To explore additional measures that could suggest evidence of complement activation in AIHA patients
  - Sera from wAIHA patients will be evaluated for evidence of autoantibodies that could initiate complement deposition on the surface of healthy human RBCs using an in vitro C4 deposition assay
  - Endogenous plasma levels of C4 will be measured in a separate wAIHA cohort to evaluate ongoing complement consumption in vivo

METHODS

In vitro complement deposition:

- Evidence for complement-depositing autoantibodies in patient sera was examined with a modified in vitro protocol by Meulenbroek et al.[4,5]
  - Healthy human type O+ RBCs were treated 2:1 with bromelain in phosphate-buffered saline (0.5% w/v) for 10 minutes at 37°C
  - Cells were washed and resuspended at 0.5% (v/v) concentration in glucose veronal buffer (GVB)++ or GVB ethylenediaminetetraacetic acid (EDTA)
  - In vitro C4 deposition was then tested by sequentially adding anti-C5-containing buffer to prevent RBC lysis, healthy/CAD/wAIHA sera, healthy serum as a source of complement components, and bromelain-treated cells to 96-well plates
  - Reactions were performed at 37°C for 90 min; cells were then washed and processed for flow cytometry
- The RBC population used in the in vitro C4 deposition protocol was identified by flow cytometry
  - Cells were then singlet gated on FSC-A and FSC-H and assessed for C4 deposition

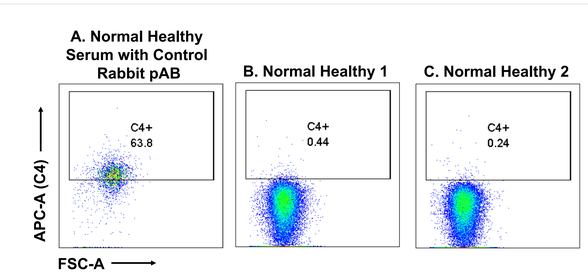
In vivo complement consumption:

- Samples from patients with wAIHA were collected after obtaining informed consent; complement component C4 assessment was performed at a central laboratory as per the manufacturer's protocol

RESULTS

- Antibody ability to sensitize RBCs was tested by incubating healthy human RBCs with 1:1500 rabbit anti-human RBC polyclonal antibody (**Figure 2A**) and compared to unsensitized cells, which displayed little to no C4 deposition (**Figure 2B and C**)
- In contrast, cells that had been rabbit polyclonal antibody-sensitized displayed both C4 deposition and moderate amounts of lysis in the presence of anti-C5 antibody

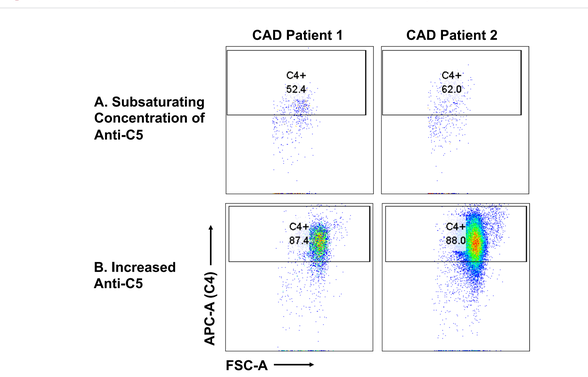
Figure 2. Example Flow Cytometry Controls for C4 Deposition Assay



pAB, polyclonal antibody.

- To determine if autoantibodies were present that could trigger C4 deposition on RBC in vitro, sera from two CAD patients were utilized in the deposition assay. This resulted in pronounced C4 deposition as well as marked RBC destruction, suggesting breakthrough hemolysis in the presence of the anti-C5 antibody (**Figure 3A**).
- Repeating the assay using 5 times the amount of anti-C5 antibody reduced hemolysis and resulted in better inhibition of cell lysis and revealed more striking in vitro C4 deposition (**Figure 3B**)

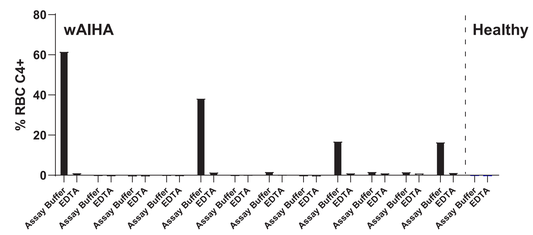
Figure 3. Complement Deposition by CAD Sera In Vitro on Healthy Human RBC



CAD, cold agglutinin disease.

