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Notes:
West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H

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The complement system, by virtue of its dual effector and priming functions, is a major host defense against pathogens. Flavivirus nonstructural protein (NS)-1 has been speculated to have immune evasion activity, because it is a secreted glycoprotein, binds back to cell surfaces, and accumulates to high levels in the serum of infected patients. Herein, we demonstrate an immunomodulatory function of West Nile virus NS1. Soluble and cell-surface-associated NS1 binds to and recruits the complement regulatory protein factor H, resulting in decreased complement activation in solution and attenuated deposition of C3 fragments and C5b–9 membrane attack complexes on cell surfaces. Accordingly, extracellular NS1 may function to minimize immune system targeting of West Nile virus by decreasing complement recognition of infected cells.

West Nile virus (WNV) is a single-stranded positive-sense-enveloped RNA flavivirus that is related closely to other major human pathogens, such as yellow fever virus, dengue virus (DENV), and Japanese encephalitis virus. The ∼11-kb genome is translated in the cytoplasm as a polyprotein and then cleaved into structural (capsid, membrane, and envelope) and nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins by virus- and host-encoded proteases. The nonstructural proteins regulate virus replication, translation, and replication and attenuate host antiviral responses. Outbreaks of WNV infection now occur annually in North America, and the elderly and immunocompromised progress more frequently to the more severe neurological forms of the disease, including paralysis, meningitis, and encephalitis.

The ability of viruses to cause disease depends on their capacity to avoid detection and targeting by the host immune response. RNA and DNA viruses use an array of strategies to evade the host immune system. Whereas large DNA viruses often acquire and modify host immunodulatory genes to avoid recognition or targeting, smaller RNA viruses have genomic size constraints that prohibit the acquisition of exogenous genes without compromising viral replication or assembly. As such, smaller viruses tend to evolve multifunctional genes that mediate essential steps of the viral life cycle and, in some cases, modulate the host response.

Flavivirus nonstructural protein (NS1) is an essential gene that generates a conserved 48-kDa glycoprotein. Within infected cells, NS1 is believed to function as a cofactor for viral RNA replication as it colocalizes with the double-stranded RNA replicative form (1), and mutations attenuate viral RNA accumulation (2–4). Unlike the other nonstructural proteins, NS1 is secreted (5–7), and high levels (up to 50 μg/ml) accumulate in the serum of WNV and DENV-infected patients and correlate with the development of severe disease (8–12). Additionally, soluble NS1 becomes associated with the cell surface through an as-yet-undetermined process (6, 7). The mechanism(s) by which soluble NS1 contribute to flavivirus pathogenesis remains uncertain, although it has been proposed to facilitate immune complex formation (9), elicit autoantibodies that react with platelet and extracellular matrix proteins (13, 14), cause endothelial cell damage (14–17), and directly enhance infection (18).

The complement system is a family of soluble and cell-surface molecules that recognize pathogen-associated molecular patterns, altered-self ligands, and immune complexes. Complement activation controls viral infections (19–26), including WNV (27, 28), through multiple mechanisms including enhanced B and T cell priming, generation of C5b–9 membrane attack complexes that lyse infected cells, and production of fragments of C3 that opsonize viral particles and chemotracr leukocytes. In response to these antiviral mechanisms, some DNA viruses have evolved specific strategies to sabotage complement activation and neutralization by producing or incorporating complement modulating or complement-blocking molecules (29–32).

Because widespread complement activation may cause pathologic tissue injury, the host expresses regulatory molecules on cell surfaces and in solution. The plasma glycoprotein factor H (fH) is the major fluid-phase regulator of the alternative pathway and, under certain circumstances, it can also act as a surface-bound inhibitor (33). fH is a 150-kDa protein composed solely of 20 short consensus repeat domains. It attenuates alternative pathway activation by inhibiting the binding of factor B to C3b, accelerating the decay of preformed C3bBb convertases and acting as a cofactor for the serine protease factor I (fI) to cleave C3b (34–36). Because fH is a key regulator of complement activation, some bacteria have manipulated its function to evade complement system detection (37, 38). Immune evasion mechanisms by viruses involving fH have not been described.

In this paper, we define an interaction between WNV NS1 and fH. We show that NS1 binds to fH in solution and promotes the fI-mediated cleavage of C3b, and cell surface-associated NS1 attenuates the deposition of C3b and C5b–9 membrane attack complexes. Because it lacks any sequence homology to known complement regulatory genes, WNV NS1 appears to have evolved specific strategies to sabotage complement activation and neutralization by producing or incorporating complement modulating or complement-blocking molecules (29–32).

