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Immunology

Discrimination between activators and nonactivators of the alternative pathway of complement: Regulation via a sialic acid/polyanion binding site on factor H

(heparin/complement component C3b/glycosaminoglycans/host vs. pathogen recognition)

SEPPO MERI* AND MICHAEL K. PANGBURN†

Department of Biochemistry, University of Texas Health Center, Tyler, TX 75710

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ABSTRACT **The alternative complement pathway is capable of discriminating human cells and tissues from a wide variety of potential pathogens. It has been recently demonstrated that attachment of complement component C3b to activator-derived molecules (e.g., small polysaccharides) restricts inactivation of C3b by factors H and I in a manner similar to activator surfaces. It is now shown that restriction is reversed by certain soluble polyanions (e.g., sialoglycopeptides, heparin, or dextran sulfate) that mimic the effects of sialic acid and glycosaminoglycans on human cells and tissues. Fluid-phase polyanions enhanced binding of factor H to C3b attached to activating particles, indicating that the effect resulted from increased affinity between C3b and factor H. The enhancement was specific for activator-bound C3b since no enhancement was observed on nonactivating particles. While several polyanions could cause this effect, some polyanions could not, indicating specificity. The active polyanions also inhibited lysis of cells via the alternative pathway. The binding site for sialic acid appears to reside on factor H, since factor H bound to heparin-agarose and to sialic acid-bearing fetuin-agarose, whereas C3b bound to neither under the same conditions. These observations suggest that occupation of a specific site on factor H by polyanions induces an increase in the C3b–H affinity, resulting in discrimination of host cells and tissues from alternative pathway-activating foreign cells.**

The alternative pathway of complement (AP) represents a natural defense system for recognition and destruction of foreign organisms in vertebrate species (1–4). It enhances phagocytosis and is cytolytic for many bacteria, parasites, and virus-infected cells and for some tumor cells. This relatively simple system is composed of only six plasma glycoproteins (C3 and factors B, D, H, I, and P) but recognizes a wide variety of foreign organisms without prior contact or memory. The molecular mechanism by which C3b and the regulatory system of complement discriminate (5) between human and foreign particles has yet to be fully described. As an approach to this problem, a fluid-phase model for the AP recognition event has been constructed (6, 7). This paper shows that the recognition process can be fully mimicked with soluble complexes.

Discrimination between activators and nonactivators of the AP begins following the covalent attachment of C3b (5). C3b deposition occurs spontaneously and indiscriminately onto host and foreign particles in contact with blood (2, 8, 9). Whether this C3b is inactivated or initiates C3b amplification depends most frequently on the affinity of bound C3b for the regulator factor H (10, 11), although other mechanisms have been described (4). The C3b–H affinity depends in turn on the interaction of these proteins with neutral and anionic polysaccharides on the surface (10–14). C3b bound to human IgG (15, 16), to AP activators (5, 10, 11), or to small polysaccharides isolated from activators (6) exhibits reduced affinity for factor H. The size and location of the restriction site responsible for reducing the C3b–H affinity has recently been described (7). We now show that the site responsible for reversing this inhibition is located on factor H. It is proposed that this site interacts with sialic acids and other polyanions on human cells and tissues and enhances binding of factor H to surface-bound C3b. This interaction protects the host from autolytic attack by the AP.

MATERIALS AND METHODS

Reagents. Dextran T-10 (M_w 9400, M_n 4600) was from Pharmacia; Hepes, fetuin (type III), dextran sulfate (average M_r 5000), colominic acid [α2-8 poly(*N*-acetylneuraminic acid) from *Escherichia coli*], and poly(aspartic acid) [poly(Asp), M_r 12,000] were from Sigma; maltoheptaose was from Boehringer Mannheim; and 8-anilino-1-naphthalenesulfonate (ANS) was from Eastman Kodak. Neuraminidase-treated fetuin was prepared by incubating 40 mg of fetuin with 30 μl of neuraminidase (0.03 unit) for 1 hr at 37°C followed by 48 hr at 4°C. Fetuin carbohydrates were isolated from Pronase-digested fetuin (17). Fetuin-agarose was prepared by coupling 130 mg of fetuin to 15 ml of CNBr-activated Sepharose (Sigma). Heparin-agarose (0.5 mg/ml) was from Pierce. C3b-coated zymosan, human erythrocytes (E_hC3b), and bovine erythrocytes (E_bC3b) were prepared as described (10, 18). Human IgG was purified on protein A-Sepharose (Sigma). Proteins were labeled with ^{125}I to 0.1–1 μCi/μg (1 μCi = 37 kBq) by using Iodo-Gen (Pierce). Veronal-buffered saline (VBS) contained 5 mM Veronal (barbital) and 145 mM NaCl at pH 7.4. Hepes-buffered saline (HBS) contained 20 mM Hepes and 140 mM NaCl at pH 7.4.

