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REGULATION OF ALTERNATIVE PATHWAY COMPLEMENT ACTIVATION BY GLYCOSAMINOGLYCANS: SPECIFICITY OF THE POLYANION BINDING SITE ON FACTOR H

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Summary. The discrimination between activators and nonactivators of the alternative pathway of complement depends on the affinity of the control factor H for C3b molecules covalently associated with the target. The affinity of factor H for the C3b-target complex is regulated by a positively charged site at or near the 13th short consensus repeat (SCR) domain of factor H. In this study we have analyzed the ability of different glycosaminoglycans and other negatively charged macromolecules to interact with the factor H polyanion recognition site and to enhance binding of H to the C3b-target complex. Strongest enhancement of factor H binding to zymosan-C3b was observed by the highly sulphated glycoconjugates dextran sulphate (m.w. = 5,000), heparin, chondroitin sulphate A and carrageenan (types III and IV). DNA also enhanced H binding. Removal of N-linked sulphates or reduction of the size of heparin decreased its enhancing effect on H binding. Little or no effect was seen with chondroitin sulphate C, keratan sulphate, hyaluronic acid, colominic acid (bacterial polysialic acid) or negatively charged polypeptides. The results show that the interaction of the polyanion binding site on factor H with glycosaminoglycans depends upon the number, orientation and polymeric arrangement of sulphate groups and suggest that most, but not all, sulphated glycosaminoglycans participate in the protection of host tissues from complement damage by promoting inactivation of tissue-bound C3b.

The antibody independent recognition function of the alternative pathway (AP) of complement (C) resides in a system of six plasma glycoproteins which include C3 and factors B, D, H, I and P (1). Of these, two components, C3b and factor H, are directly involved in the discrimination between activators and nonactivators (2-4). Nonactivator surfaces, like most human cells in contact with plasma, are protected against AP attack by sialic acid and other polyanionic substances (4-7) and by the membrane glycoproteins decay accelerating factor (8), membrane cofactor protein (9) and the C3b receptor (10).

Factor H is an elongated ≈150 kDa plasma glycoprotein composed of 20 repeating domains called short consensus repeats (SCR) or C control protein repeats (CCP) (11). Polyanions protect target cells from the AP by enhancing the interaction of C3b with factor H (3-5). Factor H interacts with polyanions through a site which has recently been localized to a region centered on CCP #13 of factor H (12). Fifteen of the 57 residues of CCP #13 are basic amino acids and modelling studies based on NMR analysis of Barlow et al. (13) suggest that most of the positively charged residues are clustered on one side of the domain. In the present study we

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have analyzed the specificity of the polyanion binding site on factor H. The results show that the ability of factor H to promote inactivation of C3b is enhanced by polyanions with specific properties. Many polyanions in addition to sialic acid and heparin may participate in the protection of human tissues from C activation.

MATERIALS AND METHODS

Reagents. The following reagents were from Sigma Chemical Co. (St. Louis, MO): Hepes, sodium sulphate, dextran sulphate (average m.w. 5,000), heparin (average m.w. 11,000), low molecular weight heparin (from porcine intestinal mucosa; average m.w. 4,000-6,000), colominic acid (α2-8 poly[N-Acetylneuraminic acid], m.w. 200,000, from *E. coli*), sialic acid (N-Acetylneuraminic acid), fetuin (type III), DNA (from herring sperm), keratan sulphate (from bovine cornea), chondroitin sulphate A (from bovine trachea), chondroitin sulphate C (from shark cartilage), hyaluronic acid (from human umbilical cord), carrageenan type III (kappa carrageenan from *Eucheuma Cottonii*), carrageenan type IV (lambda carrageenan from *Gogartina*), poly-L-aspartic acid (dp 84, m.w. 11,500), poly-L-aspartic acid (dp 93, m.w. 12,760) and dipeptides Asp-Asp and Asp-Glu. For working solutions the glycosaminoglycans and other reagents listed above were originally dissolved into VBS, GVB or water and pH adjusted to 7.2-7.8. Dextran T-10 (m.w. 9,400) was obtained from Pharmacia (Uppsala, Sweden), maltoheptaose (a seven residue polymer of α1-4 glucose) from Boehringer Mannheim (Germany) and 8-anilino-1-naphthalenesulphonate (ANS) from Eastman Kodak. Other preparations of heparin (from porcine intestinal mucosa; activity 163.6 units/mg) were obtained from Calbiochem Behring Corp. (La Jolla, CA) and from Dr. Tatsuro Irimura, MD Anderson Cancer Center, University of Texas, Houston (14). Partial or total N-desulphation of heparin was achieved by solvolysis in dimethyl sulphoxide containing 10% water (14, 15). Prior to dilution into assay buffers the heparin preparations were dissolved in distilled water at 10 mg/ml and pH adjusted to 8.0 using 1.0M NaOH/HCl. Cleavage of *E. coli* colominic acid was achieved by neuraminidase (from *V. cholerae*, Sigma) treatment, and fragments of approximately dp 7-20 were recovered by gel filtration. Fetuin carbohydrates were isolated from pronase digested fetuin according to Baenziger & Fiete (16). Veronal buffered saline (VBS) contained 5 mM barbital and 145 mM NaCl at pH 7.4. GVB was VBS containing 0.1% gelatin. Hepes buffered saline (HBS) contained 20mM Hepes and 140 mM NaCl at pH 7.4.

