EVALUATION OF BACTERIA-MEDIATED POTENTIAL "BYSTANDER" HEMOLYSIS OF PNH RED CELLS IN VITRO: NO EVIDENCE OF SIGNIFICANT COMPLEMENT CLASSICAL OR LECTIN PATHWAY-MEDIATED HEMOLYSIS INDUCED BY MICROORGANISMS

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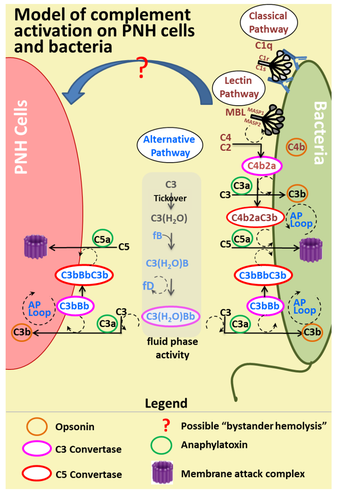
BACKGROUND

- The complement system is an enzymatic cascade of more than 30 proteins that is activated via the classical pathway (CP), lectin pathway (LP), or alternative pathway (AP) upon recognition of pathogen-associated molecular patterns that are present on the surface of pathogens.
- Activation of complement leads to the generation of biologically active fragments that play a critical role in host defense. Complement fragments opsonize pathogens, recruit and activate inflammatory cells at the site of complement activation and act as the nidus for assembly of the lytic membrane attack complex on the surface of pathogens.
- Complement activation is a well-coordinated physiological response that is highly regulated by various fluid-phase as well as membrane-bound regulatory proteins to prevent host tissue damage. Loss of membrane-bound complement regulatory proteins such as CD55 and CD59 in paroxysmal nocturnal hemoglobinuria (PNH) leads to hemolysis of PNH erythrocytes.
- Hemolysis of PNH cells occurs as a result of continuous low-level AP activation in the fluid phase (C3 "tickover"). It is unknown whether PNH cells would show enhanced hemolysis in the context of an infection where CP, LP and AP are activated on a pathogen surface. In this setting, fluid phase complement activation fragments generated from pathogen-driven CP, LP and/or AP may hypothetically bind to nearby PNH cells and cause "bystander lysis". Alternatively, pathogens may bind to PNH cells and thus complement activation on the membrane of PNH cells could occur simultaneously and cause "bystander lysis".



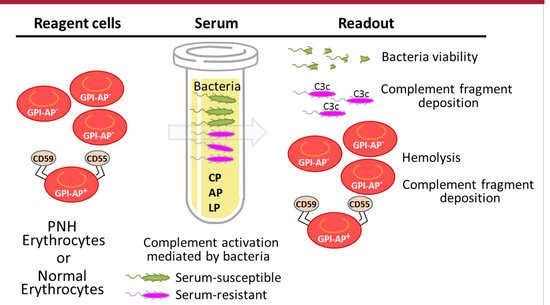
- Accordingly, using normal human sera, PNH cells, and a panel of clinically relevant bacterial strains, we undertook the studies herein to evaluate whether activation of complement by pathogens leads to enhanced hemolysis and/or complement fragment deposition on PNH cells. Moreover, we evaluated the effects of the factor D inhibitor ACH-4471 in the context of these experimental conditions.
- ACH-4471 is a novel, highly-potent and specific orally-administered small-molecule inhibitor of complement factor D (fD), a serine protease that is essential for AP activation. ACH-4471 prevents cleavage of factor B (fB) into Ba and Bb, thereby preventing C3 convertase production, AP activation, and the AP-mediated amplification of CP and LP responses. ACH-4471 is currently in phase 1 clinical study.

HYPOTHESIS

- In the context of an infection, PNH cells could be susceptible to enhanced lysis from pathogen-driven complement activation products (C3b) generated in the fluid phase, or from membrane-derived activation products (C3b) from pathogens that are directly bound to PNH cells.