Complement Proteins. C3 was purified from human plasma (19) with modifications (20) that included repurification to >95% active C3 by HPLC immediately prior to use. Factors B (21), D (22), H (23), and I (23) were prepared as described. The protein molecular weights and extinction coefficients ($A_{280}^{1\%}$) used were as described (6, 24–26).

C3b Attachment. C3b was attached covalently to proteins and carbohydrates by activating native C3 (2.8 mg/ml) with factor B (0.18 mg/ml), factor D (0.2 μg/ml), and 5 mM MgEGTA in the presence of the acceptor: human IgG (4.5 mg/ml), dextran (250 mg/ml), maltoheptaose (0.24 M), fetuin (100 mg/ml), or neuraminidase-treated fetuin (100 mg/ml).

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; AP, alternative pathway of complement; HBS, Hepes-buffered saline; VBS, Veronal-buffered saline; E_b, E_h, E_r, and E_s, bovine, human, rabbit, and sheep erythrocytes.

*Present address: Medical Research Council Centre, Hills Road, Cambridge CB2 2QH, United Kingdom.
†To whom reprint requests should be addressed.

Coupling efficiencies (percent C3b attached) were 12%, 70% (7), 80% (7), 35%, and 31%, respectively. Bound C3b was separated from free C3b and from free protein acceptors by gel filtration on TSK-400 (7.6 × 600 mm, Bio-Rad) in VBS.

Assays. Fluorimetric assays (6, 7, 24) were performed at 37°C as described. Excitation was at 386 nm (4-nm bandpass) and emission was measured at 472 nm (4-nm bandpass). Assay mixtures contained 0.5 μM–1.6 μM C3b, 40 μM ANS, 0.13 μM factor H, and 0.088 μM factor I in 1.3 ml of HBS unless otherwise stated. To cuvettes containing HBS, C3b, and ANS was added factor H (25 μg in 15 μl) followed by factor I (10 μg in 12.9 μl). Binding of ^{125}I-labeled factor H to C3b on zymosan, E_b, or E_h was measured as described (10), but at 37°C. Lysis of rabbit erythrocytes (E_r) was measured after 1.5×10^7 cells were incubated for 15 min at 37°C with 5 mM MgEGTA, VBS containing 0.1% gelatin, and normal human serum in 100 μl.

RESULTS

Fluorimetric Assay for the C3b–H Interaction. The fluorimetric assay used for Fig. 1 measures the conversion of C3b to C3bi by following the change in fluorescence of ANS as C3b is cleaved by factors H and I. Because cleavage does not occur unless C3b is bound to factor H, the rate of C3b cleavage is sensitive to the affinity between these two proteins. Fig. 1*a* compares the rate of cleavage of free C3b with that of C3b attached to a small polysaccharide (M_w 9400 dextran) or human IgG. The reduction in the rate of cleavage of C3b-dextran has been shown to be due to an ≈10-fold decrease in the affinity of factor H for C3b-dextran compared to free C3b (6). A decrease was also observed for C3b-IgG complexes (Fig. 1*a*). Particulate activators of complement such as zymosan and E_r produce similar 10-fold reductions in C3b–H affinity when C3b is bound to their surfaces (5, 10, 11).

