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Acquisition of Complement Factor H Is Important for Pathogenesis of *Streptococcus pyogenes* Infections: Evidence from Bacterial In Vitro Survival and Human Genetic Association

Karita Haapasalo,*‡ Jaana Vuopio,‡ Jaana Syrjänen,*‡ Jari Suvilehto,* Satu Massinen,‖ Matti Karppelin,¶ Irma Järvelä,‖ Seppo Meri,*‡ Juha Kere,‖¶* and T. Sakari Jokiranta*†

*Streptococcus pyogenes* (or group A *streptococcus* [GAS]) is a major human pathogen causing infections, such as tonsillitis, erysipelas, and sepsis. Several GAS strains bind host complement regulator factor H (CFH) via its domain 7 and, thereby, evade complement attack and C3b-mediated opsonophagocytosis. Importance of CFH binding for survival of GAS has been poorly studied because removal of CFH from plasma or blood causes vigorous complement activation, and specific inhibitors of the interaction have not been available. In this study, we found that activation of human complement by different GAS strains (n = 38) correlated negatively with binding of CFH via its domains 5–7. The importance of acquisition of host CFH for survival of GAS in vitro was studied next by blocking the binding with recombinant CFH5–7 lacking the regulatory domains 1–4. Using this fragment in full human blood resulted in death or radically reduced multiplication of all of the studied CFH-binding GAS strains.

To study the importance of CFH binding in vivo (i.e., for pathogenesis of streptococcal infections), we used our recent finding that GAS binding to CFH is diminished in vitro by polymorphism 402H, which is also associated with age-related macular degeneration. We showed that allele 402H is suggested to be associated with protection from erysipelas (n = 278) and streptococcal tonsillitis (n = 209) compared with controls (n = 455) (p < 0.05). Taken together, the bacterial in vitro survival data and human genetic association revealed that binding of CFH is important for pathogenesis of GAS infections and suggested that inhibition of CFH binding can be a novel therapeutic approach in GAS infections. *The Journal of Immunology*, 2012, 188: 000–000.

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treptococcus pyogenes or group A *Streptococcus* (GAS) is a Gram+ bacterium colonizing skin and mucous membranes and causing either superficial infections, such as impetigo and tonsillitis, or invading superficial or deeper tissues in erysipelas, cellulitis, necrotizing fasciitis, or sepsis. Sometimes even superficial infections can lead to poststreptococcal infection sequelae (1).

Tonsillitis and erysipelas are the two best-known infections caused by GAS. Both have a tendency to recur and need to be treated with antimicrobials to avoid the postinfection sequelae (2).

The online version of this article contains supplemental material.

Abbreviations used in this article: AMD, age-related macular degeneration; AP, alternative pathway of complement; AU, arbitrary unit; C, complement; CFH, complement factor H; CI, confidence interval; EIA, enzyme immunoassay; GAS, group A streptococcus; MF, multiplication factor; MgEGTA, 10 mM MgCl2 and 10 mM EGTA; NHS, normal human serum; PBS-T, PBS containing 0.05% Tween 20.
In domain 7 of CFH is associated with age-related macular degeneration (AMD), the most common cause of visual loss in industrialized countries (17–20). This indicates that domain 7 is important for physiological function of CFH, but it is not clear whether this is due to the role of this domain in binding to negatively charged cell surface polyanions, such as heparan sulfate, or in binding C-reactive protein (21, 22).

At least 10 pathogenic microbes protect themselves from AP attack by acquiring host CFH onto their surfaces via domains 6–7, allowing domains 1–4 to regulate C activation on the microbe (e.g., S. pyogenes and Neisseria meningitidis) (23–25). The importance of microbial CFH binding is exemplified by the only two successful vaccine candidates against N. meningitidis group B (under phase III trials), both using the meningococcal CFH-binding protein (26). The importance of CFH binding, particularly via the domains 6–7, is supported by the finding that most strains of S. pyogenes (GAS) bind CFH via these domains, and the polymorphic variant 402H impairs binding of CFH to the bacteria, reducing GAS multiplication in human blood in vitro (27, 28).

