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CLINICAL INVESTIGATION

Activation of the alternative pathway of complement by cellulosic hemodialysis membranes

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Activation of the alternative pathway of complement by cellulosic hemodialysis membranes. Compared to cellulose acetate, hemodialysis with cuprophan membranes is associated with greater activation of the alternative pathway of complement. Previous studies have shown that this difference is not due to a greater number of potential covalent binding sites for activated C3 on cuprophan. To investigate further the factors that influence complement activation by hemodialysis membranes, proteins were eluted from serum-treated cuprophan and cellulose acetate membranes with hydroxylamine at alkaline pH and analyzed by SDS-PAGE and Western blot. Approximately 23 times more total protein was removed from cellulose acetate. Virtually all the C3 in the cellulose acetate eluate was in the form of inactive fragments C3c and C3dg. In contrast, the functionally active form of C3 (C3b) was a prominent constituent of the cuprophan eluate. The binding of factor B (precursor of the catalytic subunit of the C3 convertase) and factor H (regulatory protein of C3 activation) to serum-treated membranes was also analyzed. By Scatchard's method, the affinity constant at equilibrium for factor B binding (K_B) to the two types of membranes was not significantly different; however, there were approximately four times more factor B binding sites on the cuprophan than on the cellulose acetate. For cuprophan, the number of factor B binding sites was 1.6 times greater than the number of factor H binding sites. These studies demonstrate that a portion of the C3b molecules that bind to cuprophan are protected from degradation, and suggest that the complement activating capacity of hemodialysis membranes is determined by biochemical properties that modulate both the binding of serum proteins to the membrane and the interactions of the endogenous regulatory proteins with membrane-associated C3b.

Cellulosic membranes are the types most frequently used for routine clinical hemodialysis. The prototypical cellulosic membrane, cuprophan, is prepared by regeneration of cellulose obtained from the matrix of plant cell walls [1]. Cellulose is a polymer of glucosan rings with three hydroxyl groups on each ring. Unlike cuprophan, cellulose acetate membranes have the majority (approximately 70%) of the hydroxyl groups derivatized with acetyl residues.

Hemodialysis using cellulosic membranes is associated with complement activation via the alternative pathway [2–4], and the consequent generation of biologically-active complement peptides (C3a, C5a and $C5a_{desArg}$) [3, 4] appears to contribute to dialysis-related neutropenia [3–5], pulmonary artery hypertension [6, 7] and probably to the production of interleukin-1 [8]. Compared to cuprophan, cellulose acetate membranes have been shown to be associated with significantly less complement activation [9, 10]. It has been proposed that this difference in complement activating capacity is due to the presence of a larger number of free hydroxyl groups on the cuprophan membrane that would provide a source for nucleophilic attack on the thiolester bond of nascent C3b [11]. According to this hypothesis, complement activation on cellulose acetate membranes is attenuated because derivatization of hydroxyl groups with acetyl residues blocks C3b acceptor sites. Recent investigations, however, have shown that the activated C3 which is associated with cellulosic membranes is non-covalently bound [12]. The latter observations suggest that the complement activating properties of dialysis membranes are not determined by the availability of potential covalent binding sites for activated C3b.

Activation of the alternative pathway of complement (APC) on particulate surfaces is initiated following binding of C3b [13, 14], which creates a nidus for the formation of the C3 and C5 convertase of the APC [15, 16]. The molecular basis for differences in APC activity on several biological surfaces have been reported. While greater efficiency of binding of nascent C3b may in some instances contribute to APC activation [17], in most cases the capacity of a particulate surface to support APC activation is determined primarily by biochemical properties that influence the binding of factors B and H to C3b [18–21]. Factor B is the enzymatic subunit of both the C3 (C3bBb) and the C5 (C3bBbC3b) convertase of the APC [22]. Thus binding of factor B to C3b results in activation of the APC. Factor H regulates APC activity in three ways. First by binding to C3b, factor H displaces bound Bb. Second, binding of factor H to C3b inhibits further binding of factor B. Third, factor H serves as a cofactor for the enzymatic degradation of C3b to iC3b by factor I. Thus, APC activity is inhibited under circumstances in which the affinity of C3b for factor H is greater than that for factor B.

We proposed that the interactions of the APC regulatory proteins, factors B and H, with membrane-associated C3b are primary determinants of the complement activation potential of cellulosic hemodialysis membranes. Accordingly, the binding of factors B and H to C3b associated with cuprophan and cellulose acetate membranes following incubation with serum was analyzed. The results of these studies suggest that cu-

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prophan activates more complement than cellulose acetate because a greater portion of C3b that binds to cuprophan membranes maintains its functional activity longer.

Methods

Buffers and solutions

All chemicals were of a reagent grade. The following buffers and solutions were employed: phosphate-buffered saline, consisting of 10 mM sodium phosphate and 145 mM NaCl, pH 7.4 (PBS); PBS with 5 mM $MgCl_2$ (PBS^{++}); PBS containing 5% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri, USA) and 2 mM $MgCl_2$ (PBS/A^{++}); PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co.) and 20 mM EDTA (PBS-PE); Tris-buffer consisted of 10 mM Tris, pH 7.4; 1 M hydroxylamine monohydrochloride (Sigma Chemical Co.) containing 1 mM PMSF and 20 mM EDTA, titrated to pH 10.5 (NH_2OH-PE); gelatin veronal buffered saline contained 0.1% gelatin, 140 mM NaCl, 5 mM sodium barbital, pH 7.4 (GVB); GVB containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (GVB^{++}); GVB containing 10 mM EDTA (GVB-EDTA); normal human serum (NHS); NHS supplemented with 2 mM Mg^{++}; NHS incubated with 20 mM EDTA for five minutes prior to use (NHS-EDTA).

