Comparison of Complement Functional Assays:
Differential Sensitivities of Hemolysis and Wieslab Assays to Levels of Complement Proteins C5, Factor B, and Factor D

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INTRODUCTION

- The historically important hemolytic assays for complement pathway activity have been joined in recent years by ELISA-based assays for complement function[1]. Advantages include greater standardization and the availability of specific assays for all three activation pathways: the complement classical pathway (CP), alternative pathway (AP), and mannose-binding lectin pathway (LP).
- The suitability of complement assays to screening of potential therapeutic inhibitors or monitoring clinical efficacy depends upon assay sensitivity to levels of the individual complement protein targets[2,3]. These sensitivities depend on the interplay of activation processes and their exquisite control by regulatory proteins, which in turn depend on the properties of the activating surface.
- In the present study, we used human sera selectively depleted of individual components to compare the sensitivities of hemolysis and Wieslab ELISA assays to complement factor B and factor D (fB and fD, both required for AP activation) and C5 (a terminal pathway effector of AP, CP, and LP). Specifically we assessed sensitivities to levels of fB and fD (AP assays only) and C5 (AP and CP assays) by titration of individual complement proteins into the corresponding depleted sera. In addition to the hemolysis and sC5b-9 endpoints we assessed production of activation products Bb and C5.
- We also assessed the susceptibilities of the hemolysis and Wieslab assays to inhibition by the investigational fD inhibitor ACH-4471 (AP assays only) and an antibody inhibitor of C5 (AP and CP assays). ACH-4471 is a novel small molecule fD inhibitor currently in phase I study by Achillion Pharmaceuticals as an oral therapeutic for diseases of complement dysregulation including C3 glomerulopathy (C3G) and paroxysmal nocturnal hemoglobinuria (PNH).

METHODS

- **Reagents:** The following were obtained from Complement Technology Inc. (Tyler, TX, USA): normal human serum (NHS); human sera depleted of fB, fD, and C5; purified human fB, fD, and C5; rabbit erythrocytes (Er) and Ab-sensitized sheep erythrocytes (EA), 5E8/mL; GVB^0 and GVB^{++} buffers and 100 mM Mg-EGTA. GVB^0-MgEGTA was prepared by addition of 10 mM MgEGTA to GVB^0. AP and CP Wieslab kits were obtained from EuroDiagnostica (Malmö, Sweden). Bb and C5a Microvue ELISA kits were obtained from Quidel Corp. (San Diego, CA, USA). ACH-4471 was synthesized and characterized at Achillion Pharmaceuticals, Inc. Antibody to human C5 was obtained as product number A217 from Quidel (San Diego, CA, USA).
- **Complement Assays with Depleted Serum and Purified Protein:** Depleted sera were reconstituted with corresponding purified proteins at the indicated concentrations prior to assay. AP hemolysis: 8.3% serum was incubated with 8.3E7/mL Er in GVB^0-MgEGTA at 37°C for 30 minutes. CP hemolysis: 0.5% serum was incubated with 8.3E7/mL EA in GVB^{++} at 37°C for 60 minutes. Hemolysis was quantitated from A_{405} of supernatants. AP and CP Wieslab assays were performed according to the manufacturer's instructions (final serum dilutions, 5.6% and 1% respectively). Ba and C5a levels in EDTA-terminated hemolysis supernatants were quantitated by ELISA according to the manufacturer's instructions.
- **Inhibition by ACH-4471 and an Anti-C5 Monoclonal Antibody:** ACH-4471 and anti-C5 were incubated with diluted NHS for ten minutes at room temperature prior to assay.
- **Analyses:** Concentrations of added complement proteins are expressed relative to undiluted serum prior to assay. Protein and inhibitor titrations were fitted and IC_{50} and IC_{90} values determined by four-parameter non-linear regression. Hemolysis and Wieslab activities were normalized to the activity of NHS assayed in parallel at the same dilution.

CONCLUSIONS

- AP hemolysis and AP Wieslab activity correlate with levels of AP components fB and fD, shown by protein titration and ACH-4471 inhibition.
- CP hemolysis, CP Wieslab, and AP Wieslab activity similarly correlate with levels of terminal component C5, shown by protein titration and anti-C5 inhibition.
- AP hemolysis activity in contrast shows an extremely shallow relationship with C5 concentration, shown by C5 protein titration and inhibition by anti-C5, wherein miniscule C5 levels support significant activity.
- The distinctive relationship of AP hemolysis activity to C5 modulation is a likely consequence of the nonsusceptibility of Er cells to regulation such as by factor H/factor I[4]; yet, similar behavior is not observed when the AP components fB and fD are similarly modulated.