Effect of Sialic Acid and Sialic Acid Removal on the Fluid-Phase Assay. Biological particles that are not activators of the AP frequently possess high concentrations of sialic acid (10–12, 14, 18, 27–29) or other polyanions (13, 30) on their surface and support high affinity between factor H and surface-bound C3b. To examine whether the fluid-phase assay was sensitive to the presence of sialic acid, C3b was attached to the highly sialylated protein fetuin by activating C3 in the presence of fetuin. Following purification of the C3b-fetuin complexes (C3b-F) by gel filtration, the rate of inactivation by factors H and I was found to be nearly identical to that of free C3b (Fig. 1*b*). As in many cellular systems (10–12, 28, 31), removal of sialic acid with neuraminidase led to a reduction in the affinity for factor H

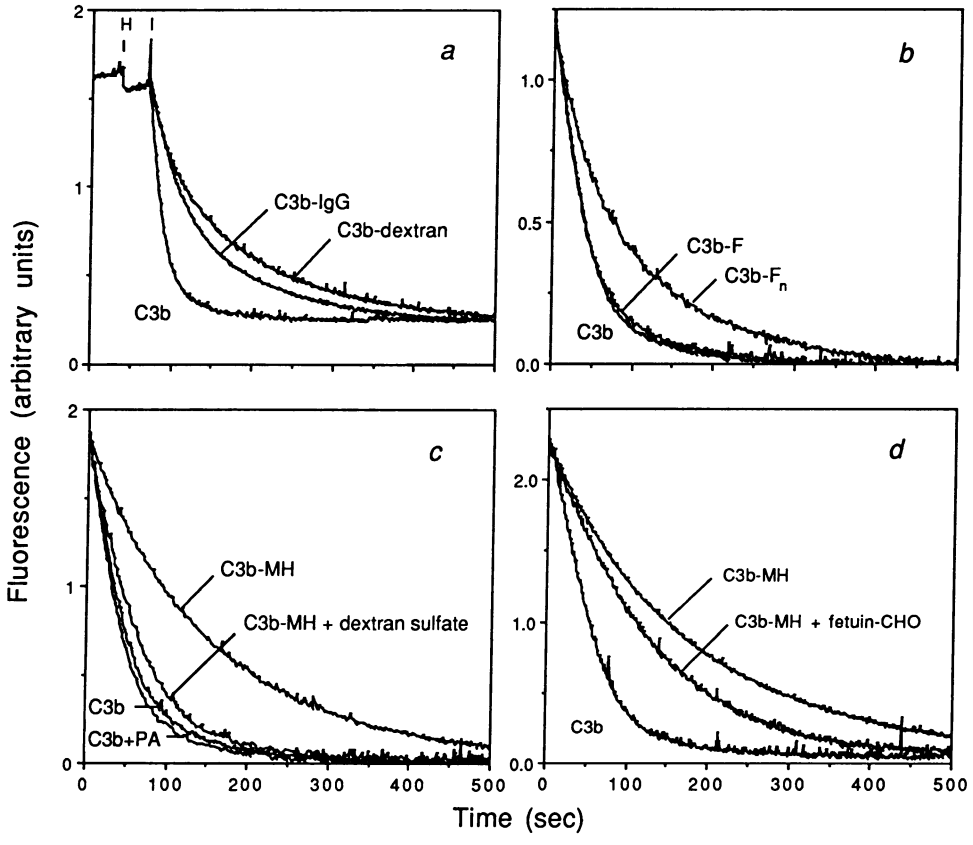

FIG. 1. Fluorimetric analysis of rates of C3b inactivation by factors H and I. (*a*) Protection of C3b from inactivation. C3b-IgG and C3b-dextran (M_w 9400) complexes were prepared and isolated as described in *Materials and Methods*. Inactivation of C3b was monitored by a fluorimetric assay (6, 7, 24) that reports cleavage of C3b as a decrease in the fluorescence of ANS. At the times indicated, factors H (47 μg/ml) and I (0.96 μg/ml) were added to a cuvette containing free or complexed C3b (0.1 mg/ml), 40 μM ANS, and HBS at 37°C. Free IgG or dextran had no effect on C3b inactivation (data not shown). The slower rate of inactivation of C3b-dextran could be approximated experimentally by using free C3b and a 10-fold lower concentration of factor H or by computer simulation of the reaction using a 10-fold decrease in C3b–H affinity (6, 7). (*b*) Fluid-phase mimicry of self surfaces, using the sialylated protein fetuin. C3b was attached to fetuin (C3b-F) or neuraminidase-treated fetuin (C3b-F_n) and its inactivation by factors H (0.13 μM) and I (0.088 μM) analyzed as in *a*. Equivalent amounts of free F, F_n, or neuraminidase did not alter the rate of inactivation of free C3b. (*c*) Restricted inactivation of C3b attached to maltoheptaose (MH), a seven-residue α1-4 polyglucose, was reversed by the addition of soluble dextran sulfate (M_r 5000) at 100 μg/ml and by heparin (data not shown). No effect on the inactivation of free C3b was observed with dextran sulfate at 100 μg/ml or heparin at 1.9 mg/ml [C3b + PA (polyanion)]. (*d*) Restricted inactivation of C3b caused by attachment to maltoheptaose was partially reversed in the presence (9 mg/ml) of a mixture of sialoglycopeptides isolated from fetuin [fetuin-CHO (carbohydrate)] (17).

