The Journal of Immunology

RESEARCH ARTICLE | APRIL 01 1998

Identification of the Second Heparin-Binding Domain in Human Complement Factor H[1] FREE

Timothy K. Blackmore; ... et. al

J Immunol (1998) 160 (7): 3342–3348.
https://doi.org/10.4049/jimmunol.160.7.3342

Related Content

Human Factor H-Related Protein 5 Has Cofactor Activity, Inhibits C3 Convertase Activity, Binds Heparin and C-Reactive Protein, and Associates with Lipoprotein

J Immunol (May,2005)

Identification of a Domain in Human Factor H and Factor H-Like Protein-1 Required for the Interaction with Streptococcal M Proteins

J Immunol (April,1998)

Complement C3b/C3d and Cell Surface Polyanions Are Recognized by Overlapping Binding Sites on the Most Carboxyl-Terminal Domain of Complement Factor H

J Immunol (December,2002)

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023

Identification of the Second Heparin-Binding Domain in Human Complement Factor H[1]

Timothy K. Blackmore,[2]* Jens Hellwage,† Tania A. Sadlon,* Naomi Higgs,* Peter F. Zipfel,† Helena M. Ward,* and David L. Gordon*

Complement factor H (fH) regulates activation of the alternative pathway of C, reducing the amount of C3b deposited on sialic acid-rich surfaces. Heparin binding has been used as a model for examining the sialic acid-binding characteristics of fH. We have previously shown that of the 20 short consensus repeat (SCR) modules of fH, SCR 7 contains an important heparin binding site, but other SCRs also play a role in heparin binding. To localize the other sites, we prepared recombinant truncated and SCR deletion mutants of fH and tested them by heparin-agarose affinity chromatography. The 5 C-terminal SCRs were found to contain a heparin binding site as an SCR 7 deletion mutant of the N terminal 15 SCRs did not bind heparin, but a construct consisting of SCRs 16–20 was shown to bind heparin. Double deletion of SCRs 7 and 20 from fH abrogated binding to heparin, indicating that SCR 20 contains a heparin binding site. This finding was confirmed with the observation that attachment of SCR 20 to a group of nonbinding SCRs produced a heparin-binding protein. A protein consisting of SCRs 19 and 20 did not bind heparin, whereas SCRs 18–20 did, indicating that, although SCR 20 contains a heparin binding site, at least two nonspecific adjacent SCRs are required. fH-related protein-3 (FHR-3) possesses an SCR homologous to SCR 7 of fH and bound heparin, whereas FHR-4, which lacks such an SCR, did not. Thus, fH contains two separate heparin binding sites, which are located in SCRs 7 and 20. ***The Journal of Immunology,*** **1998, 160: 3342–3348.**

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023

Factor H (fH)[3] is an important member of the regulators of C activation (RCA) family of genetically related proteins, which are encoded by a cluster of genes located on chromosome 1. All RCA proteins contain short consensus repeat (SCR) structural units containing approximately 60 amino acids folded into globular structures (1, 2). It has been proposed that the hypervariable region of each SCR determines ligand binding specificity (3). The tertiary structure of each SCR appears to be independent of its neighbors (4), permitting the deletion of one or more SCRs without disrupting the overall protein structure. fH and its related proteins are unique in that they are composed entirely of SCRs, whereas other members of the RCA family contain additional structural units.

fH binds C3b and controls the alternative pathway of C in two ways. Firstly, it accelerates the spontaneous slow decay of the alternative pathway C3 convertase (C3bBb) by displacing factor B (decay-acceleration) (5, 6). Second, it acts as a cofactor for the factor I-mediated cleavage of C3b to iC3b, which is unable to participate in the alternative pathway amplification loop (cofactor activity) (7). A C3b binding site has been localized to the N-terminal segment of fH, with at least three SCRs including SCRs 2 and 3 required for full cofactor activity (8, 9) and SCRs 1 through 4 for decay-acceleration (10). Additional C3b binding sites have been identified in fH, but their functional significance is unknown (11).

The C-regulatory functions of fH are modified by its binding to polyanions such as heparin and sialic acid (12–14). Surfaces rich in sialic acid such as sheep erythrocytes do not normally activate C, but can be converted to activators by the removal of sialic acid with neuraminidase (15). The ratio of fH to factor B (fB) binding has been shown to determine whether or not the alternative pathway is activated, and the fH-to-fB binding ratio is postulated, in turn, to be affected by surface sialic acid (16).

We have recently shown that SCR 7 of fH contains a major heparin binding site: a recombinant protein consisting of the seven N-terminal SCRs (H7) bound to heparin, whereas the N-terminal six-SCR protein did not. Moreover, a nine-SCR N-terminal construct (H9) bound heparin, but its SCR 7 deletion mutant (H9Δ7) did not bind. We also found that at least one heparin binding site other than SCR 7 exists in fH, but in contrast to an earlier report (17) we did not identify such a site in SCR 13, as a double deletion of SCRs 7 and 13 from fH (H20Δ7Δ13) retained heparin binding (18).

The fH-related (FHR) proteins, present in both human and mouse sera, were initially discovered by their cross-reactivity with anti-fH Abs (19). Four FHR proteins have been identified to date, and their cDNA sequences have been published (19–24). At present, their functions have not been fully described, but FHR-1, -2, and -4 have been found to associate with plasma lipoproteins, suggesting a general role as constituents of lipoproteins (24, 25). FHR-3 and FHR-4 each possesses five SCRs with amino acid sequences similar to SCRs 6, 7, 8, 19, and 20 and to SCRs 6, 8, 9, 19, and 20 of fH, respectively (20, 24). The heparin-binding characteristics of these proteins are therefore

*Department of Microbiology and Infectious Diseases, Flinders Medical Centre, Bedford Park, South Australia, Australia; and †Department of Molecular Biology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

Received for publication May 2, 1997. Accepted for publication December 1, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Australian National Health and Medical Research Council and the Deutsche Forschungsgemeinschaft Project Zi432/1-4. It is part of the doctoral thesis of J.H. at the Department of Biology, University of Hamburg, Hamburg, Germany.