In this study, we used 38 GAS strains to study correlation between streptococcal CFH binding and the resulting C activation and analyzed the importance of host CFH acquisition onto GAS by inhibiting the interaction with a recombinant CFH fragment consisting of domains 5–7 (CFH5–7). Lack of a proper animal model for GAS infections led us to analyze whether CFH binding is important for survival of GAS in vivo by studying the association between polymorphism Y402H of CFH domain 7 and clinical GAS infections. The results showed that CFH acquisition is necessary for survival and multiplication of GAS in human blood in vitro, suggested decreased susceptibility to GAS infections in individuals genetically susceptible to AMD (27), and introduced a novel idea for therapeutic intervention in GAS infections by inhibition of CFH binding.

Materials and Methods

Proteins

Cloning of the recombinant fragment CFH5–7 and its expression in Pichia pastoris were described earlier, including a figure of the fragment in both

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*aData from Haapasalo et al. (27). Categories for CFH5–7 binding (relative percentage of maximal binding): —, <14%; +, 14–30%; ++, 31–60%; ++++, >60%.

*bData from Pérez-Caballero et al. (36).

*cData from Horstmann et al. (25).

AGNs, acute glomerulonephritis skin isolate, Ethiopia; AGNth, acute glomerulonephritis throat isolate, Ethiopia; ATCC, American Type Culture Collection; Inv, invasive blood isolate, Finland; na, not assessed; NCTC, National Collection of Type Cultures; Rfs, reference strain.
SDS-PAGE gel and Western blotting. The CFH (402Y) protein was purified from plasma of individuals homozygous for the 402Y variant of CFH, essentially as described earlier (27). C4BP was purchased from Complement Technologies (Tyler, TX).

**Binding of [125I]–CFH/CFH5–7 onto GAS**

Binding of CFH5–7 to the GAS strains was described earlier using radioligand and direct-binding assays (27). Binding of full CFH and C4BP to the strains was analyzed similarly using the radioligand assay. Briefly, coating of the microtiter plates (Polyisorb BreakApart; NUNC, Thermo Scientific, Roskilde, Denmark) was done by incubating 1.2 × 10⁹ bacteria in 200 μl PBS for 24 h at 37°C. The wells were washed three times with PBS and blocked with 300 μl 3% BSA in PBS for 1 h at 37°C. After washing, 50,000 cpm of [125I]–protein in 200 μl 0.1% BSA-PBS was added to each well, and the plate was incubated for 1 h at 37°C. The wells were washed, separated from each other, and subjected to counting with a gamma counter.

For the inhibition assay, 1 × 10⁸ log-phase bacteria (per reaction) were preincubated in veronal buffered saline containing 0.1% gelatin with increasing concentrations (0, 1.2, 2.4, and 4.8 μM) of CFH5–7 and washed once with 200 μl PBS. Thereafter, [125I]–CFH (402Y) was added (50,000 cpm in 100 μl VBS containing 0.1% gelatin; sp. act. 4 × 10⁶ cpm/μg), and the mixture was incubated for 30 min under continuous horizontal rotation (Thermomixer) at 37°C. Each mixture was applied on top of 250 μl 20% sucrose in a 400-μl plastic tube; after centrifuging (10,000 × g for 3 min), radioactivity in the pellet and supernatant was measured with a gamma counter. The ratios of bound (pellet) to total activity (pellet + supernatant) were calculated. The experiment was performed three times using three parallel samples.

**Bacterial strains and growth conditions**

The strains of *S. pyogenes* (Table I) were taken from −70°C milk/glycerol suspensions and grown at 37°C under 5% CO₂ on blood agar plates supplemented with colistin and oxolinic acid.