Complement proteins and antibodies

Human C3 [23], C3c (24), C3dg [24], factors B [25], H [26], and D [26] and cobra venom factor (CVF) [27, 28] were isolated according to published methods. Polyclonal goat anti-human C3 IgG, goat anti-human factor H IgG and goat anti-human factor B IgG were purchased from Atlantic Antibodies (Scarborough, Maine, USA). Affinity-purified peroxidase-conjugated porcine anti-goat IgG was purchased from TAGO, Inc. (Burlingame, California, USA).

Cellulosic hemodialysis membranes

Cuprophan and cellulose acetate hemodialysis membranes (in sheet format) were supplied by Dr. U. Baurmeister and Dr. J. Vienken (Akzo, formerly Enka AG, Wuppertal, FRG). Circular discs of 1 cm diameter were used in the experiments described herein. Surface analysis by electron spectrometer (HP 5950B, Hewlett Packard, Palo Alto, California, USA) showed that approximately 70% of the hydroxyl groups were acetylated on the cellulose acetate membranes.

Analysis of proteins eluted from hemodialysis membranes

One hundred pieces of 1 cm diameter disc of cuprophan or cellulose acetate were incubated at 37°C with 2.5 ml of NHS^{++}. After 30 minutes, the membranes were washed three times with PBS-PE. Proteins were eluted by incubating the membranes at 37°C with 4 ml of NH_2OH-PE. After 30 minutes the supernate was dialyzed at 4°C against Tris buffer using Spectropor dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, California, USA) with a molecular weight exclusion limit of 2 kDa. After subsequent lyophilization, the proteins were resuspended in 250 μl of Tris buffer and analyzed by SDS-PAGE [29] under reducing conditions in a 5 to 15% linear gradient gel. After fixation, the gels were stained with Coomassie Brilliant blue R-250 (Bio-Rad Laboratories, Richmond, California, USA). Western blots were developed with anti-C3, anti-factor B or anti-factor H as the primary antibodies as previously described [30]. For consistency, a fixed quantity (35 μg) of NHS or membrane eluates were analyzed in the different blots.

Apparent molecular weights were calculated according to the method of Weber and Osborn [31] using standards purchased from Bio-Rad Laboratories, and the established M_r of the α and β chains of C3 (125 and 75 kDa, respectively) [22], the α chain fragments of C3c (43 and 40 kDa) [32], C3dg (41 kDa) [32], factor H (150 kDa) [26], factor B (93 kDa) and the Bb (60 kDa) and Ba (33 kDa) fragments of factor B [25].

Generation of factor B fragments

An aliquot of 100 μl of factor B (200 μg/ml) was incubated for 30 minutes at 37°C with 100 μl of CVF (200 μg/ml) and either 25 μl factor D or 25 μl PBS. The final volume of the reaction mixture was adjusted to 275 μl by the addition of 50 μl PBS^{++}. A third reaction mixture contained 100 μl of factor B and 175 μl of PBS^{++}

Determination of residual C3 convertase activity on hemodialysis membranes following incubation with NHS

Five pieces of membrane were incubated at 37°C with 500 μl of either NHS^{++} or NHS-EDTA. After 30 minutes, the membranes were washed three times with PBS. The NHS-treated membranes were then incubated at 37°C with 50 μg of purified native C3 in 200 μl GVB^{++}; the NHS-EDTA-treated membranes were incubated with the same amount of C3 in 200 μl of GVB-EDTA. After 30 minutes, the C3a present in the supernate was quantitated by radioimmunoassay (Amersham, Arlington Heights, Illinois, USA). Specific C3a generation by the membranes during the incubation with NHS was calculated by subtracting the background value (incubation with NHS-EDTA) from the total amount generated (incubation with NHS).

Binding of factor B and factor H to NHS-treated membranes

Factors B and H were radiolabeled to a specific activity of 2 to 8×10^5 cpm/μg with ^{125}I as NaI (Amersham Corp.) using the lactose peroxidase method [33]. To determine if their binding affinities were affected by the iodination, labeled and unlabeled factor B or factor H were incubated with sheep erythrocytes bearing human C3b (EC3b) [34]. The concentration of factor B or H was kept constant, but the proportion of the total input that was radiolabeled was varied. For both factor B and factor H, the amount of bound radiolabeled protein increased linearly when the ratio of labeled: unlabeled protein was increased in the input. These results indicated that the affinities of the labeled and unlabeled proteins for C3b were equivalent.

Initial attempts to analyze binding of factors B and H to C3b deposited onto cuprophan and cellulose acetate membranes using isolated components of the APC were unsuccessful because, unlike the situation with erythrocytes [35] and numerous other biological surfaces [36], specific binding of C3b to the dialysis membranes was not observed.

Inasmuch as activated C3 is deposited onto cellulosic membranes following incubation with NHS [12], binding of radiolabeled factors B and H to NHS-treated cuprophan and cellulose acetate membranes was examined. One circular disc of membrane was incubated with 200 μl of NHS at 37°C. Membranes