($C3b-F_n$; Fig. 1*b*). These results support the use of the fluid-phase model to study AP recognition.

Effect of Fluid-Phase Polyanions. The effect of sulfated and sialic acid-bearing polyanions on the C3b–H interaction was examined by adding the soluble polyanions to fluorescence-assay mixtures containing C3b-maltoheptaose (C3b-MH; Fig. 1 *c* and *d*). Attachment of C3b to maltoheptaose (a seven-residue α1-4 polyglucose) restricts the binding of factor H ≈3-fold (7). Dextran sulfate (100 μg/ml) almost completely reversed the restrictive effects of maltoheptaose (Fig. 1*c*). Similar results were seen with heparin and DNA. None of the polyanions affected the rate of cleavage of free C3b, even at higher concentrations (e.g., heparin at 1.9 mg/ml; Fig. 1*c*). At very high concentrations the C3b–H interaction was inhibited by all polyanions tested, but at these concentrations most complement reactions are inhibited (32–35). A weak effect was also found with the fluid-phase sialoglycopeptides isolated from fetuin (Fig. 1*d*). Although several polyanions had similar effects, neither poly(Asp) nor colominic acid (*E. coli* α2-8 polysialic acid) caused increases in affinity. Free sialic acid had no detectable effect except at concentrations above 50 mM, where salt effects predominated.

Effect of Polyanions on the Affinity of Factor H for Cell-Bound C3b. Kazatchkine *et al.* (13) demonstrated that the affinity of factor H for C3b on zymosan (yeast cell walls) could be enhanced by covalently conjugating heparin to the zymosan. Fig. 2 shows that soluble heparin was able to increase the binding of factor H to zymosan-C3b 3-fold and that dextran sulfate increased it 5-fold. No enhancement was observed when C3b was bound to nonactivators such as E_h (Fig. 2) or E_b (data not shown), which bear high densities of surface sialic acid and which naturally support a high-affinity C3b–H interaction. An inhibitory effect seen at high concentrations may have prevented the full 10-fold reversal from being observed.

Inhibition of Cell Lysis. The effect of dextran sulfate on the AP-mediated lysis of E_r was examined (Fig. 3). The effective concentration was dependent on the concentration of serum, indicating significant adsorption of the polyanion by plasma proteins. At low serum concentrations the concentration of dextran sulfate causing inhibition was similar to that causing increased affinity between cell-bound C3b and factor H (Fig. 2). Colominic acid required 300-fold higher concentrations for comparable inhibition (data not shown).

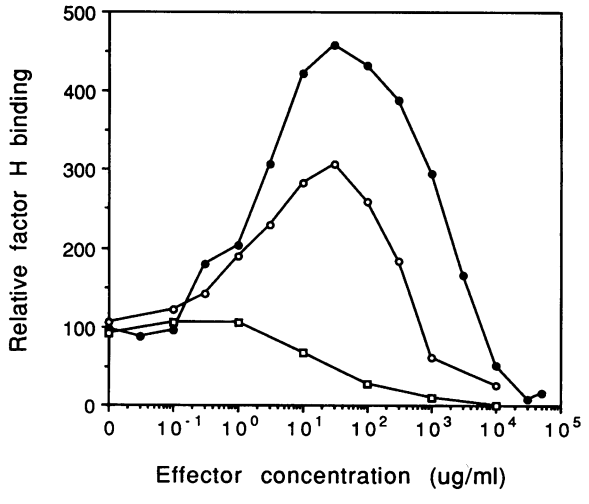

FIG. 2. Effect of soluble polyanions on factor H binding to C3b on an activator or a nonactivator. Results are shown for zymosan-C3b plus dextran sulfate (●), zymosan-C3b plus heparin (○), and E_hC3b plus dextran sulfate (□). Zymosan-C3b and E_hC3b were prepared with approximately 30,000 or 58,000 C3b molecules per particle, respectively. Binding of factor H was measured by incubating ^{125}I-labeled factor H (0.05 μg, 30,000 cpm) with various concentrations of dextran sulfate or heparin and 25×10^6 zymosan-C3b or 2.5×10^6 E_hC3b in 100 μl of VBS containing 0.1% gelatin for 10 min at 37°C (10). Binding values have been normalized for comparison. The percentage of ^{125}I-labeled factor H bound without polyanion was 4% for zymosan-C3b and 12% for E_hC3b.