**Study populations**

Study populations consisted of Finnish patients (*n* = 487) suffering from erysipelas (*n* = 278) or tonsillitis (*n* = 209). The erysipelas patients were either treated in Tampere University Hospital or Hatanpää City Hospital, Tampere, for an acute episode of erysipelas (*n* = 90) or they were on benzathine penicillin prophylaxis because of recurrent erysipelas (*n* = 188). Tonsillitis cases were pediatric patients referred to Helsinki University Central Hospital for scheduled tonsillectomy due to chronic or recurrent tonsillitis and/or pharyngitis (*n* = 209). Inclusion criterion for tonsillitis patients was recurrent tonsillitis (at least six episodes/year or three episodes/year for two consecutive years, with at least one positive history of erysipelas). Prolonged tonsillar infection refractory to antimicrobial therapy, or tonsillar hyperplasia with symptoms (29). Control group (*n* = 455) consisted of Finnish blood donors (*n* = 350) and individuals without history of erysipelas (*n* = 105).

**Ethics statement**

The studies on the erysipelas patients were approved by the Ethical Review Board of the Hospital District of Helsinki and Uusimaa. A written informed consent was provided by the study participants and/or their legal guardians.

**Analysis of AP activation in plasma**

GAS strains were grown in 10 ml Todd–Hewitt broth at 37°C under 5% CO₂ atmosphere until late log phase OD₆₀₀ = 0.6, and the pellet was frozen at −70°C in the broth containing 30% glycerol. After thawing, the aliquots were washed three times with PBS, OD₆₀₀ was adjusted to 0.6 (2 × 10⁶ bacteria/ml), 1 ml the suspension was pelleted, and each pellet was resuspended into 200 μl 50% normal human serum (NHS) diluted using ice-cold PBS with 10 mM MgCl₂ and 10 mM EGTA (MgEGTA) on ice. As a negative control for the C3a assay, the pellet was resuspended into 200 μl 50% NHS diluted in ice-cold PBS containing 10 mM EDTA. The NHS used was obtained by pooling serum from six individuals. The mixtures were incubated at 37°C for 30 min under continuous shaking, and the reactions were stopped on ice with 15 μl 0.2 M EDTA. The bacteria were pelleted, and the supernatants were frozen at −70°C for C3a, Bb, and SC5b-9 analyses. C3a and Bb concentrations were measured using MicroVue C3a and Bb Plus ELISA kits (Quadel, San Diego, CA), according to the manufacturer’s instructions, using dilutions of 1:2000 (C3a ELISA) or 1:200 (Bb ELISA).

For the SC5b-9 ELISA, anti-C9 neoepitope Ab WU13-15 (2 μg/ml; Hyclut Biotech, Uden, The Netherlands) in 100 μl 0.2 M Na₂CO₃ (pH 10.6) was added to wells of a microtiter plate (Maxisorp NUNC, Thermo Scientific) and incubated for 17 h at 4°C. For blocking, 0.5% BSA (Sigma-Aldrich, Steinheim, Germany) in PBS was incubated for 2 h at 22°C, followed by three washes with PBS containing 0.05% Tween 20 (PBS-T). The plasma samples (100 μl 1:400 in PBS-T) were added, incubated for 2 h at 4°C, washed five times, and incubated for 1 h at 22°C with 1:10000 dilution goat-anti-C7 (Organon Teknika, West Chester, PA). After five washes, HRP-conjugated donkey anti-goat IgG (1:3000; Jackson ImmunoResearch, West Grove, PA) in PBS-T was incubated for 1 h at 22°C, followed by five washes and the addition of HRP substrate (OPD; DAKO, Glostrup, Denmark). The reaction was stopped with 100 μl 0.5 M H₂SO₄, and absorbance was detected at 490 nm. Arbitrary units (AU)/ml were calculated using a standard curve obtained by activating NHS with Zymosan A (Sigma-Aldrich; 1 mg/ml, 60 min at 37°C) (normalized to 1000 AU/ml).