The authors declare no conflict of interest.

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Abbreviations: WNV, West Nile virus; DENV, dengue virus; fH, plasma glycoprotein factor H; fI, serine protease factor I; NHS, normal human serum; BHK, baby hamster kidney; C7d-HS, C7-deficient human serum.

See Commentary on page 18879.

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evolved a novel viral immune evasion activity, inhibiting the inflammatory and effector functions of complement.

### Results

#### Identification of a Complex with WNV NS1

In a previous study, recombinant WNV NS1 was purified to near homogeneity from baculovirus-infected SF9 cells that were cultivated under serum-free conditions (39). When NS1 was purified from infected SF9 cell supernatants containing 10% FBS, despite several sequential purification steps, NS1 was enriched along with one major and several minor contaminant proteins (Fig. 1). We hypothesized that some of the contaminating bands could represent serum ligands for NS1. We identified the dominant 150-kDa protein by tandem MS (MALDI-TOF/TOF). Peptide fragmentation spectra from tryptic peptides identified the 150-kDa band as bovine complement fH [Table 1 and supporting information (SI) Table 1: MS analysis of tryptic peptides of the high-molecular-weight protein that copurified with WNV NS1, which correspond to bovine complement fH (gi/1419424 and gi/61834503)].

#### WNV NS1 Directly Binds fH

Coimmunoprecipitation studies were designed to establish a direct interaction between WNV NS1 and fH. The presence of fH in immunoprecipitates was evaluated by Western blotting with a polyclonal antibody against human fH. The experiments were performed several ways with normal human serum (NHS), NS1 of mammalian or insect origin, and purified fH (Fig. 2A–D). Initially, we used a baby hamster kidney (BHK) cell line that stably propagates a WNV subgenomic replicon and expresses the viral proteins NS1–NS5 (40). Using a quantitative capture ELISA with two anti-NS1 mAbs (3NS1 and 10NS1; ref. 39), we confirmed these cells efficiently secrete NS1 (data not shown). Supernatants from these cells or uninfected control BHK cells were immunoprecipitated with anti-NS1-mAb-Sepharose, washed, incubated with NHS, washed again, and subjected to SDS/PAGE and Western blotting. Only anti-NS1-mAb-Sepharose that was incubated with NS1-containing supernatants coprecipitated a 150-kDa band that was recognized by the anti-fH antibody (Fig. 2A). When these studies were repeated with NS1 purified from baculovirus-infected insect cells, similar results were observed (Fig. 2B). As an additional confirmation, the immunoprecipitation studies with NS1 obtained from mammalian (Fig. 2C) or insect cells (Fig. 2D) were repeated with commercially available purified human fH. Importantly, the concentrations of NS1 (8 μg/ml) and fH (~50 μg/ml) were varied over a range of 100-fold, and no other bands were recognized by the antibody. Coimmunoprecipitation studies with NS1 purified from baculovirus-infected insect cells were also performed with commercially available human fH. Importantly, the concentrations of NS1 (8 μg/ml) and fH (~50 μg/ml) were varied over a range of 100-fold, and no other bands were recognized by the antibody.
Increasing concentrations of fH were added to NS1 on microtiter plates and evaluated for binding by ELISA. The curve is representative of three independent experiments performed in duplicate. The error bars indicate standard deviations. (A) NS1 with purified fH was added and detected with a polyclonal antibody against human fH. Results are representative of at least three independent experiments performed in duplicate. Human fH (lane 2) was commercially purchased. The positions of marker proteins are indicated to the left of the gel. (B) fH binds to solid-phase WNV NS1. Microtiter plates were coated overnight with purified NS1, WNV envelope (Ecto-E), or BSA. After blocking, purified fH was added and detected with a polyclonal antibody against human fH. Results are representative of at least three independent experiments. (C) NS1 binds to solid-phase human fH. The microtiter plate was coated with human fH or BSA as control, blocked, and then incubated with purified WNV NS1. Subsequently, after washing, NS1 binding was detected with mAb 3NS1 or 22NS1. Experiments were performed in duplicate, and the results are representative of at least three independent experiments. (D) Saturation binding of fH and NS1. Increasing concentrations of fH were added to NS1 on microtiter plates and evaluated for binding by ELISA. The curve is representative of three independent experiments performed in duplicate.