[2] Address correspondence and reprint requests to Dr. Timothy K. Blackmore, at his current address, The Department of Microbiology and Infectious Diseases, Flinders Medical Centre, Bedford Park, South Australia 5042. E-mail address: tim.blackmore@flinders.edu.au

[3] Abbreviations used in this paper: fH, factor H; CHO, Chinese hamster ovary; FHR-3 and -4, factor H-related proteins 3 and 4; H20, recombinant factor H; HT, N-terminal tryptic fragment of fH; RCA, regulators of complement activation; SCR, short consensus repeat.

Copyright © 1998 by The American Association of Immunologists 0022-1767/98/$02.00

of great interest, as they differ in their possession of an SCR similar to SCR 7 of fH, yet both contain SCRs similar to fH SCRs 19 and 20. SCRs similar to SCRs 19 and 20 of fH are present in all of the FHR proteins, and it has been postulated that these SCRs may mediate important biologic functions (20).

In this paper, we identify the second heparin binding site in fH, using truncation and deletion mutants of fH, and determine the heparin-binding characteristics of recombinant FHR proteins. Attachment of SCR 20 to the first five SCRs of fH converted it from a nonbinding to a heparin-binding protein. The heparin-binding characteristics of FHR-3 and -4 proteins correlate with the possession of an SCR similar to SCR 7 of fH.

Materials and Methods

DNA constructs

cDNA encoding SCRs 1 through 15 (H15) and an SCR 7 deletion of full-length fH (H20Δ7) were prepared and cloned into the BSRαEN eukaryotic expression vector as previously described (18). *Xho*I and *Xba*I restriction sites were incorporated at the 5′ and 3′ terminus, respectively.

C-terminal truncated proteins from which SCR 7 had been deleted were produced utilizing a unique *Ppu*MI site at nucleotide 2072 of the fH sequence. Digesting BSRαEN-H20Δ7 with *Ppu*MI and *Xba*I released a fragment corresponding to SCRs 11–20. When this was replaced with truncated cDNA encoding SCRs 11–15, 11–18, or 11–19, cDNA encoding H15Δ7, H18Δ7 or H19Δ7, respectively, was produced. cDNA encoding SCRs 11–15 originated from BSRαEN-H15 digested with *Ppu*MI and *Xba*I. cDNA encoding SCRs 11–18 and 11–19 was prepared by PCR using Vent polymerase (New England Biolabs, Beverly, MA) from the BSRαEN-H20 template. The forward primer was designed to anneal to nucleotides 2021–2040 in SCR 11, 5′ to the *Ppu*MI site. Reverse primers annealed to the C-termini of SCRs 18 and 19 and were selected on the basis of the SCR junction analysis by Zipfel and Skerka (20). These primers also incorporated stop and *Xba*I sites. After digestion with *Ppu*MI and *Xba*I, the PCR products were then cloned into BSRαEN-H20Δ7.

A mutant fH protein consisting of SCRs 16–20 (H16–20) was prepared by ligating cDNA encoding the fH signal peptide via a *Mlu*I restriction site. This was achieved by incorporating an *Xho*I site into the fH signal forward primer, a *Mlu*I site into the fH signal reverse and the H16–20 forward primers, and an *Xba*I site in the SCR 20 reverse primer. The PCR product encoding the signal peptide was restricted with *Xho*I and *Mlu*I and the product encoding SCRs 16–20 with *Mlu*I and *Xba*I. The digested PCR products were then ligated into the *Xho*I and *Xba*I sites of BSRαEN in a three-way ligation reaction.

cDNA encoding SCRs 18–20 was amplified using a forward primer that annealed to the beginning of SCR 18 and introduced a *Mlu*I restriction site. The same SCR 20 reverse primer described above was used, and the PCR product was cloned into the *Mlu*I and *Xba*I sites of BSRαEN-H16–20.

SCR 20 was attached to SCRs 1–5 (H5 + 20) by splice overlap extension PCR. cDNA encoding SCRs 1–5 and SCR 20 was amplified in the first and second primary reactions, respectively. The forward primer for the second primary reaction contained nucleotides complementary to the last 15 nucleotides of SCR 5 adjacent to the first 18 nucleotides of SCR 20. The secondary reaction contained the primary PCR products and the forward and reverse primers used to amplify full-length fH, containing *Xho*I and *Xba*I restriction sites. cDNA constructs were confirmed by DNA sequencing.

Chinese hamster ovary cell-expressed proteins

CHO cells were transfected with 5 μg cDNA, 35 μl Lipofectamine (Life Technologies, Gaithersburg, MD), and 1.5 ml Opti-MEM serum-free medium (Life Technologies) per 25-cm^2 cell culture dish. Ham's F12 medium (Trace Biosciences, Castle Hill, New South Wales, Australia) supplemented with 0.5% FCS and 250 μg/ml G418 (Life Technologies) was used for selection and maintenance of stable transfectants. Cell supernatants were harvested twice weekly, clarified by centrifugation, and stored at −70°C.

Baculovirus-expressed proteins

Recombinant human FHR-3 and -4 and SCRs 19–20 of fH (H19–20) were prepared in the pBSV-8His baculovirus expression system as previously described (26). Eight C-terminal histidine residues were included and used to purify expressed proteins by Ni^{2+}-NTA-agarose affinity chromatography. An enterokinase site incorporated immediately N terminal to these His residues allowed their removal with EnterokinaseMax (Invitrogen, San Diego, CA). The His residues were removed because it was found that His tagging weakly affected heparin-binding characteristics (data not shown).