**Analysis of AP activation in full blood**

Bacteria grown overnight in Todd–Hewitt broth were diluted in the same broth (1:50) and grown to OD₆₀₀ of 0.15. The suspension was then diluted 1:10,000 in 100 μl PBS (to obtain 200 CFU) and incubated under continuous horizontal rotation (Thermomixer) for 15 min at 37°C in the presence or absence of 20 μg CFH5–7 (final concentration 4.8 μM). The samples were mixed with 1.2 ml human blood from healthy volunteers and incubated at 37°C for 1 h under continuous rotation (resulting in CFH5–7/CFH molar ratio = 1:3). We chose the CFH5–7 concentration to inhibit, but not to activate complement (27).

**FIGURE 1.** Relationship between CFH binding by GAS and generation of C-activation marker C3a upon exposure of 38 GAS strains to serum. Each GAS strain was incubated in 50% NHS in the presence of Mg²⁺ and EGTA (each 10 mM), and C3a release was quantified using an EIA. The results were compared with binding of each strain to [¹²⁵I]-labeled CFH (using a radioligand assay) (A) or previously published data on binding dilution strain to [¹²⁵I]-C5a (B) (27). Five values indicate relative binding of CFH compared with a negative and positive control in the CFH radioligand assay or the percentage of radioactivity in pellet versus total radioactivity in pellet and supernatant in the direct CFH5–7 binding assay. Pearson correlations were calculated, showing statistically significant negative correlations between binding of each strain to either CFH or CFH5–7 and generation of complement AP-activation marker C3a (*p* < 0.05). The C3a results are presented as mean of triplicate samples in an EIA in which an NHS pool from six individuals was used.
not to block, binding of CFH to the GAS surface to avoid possible complement attack against red cells if CFH binding to blood cells was blocked with high CFH5–7 concentration. The blood samples (n = 2) were anti-coagulated with lepirudin (Refludan, Schering, Berlin, Germany) and drawn from individuals who were genotyped CFH402Y homozygous or CFH402Y/CFH402H heterozygous. At 0, 30, and 60 min, 400-, 50-, and 50-μl samples were transferred into tubes containing 50, 15, or 15 μl 0.2 M EDTA, respectively. The cells were separated by centrifugation, and the supernatant was stored at −70°C before measuring the amounts of Bb and SC5b-9, as described above. Blood from different individuals was used in the two experiments performed for calculating the statistics.

**Multiplication assay**

Bacteria were prepared and incubated in lepirudin-anticoagulated blood, as described above. The assay was performed from blood obtained from three individuals (n = 3). The multiplication factor (MF) of the bacteria in blood was measured, as described previously (30, 31). Tubes were incubated at 37°C for 3 h under continuous orbital rotation. At 0, 60, 120, and 180 min, 400-μl samples were transferred into tubes containing 50 μl 0.2 M EDTA. At 60 and 120 min, 400 μl the same blood was added after taking the samples. For each 400-μl sample, 100 μl the mixture (or its 1:10–1:1000 dilution) was inoculated on blood agar plates and incubated for 17 h (37°C, 5% CO2). The CFU were counted using the pour-plate method, and MF was calculated for each time point. In each single assay, two parallel samples with two parallel inoculations were tested; in each of the three experiments used for calculating the statistics, blood from a different individual was used.

To monitor whether addition of CFH5–7 disturbs C activation in blood, the assay was performed by mixing 1.2 ml blood with 200 μl PBS or 20 μg CFH5–7 in PBS (final concentration of CFH5–7 was 0.7 μM, and the CFH5–7/CFH ratio was ∼1:3). At three time points, 200-μl blood samples were taken and centrifuged for 3 min at 10,000 × g, and the C activation in the separated supernatant was analyzed using the C3a EIA.

**Factor H Y402H genotyping**

The sample DNA was isolated and genotyped for Y402H polymorphism (T1277C, rs1061170) by sequencing, as previously described (32).