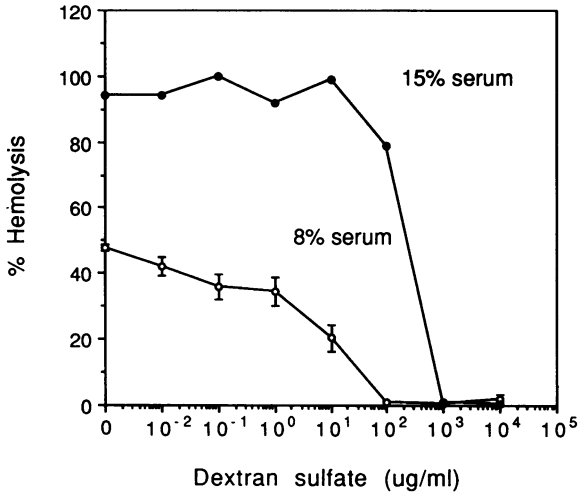

FIG. 3. Effect of various concentrations of dextran sulfate on the activation of the AP and lysis of E_r. The final concentration of serum in the assays was 15% (●) or 8% (○).

Affinity Chromatography on Heparin-Agarose and Fetuin-Agarose. To identify which protein was responsible for recognizing the presence of sialic acid or polyanions on nonactivators, both C3b and factor H were chromatographed on heparin-agarose and fetuin-agarose. Fig. 4 shows that factor H bound selectively to both columns, whereas C3b was eluted in the breakthrough. Binding to fetuin-agarose required lower ionic strength (2 mS) than binding to heparin-agarose (10 mS). Binding of C3b could be demonstrated at lower ionic strengths, but gradient elution resolved the proteins with factor H always exhibiting the highest affinity. Specificity of the interaction was indicated by the failure of factor H to bind to heparin-agarose in the presence of heparin at 0.17 mg/ml (13 μM).

Demonstration of Direct, Sialic Acid-Dependent Binding of Factor H to Nonactivating Cell Surfaces. Utilizing the conditions found for binding factor H to sialic acid on fetuin-agarose, we examined binding of ^{125}I-labeled factor H to activators and nonactivators of the AP. The results (Table 1) show that binding of factor H to sheep and human erythrocytes (E_s and E_h), which bear high densities of surface sialic acid, was 5–10 times higher than binding to cells with little or no sialic acid (E_r, neuraminidase-treated E_s, and zymosan). Binding of C3b was <2% on all cells. Direct factor H binding to cell surfaces correlated well with the restriction index (14), which is related to the affinity of factor H for C3b on those surfaces.

DISCUSSION

It has been assumed that recognition of particles as activators or nonactivators of the AP was a surface-restricted phenomenon, but it has recently been shown that C3b attached to small fragments of activators consisting of polysaccharides or small oligosaccharides exhibits restricted affinity for factor H (6, 7). Although the activator-like properties of these molecules are recognized by the C3b–H system, they are not activators of complement since they are generally too small to sustain amplification. Examination of soluble complexes of C3b and activator- or nonactivator-like molecules has