Purified proteins. C3 was purified from human plasma by described methods (17) that included a mono S cation exchange column as a final step to obtain >95% active C3. Factors B (18), D (19), C5 (20), H and I (21) were purified as described. The Fab fragment of the anti-C3d monoclonal antibody 4C2 (22) was a gift of Dr. V. Koistinen, Finnish Red Cross, Helsinki, Finland). Proteins were radiolabeled with ^{125}I to specific activities of 0.1-1.0 μCi/μg by using the Iodogen method (Pierce). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to Laemmli (23).

Covalent coupling of C3b to soluble molecules and particles. C3b was attached to soluble acceptors as described (4, 24, 25). C3b-coated zymosan (ZC3b) was prepared by using factors B, D, C3 and $NiCl_2$ to deposit and amplify C3b (3). Fluorimetric analysis of C3b inactivation using the ANS probe was performed as described (4, 24). Excitation was at 386 nm (4 nm bandpass) and emission was measured at 472 nm (4 nm bandpass).

Factor H binding assays. Binding of ^{125}I-H to ZC3b was analyzed as described previously (3, 4). To study the effects of various glycosaminoglycans and other reagents on factor H binding, the reagents were equilibrated (10 min at 37°C) with ^{125}I-H prior to addition of ZC3b. To separate bound and free ^{125}I-H the mixtures were centrifuged through dibutyl phthalate oil. All assays were performed in duplicate.

RESULTS

Activator-induced restriction of C3b inactivation and its reversal by polyanions. In the assay used the fluorescence of the ANS probe decreases when C3b is converted to C3bi by factors H and I. Small polysaccharides, when in complex with C3b, are

capable of restricting the inactivation of C3b by factors H and I (24). Quantitatively, this restriction can be expressed as a restriction index (RI), a parameter related to the relative affinity of factor H for bound C3b. RI is calculated as the ratio of factor H concentrations required to inactivate 50% of free C3b vs. complex-bound C3b under standard conditions (+37°C, Hepes buffer, pH 7.4, 1.1 μM C3b). For AP nonactivators RI is 1.0 (4) indicating that the affinity of nonactivator-bound C3b for factor H is identical to that of free C3b. The degree of activator-induced restriction of C3b inactivation is dependent on the size of the activator molecule with maximum restriction (a 10-fold reduction in the affinity of factor H for C3b or RI = 0.1) being reached at the length of ≈16 glucose residues (26). Using the fluorimetric assay RI values of 0.3 and 1.0 for maltoheptaose (m.w. 1,153) and dextran (m.w. 9,400) were obtained, respectively (Fig. 1). The activator-induced restriction of C3b can be reversed by polyanions (4). To determine if the reversal was dependent on the size of the activator-like polysaccharide bound to C3b, the effects of heparin on the inactivation of C3b-maltoheptaose and on C3b-dextran were compared (Fig. 1). Heparin increased the rate of C3b inactivation of both C3b-maltoheptaose and C3b-dextran, but the reversal was less complete with the high RI C3b-dextran complexes.

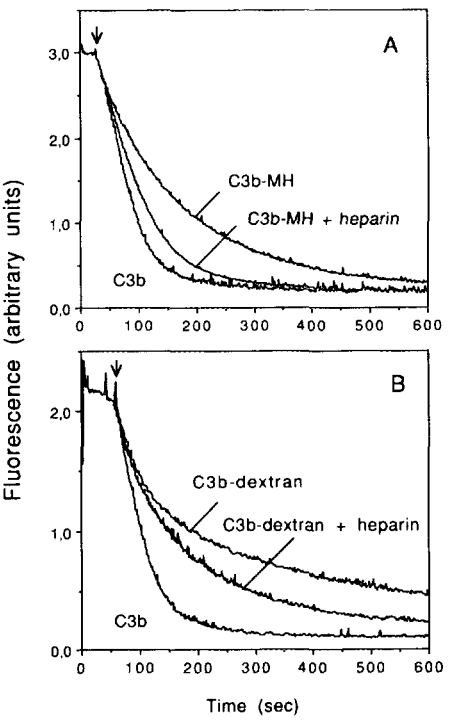

Fig. 1. (A) Retardation of the rate of inactivation of C3b by attachment to maltoheptaose (C3b-MH) compared to free C3b and the accelerating effect of heparin on the rate of inactivation (C3b-MH + heparin). C3b and C3b covalently coupled to maltoheptaose (C3b at 1.1 μM) in the presence or absence of 1.7×10^{-7} M heparin (average m.w. 11,000) were subjected to inactivation by factors H (61 nM) and I (88 nM). The time of adding factors H and I is indicated by an arrow. The concentration of heparin used did not significantly affect the inactivation of free C3b and is therefore not shown. (B) Similar analysis of C3b bound to dextran (m.w. 9,400).