**Statistical methods**

Pearson correlation was used to measure the dependence between C activation and binding of CFH5–7, CFH, or C4BP, as well as among SC5b-9, Bb, and C3a concentrations (version 15.0 of SPSS for Windows; Analytical Software, IBM, Chicago, IL). For the CFH-inhibition assay, multiplication assay, and the assay measuring Bb or SC5b-9 formation in blood, the difference between means of each experiment within one, two, or three individual experiments was compared using the Student t test. Genetic associations between different patient and control groups were analyzed by the Pearson χ2 test, and the relative risk and the 95% confidence interval (CI) for each comparison were calculated using SPSS software. Deviations from Hardy–Weinberg equilibrium were analyzed using the χ2 test of Microsoft Excel software.

**Results**

**Binding of CFH via CFH5–7 protects GAS from complement activation**

To determine whether acquisition of CFH, via its domains 5–7, by GAS is associated with decreased AP activation, we subjected 38 GAS strains (Table I) to AP attack in serum and quantified the resulting activation products C3a and C5b-9 (SC5b-9). Our previous data on binding of CFH5–7 to those strains (27) and previously unpublished data on binding of full-length CFH to those strains correlated negatively with the new data for which the production of both C3a (Pearson correlations = −0.38 and −0.44 for CFH and CFH5–7, p = 0.017 and 0.007, respectively) (Fig. 1) and SC5b-9 (Pearson correlation = −0.30 and −0.31, p = 0.034 and 0.034, respectively) was quantified (Fig. 2A, 2B). Binding of a regulator of the classical pathway, C4b-binding protein (C4BP), to GAS showed no correlation to C3a or SC5b-9 production (p = 0.163 and 0.596, respectively; Fig. 3). Because all of the activation pathways may act simultaneously in vivo, the C3a assay was also performed in NHS where all of the pathways were active (in the absence of MgEGTA). The correlation analyses showed similarly that C3a formation is negatively correlated with binding of CFH via domains 5–7 (Pearson correlation = −0.42; p = 0.009) but not with C4BP binding to the bacteria (p = 0.541) (Supplemental Fig. 1).

To verify that SC5b-9 resulted from AP activation, we compared SC5b-9 with C3a and Bb concentrations and found a significant positive correlation between SC5b-9 and C3a (Pearson correlation = +0.4, p = 0.04) (Fig. 2C) or Bb (Pearson correlation = +0.7, p = 0.002) concentration (Fig. 2D). Unlike the others, four strains (emm55, emm66, st221, and stG653) showed practically no C3a
formation, although they failed to bind CFH5–7; however, these strains bind full-length CFH (Table II).

**Innate immune attack against GAS is enhanced by CFH5–7**

Next, the fragment CFH5–7 was used to inhibit binding of whole CFH on the bacteria in an assay in which a representative CFH-binding strain (st369) was preincubated with increasing concentrations of CFH5–7. Preincubation with CFH5–7 resulted in significant dose-dependent inhibition of CFH binding on GAS surface (Fig. 4A). To further investigate whether the inhibition of CFH binding could have an effect on GAS survival, the bacteria were exposed to CFH5–7 in a full-blood survival assay (i.e., an ex vivo sepsis model). Preincubation of a representative CFH-binding GAS strain (st369) with recombinant fragment CFH5–7 (lacking the regulatory domains 1–4) resulted in increased Bb and SC5b-9 release upon exposure of the bacteria to blood (Fig. 4B, 4C). As expected, when the same amount of CFH5–7 was incubated in whole blood in the absence of bacteria, no increase in the C-activation products was seen.

Because incubation of the bacteria in blood with the CFH5–7 fragment resulted in increased C-activation products, we next determined whether five of the GAS strains binding both CFH and CFH5–7 (Fig. 5A) could be more easily opsonophagocytosed in the presence of the CFH5–7 fragment. Using fresh anticoagulated human blood, we could evaluate C-mediated inhibition of GAS multiplication because the Gram+ bacteria are resistant to direct killing by complement. By counting CFU using the pour-plate method, we observed a significant decrease in MF of all of the studied strains when the bacteria were incubated in the presence, versus the absence, of CFH5–7 (p < 0.001, for all strains by the 180-min time point) (Fig. 5B–F).