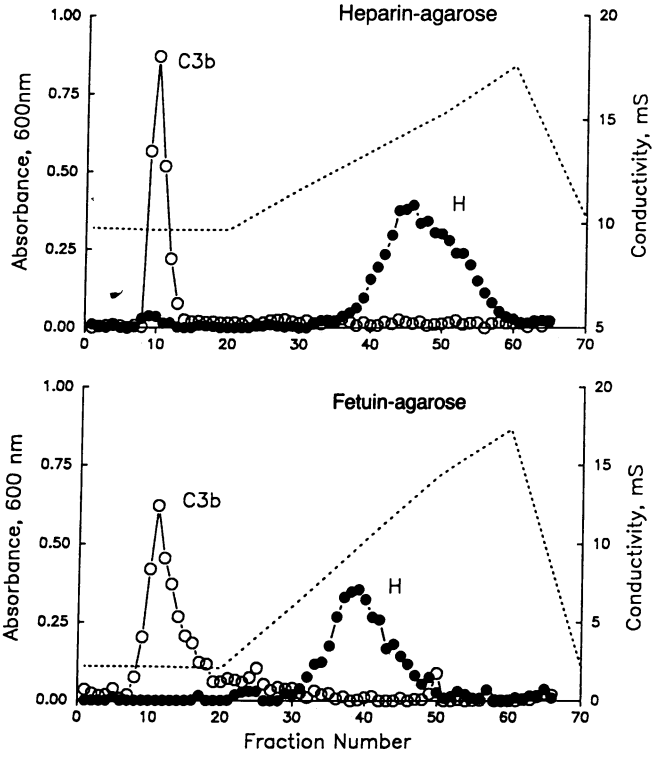

FIG. 4. Affinity chromatography of factor H and C3b on heparin-agarose or fetuin-agarose. Factor H and C3b (300 μg) were loaded separately onto a heparin-agarose column (1 × 12 cm) in 10 mM sodium phosphate/70 mM NaCl, pH 7.4, at 22°C. The column was washed with 10 ml of buffer and bound material was eluted with a 40-ml gradient to 10 mM sodium phosphate/150 mM NaCl, pH 7.4. Fractions (1 ml) were tested for protein (A_{600}) with the Coomassie dye assay (Bio-Rad). Fetuin-agarose chromatography was run similarly except that the samples and the column were initially in 10 mM sodium phosphate, pH 7.4. The conductivity (dashed line) was measured at 22°C.

allowed a more detailed analysis of the recognition process itself, and we now show that these fluid-phase complexes fully mimic the behavior of particle-bound C3b and that the recognition of sialic acid on surfaces occurs through a site on factor H.

The affinity of factor H for C3b is reduced as much as 10-fold by attachment of C3b to large polysaccharides (6). Fries *et al.* (15) showed that attachment of C3b to IgG

Table 1. Direct binding of ^{125}I-labeled factor H to cells bearing sialic acid

Surface	Factor H bound, %	RI* for C3b–H binding
E_h	21 ± 3	1.0
E_s	19 ± 2	1.0
$E_sNA^\dagger$	3.9 ± 0.2	0.3
E_r	2.0 ± 0.2	0.1
Zymosan	2.9 ± 0.2	0.1

Binding was measured at 1/10 physiological ionic strength in 10 mM sodium phosphate/0.1% gelatin/5% glucose after 2 × 10^7 cells were equilibrated with 0.1 μCi (1.8 μg) of labeled factor H in 100 μl for 10 min at 22°C. Bound factor H was separated from free by sedimenting cells through a 2-cm column of dibutyl phthalate oil at 9000 × *g* for 2 min. Values shown are mean ± SD (n = 3 or 4).

*Restriction index (14) refers to the relative affinity of factor H for C3b on various cells or surfaces. The average affinity of factor H for C3b on E_h is 10-fold higher than that for C3b bound to E_r or zymosan (yeast cell walls).

†Neuraminidase-treated E_s. This treatment (10) removed 85% of the sialic acid from the cells.

protects C3b. Fig. 1*a* shows that this restriction is quantitatively similar to that observed on activator surfaces. Of the proteins examined thus far, all restrict factor H binding unless they bear sialic acid. Fetuin is a small protein (M_r 48,000) composed of >40% carbohydrate with six oligosaccharide chains per molecule (36, 37). Each of these chains possesses from one to three sialic acid residues. C3b attached to fetuin did not exhibit restricted binding of factor H (Fig. 1*b*). The rates of inactivation of C3b-fetuin and C3b were nearly identical. Removal of sialic acid from fetuin resulted in restriction of factor H binding (C3b-F_n; Fig. 1*b*), indicating that the sialic acid residues were responsible for reversing or preventing the restriction of factor H binding.

It is well established that surface-bound polyanions such as sialic acid or heparin can regulate the affinity of factor H for C3b (4, 10–13, 38). What has not been shown before is that the polyanion need not be attached to the surface bearing C3b to increase the affinity. Fig. 1*c* shows that the presence of low molecular weight dextran sulfate (M_r 5000) in the assays reversed the restriction produced by attachment of C3b to maltoheptaose. A variety of polyanions including heparin and DNA exhibited the same effect. Even the sialoglycopeptides isolated from Pronase-digested fetuin reduced the restriction (Fig. 1*d*). The effect showed a degree of specificity, since neither poly(Asp) nor poly(sialic acid) had any effect. The latter observation suggests that inhibition of complement activation by poly(sialic acid) on bacteria may occur via another mechanism (4).