µg/ml) used in these studies were less than that (50 and 500 µg/ml, respectively) observed in human plasma (8, 11, 41).

To verify the interaction between fH and NS1, a direct binding assay was developed. Initially, insect cell-generated NS1 that was cultivated in serum-free medium was purified to homogeneity (Fig. 3A) and adsorbed to plastic. As negative controls, insect cell-generated soluble WNV envelope protein (Ecto-E; ref. 42) and BSA were adsorbed at similar concentrations in parallel. After blocking, purified fH was added, and bound fH was detected with a polyclonal antibody to human fH. Notably, significant and reproducible binding of fH was observed with NS1 but not Ecto-E or BSA proteins (Fig. 3B). The assay was also performed in the reverse order: fH was adsorbed to plastic, and soluble NS1 was added. With this assay, significant binding was observed between fH and NS1 (Fig. 3C) compared with BSA controls. Binding between fH and NS1 was saturable although a true affinity constant could not be determined, because Scatchard analysis failed to yield a model of single-site interaction, likely because both NS1 and fH exist as oligomers (Fig. 3D and data not shown). Taken together, these experiments establish a biochemical interaction between WNV NS1 and fH.

**WNV NS1 Recruits fH to Degradate C3b in Solution.** Cofactor assays were conducted to determine whether NS1 could associate with fH and activate fH-mediated cleavage of C3b in solution. Experiments were performed by using biotinylated human C3b, and cleavage products were identified by Western blot (Fig. 4). NHS was mixed with purified WNV NS1 or BSA and immunoprecipitated with an anti-NS1-mAb-Sepharose. After extensive washing, purified human fH and biotinylated-C3b were added. As a positive control, purified human fH was added directly to fI and biotinylated-C3b to induce the cleavage and generation of iC3b fragments (Fig. 4A, lanes 4 and 8), in contrast to the negative control (Fig. 4A, lanes 3 and 7). Anti-NS1-mAb-Sepharose incubated with purified NS1 but not BSA precipitated a protein or proteins from NHS with cofactor activity for C3b cleavage (Fig. 4A, lanes 2 and 6). Similar results were obtained in a heterologous system in which anti-NS1-mAb-Sepharose charged with NS1 precipitated cofactor activity from mouse serum for human C3b (Fig. 4C, lanes 2 and 6). NS1 itself lacked direct cofactor activity; addition of NS1 to fI and C3b did not generate iC3b fragments, even after prolonged incubation (Fig. 4E). NS1, however, modestly enhanced the cofactor activity of fH (SI Fig. 7). To confirm that cofactor activity associated with NS1 was due to its ability to bind fH, purified human fH was substituted for NHS and mixed with purified WNV NS1 or BSA. After immunoprecipitation, cofactor activity and production of iC3b were markedly enhanced in the presence of NS1 (Fig. 4B, lane 2). Analogously, when serum from fH−/− mice was used, NS1-
charged Sepharose no longer cleaved human C3b to iC3b (Fig. 4D, lane 2). Collectively, these experiments establish that NS1 recruits the cofactor activity of fH to degrade C3b in solution.