### Genetic association between CFH polymorphism and GAS infections

Next, we wanted to analyze whether CFH binding is important for complement evasion of GAS in vivo. Unfortunately murine CFH does not bind to any of the tested GAS strains (Supplemental Fig. 2A), compromising the ability to use a mouse model. Therefore, we designed an analysis of the importance of streptococcal CFH binding during human infections based on the data that the polymorphism Y402H within domain 7 of CFH leads to impaired binding of the variant 402H to GAS (27). To test possible association of the polymorphism with human GAS infections, we analyzed the Y402H genotype from patients with a history of erysipelas (n = 278) or recurrent tonsillitis (n = 209), as well as from 455 control subjects (350 blood donors and 105 nonerysipelas controls). The allele frequencies in both patient groups and both control groups were consistent with the Hardy–Weinberg equilibrium, as expected (Table III). The frequency of the variant allele responsible for the expression of 402H (1277C) in the combined patient group (all cases) was significantly lower than in the combined group of control subjects (p < 0.05); the odds ratio (OR) for the risk for erysipelas or tonsillitis in carriers of the allele was 0.831 (95% CI: 0.693–0.998) (Table IV). A significant difference (p < 0.05) between the corresponding allele frequencies was also found when all of the cases were compared with the nonerysipelas controls (OR, 0.735; 95% CI: 0.545–0.991).

Next, genotype frequencies were compared; the frequency of the CC genotype (homozygosity for the allele C coding for the 402H allele) was significantly lower in the patients than in the control subjects (p = 0.029; OR, 0.653; 95% CI: 0.446–0.958) (Table IV). Also, when all of the cases were compared with the nonerysipelas controls, the frequency of the CC genotype was significantly lower (p < 0.05; OR, 0.513; 95% CI: 0.274–0.958).

### Discussion

In this study, we showed that acquisition of host CFH is important for immune evasion of GAS in blood ex vivo, and it could be blocked by using CFH5–7, resulting in impaired survival of the bacteria. We also showed that the impaired GAS–CFH interaction mediating allele 402H (1277C) is suggested to be associated with protection from human erysipelas and recurrent tonsillitis. This allele was shown to be associated with AMD (17–20), and diminished binding of 402H variant to heparin and/or C-reactive protein was suggested to explain the biological basis for the association (33–35).

Binding of CFH by different clinical GAS isolates has been studied, but at least two studies showed a significant variation in CFH binding between different strains and isolates (29, 36). Therefore, the effect of CFH binding on complement evasion of GAS was analyzed by comparing CFH binding and markers of complement activation upon exposure of the bacteria to serum. When 38 GAS strains that were previously analyzed for CFH5–7 binding (27) were subjected to serum, a significant negative correlation was seen between CFH5–7 binding and an increase in C-activation markers C3a (Fig. 1B) and SC5b-9 (Fig. 2B). There were four strains that did not bind CFH5–7 but failed to cause any
increase in C3a concentration (Table II). In principle, this could result from expression of C5a-peptidase by these strains, because it might also cleave C3a, which is very similar to C5a (37). Because three of these strains also did not lead to generation of SC5b-9, a more probable explanation is that these GAS strains could explain why the observed CFH binding does not protect recognition and regulation of surface-bound C3b by CFH. This could explain why the observed CFH binding does not protect GAS of emm5 type from opsonophagocytosis, as described previously (30).

We analyzed the effect of CFH5–7 on CFH binding of GAS in a direct binding assay, using purified proteins, as well as on the survival of GAS in fresh full blood, not in serum only, because the thick cell wall protects Gram+ bacteria from direct C-mediated lysis. To avoid interpretation of the results on the basis of one genotype only, we used blood from three individuals, one of whom was homozygous for CFH402Y and another was heterozygous (genotype of the third was not assessed). Preincubation of either domains 6–7 or 19–20 and only one via domains 8–10 and 13–15 (39). Acquisition via other domains might disturb proper recognition and regulation of surface-bound C3b by CFH. This could explain why the observed CFH binding does not protect GAS of emm5 type from opsonophagocytosis, as described previously (30).