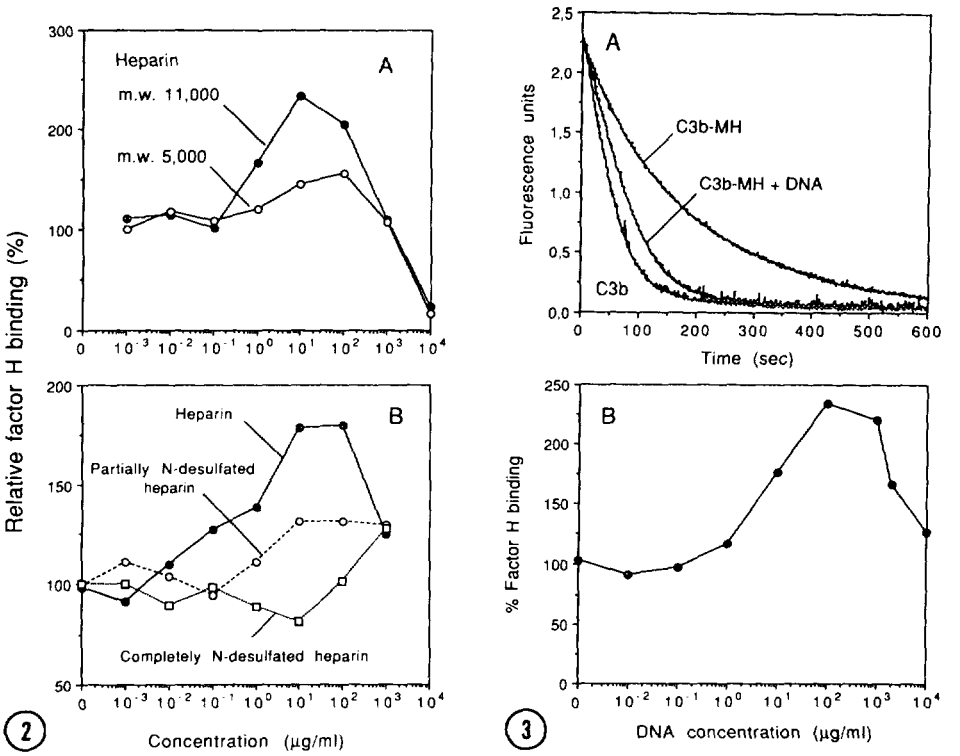

Fig. 2. The effect of heparin size (A) and N-desulphation (B) on its ability to enhance factor H binding to ZC3b. (A) Increasing concentrations of heparin preparations of average molecular weights of 5,000 and 11,000 were incubated with ^{125}I-H (0.05 µg, 40,000 cpm) and ZC3b (25 x 10^6 particles; ≈30,000 C3b molecules/particle) for 10 min at +37°C prior to separation of bound radioactivity by spinning through dibutyl phthalate. Factor H binding is expressed as a percentage of initial binding that occurred in the absence of heparin (≈ 5% of input). (B) Comparison of the ability of intact and chemically modified heparins (m.w. 11,000) to enhance factor H binding to ZC3b. Each data point represents the mean of duplicate determinations.

Fig. 3. The effect of DNA on the cleavage of maltoheptaose-C3b in the fluid phase (A) and on the binding of factor H to ZC3b (B). In (A) the DNA (herring sperm DNA in Hepes buffer, pH 7.4) concentration was 0.38 mg/ml and otherwise the conditions were as in Fig. 1. In (B) increasing concentrations of DNA (pH adjusted to 7.4) were mixed with ^{125}I-H and ZC3b and factor H binding analyzed as in Fig. 2.

The effect of heparin size and degree of N-sulphation on its ability to enhance factor H binding to zymosan-C3b (ZC3b). Fig. 2 demonstrates that the affinity of factor H for ZC3b (RI = 0.1) could be increased by heparin and that the magnitude of the effect was significantly less with low molecular weight heparin (Fig. 2A). Removal of the N-linked sulphate groups decreased the effect in a dose-dependent manner indicating the need for negatively charged N-linked substitutions in the activity of heparin (Fig. 2B).