**WNV NS1 Attenuates C3b and C5b–9 Deposition on Cell Surfaces.** In addition to accumulating in serum of WNV-infected patients (11), NS1 also binds back to the cell surface of infected cells. We hypothesized that cell surface-associated NS1 could recruit fH to accelerate the decay of the alternative pathway C3bB convertase; this would reduce deposition of C3 ligands and inhibit formation of the C5b–9 membrane attack complex. To test this, we modified an established CHO cell assay of complement activation and C3 deposition that requires classical pathway initiation and alternative pathway amplification (43–45). CHO cells were transfected with an empty retroviral vector or one that encoded for full-length secreted WNV NS1. Several NS1-expressing (CHO-NS1) and vector alone (CHO-V) lines were identified and grown in serum-free media. As expected, WNV NS1 was detected in supernatants and on the cell surface of CHO-NS1 transfectants but not in vector-transfected cells (Fig. 5A). The level of NS1 on the CHO cell surface was equivalent to that observed on several cell types infected with WNV (data not shown). To assess whether cell-associated NS1 modulated C3 deposition, CHO-NS1 or CHO-V cells were preincubated with C7-deficient human serum (C7d-HS) to enhance interaction with fH. These cells were sensitized with a polyclonal antibody against CHO cell surface antigens, incubated with C7d-HS as a complement source, and analyzed for C3b deposition by flow cytometry. Notably, cell surface-associated NS1 decreased the C3b deposited on the cell surface (Fig. 5B, 11 ± 2% on CHO-NS1 vs. 22 ± 3% on CHO-V, P < 0.01). This effect was not due to NS1-dependent blockade of CHO surface antigen sites, because no change in antibody binding was observed in the presence or absence of NS1 (data not shown). Importantly, after a short incubation with human serum, markedly higher levels of fH were bound by CHO-NS1 compared with CHO-V cells (Fig. 5C). Thus, a decrease in C3b deposition on CHO-NS1 cells was associated with recruitment of soluble fH. Because the lower levels of C3b on CHO-NS1 cells could translate into decreased complement-dependent lysis, experiments were performed to determine the effect of NS1 on deposition of the C5b–9 membrane attack complex. CHO-NS1 or CHO-V cells were preincubated with NHS, sensitized with polyclonal antibody against CHO cell antigens, incubated with NHS as a complement source, and analyzed for C5b–9 deposition. After incubating cells with 10% NHS, we observed that expression of cell surface-associated NS1 on CHO cells resulted in a 4- to 5-fold decrease in detectable C5b–9 deposition (2.5 ± 0.6% vs. 11.4 ± 3%, P < 0.01; Fig. 5D). Similar results were observed when cells were incubated with 25% NHS (8 ± 1% for CHO-NS1 and 27 ± 5% for CHO-V, P < 0.01). Overall, our data suggest that cell surface-associated NS1 attenuates C3b deposition and C5b–9 membrane attack complex formation.

**Discussion**

For viruses to infect mammalian hosts productively, they evolve or acquire genes that attenuate or disable innate and adaptive immune responses. Because flavivirus NS1 is a secreted glycoprotein that binds back to cell surfaces and accumulates in serum, it has been speculated to have an immune evasive function. In this regard, our data suggest an immunomodulatory function for WNV NS1: it inhibits complement activation in solution and on cell surfaces. We used several experimental approaches to demonstrate a functional interaction between WNV NS1 and fH: (i) NS1 efficiently bound fH in ELISA and coimmunoprecipitation experiments; and (ii) NS1, by itself, lacked cofactor activity for fH-mediated cleavage of C3b; however, fH–NS1 complexes provide cofactor activity for the degradation of C3b in solution and (iii) cell surface NS1 bound fH and decreased the deposition of C3 fragments and C5b–9 complexes upon complement challenge.

Our finding that WNV NS1 interacts with a complement regulatory protein initially surprised us. Several decades ago, flavivirus NS1 was originally identified as a “soluble complement fixing antigen” (46, 47), and vascular leakage associated with severe DENV infection correlated with complement consumption and the accumulation of NS1 in serum (8–10, 12, 48). Moreover, soluble DENV NS1 appeared to activate complement in vitro and was associated with increased levels of C5b–9 terminal complexes in serum (12). Subsequent experiments...
indicate that DENV NS1 does not activate complement directly but instead forms immune complexes with natural or DENV immune antibody, which then lead to complement consumption and C5b–9 complex formation (P. Avirutnam, J.P.A., and M.S.D., unpublished results). It is possible that WNV and DENV NS1 have evolved distinct abilities to regulate complement activation. Consistent with this, complement activation and C3 consumption after WNV infection in mice peaks in serum within 2 days and gradually normalizes with kinetics that parallel the detection and accumulation of NS1 in serum (27). In comparison, C3 and C4 consumption after DENV infection in humans increases over time and correlates with the most severe clinical phenotype, the capillary leak syndrome of dengue hemorrhagic fever (48). Future studies are required to resolve whether NS1 from other flaviviruses interacts similarly with fH.

The mechanism by which WNV NS1 modulates complement activation is novel among viruses. Herpesviruses and poxviruses sabotage complement activation by incorporating and modulating complement modulating or complement-blocking molecules (29–32). In contrast, NS1 itself lacks any sequence homology to known complement regulatory genes and indeed lacked direct cofactor activity. Instead, NS1 binds to and recruits fH to attenuate complement activation in solution and on cell surfaces. This latter function of converting fH into a membrane-associated complement regulatory protein is reminiscent of the immunomodulatory activities of surface proteins and carbohydrates of Streptococcus, Neisseria, Yersinia, and Borrelia, which bind fH to regulate complement activation and reduce opsonization and phagocytosis (49–52).