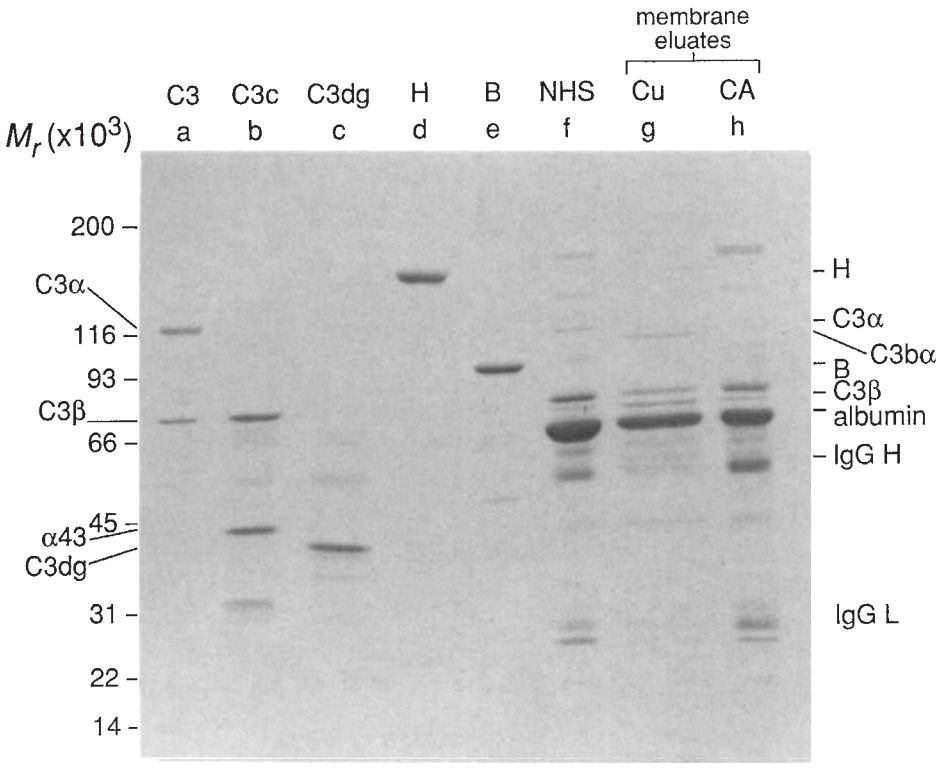

Fig. 1. *Analysis of proteins eluted from cuprophan and cellulose acetate.* Membranes were incubated with NHS. After washing, membrane-associated proteins were eluted with 1 M NH_2OH, pH 10.5. The samples were dialyzed and concentrated by lyophilization. Aliquots of 35 μg of protein eluted from cuprophan (lane g) and cellulose acetate (lane h) were subjected to SDS-PAGE under reducing conditions. The gels were stained with Coomassie blue. Lane a, isolated C3 (5 μg); lane b, isolated C3c (12 μg); lane c, isolated C3dg (9 μg); lane d, isolated factor H (3 μg); lane e, isolated factor B (3 μg); lane f, NHS (35 μg). The positions of the molecular weight standards are indicated on the left. There were qualitative and quantitative differences in the proteins eluted from the two types of dialysis membranes.

incubated with NHS-EDTA served as controls. After 30 minutes, the membrane was washed three times with PBS, and incubated at 37°C with 200 μl of PBS/A^{++} containing incremental concentrations of either radiolabeled factor B or H. After 30 minutes, the membrane was rapidly washed three times with PBS in the test tube, transferred to a clean tube and membrane-associated radioactivity was quantified in a gamma counter. The amount of ^{125}I-factor B or H bound was calculated based on the specific activity of the radiolabeled protein and an M_r of 93 kDa and 150 kDa, respectively. Binding to the NHS-EDTA-treated membranes was subtracted from binding to NHS-treated membranes (total binding) to calculate specific binding.

Pilot studies showed that the specific binding of both radiolabeled factor B and factor H to cuprophan and cellulose acetate membranes was reversible, and that maximum specific binding was reached within 20 minutes and remained constant through 30 minutes of observation.

Protein concentrations

The concentration of protein eluted from the membranes was determined by Bio-Rad Protein Assay (Bio Rad Laboratories) using bovine IgG as the standard. The concentrations of purified C3 [37], factor B [28], factor H [38] and CVF [28] were determined spectrophotometrically. C3c and C3dg concentrations were estimated using an absorbance coefficient at 280 nm of 10.0.

Statistical analysis

Results are shown as the mean ± SEM. Unpaired data were analyzed by the two-tailed *t*-test of Student [39]. Linear regression analysis was performed using the method of least mean squares. Equilibrium binding of factors B and factor H to

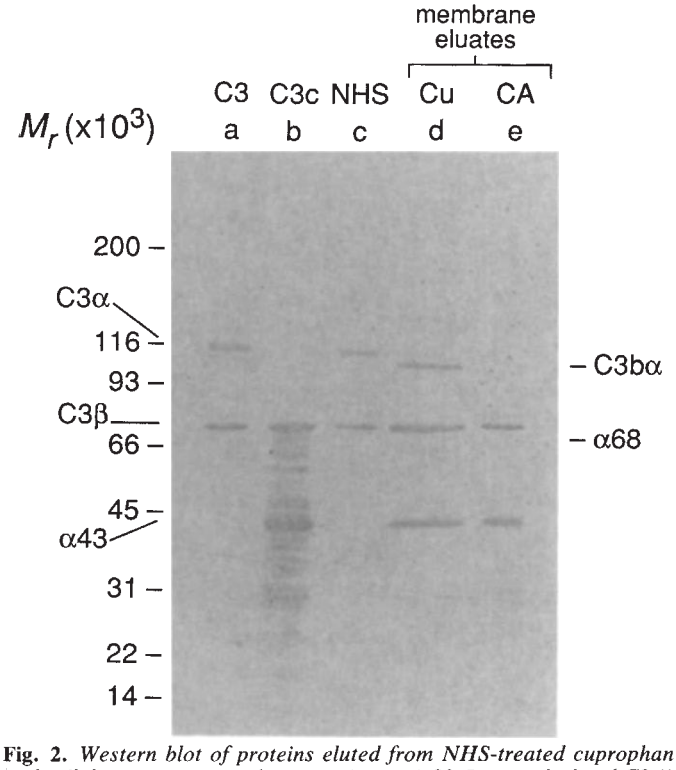

Fig. 2. *Western blot of proteins eluted from NHS-treated cuprophan and cellulose acetate membranes using anti-C3.* Lane a, isolated C3 (1 μg); lane b, isolated C3c (12 μg); lane c, NHS (35 μg); lane d, cuprophan eluate (35 μg); lane e, cellulose acetate eluate (35 μg). The putative α chain of C3b is a prominent constituent of the cuprophan eluate, but it is not visible in the cellulose acetate eluate. The α43 fragment of C3c or iC3b (degradation products of C3b) can be seen in both eluates.