METHODS

80 µL NHS was pre-incubated with ACH-4471 for 5 minutes on ice. 10 µL PNH erythrocytes (5×10^8 /mL in GVB^{++}) and 10 µL bacteria (5×10^7 /mL or 5×10^8 /mL in GVB^{++}) were added. Reactions were incubated at 37°C for 1 h and supernatants and cell pellets were separated by differential centrifugation. Absorbance of supernatants was measured at 405 nm. Erythrocytes and/or bacteria recovered from pellets were labeled with FITC-conjugated anti-C4d or anti-C3c antibody and cell surface fluorescence was examined by flow cytometry. PNH erythrocytes were identified by flow cytometry with anti-CD59 antibody. Bacteria: *Streptococcus pneumoniae* (ATCC 49619), *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 19213), *Haemophilus influenzae* type b strain Eagan, *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* lab strain, *Neisseria meningitidis* A (ATCC 13077), *Neisseria meningitidis* B (ATCC 700112).



RESULTS

PNH Patient Demographics

Table 1: PNH Patient Hematologic Characteristics

Patient ID	Age, Gender	Blood type	Erythrocyte clone size Type II/III (%)	Granulocyte Clone size (%)	LDH (U/L)	Hemoglobin (g/dL)	Direct Coombs C3	Direct Coombs IgG	Absolute reticulocyte count (K/cu mm)	Start of Eculizumab use
A	52, F	B	3.1/88	99	293	9.8	Pos	Neg	288.3	(started 3/2011)
B	58, M	O	1/98	99	301	11.3	Pos	Neg	311.7	(started 8/2005)
D	39, M	O	21/23	31	210	11.5	N/A	N/A	155.4	(started 2/2014)
E	23, F	O	25/13	95	2015	8.8	N/A	N/A	320.8	N/A

PNH blood samples obtained following informed consent
N/A, not available; Pos, positive; Neg, negative

Targeted Inhibition of Factor D Prevents Hemolysis of PNH Cells In Vitro

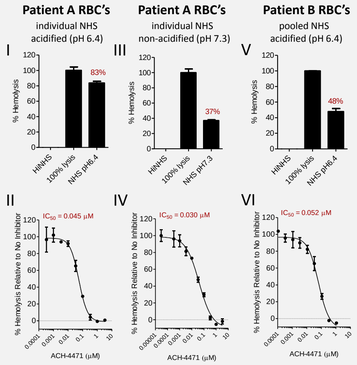


Table 2: Hemolysis of PNH Cells and ACH-4471 Potency

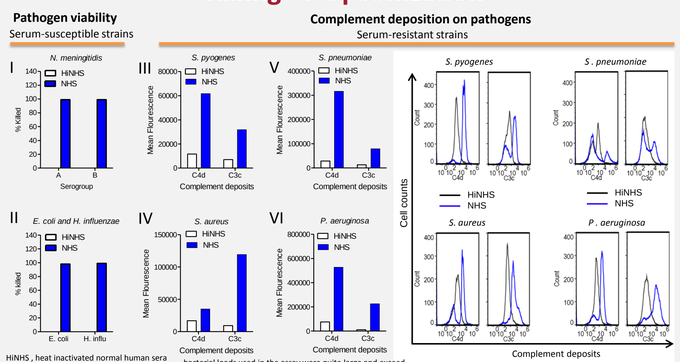
Patient ID	NHS/pH	% Hemolysis no Inhibitor	IC_{50} µM ± SE	N
A	Individual 6.4	76%	0.047 ± 0.007	2
A	individual 7.3	37%	0.030	1
B	pooled 6.4	53%	0.041 ± 0.005	8
B	individual 6.4	87%	0.010	1
D	pooled 6.4	28%	0.035	1
E	pooled 6.4	21%	0.026	1

Test reactions included serum and PNH cells as indicated and incubated for 1 hr at 37°C. PNH cells are more susceptible to lysis at pH 6.4 (Ham test)

ACH-4471, small molecule fD inhibitor
HiNHS , heat inactivated normal human sera
NHS, normal human sera
Individual NHS, serum from one donor (ABO Blood Type: AB)
Pooled NHS, sera obtained from a number of individuals and pooled together
NHS pH 6.4 or 7.3 in the absence of EGTA (All complement pathways are open)
N, the number of independent experiments or a representative experiment

The small molecule factor D inhibitor ACH-4471 inhibits complement-mediated hemolysis of PNH cells.