The present study demonstrates that WNV NS1 on cell surfaces inhibits deposition of C3b and the C5b–9 membrane attack complex. A mechanistic appreciation of how and why soluble flavivirus NS1 binds to cell surfaces has been lacking, although a recent study suggested that it might directly enhance DENV infection in hepatocytes (18). Our study suggests that cell surface-associated NS1 could minimize immune system activation and priming and the generation of inflammatory ligands (e.g., C3a or C5a) and opsonins (e.g., C3b or iC3b). By decreasing complement activation and therefore the number of C5b–9 membrane attack complexes on infected cells, NS1 also could delay or prevent complement-dependent lysis of infected cells, thereby allowing for continued production and secretion of virus. Although structural studies are lacking, prior mapping studies have suggested that NS1 may have distinct domains (14, 39, 53); studies are under way to identify the region of WNV NS1 that interacts with fH and analogously the region on fH that binds WNV NS1. A detailed molecular understanding of the interaction between WNV NS1 and fH may, in part, explain differences in pathogenesis and disease among flaviviruses and facilitate the development of inhibitors that block immune evasion.

Materials and Methods

**Purified Proteins.** Expression and purification of recombinant WNV NS1 from insect cells were described previously (39). In some experiments, SF9 insect cells were cultivated in Grace’s media (JHR Bioscience, Lenexa, KS) with 10% FBS. Purified human fH, C3b, and fI were obtained commercially (Advanced Research Technologies, San Diego, CA), aliquotted, and stored at −80°C.

**MALDI-TOF MS.** Peptide pools were prepared from SDS/PAGE gels by using a previously described method (54). Gel bands from 1D gels were transferred to a 96-well plate (Axygen Scientific, Union City, CA). After trypsin digestion, an aliquot was removed from each well and placed in a microfuge tube containing MALDI Matrix (Agilent Technologies, Palo Alto, CA). The tubes were vortexed and microfuged, and 1 μl was spotted onto a stainless-steel target (192 spot plate) for MS, as described (55).

**NS1-Binding Assays. Complementprecipitation.** Complementprecipitations were performed with NHS, NS1 generated from baculovirus-infected SF9 cells or a BHK cell line that stably propagates a subgenomic replicon of WNV (40), and purified human fH.

**NS1 from WNV replicon and NHS or purified fH.** BHK cells expressing the WNV replicon or uninfected control BHK cells were cultured in serum-free DMEM. Supernatants were immunoprecipitated with anti-NS1-Sepharose 4B beads [9NS1-Sephr4B (39)]. After washing, the beads were incubated with NHS (70 μl) or purified fH (35 μg) in serum-free DMEM or GVB-Mg2+–EGTA overnight at 4°C. The beads were washed four times with GVB-Mg2+–EGTA or serum-free DMEM media at 4°C.

**NS1 from baculovirus-infected SF9 insect cells and NHS.** NHS (70 μl) and purified NS1 (70 μl, 100 μg/ml) from baculovirus-infected SF9 insect cells, cultivated without FBS, were mixed, incubated overnight at 4°C, and immunoprecipitated for 5 h at 4°C with 9NS1-Sephr4B. The beads were washed four times with serum-free DMEM media at 4°C. Purified NS1 and fH. Purified NS1 (7 μg) or BSA (7 μg) was immunoprecipitated with 22NS1-Sephr4B (39). Beads were washed, incubated with purified fH (55 μg) in GVB-Mg2+–EGTA overnight at 4°C, and washed four times. All immunoprecipitates were boiled in SDS sample buffer and separated by reducing 8% SDS/PAGE. Western blot analysis was performed as described (39) by using a 1/2,000 dilution of a sheep anti-human fH antibody (Biodesign, Saco, ME) and a 1/3,000 dilution of secondary antibody conjugated with HRP.

**ELISA. fH binding to NS1.** Insect cell-derived WNV NS1 (~0.5 μg) was adsorbed overnight at 4°C to microtiter plates. Non specific sites were blocked [150 mM NaCl/25 mM Tris-HCl (pH 7.5)/2% BSA/0.025% Nonidet P-40/0.025% NaN3]. Human fH (50 μl of 10 μg/ml stock) was added to the wells and incubated for 1.5 h at room temperature. After washing four times [150 mM NaCl/25 mM Tris-Cl (pH 7.5)/0.5% BSA/0.025% Nonidet P-40/0.025% NaN3], plates were incubated with a 1/200 diluted sheep anti-human fH antibody for 1 h at room temperature. After an additional wash, plates were incubated with HRP-conjugated rabbit anti-sheep IgG and developed after addition of 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate.