We analyzed the effect of CFH5–7 on CFH binding of GAS in a direct binding assay, using purified proteins, as well as on the survival of GAS in fresh full blood, not in serum only, because the thick cell wall protects Gram+ bacteria from direct C-mediated lysis. To avoid interpretation of the results on the basis of one genotype only, we used blood from three individuals, one of whom was homozygous for CFH402Y and another was heterozygous (genotype of the third was not assessed). Preincubation of bacteria with CFH5–7 resulted in significant reduction in CFH binding (Fig. 4A) and significant increase in SC5b-9 and Bb (Fig. 4B, 4C) within 60 min, indicating that CFH5–7 interferes with
CFH binding and AP evasion of GAS. An increase in Bb concentration was already detected at the 30-min time point, whereas the increase in C5b-9 concentration was seen only at 60 min. This could result, for example, from the possible role of erythrocyte or leukocyte CD59 in eliminating small amounts of C5b-8 or C5b-9 complexes that have not been assembled on the bacteria and, therefore, released to the fluid phase.

All of the strains multiplied well in blood in the absence of CFH5–7 but failed to do so in the presence of CFH5–7 (Fig. 5). Of the five tested GAS strains, multiplication of strain emm89, which binds CFH less efficiently than the other four strains, was totally inhibited. This showed for the first time, to our knowledge, that CFH binding via domains 5–7 is a very important innate immune-evasion strategy for GAS. This probably was not shown previously, because the impact of CFH binding to the bacteria cannot be measured by removing CFH from serum or blood because of the resulting vigorous C activation, and removal of the M protein by bacterial mutagenesis could also impair its other functions, such as C4BP binding (40). Our approach of showing the impact of CFH acquisition for the bacteria is novel and free of these biases. Furthermore, some GAS strains were shown to express other CFH-binding ligands, such as FbaA, which might also interact with CFH via CFH5–7, because it binds FHL-1 containing the CFH domains 1–7 (41). One study also questioned the relevance of the interaction between CFH and GAS, because binding of full-length CFH to some GAS strains is affected by physiological salt concentration, unlike binding of FHL-1 and C4BP (36). In our study, we did not analyze the effects of CFH versus FHL-1 in protection of GAS, because CFH5–7 could inhibit binding of both of these proteins. Although C4BP was also shown to enhance complement and phagocytosis resistance of some GAS strains (M22, M60) (40, 42), we found no correlation between C4BP binding and generation of C-activation markers. This could be explained by the fact that C4BP is a regulator of the classical pathway in which Abs play a major role in initiation of activation; therefore, the binding of C4BP by GAS is probably more relevant when specific Abs are present. However, the contribution of C4BP on AP evasion of some strains cannot be excluded on the basis of our results, but the lack of correlation between C3a release and C4BP binding indicates that this is not a common phenomenon.

This study showed that recombinant CFH5–7 interferes with C evasion by GAS in vitro by inhibiting binding of CFH to GAS surface (Fig. 4A). CFH binding on GAS emm1 surface was studied previously using a mAb against FbaA protein, supporting our hypothesis that competitive inhibition of CFH binding to GAS could enhance elimination of GAS (43). Inhibition of CFH binding to GAS by CFH5–7 in our study was tested using the CFH402Y allotype, which was shown to interact better with GAS than the CFH402H allotype (27). It is very likely that the inhibitory effect of CFH5–7 on the binding of the CFH402H allotype would be similar or better than on the binding of the CFH402Y allotype. It is also obvious that 100% inhibition of CFH binding to GAS surface is not required for elimination of the bacterium; in our assays, the CFH5–7 concentration was approximately one third of the CFH concentration. The dramatic effect of partial inhibition of CFH binding on reduced survival of GAS probably results from changing the balance between the binding of factor B versus CFH to the surface-bound C3b, because relatively increased factor B binding can lead to activation of the AP. This kind of effect is also caused by several known mutations in the surface-recognition site on CFH domains 19–20, where only a minor change in affinity of CFH to C3b leads to severe tissue damage in atypical hemolytic uremic syndrome (44).