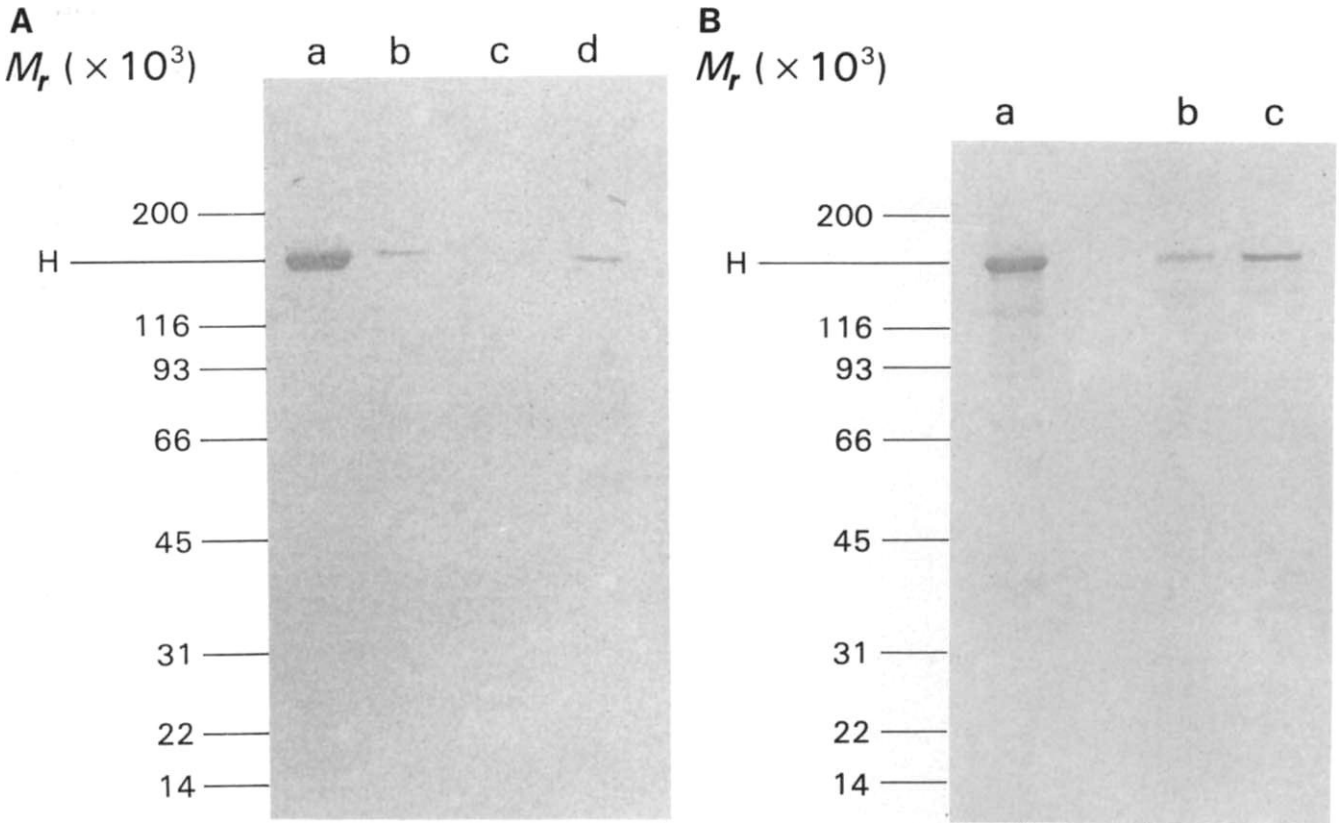

Fig. 3. *Western blot of proteins eluted from NHS-treated cuprophan and cellulose acetate membranes using polyclonal anti-H.* **A.** Lane a, isolated factor H (2 μg); lane b, NHS (35 μg); lane c, cuprophan eluate (35 μg); lane d, cellulose acetate eluate (35 μg). The concentration of factor H present in the cellulose acetate eluate (lane d) appears similar to that in NHS (lane b), while no factor H antigens are visible in the cuprophan eluate (lane c). **B.** In a separate experiment, a greater amount (50 μg) of protein was analyzed. Factor H is present in the cuprophan eluate (lane a); however, the concentration appears to be less than that in the cellulose acetate eluate (lane b).

