INTRODUCTION

The historically important hemolytic assays for complement pathway activity have been joined in recent years by ELISA-based assays for complement function1. 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 targets2-4. These sensitivities depend on the interplay of activation proteins and the number of requisites for 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 assay. In addition to the hemolysis and sc5b-9 endpoints we assessed production of activation products FB 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 deregulation including atypical hemolytic uremic syndrome (aHUS) and paroxysmal nocturnal hemoglobinuria (PNH).

METHODS

Reagents: The following were obtained from Complement Technology Inc. (Tylertown, TX, USA): normal human serum (NHS); human sera depleted of FB, FD, and CP, purified human serum FB, FD, and, rabbit erythrocytes (Er) and Ab-sensitized sheep erythrocytes (SEA); ELISA kits (GB, VGB, and 100 nM MgEGTA. VGB+MgEGTA was prepared by addition of 10 mM MgEGTA to VGB. AP and CP Wieslab kits were obtained from EuroDiagnostics (Malmø, Sweden); Bb and Csa Micro-EUSA 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 protein sample A2171 from Quidel (San Diego, CA, USA).

Complement Assays with Depleted Serum and Purified Proteins: Depleted sera were reconstituted with corresponding purified proteins at the indicated concentrations prior to assay. AP hemolysis: 8.3% serum was incubated with 8.87/µL Er in GB+MgEGTA at 37°C for 30 minutes. CP hemolysis: 0.5% serum was incubated with 8.87/µL Er in GB at 37°C for 60 minutes. Hemolysis was quantified from A540 of supernatant. AP and CP Wieslab assays were performed according to the manufacturer’s instructions (final serum dilutions, 5.6% and 1% respectively). Bb and Csa 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 to Hill equations (Hill coefficients determined by four-parameter non-linear regression).

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 CP, shown by protein titration and inhibition by anti-C5, wherein CP levels significantly exceed activity.

The distinctive relationship of AP hemolysis activity to C5 modulation is a likely consequence of the nonsusceptibility of B cells to regulation such as by factor H/factor I, yet, similar behavior is not observed when the AP components FB and FD are similarly modulated.

REFERENCES


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

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

Fig 3: FB, FD, and C5 are essential for AP activity by hemolysis or Wieslab.

Fig 4: Inhibition by ACH-4471 (Small Molecule Fd Inhibitor) and Anti-CS

*With FD reconstitution, Bb and Csa generation correlate with AP hemolysis.

**With C5 reconstitution, Csa 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-CS

**The small molecular Fd inhibitor ACH-4471 inhibits equally well in AP hemolysis and AP Wieslab assays.

The anti-CS antibody inhibits similarly in AP Wieslab and 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 CS protein established above.

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