Effect of the CFH5–7 fragment in vivo was not easy to assess, because we could not find any GAS strains that bound murine CFH (Supplemental Fig. 2A), although majority of the tested GAS strains binds human CFH (Supplemental Fig. 2B). This is not unexpected, because GAS is not a natural pathogen for mice. Species specificity of GAS could be at least partially dependent on CFH binding, similar to Neisseria gonorrhoeae, which does not infect or bind CFH of primates other than humans (45). It might be possible to use recombinant CFH5–7 as a biological antimicrobial agent against GAS or, preferably, against CFH-binding microbes that are more resistant to antibiotics (e.g., Pseudomonas aeruginosa). In principle, the strategy is very good for an antimicrobial...
agent, because the later-emerging resistance to CFH5–7 (i.e., failure to bind CFH) would simultaneously make the CFH5–7-resistant strain prone to C attack.

Our results suggested that CFH variant 402Y is associated with a patient history of erysipelas or recurrent tonsillitis. It is known that GAS is the main pathogen causing these infections. The main reasons for selecting these infections in this study were that the patients are readily available because of the high incidence, and susceptibility to these infections seems to have a genetic component, as evidenced by a high recurrence rate and occasional familial occurrence. When all of the patients were pooled and compared with the controls, statistically significant differences in the frequency of the 402H (1277C) allele and the CC genotype (homozygosity for the allele C coding for 402H allotype) were seen. This was not the case when either of the patient groups (erysipelas or recurrent tonsillitis) was compared separately with the control groups. In the erysipelas group, the reason for the relatively small differences between the patient and control groups might be dilution of the association because of indirect risk factors, such as interdigital skin ulceration, diabetes, or impaired lymphatic drainage in extremities, or because these infections can also be caused by group G Streptococcus (46) not necessarily binding CFH. Dilution of the association could also be explained by the lack of clinical history of erysipelas or tonsillitis of the blood donor control subjects. Because interview-based information on previous streptococcal tonsillitis is often unreliable, we did not

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want to create a possible bias in selecting the controls on the basis of tonsillitis in general. Although we did not exclude history of streptococcal tonsillitis in the control group, we could demonstrate that 402Y (1277T) allele and the TT genotype were suggested to be associated with an increased risk for erysipelas/tonsillitis.

In summary, our study shows for the first time, to our knowledge, that CFH binding by GAS is essential for bacterial survival in human blood and that a recombinant fragment of CFH that lacks the regulatory functions can be used to inhibit CFH binding on bacterial surface and impair bacterial survival in blood ex vivo. The latter suggests a novel possible strategy for treating streptococcal infections. In addition, we suggest that a CFH variant 402H, which impairs binding of CFH onto GAS, is associated with decreased susceptibility to erysipelas and recurrent tonsillitis. Our finding suggested that individuals who are at risk for AMD are protected against streptococcal infections during childhood and early adult life, despite its harmful effect in predisposing to visual loss in the elderly.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Legends

Supplemental Figure S1. Correlation analysis in NHS. Relation between (A) CFH5-7 or (B) C4BP binding by GAS and generation of complement activation marker C3a upon exposure of 38 GAS strains to serum without MgEGTA

Supplemental Figure S2. Analysis of mouse and human factor H binding to GAS. For the assay 1 x 10^8 bacteria of the GAS strains were incubated with 10 % (A) serum of C3-/- C57Bl/6 mice or (B) NHS. (A) Supernatants of the given mouse serum first and third wash and the eluate or (B) only the eluate of GAS strains incubated in NHS were analyzed by western blotting. Molecular weights are shown in the left side. Factor H can be seen as a 150 kDa band in both figures A and B.
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A

B
Fig. S2. Haapasalo et al.