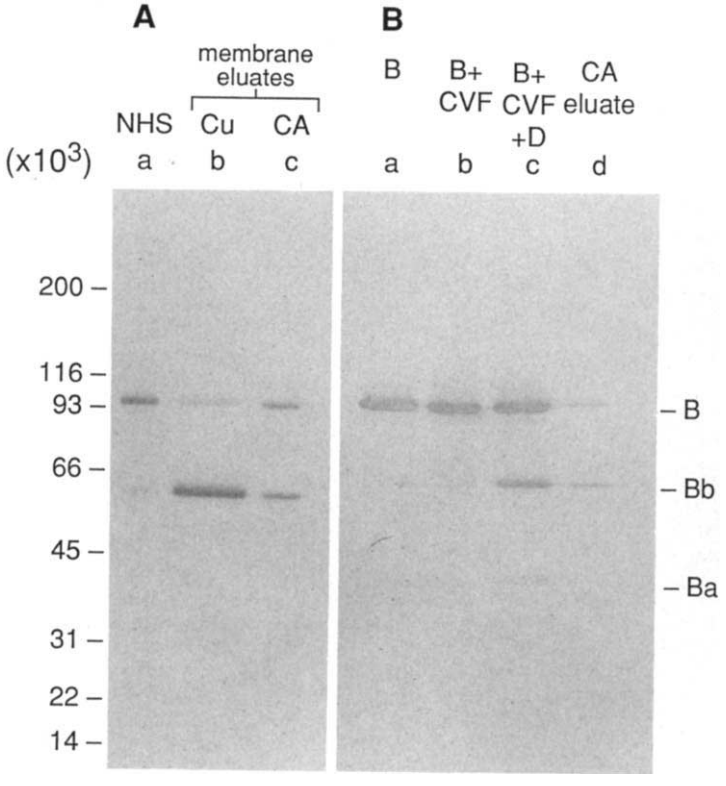

Fig. 4. A. *Western blot of proteins eluted from NHS-treated cuprophan and cellulose acetate membranes using polyclonal anti-B.* Lane a, NHS (35 μg); lane b, cuprophan eluate (35 μg); lane c, cellulose acetate eluate (35 μg). The majority of the protein eluted from cuprophan that expressed factor B antigenicity was in the form of Bb (lane b) while B and Bb appeared equally represented in the cellulose acetate eluate (lane c). **B.** Western blot of isolated factor B. Purified factor B (2.7 μg) was incubated with buffer (lane a), with CVF (lane b), or with CVF and factor D (lane c). Lane d contained the cellulose acetate eluate (35 μg). These experiments allow conclusive identification of the bands representing factor B, Bb and Ba.