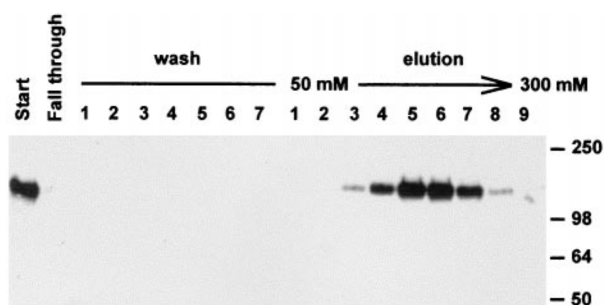

FIGURE 1. Heparin binding of an SCR 7 deletion mutant of fH (H20Δ7). Supernatant from transfected CHO cells ("start") was dialyzed into 50 mM phosphate buffer, pH 7.4, and then passed at least five times over a heparin-agarose affinity column. A sample of fall-through was collected before the column was washed in the same buffer with 5-ml fractions collected to at least a 50-ml wash volume. Bound protein was eluted in a linear salt gradient to 300 mM sodium chloride-phosphate buffer, and 4-ml fractions were collected. Samples of the start, fall-through, first seven wash fractions, and all elution fractions were separated on a 7.5% SDS-PAGE gel and analyzed by Western blotting with a chemiluminescence detection system. Molecular mass is indicated on the *right* in kDa.

Western blotting

The correct identity of expressed proteins was confirmed by SDS-PAGE and Western blotting. Proteins were electrophoresed under nonreducing conditions and transferred to nitrocellulose. Polyclonal goat Ab to human fH (Calbiochem, San Diego, CA) followed by donkey anti-goat Ab conjugated to horseradish peroxidase (Silenus, Hawthorn, Australia) was used to detect most proteins. FHR-3 and FHR-4 were detected by polyclonal Ab produced in rabbits, followed by sheep anti-rabbit Ab conjugated to horseradish peroxidase (Silenus). Proteins were detected with the enhanced chemiluminescence system (Amersham, Buckinghamshire, U.K.). All proteins migrated on SDS-PAGE according to their predicted m.w. and degree of glycosylation.

Heparin-agarose affinity chromatography

All heparin-binding experiments used 50 mM phosphate buffer, pH 7.4, for dialysis or dilution of samples and equilibration and washing of the columns (27). Ten to twenty milliliters of transfected CHO cell culture supernatant or 5 to 10 μg of diluted baculovirus-expressed protein was applied to 1 × 1 cm heparin-agarose columns (Pierce, Rockford, IL). The sample was passed over the column at least five times and a sample of the fall-through collected. The column was washed with >40 ml of buffer, and 2.5-ml fractions were collected for testing. Bound protein was then eluted by a linear salt gradient to 300 mM sodium chloride-phosphate buffer with 2-ml fractions collected. An automated fraction collector and a flow rate of 1 ml/min was used for all experiments. Conductivity was measured at 24°C, using a portable meter (Activon model 301, Sydney, Australia), and a consistent salt gradient was obtained for all experiments. Columns were reconstituted by washing afterward with 1 M NaCl, then stored in 50 mM phosphate buffer with 0.02 M sodium azide.

All experiments were performed at least three times, and reproducible results were obtained in all cases.

Results

Expression and binding of H20Δ7 and H15Δ7

SCR 7 was shown in a previous report to contain a heparin binding site (18). However, an SCR 7 deletion mutant of fH (H20Δ7) still bound to heparin and eluted in a linear salt gradient at approximately 150 mM (Fig. 1). As H15 also binds to heparin (18), we first prepared a deletion of SCR 7 from H15 (H15Δ7) to assess whether a second heparin binding site existed in SCRs 1–15.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023

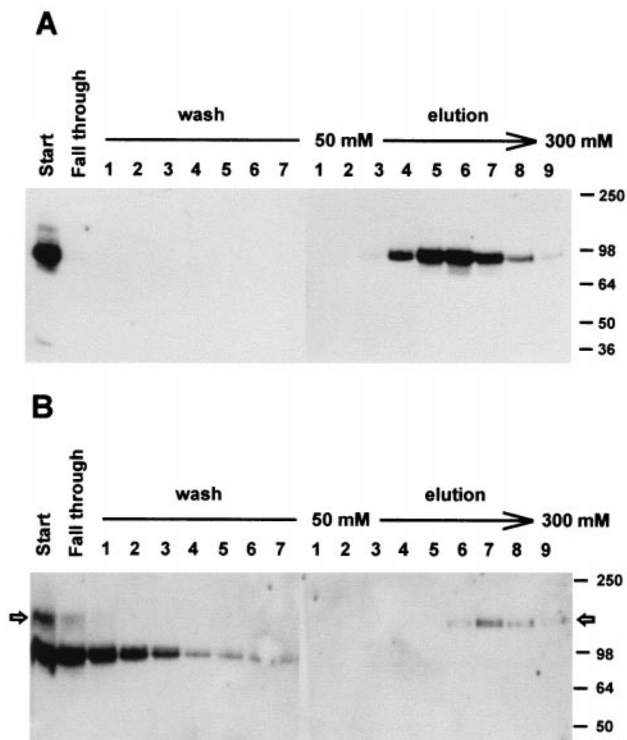

FIGURE 2. Heparin-binding characteristics of H15 (*A*) and an H15Δ7 (*B*). Supernatants from transfected CHO cells were applied to heparin-agarose affinity columns, which were then washed and the bound proteins eluted, as described in the legend to Figure 1. Proteins were similarly detected by Western blotting after separation by 7.5% SDS-PAGE. Note that deletion of SCR 7 from H15 (*B*) abrogates binding to heparin, as shown by the presence of protein in fall-through and wash fractions of H15Δ7. The higher band seen on gel B (open arrow) represents contaminating fH from FCS or endogenous CHO cell production.

H15Δ7 expressed well and migrated slightly faster on SDS-PAGE than H15 did (data not shown). H15Δ7 did not bind heparin, with a similar amount of protein seen in the start and fall-through fractions. Moreover, the small amount of protein remaining associated with the column was washed off in 50 mM of phosphate equilibration buffer. No protein eluted from the column in the linear salt gradient. Figure 2 shows the heparin-binding and elution profiles of H15 and H15Δ7, demonstrating the clear difference in behavior of the two proteins. These results show that SCR 7 contains the only heparin binding site within SCRs 1–15.