**NS1 binding to fH.** Microtiter plates were coated with 2.5 μg of human fH in PBS overnight at 4°C. After blocking, WNV NS1 (0.5 μg) was added and incubated for 1.5 h at room temperature. After washing, the plates were incubated serially with 10 μg/ml anti-WNV mAbs 3NS1 or 22NS1 (39) and donkey anti-mouse IgG-conjugated HRP (1/600 dilution) and developed with tetramethyl-benzidine substrate.

**C3b Cofactor Assay.** C3b cofactor activity was assessed as described (43, 44). This assay analyzes the breakdown products of biotinylated human C3b in solution. The fragments of the cleavage reaction, after addition of human fH and NS1 coinmunoprecipitates, were assessed by Western blot analysis of the samples as described (43, 44). For the cofactor assay, all samples were treated with proteinase K at 37°C and then extracted with chloroform, and the nucleic acid was removed. The reaction mixtures were added to 10 μg/ml fH and 200 μg/ml C3b Cofactor Assay.

**Coimmunoprecipitation.** Coimmunoprecipitations were performed with NHS, NS1 generated from baculovirus-infected SF9 cells or a BHK cell line that stably propagates a subgenomic replicon of WNV (40), and purified human fH.

**NS1 from WNV replicon and NHS or purified fH.** BHK cells expressing the WNV replicon or uninfected control BHK cells were cultured in serum-free DMEM. Supernatants were immunoprecipitated with anti-NS1-Sepharose 4B beads [9NS1-Sephr4B (39)]. After washing, the beads were incubated with NHS (70 μl) or purified fH (35 μg) in serum-free DMEM or GVB-Mg2+–EGTA overnight at 4°C. The beads were washed four times with GVB-Mg2+–EGTA or serum-free DMEM media at 4°C.

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Cho Cell Lines. Cho-NS1 stable transfectants were generated as follows: an initiator methionine, the last 72 nucleotides of WNV E (endogenous signal sequence), and entire NS1 protein from the New York 1999 strain were amplified from the infectious cDNA clone by high-fidelity PCR. The PCR product was digested and inserted in the BamHI and SalI site of pBabe-puro vector (Clonetech). Stable transfectants were selected into Ham’s F-12 medium supplemented with puromycin (10 μg/ml). Clones of NS1 (CHO-NS1) or vector-containing (CHO-V) cells were isolated and adapted into serum-free CHO-S-SFM II (In Vitrogen, Carlsbad, CA) supplemented with puromycin (8 μg/ml).

Complement Activation on the Surface of Cho Cells. Complement activation and detection of split products on CHO cells were a modification of published protocols (43–45). To measure cell surface C3b or C5b–9 deposition, CHO cells were resuspended in Ham’s F-12 medium and preincubated with 10% C7-dHS (gift of P. Densen, University of Iowa, Iowa City, IA). After a 1-hr incubation in ice, the cells were washed and incubated with goat anti-CHO surface antigen IgG (Cygnus Technologies, Southport, NC; 10 μg/ml) for 20 min on ice. Subsequently, the cells were washed and resuspended in 75 μl of GVB-Mg2+/EGTA, and 75 μl of 18% C7-dHS was added. Cells were incubated at 37°C for 30 min and washed, and C3b deposition was detected with a murine mAb to human C3d (5 μg/ml; Quidel, San Diego, CA). To measure C5b–9 deposition, cells were pretreated with 10% NHS, incubated with 0.5 mg/ml goat anti-CHO IgG, and then incubated with 10% or 25% of NHS as the complement source. CHO cells were immunostained with 10 μg/ml murine mAb against human C5b–9 and analyzed by flow cytometry.

Fh Binding to Cho Cell Lines. Cho-NS1 or Cho-V cells were harvested, resuspended in 10% NHS in Ham’s F12 medium, and incubated on ice. Cells were washed four times and lysed with SDS sample buffer, and lysates were separated by SDS/PAGE. The level of bound Fh was analyzed by Western blot with a sheep anti-human Fh antibody.

Statistical Analysis. All data were analyzed with Prism software (GraphPad, San Diego, CA). Statistical significance was determined by using a two-tailed nonpaired Student’s t test.

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