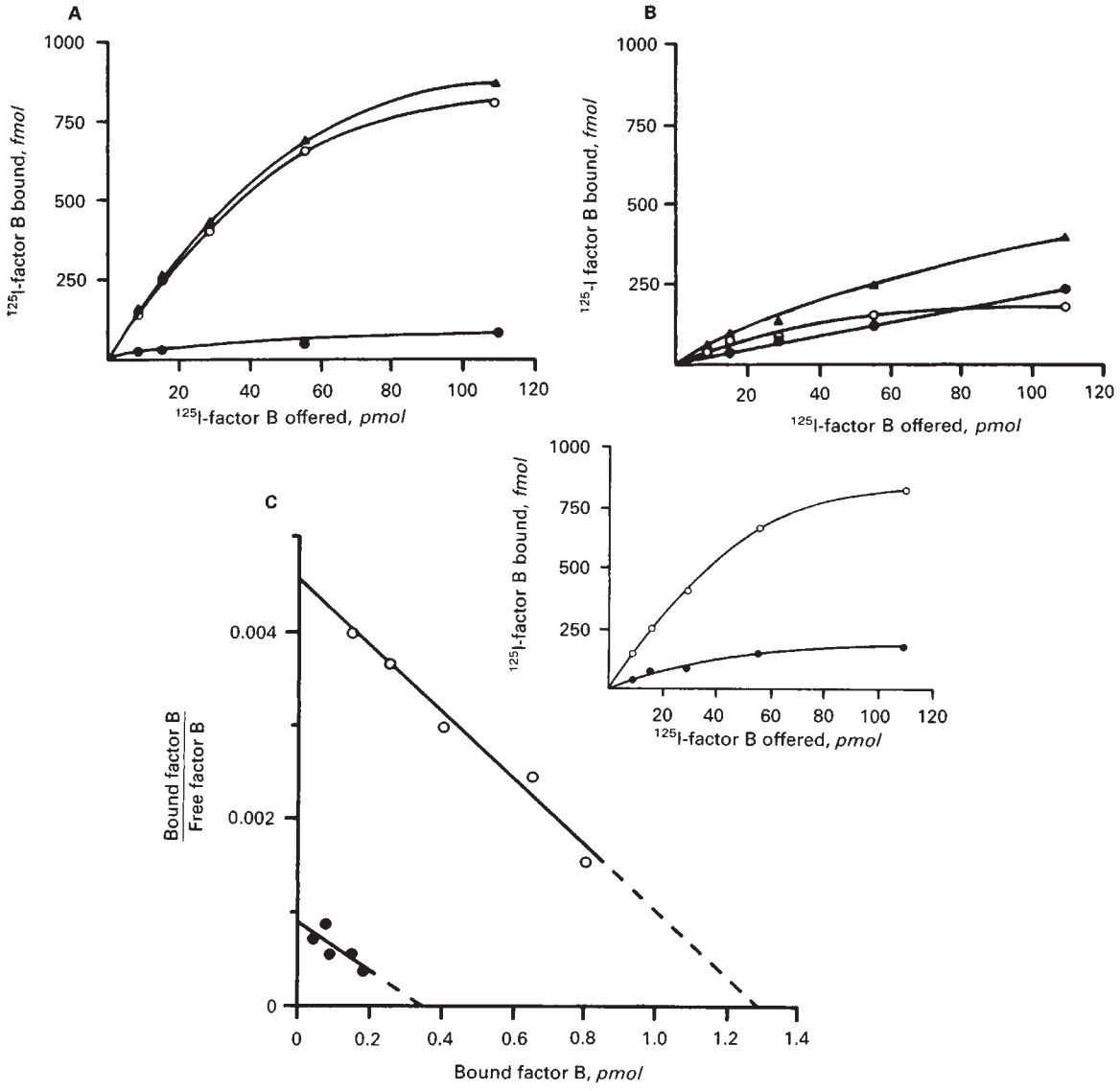

Fig. 5. *Binding of radiolabeled factor B to NHS-treated cuprophan and cellulose acetate membranes.* Membranes were incubated with NHS or NHS-EDTA. After washing, the membranes were incubated with incremental concentrations of ^{125}I-factor B. The membranes were washed and factor B binding was quantified based on the radioactivity of the membranes. Non-specific binding (●, membranes incubated with NHS-EDTA) was subtracted from total binding (▲, membranes incubated with NHS) in order to calculate specific binding (○). **A.** Isotherm of factor B binding to cuprophan membranes. The results are representative of 5 separate experiments. The shape of the dose-response curve for specific binding is consistent with a saturable reaction. **B.** Isotherm for factor B binding to cellulose acetate membranes. The results are representative of 5 separate experiments. The shape of the dose-response curve for specific binding is consistent with a saturable reaction. Maximum binding, however, appears to be less than that for cuprophan. **C.** Analysis by Scatchard's method of the specific binding of factor B to cuprophan (○) and cellulose acetate (●) membranes. The results were derived from the data depicted in panels A and B (presented simultaneously in the inset). The affinity constant for ^{125}I-factor B binding (the negative of the slope) to the two membranes was similar (3.53×10^6 M^{-1} for cuprophan compared to 2.61×10^6 M^{-1} for cellulose acetate). The maximum binding of factor B (the intercept of the line with the abscissa) to cuprophan, however, was approximately four times greater than that for cellulose acetate.

membranes were analyzed by the method of Scatchard [40]. The affinity constants at equilibrium for factor B (K_B) and factor H (K_H) binding to NHS-treated membranes were compared by using the *t*-test on the sets of individual values.

Results

Analysis of the proteins eluted from membranes

Following incubation in NHS, membrane-bound proteins were dissociated with NH_2OH at alkaline pH. Approximately 23 times more protein was eluted from the cellulose acetate than from cuprophan (27.9 ± 5.7 μg/piece vs. 1.2 ± 0.5 μg/piece, $N = 5$, $P < 0.001$). Analysis by SDS-PAGE under reducing conditions (Fig. 1) showed that the proteins eluted from the cellulose acetate (lane h) were quantitatively and qualitatively similar to serum proteins (lane f) except that the α chain of C3 was apparently missing from cellulose acetate eluates. In the cuprophan eluates (lane g), the putative albumin band was prominent, but the putative heavy and light chains of IgG were

Table 1. Affinity constants at equilibrium for factor B (K_B) and factor H (K_H) binding to cuprophan and cellulose acetate membranes

K_B		K_H[a]
Cuprophan	Cellulose acetate	Cuprophan
4.64	6.84	1.99
3.53	2.61	3.03
11.00	9.40	4.64
4.11	3.15	4.38
5.31	3.87	3.51
		2.37
		1.74
5.72 ± 1.21	5.17 ± 1.15[b]	3.09 ± 0.04[c]

The affinity constants are reported as the mean ± SEM × 10^6 M^{-1}. Membranes were incubated with serum and then washed. Binding of radiolabeled factor B and factor H to the serum-treated membranes was analyzed by the method of Scatchard.

[a] Binding of factor H to cellulose acetate membranes did not demonstrate saturation binding characteristic; therefore analysis by Scatchard's method was not possible.

[b] The difference between the K_B to cuprophan and cellulose acetate was not statistically significant ($P = 0.774$).

[c] The difference between the K_H and K_B to cuprophan was not statistically significant ($P = 0.057$).

Table 2. Maximum binding of factor B and factor H to cuprophan and cellulose acetate membranes

Factor B		Factor H[a]
Cuprophan	Cellulose acetate	Cuprophan
1.20	0.20	0.81
1.24	0.29	1.10
1.30	0.42	0.21
1.14	0.26	0.37
1.29	0.34	0.68
		1.00
		1.34
1.24 ± 0.03	0.30 ± 0.03[b]	0.77 ± 0.14[c]

The results are reported as the mean ± SEM (pmol/piece of 1 cm diameter circular membrane). Membranes were incubated with serum and then washed. Binding of radiolabeled factor B and factor H to the serum-treated membranes was analyzed by the method of Scatchard.

[a] Binding of factor H to cellulose acetate membranes did not demonstrate saturation binding characteristic; therefore analysis by Scatchard's method was not possible.

[b] The difference between the maximum binding of factor B to cuprophan and cellulose acetate was statistically significant ($P < 0.001$).

[c] The difference between the maximum binding of factor H and factor B to cuprophan was statistically significant ($P = 0.027$).

much less conspicuous compared to either serum (lane f) or the cellulose acetate eluate (lane h). In addition, C3bα was apparent in the cuprophan eluate but not in serum or the cellulose acetate eluate.

Membrane-associated proteins expressing C3 antigens were identified by immunoblotting of the eluates (Fig. 2). In addition to its degradation fragments (α68 of iC3b and α43 of iC3b or C3c), the α chain of C3b was conspicuous in the cuprophan eluate (lane d). In contrast, only degradation fragments of C3b were observed in the cellulose acetate eluate (lane e).

Immunoblotting for factor H antigen showed that there was proportionally more of this APC inhibitory protein in the cellulose acetate eluate compared to the cuprophan eluate (Fig. 3). Inasmuch as cellulose acetate bound over 20 times more proteins than did cuprophan, the total amount of factor H bound to cellulose acetate was much greater than that bound to cuprophan.

The Western blot of the eluates in which anti-factor B was used as the primary antibody (Fig. 4A) showed that for cuprophan, the majority of the protein expressing factor B antigens was in the form of the enzymatically active Bb fragment (lane b). In contrast, the cellulose acetate eluate appeared to contain equivalent amounts of native factor B and Bb (lane c). In a control experiment to confirm the identity of Bb ($M_r \sim 60$ kDa), factor B was incubated with CVF and factor D in the presence of $MgCl_2$ (Fig. 4B, lane c). Under these conditions, factor B binds to CVF and is subsequently cleaved by factor D. In the absence of factor D, no Bb was observed (lane b). The Ba fragment was also observed in lane c of Fig. 4B. Its absence from lanes b and c of Fig. 4A indicated that the fragment did not bind to either membrane.