- These data confirmed that CAD sera has autoantibodies capable of initiating C4 deposition in vitro and that RBC destruction at lower anti-C5 levels was likely complement-mediated
- When serum samples collected from twelve wAIHA patients were assessed in the C4 deposition assay in the absence and presence of EDTA, which blocks classical complement pathway activity, four out of twelve wAIHA sera samples displayed complement deposition activity, indicating classical pathway involvement (**Figure 4**)

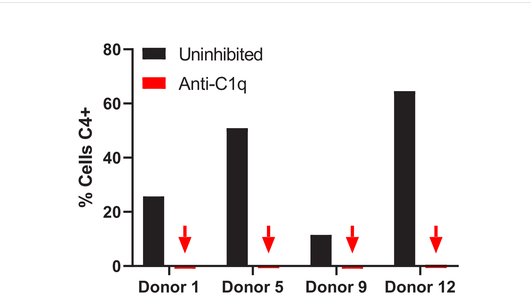
Figure 4. Complement Deposition by a Subset of wAIHA Sera In Vitro on Healthy Human RBC



EDTA, ethylenediaminetetraacetic acid; RBC, red blood cell; wAIHA, warm auto-immune hemolytic anemia.

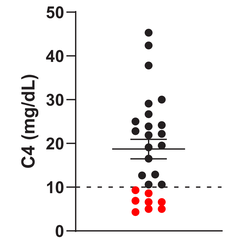
- Repeating deposition assays for the four patients displaying complement deposition activity in the presence or absence of an inhibitory anti-C1q antibody abrogated C4 deposition (**Figure 5**)

Figure 5. Blockade of C4 Deposition in wAIHA Sera on Healthy Human RBC by Anti-C1q



- These results suggest robust complement activation by autoantibodies in a subset of wAIHA patient sera, and that this activity occurs through the classical complement pathway
- C4 deposition in the in vitro analysis (**Figure 4 and 5**) could potentially be modulated by multiple factors, such as antibody isotype, antibody concentration, target of anti-RBC polyclonal antibody, and presence of pentraxins. These may or may not correlate with a given patient's clinical disease pathogenesis.
- To further evaluate circulating levels of complement pathway factor C4, multiple proximal measures of complement pathway activity are under investigation in an ongoing phase 0 wAIHA trial
- To date, we have assessed 27 individual samples and found that 8 (~30%) displayed C4 levels below the lower limit of normal, suggesting that ongoing classical complement activation/C4 consumption may be occurring in vivo in a subset of wAIHA patients (**Figure 6**)

Figure 6. Complement Depletion in wAIHA Patients



Dashed line represents C4 normal range lower cutoff.

CONCLUSIONS

- **Using a modified in vitro complement deposition assay described by Meulenbroek et al., we have shown that sera from CAD patients and a subset of wAIHA patients possess autoantibodies capable of triggering classical pathway C4 deposition on the surface of healthy human RBCs[4,5]**
- **Multiple factors may affect complement deposition in this assay, which may be contributing to our findings. These include the isotype of the autoantibody within the serum, its concentration, the antigenic bias of polyclonal sera from different patients, or the donor of the RBCs used for deposition.**
- **We are currently ascertaining how these and other in vitro assay results may be translated to demonstrate classical complement pathway involvement in AIHA patients in vivo in an ongoing phase 0 non-interventional trial**
- **The therapeutic potential of classical complement pathway blockade in wAIHA patients who have evidence of classical complement activity is currently being evaluated in an ongoing phase 2 interventional trial (NCT04691570)**

References

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Disclosures

JT, JL, SR, ECM, TY, HAK, SK: Annexon Inc: Current Employment, Current Equity Holder in publicly traded company

RSG: None

WB: Novartis: Honoraria; Bioverativ: Membership on an entity's Board of Directors or Advisory Committees; Agios: Honoraria, Research Funding; Alexion Pharmaceuticals: Honoraria; Incyte: Membership on an entity's Board of Directors or Advisory Committees

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