A direct effect of polyanions on the affinity between C3b and factor H was demonstrated by measuring the binding of radiolabeled factor H to activator-bound C3b (Fig. 2). Binding was increased 3- to 5-fold by a variety of polyanions. No effect of polyanions on the C3b–H interaction was observed when C3b was on nonactivators (E_h or E_b), presumably because the sialic acid on these cells had already increased the affinity to a maximum. As in the fluid-phase assay neither poly(Asp) nor poly(sialic acid) had any effect.

It has been reported that a variety of polyanions activate both pathways of complement (39–42). It has also been reported that similar polyanions inhibit AP activation (32, 34, 35, 39, 40, 43). These observations are not inconsistent. Polyanions on surfaces can prevent activation of the AP via enhanced binding of factor H to C3b. Fig. 2 shows that soluble polyanions may also prevent activation via this mechanism. Fig. 3 shows that inhibition of AP activation by fluid-phase dextran sulfate occurred at concentrations that do not inactivate other complement reactions. Numerous studies (33–35, 43) have shown that soluble polyanions inhibit many complement reactions, thus directly blocking activation. Such an inhibitory effect was seen with factor H binding in the presence of high concentrations of polyanions (Fig. 2). Particulate polyanions may also activate the AP by adsorbing factor H (Fig. 4) and allowing uncontrolled amplification to occur in solution (39). Since a partial reduction in factor H concentration can enhance AP activation on nearby particles, this phenomenon can have mixed effects. Which mechanism predominates under a given set of circumstances with a given polyanion will determine whether complement activation or inhibition is observed.

The lack of an effect of poly(sialic acid) suggests that the binding site for clusters of sialic acids has particular three-dimensional requirements including a requirement for terminal sialic acid residues, as has been noted before (12, 44). The three-dimensional structure of colominic acid [a linear α2-8 poly(sialic acid)] would not resemble that of oligosaccharides terminating in sialic acid. The sialic acid recognition site has also been shown to have strict requirements for the structure of the sialic acid itself (12, 44).

Characterization of the sialic acid recognition site of the AP has been difficult due to its low affinity for sialic acid. The

results of the fluid-phase and zymosan–C3b binding assays suggested, however, that not only were the biological effects of heparin and dextran sulfate similar to those of sialic acid, but the affinities were much higher. Affinity chromatography on heparin-agarose subsequently revealed that factor H bound with higher affinity than C3b, suggesting that the site was on factor H. This conclusion is supported by the finding that at low ionic strength, sialic acid-bearing fetuin-agarose bound factor H but not C3b (Fig. 4). Similarly, factor H, but not C3b, bound to cells bearing high densities of surface sialic acid (Table 1). Factor H has been shown to be one of the two major DNA-binding proteins in human plasma (45) and can be selectively depleted from serum with sulfated Sephadex (39).

A variety of models can be constructed that are consistent with the present data. The simplest is that attachment of C3b to polysaccharides or proteins causes, through occupation of a restriction site, at most a 10-fold reduction in the affinity for factor H. Whether this is due to a conformational change in C3b, steric hinderance, or partial competition for the factor H site on C3b is unknown. Reports of neoantigenic sites on bound C3b not present on fluid-phase C3b favor a conformational change (46). Occupation of a cationic site on factor H by an appropriate polyanion would increase its affinity for attached C3b ≈10-fold, exactly overcoming the inhibitory effect of the polysaccharide or protein. On human cells and tissues, polyanions such as sialoglycoproteins and glycosaminoglycans would be recognized by factor H and the increased affinity for C3b would result in rapid C3b inactivation (47). A two-point attachment model where factor H binds with higher affinity because it binds to C3b and to surface anions can be ruled out since *fluid-phase* polyanions enhance C3b–H binding on cells and in the fluid phase. Other models may be described that are also consistent with the data. Perhaps fluid-phase C3b expresses a polyanion site for factor H, but this site is hidden on attached C3b, necessitating the presence of another polyanion to permit high-affinity binding.

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