The presence of C3b and Bb on cuprophan membranes suggested that residual C3 convertase activity might be present after exposure to NHS for 30 minutes. To test this hypothesis, membranes were incubated with NHS. After washing, isolated C3 was added, and the capacity of the membranes to mediate C3 activation was assessed by measuring the generation of C3a. Greater than 2.5 times more C3a was present in the supernate of the serum-treated cuprophan membranes (1677 ± 199 ng/ml compared to 608 ± 77 ng/ml for cellulose acetate, $N = 5$, $P = 0.001$).

Analysis of the binding of radiolabeled factor B and factor H to membranes

The apparent differences in C3b integrity when bound to cuprophan and cellulose acetate membranes (Fig. 2) prompted the investigation of the binding of ^{125}I-factor B and ^{125}I-factor H to C3b on the two membranes following incubation with NHS. Binding of factor B to both membranes appeared to be saturable (Fig. 5 A and B). When the results were analyzed by Scatchard's method, the data points showed a linear relationship that is consistent with a homogeneous reaction involving a single class of binding sites. There was no difference between the affinity constant for factor B binding (K_B) to the two membranes (Fig. 5C, Table 1). The maximum number of factor B binding sites (determined by the intercept of the line with the abscissa) on cuprophan, however, was approximately four times greater than that on cellulose acetate (Fig. 5C, Table 2).

The isotherm for ^{125}I-factor H binding to cuprophan membranes was also consistent with a saturable reaction (Fig. 6A). Scatchard's analysis indicated the existence of a single class of binding sites (Fig. 6C). The affinity constant for factor H binding (K_H) to cuprophan membranes was less than the K_B (Table 1) but the difference did not achieve statistical significance ($P = 0.057$). The maximum number of factor H binding sites was significantly lower than the maximum binding sites for factor B (Table 2).

The dose-response curve for factor H binding to cellulose acetate was complex (Fig. 6B). The total binding appeared to reach a plateau, however, so did the nonspecific binding. As a result, the specific binding curve appeared biphasic. Inasmuch as factor H binding did not demonstrate saturation binding characteristics, analysis by Scatchard's method was invalid.