Binding of H16–20 to heparin

The binding of H20Δ7 to heparin and the lack of binding of H15Δ7 together indicate that a second heparin binding site is located in SCRs 16–20 of human fH. To investigate this possibility, we prepared a construct in which the fH signal sequence was attached to cDNA encoding SCRs 16–20. An ~40-kDa protein was identified by Western blot in supernatants of transfected CHO cells. Heparin binding analysis showed that H16–20 bound to immobilized heparin and was not washed off in 50 mM phosphate buffer. H16–20 eluted in a lower concentration of salt than H15Δ7 (Fig. 3). These results indicate that a second heparin binding site resides within SCRs 16–20, and that this site may be of lower affinity than that contained in SCR 7.

Localization of the second heparin binding site in fH

To further localize the second heparin binding site, we next produced SCR 7 deletion mutant proteins of SCRs 1–18 and 1–19 (H18Δ7 and H19Δ7).

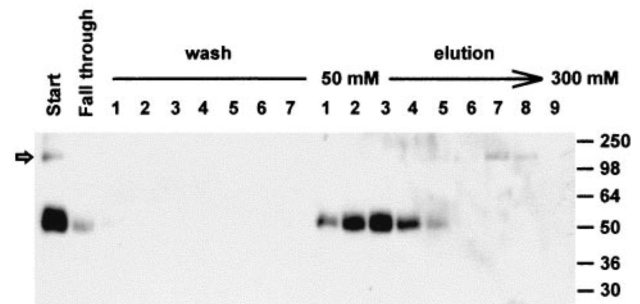

FIGURE 3. Heparin-binding characteristics of H16–20. Supernatants from transfected CHO cells were applied to heparin-agarose affinity columns, which were then washed and bound proteins eluted, exactly as described in the legend to Figure 1. Protein was similarly detected by Western blotting after separation by 10% SDS-PAGE. The higher m.w. band (open arrow), representing contaminating fH from FCS or endogenous CHO cell production, is seen to elute in a higher salt concentration than H16–20.

Expression of the proteins was determined by Western blot with both proteins migrating on SDS-PAGE at their predicted m.w. In contrast to H20Δ7, neither H18Δ7 nor H19Δ7 demonstrated significant binding to heparin. A small amount of protein associated with the heparin-agarose affinity column, but was washed off in 50 mM of phosphate buffer (Fig. 4).

These results suggest that SCR 20 is the essential component of the second heparin binding site of human fH. To confirm this opinion, SCR 20 was attached to the N-terminal five SCRs of fH (H5 + 20) and its heparin-binding characteristics examined. The five N-terminal SCRs of fH do not contain a heparin binding site (18).

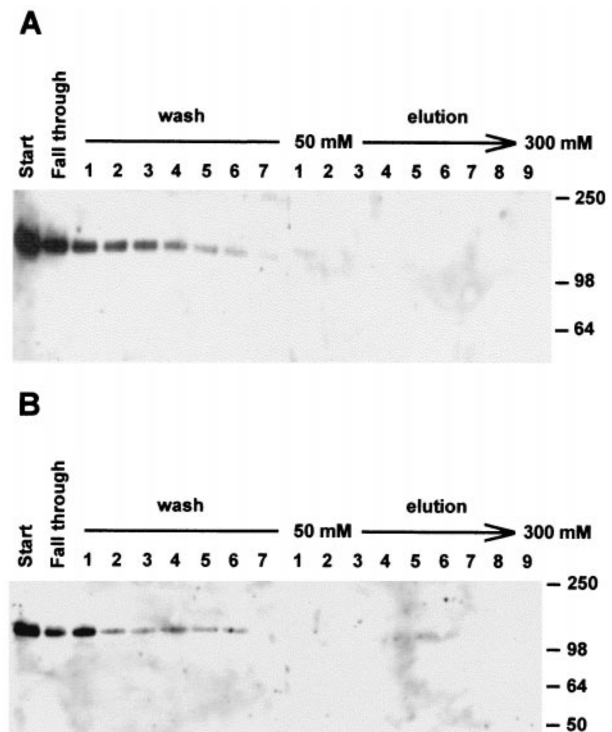

FIGURE 4. Heparin-binding characteristics of H18Δ7 (*A*) and H19Δ7 (*B*). Supernatants from transfected CHO cells were applied to heparin-agarose affinity columns, which were washed and bound proteins eluted, as described in the legend to Figure 1. Proteins were similarly detected by Western blotting after separation by 7.5% SDS-PAGE.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023

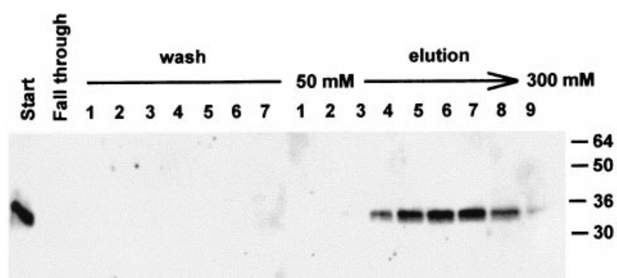

FIGURE 5. Heparin-binding characteristics of H5 + 20. cDNA encoding SCRs 1–5 and SCR 20 was prepared by splice overlap extension PCR. Supernatant from transfected CHO cells was applied to a heparin-agarose affinity column, which was washed and bound proteins eluted, as described in the legend to Figure 1. Protein was detected by Western blotting after separation by 12.5% SDS-PAGE.