REFERENCES

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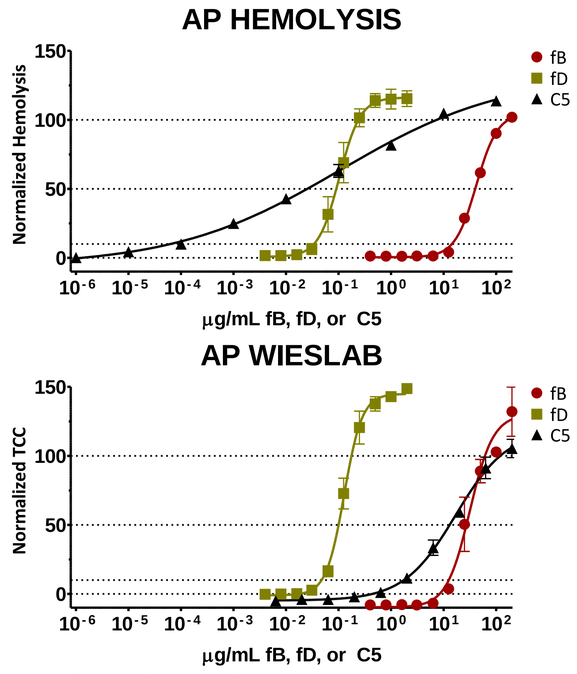
4. **Pangburn MK and HJ Müller-Eberhard**. Complement C3 convertase: Cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. *Proc. Natl. Acad. Sci. USA* 1978, 75:2416-2420.

RESULTS

Requirement for AP and Terminal Components: AP Hemolysis vs AP Wieslab

Fig 1: Purified fB, fD, and C5 Titrations into Depleted Sera: AP Activity by Hemolysis vs Wieslab Assays



- Curves from single or representative assays

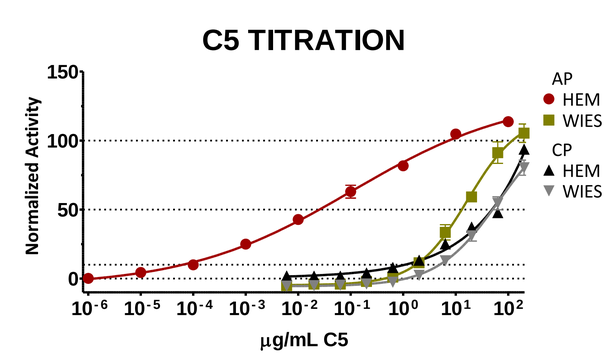
	ASSAY &/or RATIO	µg/mL PROTEIN for 50% ACTIVITY	µg/mL PROTEIN for 10% ACTIVITY
fB	HEM	40	14
	WIES	27	13
	W:H	0.68	1.0
fD	HEM	0.091 - 0.091	0.034 – 0.037
	WIES	0.10	0.048
	W:H	1.1	1.3
C5	HEM	0.0085 – 0.025	1.9E-5 – 6.3E-5
	WIES	13	1.75
	W:H	1,000	60,000

- HEM = hemolysis; WIES = Wieslab; W:H = WIES:HEM ratio.
- Values from single or duplicate independent assays

- fB, fD, and C5 are essential for AP activity by hemolysis or Wieslab assay.
- Purified fB and fD show equivalent concentration-activity relationships in AP hemolysis and AP Wieslab assays, with steep linear ranges, and 50% activity when protein is supplied at ~10-20% normal blood levels.
- Purified C5 shows a much shallower concentration-activity relationship by AP hemolysis than by AP Wieslab assay, supporting 50% AP hemolysis activity at miniscule levels (~0.01% normal blood levels).

Requirement for Terminal Component C5: AP vs CP Assays

Fig 2: Purified C5 Titration into Depleted Serum: AP and CP Activity by Hemolysis vs Wieslab Assays