Activation of Complement by Pathogens Leads to Pathogen Killing or Opsonization

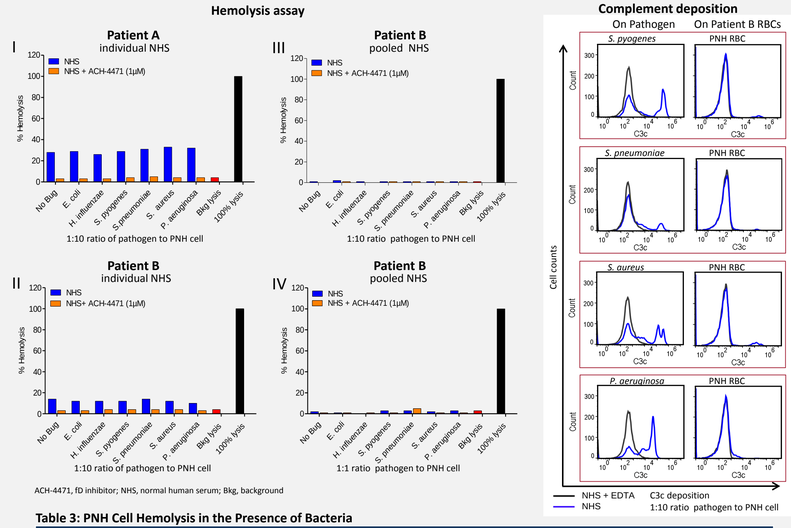


HiNHS , heat inactivated normal human sera
NHS, normal human sera
H. influe, H. influenzae

bacterial loads used in the assay were quite large and exceed conditions expected to be seen during human infections or sepsis.

All test pathogens activate complement (CP, LP and or/AP) on their surface , as shown by killing (serum-susceptible strains) or complement deposition (serum-resistant strains).

PNH Cells Show No Increase in Complement-Mediated Hemolysis or Opsonization When Co-incubated with Test Pathogens



ACH-4471, fD inhibitor; NHS, normal human serum; Bkg, background

Table 3: PNH Cell Hemolysis in the Presence of Bacteria

		% Hemolysis of PNH Cells								
Patient ID	NHS	*No bug*	*E. coli*	*H. influenzae*	*S. pyogenes*	*S. pneumonia*	*S. aureus*	*P. aeruginosa*	*Men. A*	*Men. B*
A	individual	**28**	32	26	29	31	33	32	ND	ND
B	pooled	**1**	2	1	1	1	1	1	ND	ND
B	individual	**14**	12	12	12	14	12	10	0	0
D	pooled	**0**	0	0	0	0	1	1.5	0*	0*
E	pooled	**0**	ND	0	1	0	ND	ND	ND	ND
Normal	pooled	**2**	1	1	2	2	1	1	ND	ND

Test reactions included serum, PNH cells and pathogens as indicated and were incubated for 1 hr at 37°C. 1:10 ratio of pathogen to PNH cell. *assayed with individual NHS

PNH cells show no enhancement in complement-mediated lysis or C3 deposition when co-incubated with pathogens under conditions that allow activation of complement CP, LP and AP.

CONCLUSIONS

- **The fD inhibitor ACH-4471 is a potent inhibitor of complement-mediated hemolysis of PNH cells under conditions that activate the classical or alternative pathways.**
- **Complement activation by pathogens leads to pathogen lysis and opsonization but does not cause "bystander" hemolysis or opsonization of PNH cells under these assay conditions in vitro.**
- **Our in vitro results suggest that bacterial infections and accompanying complement activation in the setting of ACH-4471 therapy are unlikely to elicit complement-mediated breakthrough hemolysis by the "bystander" mechanism.**

References

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Disclosures

RB is on the advisory board of Achillion; GY, JT, MG, SP and MH are employees and share holders of Achillion.

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