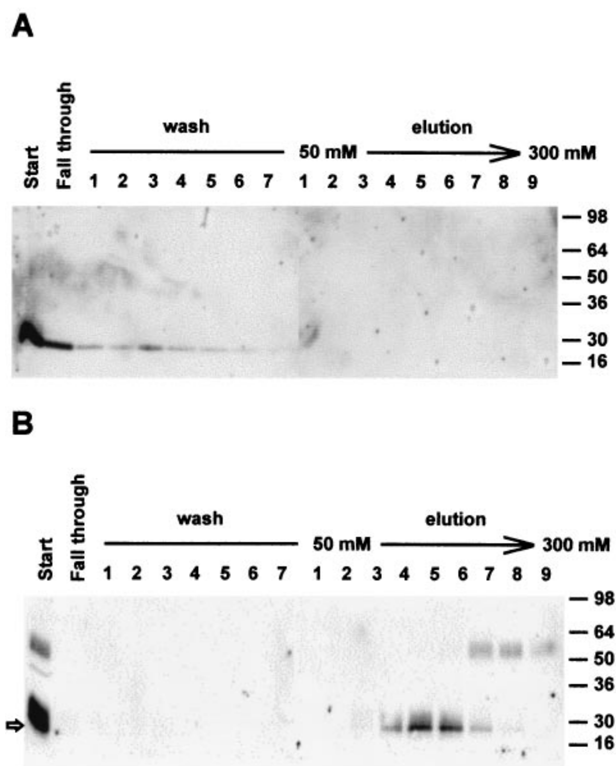

FIGURE 6. Heparin-binding characteristics of H19–20 (*A*) and H18–20 (*B*). Recombinant protein was applied to a heparin-agarose affinity column, which was washed and bound protein eluted, as described in the legend to Figure 1. Proteins were detected by Western blotting after separation by 12.5% SDS-PAGE. H18–20 (*B*) is indicated by an open arrow. The higher m.w. band represents an uncharacterized protein that cross-reacts with anti-fH Ab. This protein was consistently present in the supernatant of CHO cells transfected with both H18–20 and H17–20 (data not shown).

The addition of SCR 20 to SCRs 1–5 converted a non-heparin-binding protein to a heparin-binding protein, clearly demonstrating that the second heparin binding site is located in SCR 20 (Fig. 5).

Assessment of the role of adjacent SCRs

The above results indicate that SCR 20 is an essential component of the second heparin binding site, but do not address whether SCR 20 alone is sufficient for heparin binding. Recombinant proteins consisting of SCRs 19 and 20 (H19–20) and SCRs 18–20 (H18–20) were prepared. H19–20 did not bind heparin, because protein was present in the fall-through and wash fractions and none was eluted (Fig. 6). In contrast, H18–20 was not detected in the fall-through or wash fractions, and bound protein eluted in the salt gradient (Fig. 6). Similar results were found for a protein containing SCRs 17–20 (data not shown). Therefore, while SCR 20 is an essential component of the second heparin binding site, at least two adjacent SCRs are required. The adjacent SCRs may be nonspecific, since H5 + 20 also bound to heparin.

Analysis of heparin binding by FHR-3 and FHR-4

Human FHR-3 and -4 are both composed of five SCRs, with each SCR similar to certain SCRs of fH. SCRs 1–3 of FHR-3 and FHR-4 share partial identity with fH SCRs 6, 7, and 8 and SCRs 6, 8, and 9, respectively. SCRs 4 and 5 of both FHR-3 and FHR-4 are almost identical to each other and have partial similarity with SCRs 19 and 20 of fH (20, 24). Therefore, we used these proteins as "natural mutants" of fH and examined their heparin-binding characteristics. These results were then correlated with the amino acid sequences of SCRs 7 and 20 in an attempt to identify potential heparin binding sites. FHR-3 was expected to bind heparin because its third SCR is so similar to SCR 7 of fH. Binding of FHR-4 to heparin would most likely depend on whether there is sufficient similarity between SCR 5 of FHR-4 and SCR 20 of fH to preserve the heparin binding site.

The heparin-binding characteristics of FHR-3 and FHR-4 are shown in Figure 7. As expected, FHR-3 binds to heparin. FHR-4 does not bind, indicating that its SCR 5 is not sufficiently similar to SCR 20 of fH to result in heparin binding.

Summary of recombinant proteins and their heparin-binding characteristics

A schematic representation of the proteins produced and analyzed in this study is shown in Figure 8. SCRs are numbered in the figure on the basis of similarity to native human fH. The heparin-binding properties of each construct are also shown.

Discussion

The heparin-binding characteristics of fH are of great interest because of the central role that fH plays in controlling the alternative pathway (28) and the influence that polyanions have on fH binding to surfaces (16). Our previously reported experiments demonstrated the presence of a heparin binding site in SCR 7 of human fH, but it was apparent that at least one other site was present in SCRs 10–20 (18). The purpose of the present study was to determine the location of the additional site(s). Recombinant DNA techniques allow construction of truncated proteins and deletion mutants and have been used by several groups to examine the functional domains of fH (8–11, 18, 29) and other SCR containing proteins (30–32). These techniques were used, therefore, to determine the location of the second heparin binding site in human fH.

A series of recombinant mutant proteins was initially expressed in transfected CHO cells. Recombinant fH (H20) produced in this system has full cofactor activity and migrates on SDS-PAGE in a fashion identical to the native protein (18). This suggests that transfected CHO cells produce correctly glycosylated recombinant proteins.

Deleting SCR 7 from H15 (H15Δ7) resulted in the loss of heparin binding, while H20Δ7 retained its binding to heparin (Figs. 1 and 2). These findings identified a second heparin-binding domain and localized it to SCRs 16–20. H16–20 was produced by attaching the fH signal sequence to cDNA encoding SCRs 16–20 in the

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023

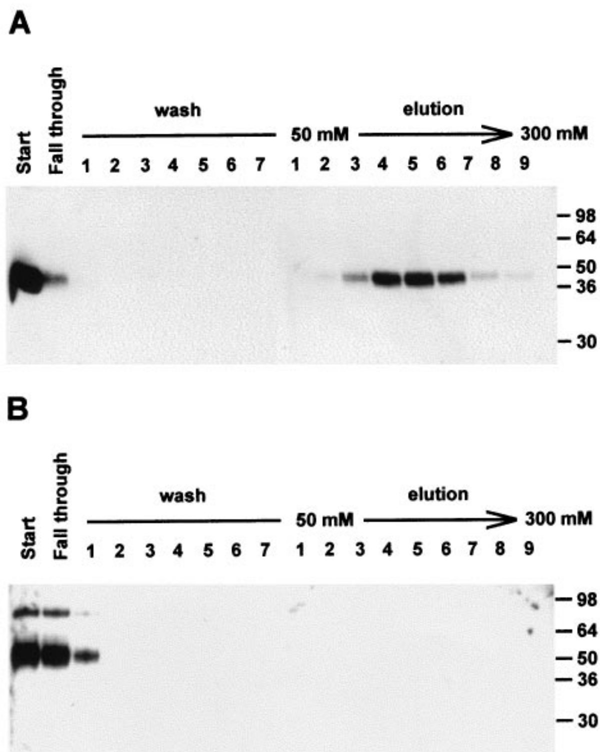

FIGURE 7. Heparin binding of factor H-related proteins FHR-3 (*A*) and FHR-4 (*B*). Baculovirus-expressed proteins were applied to heparin-agarose affinity columns, which were then washed and bound proteins eluted, as described in the legend to Figure 1. Proteins were detected by Western blotting after separation by 10% SDS-PAGE.