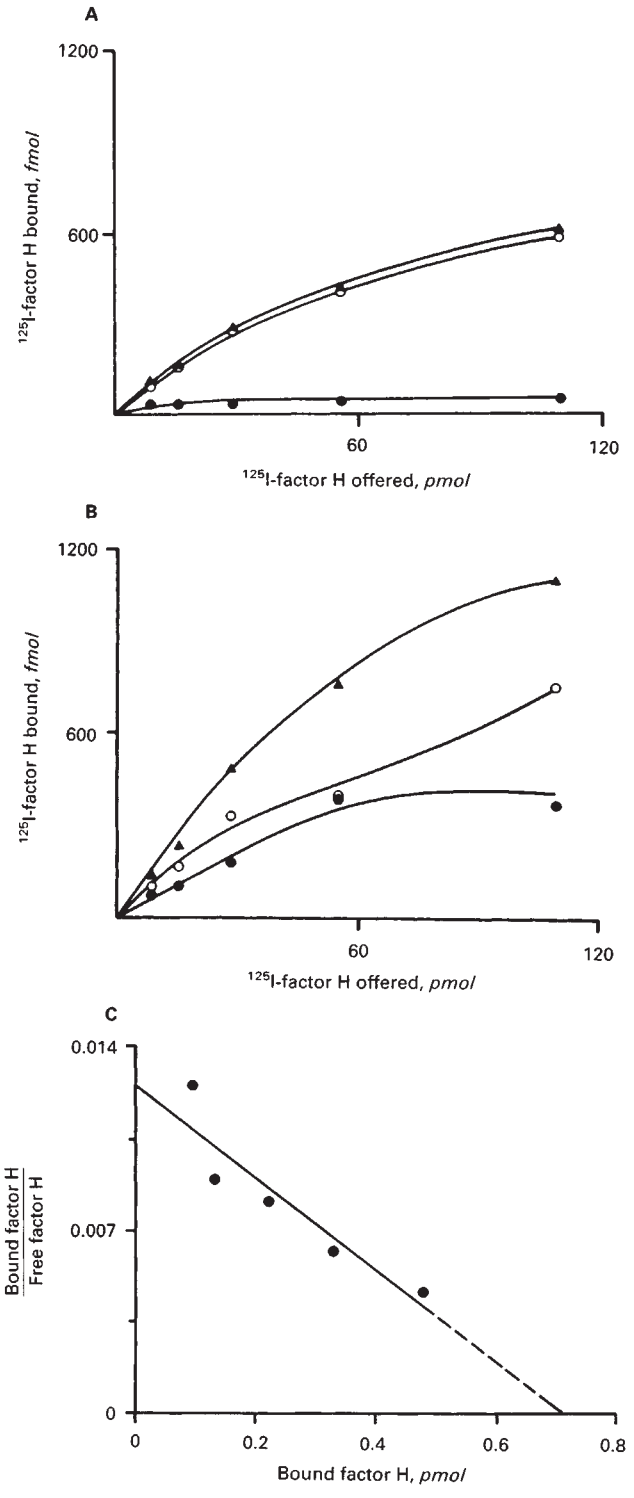

Fig. 6. *Binding of radiolabeled factor H to NHS-treated cuprophan and cellulose acetate membranes.* Membranes were incubated with NHS or NHS-EDTA. After washing, the membranes were incubated with incremental concentrations of ^{125}I-factor H, the membranes were washed and factor H binding was quantified based on the radioactivity of the membranes. Non-specific binding (●, membranes incubated with NHS-EDTA) was subtracted from total binding (▲, membranes incubated with NHS) in order to calculate specific (○) binding. **A.** Isotherm of factor H binding to cuprophan membranes. The results are representative of seven separate experiments. The shape of the dose-response curve for specific binding is consistent with a saturable reaction. **B.** Isotherm for factor H binding to cellulose acetate membranes. The results are representative of seven separate experiments. The shape of the dose-response curve for specific binding is inconsistent with a saturable reaction. **C.** Analysis by Scatchard's method of the specific binding of factor H to cuprophan membranes (●). The regression analysis curve was linear suggesting a single class of binding sites for factor H on the NHS-treated cuprophan membranes. Binding of factor H to NHS-treated cellulose acetate membranes could not be analyzed by Scatchard's method because the isotherm did not demonstrate characteristics of saturation.

Discussion

The results of the studies reported herein indicate that the greater activation of the APC by cuprophan membranes compared to cellulose acetate membranes is due to the fact that a greater portion of the C3b that binds to the cuprophan is protected from inactivation by factor H (Fig. 2). As a result, the formation and stability of the C3 convertase is favored.

Compared to cuprophan, cellulose acetate membranes bind much greater amounts of serum proteins, but the C3b that is bound is rapidly degraded into inactive forms (Fig. 2). The binding of factor H (Fig. 3), along with other serum proteins, may create an environment on the membrane that is similar to that in NHS. Under these circumstances, the presence of relatively large concentrations of factor H on the membrane could inhibit C3 convertase formation and facilitate the enzymatic degradation of C3b by factor I. That membrane-bound factor H may function as an inhibitor of APC activity has been suggested by the recent report of Horstman et al [21]. In those studies, *streptococci* bearing the M protein were shown to bind factor H much more avidly than *streptococci* lacking the M protein. These results suggested the possibility that inhibition of APC activity by M-positive *streptococci* is due to the binding of factor H to a membrane constituent, thus placing the regulatory protein in close proximity to cell-bound C3b. Factor H bound to cellulose acetate membranes may function in an analogous fashion. The mechanism by which acetylation enhances protein binding, however, remains to be determined.

The protein expressing factor B antigens on cuprophan membranes was primarily in the form of Bb, the enzymatic subunit of the C3 and C5 convertases of the APC (Fig. 3). On cellulose acetate membranes, native factor B and Bb appeared to be represented in equivalent amounts. Since cellulose acetate membranes have bound 23 times more protein than cuprophan membranes, it appeared that there were more Bb molecules on cellulose acetate than on cuprophan membranes. The residual C3 convertase activity on the cuprophan membranes was, however, approximately three times greater than that of cellulose acetate. These results suggested the majority of the Bb on cuprophan membranes was associated with C3b and was therefore functionally active, while most of the Bb on cellulose acetate was bound to the membrane but not in association with C3b.

The efficacy of hydroxylamine at alkaline pH in dissociating membrane-bound C3 has been previously demonstrated [12, 41] and was further substantiated by incubating cellulosic membranes with C3-depleted human serum repleted with radiolabeled C3. The results of these experiments showed that 1 M hydroxylamine, pH 10.5 removed > 97% of the radiolabeled C3

from both cuprophan and cellulose acetate membranes (data not shown).

In order to determine the efficiency of binding of activated C3b and to characterize the binding of factor B and factor H to membrane-bound C3b, attempts were made to deposit C3b onto cuprophan and cellulose acetate membranes using isolated complement components. Unlike the situation with erythrocytes [35] and numerous other biological surfaces [36], however, specific binding of C3b to cellulosic membranes was not observed. These findings are consistent with previous observations that activated C3b does not bind covalently to cellulosic membranes [12], and indicate that other serum constituents are required for the binding of activated C3 to occur.

The K_B to cuprophan and cellulose acetate was equivalent (Fig. 5C, Table 1), and these values are similar to those reported for factor B binding to fluid-phase C3b and to C3b on other particulate surfaces [42]. The maximum number of factor B binding sites on cuprophan, however was four times greater than that on cellulose acetate (Fig. 5C, Table 2). Since C3b is the ligand for factor B, these results are consistent with the anti-C3 Western blot (Fig. 2) which showed more C3b on the cuprophan eluate. The K_H to cuprophan was lower than the K_B but the difference was not statistically significant (Table 1). The maximum number of factor B binding sites, however, was approximately twice as great as that of factor H binding sites (Table 2). These results suggest that a portion of the C3b binding site on cuprophan are inaccessible to binding by factor H. As a result, these C3b molecules may be protected from inactivation.

The binding of factor H to NHS-treated cellulose acetate membranes could not be analyzed by Scatchard's method because saturation binding characteristics were not observed (Fig. 6B). The total binding of factor H to cellulose acetate membranes was greater than that to cuprophan membranes, in part because the non-specific binding was greater (Fig. 6 A and B). Nonspecific binding is defined as binding to membranes that have been incubated with NHS-EDTA. It is possible, however, that as discussed above, the binding of factor H to the membrane may be of functional importance. Under these circumstances, the term non-specific may be inappropriate.

The present in vitro studies demonstrated a difference between the binding of APC regulatory proteins to cuprophan and cellulose acetate and provide a plausible explanation for the difference in complement activation potential between these two membranes. Conceivably, in the clinical setting, complement activation on hemodialysis membranes may be influenced by blood cells and blood flow rates. Further studies aimed at analyzing the effects of these interactions should be pursued.

Extracorporeal membranes are employed in a variety of clinical situations. An understanding of the molecular basis by which their biochemical and biophysical properties influence bioactive systems should lead to the development of artificial membranes with improved biocompatibility characteristics.

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