- Curves from single or representative assays

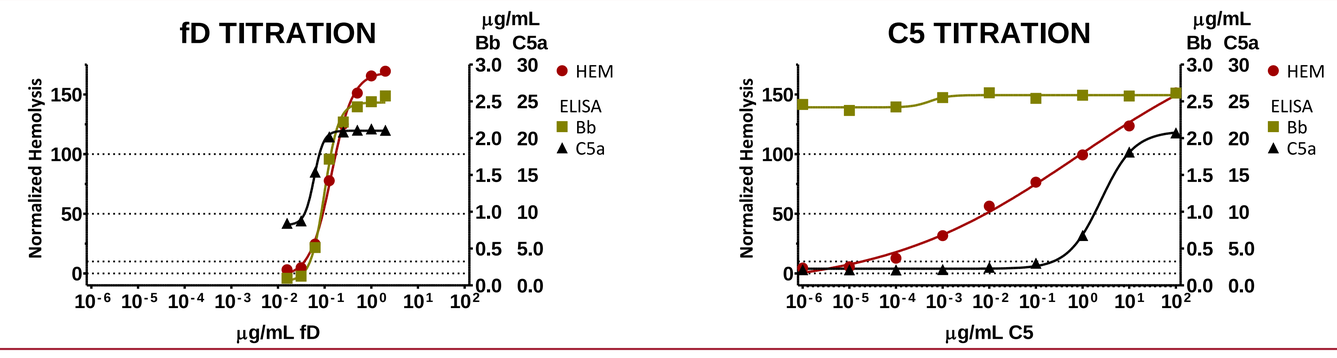
	ASSAY &/or RATIO	µg/mL C5 for 50% ACTIVITY	µg/mL C5 for 10% ACTIVITY
AP	HEM	0.0085 - 0.025	1.9E-5 – 6.3E-5
	WIES	13	1.75
	W:H	1,000	60,000
CP	HEM	49	1.1
	WIES	50	4.9
	W:H	1.0	4.6

- HEM = hemolysis; WIES = Wieslab; W:H = WIES:HEM ratio.
- Values from single or duplicate independent assays

- Greater sensitivity to added C5 is restricted to AP hemolysis assay and is not observed in either CP assay.

Component Requirements in AP Hemolysis: Activation Products Bb and C5a

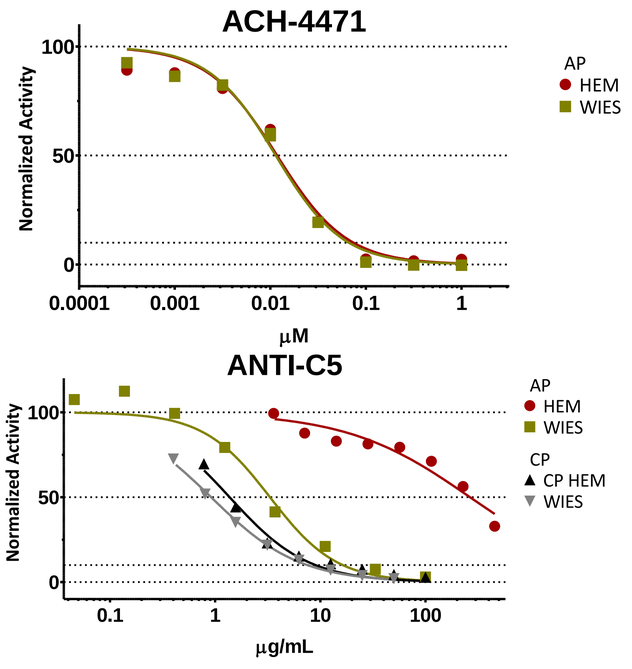
Fig 3: fD and C5 Titrations into Depleted Sera: Split Product Generation in AP Hemolysis Assay



- With fD reconstitution, Bb and C5a generation correlate with AP hemolysis.
- With C5 reconstitution, C5a production shows a much steeper curve than AP hemolysis; C5 cleavage appears nearly complete at high hemolysis yet undetectable 50% hemolysis.

Pathway Inhibition by ACH-4471 (Small Molecule fD Inhibitor) and Anti-C5

Fig 4: Inhibition by ACH-4471 and Anti-C5: AP and CP Pathways by Hemolysis and Wieslab Assays



- Curves from single or representative assays

		ASSAY &/or RATIO	INHIBITION IC_{50}	INHIBITION IC_{90}
ACH-4471 (µM)	AP	HEM	0.027 ± 0.013	0.092 ± 0.026
		WIES	0.023 ± 0.014	0.080 ± 0.035
		H:W	1.2	1.2
ANTI-C5 (µg/mL)	AP	HEM	265	>455
		WIES	8.2 ± 3.4	57 ± 44
		H:W	32	>8.0
	CP	HEM	0.38 – 1.1	5.0 – 27
		WIES	1.2 – 1.8	10 - 11
		H:W	0.5	1.5

- HEM = hemolysis; WIES = Wieslab; H:W = HEM:WIES ratio.
- ACH-4471: mean ± SD from N = 16 (AP HEM) or N = 4 (AP WIES)
- anti-C5: individual values (AP HEM), duplicate values (CP HEM, CP WIES), or mean ± SD from N = 5 (AP WIES) assays.

- The small molecule fD inhibitor ACH-4471 inhibits equally well in AP hemolysis and AP Wieslab assays.
- The anti-C5 antibody inhibits similarly in AP Wieslab and in CP assays, but AP hemolysis is uniquely non-susceptible and shows an unusually shallow inhibition curve.
- Assay susceptibilities mirror the respective requirements for fD and C5 protein established above.

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