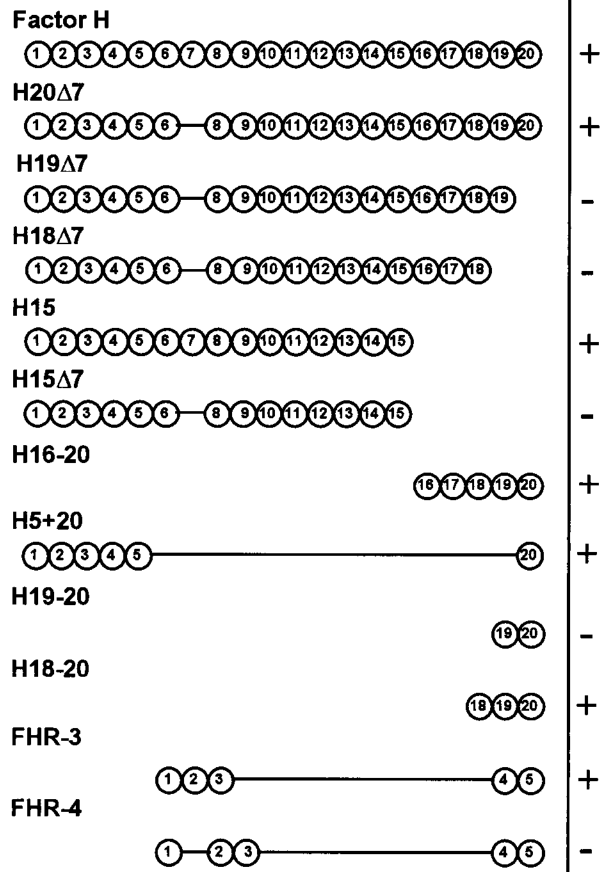

FIGURE 8. Schematic representation of native fH and the recombinant proteins used in this study and summary of their heparin-binding characteristics. Each circle represents an SCR; SCR deletions from fH are shown as a solid line. For all proteins other than FHR-3 and FHR-4, SCRs are numbered and aligned on the basis of amino acid sequence identity to fH. The SCRs of FHR-3 and FHR-4 are aligned to the SCRs of fH with which they share greatest similarity.

expression vector BSRαEN. This approach has been used previously in our laboratory to produce recombinant N-terminal truncations of fH (8), domains of the HIV gp120 glycoprotein, and recombinant rubella virus proteins (unpublished data). H16–20 bound heparin, but eluted earlier in a salt gradient (Fig. 3). Therefore, it is possible that the binding site in this region is of lower affinity.

We next prepared truncations of the C-terminal SCRs from H20Δ7 to determine the location of the second heparin binding site. In contrast to H20Δ7, neither H18Δ7 nor H19Δ7 bound to heparin (Fig. 4). These findings indicate that SCR 20 contains an essential component of the second heparin binding site of fH. To confirm that it contains a heparin binding site, SCR 20 was attached to a non-heparin-binding protein. The addition of SCR 20 to the N-terminal five SCRs (H5+20) generated a heparin-binding protein (Fig. 5), conclusively demonstrating that SCR 20 contains a heparin binding site.

These results indicate that SCR 20 is an essential part of the second heparin binding site, but the role of adjacent SCRs remained to be determined. Therefore, the heparin-binding characteristics of SCRs 19 and 20 construct (H19–20) and SCRs 18–20 (H18–20) were examined (Fig. 6). H19–20 did not bind heparin, whereas H18–20 did. Therefore, adjacent SCRs in spacer or structural roles are required for heparin binding. SCR 20 will mediate heparin binding even when attached to nonspecific SCRs, as shown by the binding of H5 + 20. This indicates that the second heparin binding site consists of SCR 20 and at least another two nonspecific SCRs. Three to four SCRs are also necessary for maximal C3 regulatory activity of RCA proteins including CR1 (31) and fH (8, 9). Within the three- to four-SCR unit, one to two SCRs are essential, with the flanking SCRs required for full functional activity.

We took advantage of the similarity of the FHR proteins FHR-3 and FHR-4, both of which were cloned and expressed in a baculovirus system. FHR-3 and FHR-4 contain SCRs similar to particular SCRs of fH. They differ in that only FHR-3 contains an SCR similar to SCR 7 of fH; SCR 2 of FHR-3 has 88% amino acid identity to SCR 7 of fH. SCRs 4 and 5 of FHR-3 are almost identical to the comparable SCRs of FHR-4, with a total of only four amino acids different between the four SCRs (24). Therefore, finding that FHR-3 binds to heparin and that FHR-4 does not (Fig. 7) suggests that SCR 2 of FHR-3 is likely to be responsible for the binding of FHR-3. The highly conserved amino acid sequences of SCR 7 of human fH and SCR 3 of human FHR-3 make it difficult to identify putative heparin-binding domains within these SCRs. Using two-dimensional nuclear magnetic resonance (2D NMR), a "hypervariable loop" within each SCR was postulated to be the

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023

region responsible for specific ligand binding (4, 33–35). The hypervariable loop of SCR 7 of fH consists of the sequence (H/Y)GRK (the first amino acid is the site of H/Y polymorphism) (36, 37) with YGRK in SCR 3 of human FHR-3 (20). Despite the fact that this site contains two or three basic amino acids, it would be surprising if it alone is responsible for heparin binding, because in other proteins the heparin-binding domain may be spread over a discontinuous sequence of many amino acids (38). It would also be interesting to examine whether a heparin-binding domain exists in SCR 7 of murine and bovine fH, as each shares only 57% identity with the human counterpart. It may then be possible to identify likely amino acid sequences responsible for heparin binding and to determine the functional significance of the hypervariable loop of SCR 7. The removal of SCRs 7 and 20 from fH completely abrogates its capacity to bind heparin, which rules out a significant contribution from SCR 13 in our assay system. It is surprising that no heparin-binding activity was observed for SCR 13 of fH, because based on the model of Barlow et al. (3), the hypervariable loop of SCR 13 contains many basic amino acids (HLKNKKEF). This suggests that the hypervariable loop model may not be applicable to every SCR.

As demonstrated by the lack of binding of FHR-4, the heparin binding site in SCR 20 of fH is not conserved in SCR 5 of FHR-4. The amino acid sequence of SCR 20 of fH (37) was analyzed for clusters of basic residues not present in SCR 5 of FHR-4 (24). No such areas were apparent, making it difficult to identify putative heparin binding sites within SCR 20 of fH.

We plan to use H19Δ7 to establish the role that fH plays in regulating C activation by sialic acid-rich bacteria and cells. Important pathogens that may utilize fH binding as a way of avoiding alternative pathway activation include *Streptococcus agalactiae* (39, 40), *Neisseria meningitidis*, *Neisseria gonorrhoeae* (41–44), and *Escherichia coli* K1 (45). By comparing the protective effects against C deposition of H19Δ7 and H20, it will be possible to test the hypothesis that the heparin/sialic acid-binding capacity of fH is essential to self/nonself recognition by the alternative pathway. C activation is controlled on host cells to which fH is bound (17), and the degree of fH binding to cell surfaces is related to the amount of membrane-associated sialic acid (12–14). Sheep erythrocytes, which are rich in sialic acid, may be converted by neuraminidase treatment from nonactivators to activators of the alternative pathway, accompanied by a reduction in fH binding (15). Therefore, it would be expected that H19Δ7 added to an alternative pathway lacking fH would not protect sheep erythrocytes from C-mediated lysis. Such an approach would examine the possibility that other regulators, such as those found on sheep erythrocytes (46), are also playing an important C-regulatory role.

In summary, we have identified the second heparin binding site of human fH and produced a mutant fH containing all SCRs except those required for heparin binding. This molecule (H19Δ7) will be invaluable for studying the significance of polyanion binding to the C-regulatory functions of fH.

References

1. Hourcade, D., V. M. Holers, and J. P. Atkinson. 1989. The regulators of complement activation (RCA) gene cluster. *Adv. Immunol. 45:381.*
2. Liszewski, M. K., T. C. Farries, D. M. Lublin, I. A. Rooney, and J. P. Atkinson. 1996. Control of the complement system. *Adv. Immunol. 61:201.*
3. Barlow, P. N., and I. D. Campbell. 1994. Strategy for studying modular proteins: application to complement modules. *Methods Enzymol. 239:465.*
4. Barlow, P. N., A. Steinkasserer, D. G. Norman, B. Kieffer, A. P. Wiles, R. B. Sim, and I. D. Campbell. 1993. Solution structure of a pair of complement modules by nuclear magnetic resonance. *J. Mol. Biol. 232:268.*
5. Whaley, K., and S. Ruddy. 1976. Modulation of the alternative complement pathway by β1H globulin. *J. Exp. Med. 144:1147.*
6. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein β1H. *Proc. Natl. Acad. Sci. USA 73:3268.*
7. Pangburn, M. K., R. D. Schreiber, and H. J. Müller-Eberhard. 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein β1H for cleavage of C3b and C4b in solution. *J. Exp. Med. 146:257.*
8. Gordon, D. L., R. M. Kaufman, T. K. Blackmore, J. Kwong, and D. M. Lublin. 1995. Identification of complement regulatory domains in human factor H. *J. Immunol. 155:348.*
9. Kühn, S., C. Skerka, and P. F. Zipfel. 1995. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H. *J. Immunol. 155:5663.*
10. Kühn, S., and P. F. Zipfel. 1996. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *Eur. J. Immunol. 26:2383.*
11. Sharma, A. K., and M. K. Pangburn. 1996. Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. *Proc. Natl. Acad. Sci. USA 93:10996.*
12. Pangburn, M. K., D. C. Morrison, R. D. Schreiber, and H. J. Müller-Eberhard. 1980. Activation of the alternative complement pathway: recognition of surface structures on activators by bound C3b. *J. Immunol. 124:977.*
13. Kazatchkine, M. D., D. T. Fearon, and K. F. Austen. 1979. Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and β1H for cell-bound C3b. *J. Immunol. 122:75.*
14. Fearon, D. T. 1978. Regulation by membrane sialic acid of β1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc. Natl. Acad. Sci. USA 75:1971.*
15. Pangburn, M. K., and H. J. Müller-Eberhard. 1978. Complement C3 convertase: cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. *Proc. Natl. Acad. Sci. USA 75:2416.*
16. Horstmann, R. D., M. K. Pangburn, and H. J. Müller-Eberhard. 1985. Species specificity of recognition by the alternative pathway of complement. *J. Immunol. 134:1101.*
17. Pangburn, M. K., M. A. L. Atkinson, and S. Meri. 1991. Localization of the heparin-binding site on complement factor H. *J. Biol. Chem. 266:16847.*
18. Blackmore, T. K., T. A. Sadlon, H. M. Ward, D. M. Lublin, and D. L. Gordon. 1996. Identification of a heparin binding domain in the seventh short consensus repeat of complement factor H. *J. Immunol. 157:5422.*
19. Vik, D. P., P. Muñoz-Cánoves, H. Kozono, L. G. Martin, B. F. Tack, and D. D. Chaplin. 1990. Identification and sequence analysis of four complement factor H-related transcripts in mouse liver. *J. Biol. Chem. 265:3193.*
20. Zipfel, P. F., and C. Skerka. 1994. Complement factor H and related proteins: an expanding family of complement-regulatory proteins? *Immunol. Today 15:121.*
21. Skerka, C., R. D. Horstmann, and P. F. Zipfel. 1991. Molecular cloning of a human serum protein structurally related to complement factor H. *J. Biol. Chem. 266:12015.*
22. Estaller, C., V. Koistinen, W. Schwaeble, M. P. Dierich, and E. H. Weiss. 1991. Cloning of the 1.4-kb mRNA species of human complement factor H reveals a novel member of the short consensus repeat family related to the carboxy terminal of the classical 150-kDa molecule. *J. Immunol. 146:3190.*
23. Skerka, C., C. Timmann, R. D. Horstmann, and P. F. Zipfel. 1992. Two additional human serum proteins structurally related to complement factor H. Evidence for a family of factor H-related genes. *J. Immunol. 148:3313.*
24. Skerka, C., J. Hellwage, W. Weber, A. Tilkorn, F. Buck, T. Marti, E. Kampen, U. Beisiegel, and P. F. Zipfel. 1997. The human factor H-related protein 4 (FHR-4)—a novel short consensus repeat-containing protein is associated with human triglyceride-rich lipoproteins. *J. Biol. Chem. 272:5627.*
25. Park, C. T., and S. D. Wright. 1996. Plasma lipopolysaccharide-binding protein is found associated with a particle containing apolipoprotein a-I, phospholipid, and factor H-related proteins. *J. Biol. Chem. 271:18054.*
26. Kühn, S., and P. F. Zipfel. 1995. The baculovirus expression vector pBSV-8His directs secretion of histidine-tagged proteins. *Gene 162:225.*
27. Zammit, A., D. S. Pepper, and J. Dawes. 1993. Interaction of immobilised unfractionated and LMW heparins with proteins in whole human plasma. *Thromb. Haemost. 70:951.*
28. Vik, D. P., P. Muñoz-Cánoves, D. D. Chaplin, and B. F. Tack. 1989. Factor H. *Curr. Top. Microbiol. Immunol. 153:147.*
29. Sharma, A. K., and M. K. Pangburn. 1997. Localization by site-directed mutagenesis of the site in human complement factor h that binds to *Streptococcus pyogenes* M protein. *Infect. Immun. 65:484.*
30. Coyne, K. E., S. E. Hall, S. Thompson, M. A. Arce, T. Kinoshita, T. Fujita, D. J. Anstee, W. Rosse, and D. M. Lublin. 1992. Mapping of epitopes, glycosylation sites, and complement regulatory domains in human decay accelerating factor. *J. Immunol. 149:2906.*
31. Kalli, K. R., P. H. Hsu, T. J. Bartow, J. M. Ahearn, A. K. Matsumoto, L. B. Klickstein, and D. T. Fearon. 1991. Mapping of the C3b-binding site of CR1 and construction of a (CR1)2-F$(ab')_2$ chimeric complement inhibitor. *J. Exp. Med. 174:1451.*
32. Kalli, K. R., J. M. Ahearn, and D. T. Fearon. 1991. Interaction of iC3b with recombinant isotypic and chimeric forms of CR2. *J. Immunol. 147:590.*
33. Barlow, P. N., D. G. Norman, A. Steinkasserer, T. J. Horne, J. Pearce, P. C. Driscoll, R. B. Sim, and I. D. Campbell. 1992. Solution structure of the fifth repeat of factor H: a second example of the complement control protein module. *Biochemistry 31:3626.*

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023

34. Norman, D. G., P. N. Barlow, M. Baron, A. J. Day, R. B. Sim, and I. D. Campbell. 1991. Three-dimensional structure of a complement control protein module in solution. *J. Mol. Biol. 219:717.*
35. Barlow, P. N., M. Baron, D. G. Norman, A. J. Day, A. C. Willis, R. B. Sim, and I. D. Campbell. 1991. Secondary structure of a complement control protein module by two-dimensional 1H NMR. *Biochemistry 30:997.*
36. Soames, C. J., A. J. Day, and R. B. Sim. 1996. Prediction from sequence comparisons of residues of factor H involved in the interaction with complement component C3b. *Biochem. J. 315:523.*
37. Ripoche, J., A. J. Day, T. J. R. Harris, and R. B. Sim. 1988. The complete amino acid sequence of human complement factor H. *Biochem. J. 249:593.*
38. Faham, S., R. E. Hileman, J. R. Fromm, R. J. Linhardt, and D. C. Rees. 1996. Heparin structure and interactions with basic fibroblast growth factor. *Science 271:1116.*
39. Edwards, M. S., D. L. Kasper, H. J. Jennings, C. J. Baker, and A. Nicholson-Weller. 1982. Capsular sialic acid prevents activation of the alternative complement pathway by type III, group B streptococci. *J. Immunol. 128:1278.*
40. Marques, M. B., D. L. Kasper, M. K. Pangburn, and M. R. Wessels. 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. *Infect. Immun. 60:3986.*
41. Jarvis, G. A. 1994. Analysis of C3 deposition and degradation on *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Infect. Immun. 62:1755.*
42. Alcorn, T. M., and M. S. Cohen. 1994. Gonococcal pathogenesis: adaptation and immune evasion in the human host. *Curr. Opin. Infect. Dis. 7:310.*
43. Mandrell, R. E., A. J. Lesse, J. V. Sugai, M. Shero, J. Griffis, J. A. Cole, N. J. Parsons, H. Smith, S. A. Morse, and M. A. Apicella. 1990. In vitro and vivo modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. *J. Exp. Med. 171:1649.*
44. Smith, H., N. J. Parsons, and J. A. Cole. 1995. Sialylation of neisserial lipopolysaccharide: a major influence on pathogenicity. *Microb. Pathog. 19:365.*
45. Stevens, P., S. N.-Y. Huang, W. D. Welch, and L. S. Young. 1978. Restricted complement activation by *Escherichia coli* with the K-1 capsular serotype: a possible role in pathogenicity. *J. Immunol. 121:2174.*
46. Ezzell, J. L., and C. J. Parker. 1992. Cell-surface regulation of the human alternative pathway of complement: sheep but not rabbit erythrocytes express factor I-dependent cofactor activity. *Scand. J. Immunol. 36:79.*

Downloaded from http://journals.aai.org/jimmunol/article-pdf/160/7/3342/1088144/im0798003342o.pdf by guest on 05 February 2023