Augmentation of factor H cofactor function by DNA. DNA was found to have an effect on inactivation of maltoheptaose bound C3b similar to that of dextran sulphate and heparin (Fig. 3A). The enhancing effect of DNA on factor H binding to ZC3b was also demonstrated with the optimal DNA concentration being ≈100 µg/ml (Fig. 3B). This result showed that the augmentation of factor H cofactor function was not restricted to sulphated glycoconjugates, but could be induced by polymeric negatively charged phosphate groups, too.

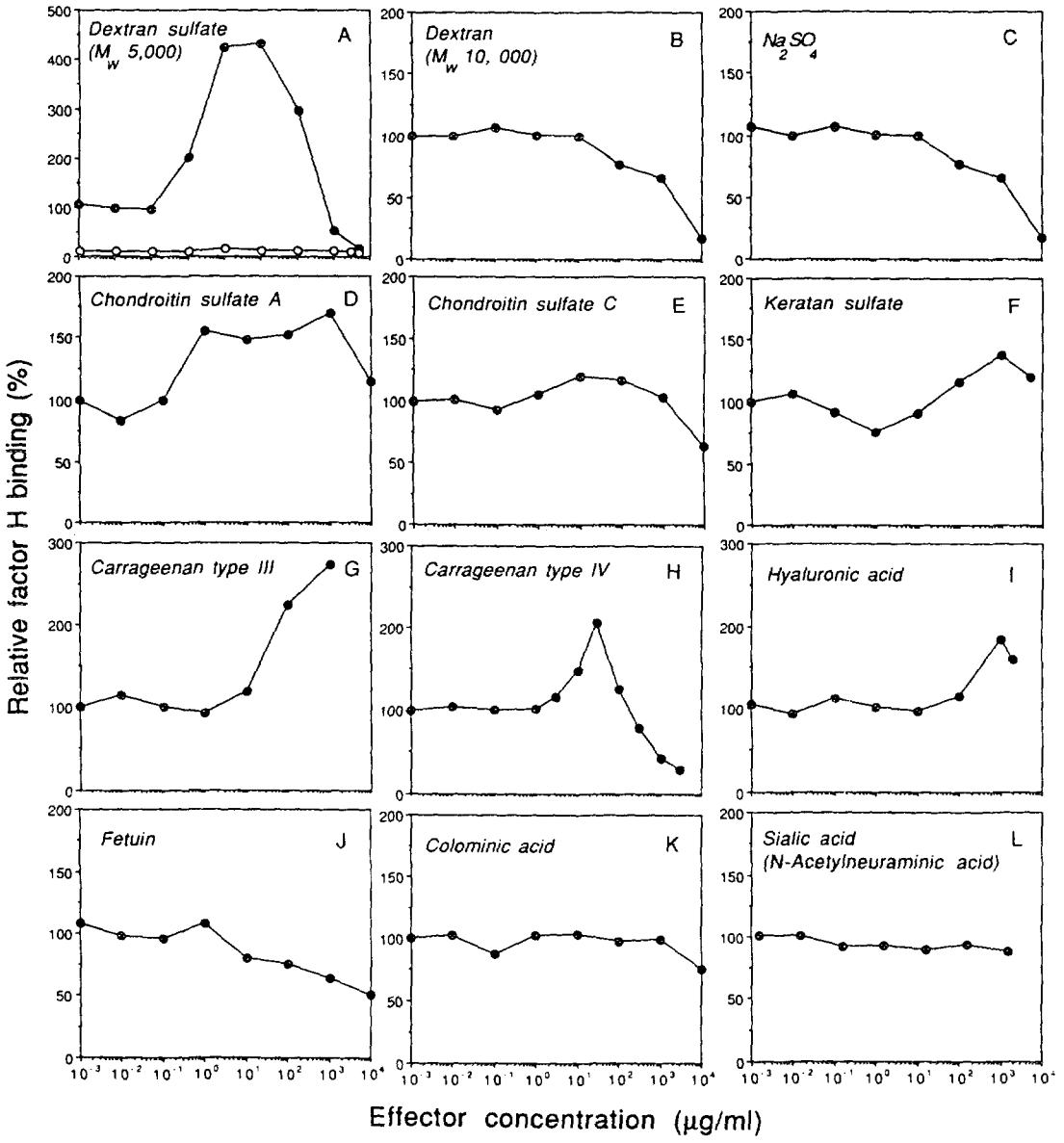

Fig. 4. Comparison of the effects of sulphated or sialylated glycoconjugates on factor H binding to ZC3b. The glycoconjugates were diluted (after adjustment of pH) into GVB and mixed with ^{125}I-H (0.05 µg) and ZC3b (●; 25 x 10^6 particles; ≈ 30,000 C3b molecules/particle) or plain Z (o; 25 x 10^6 particles) in a final volume of 100 µl. After a 10 min incubation at +37°C mixtures were spun through dibutyl phthalate oil and bound radioactivity determined from pellets. Binding percentages have been normalized for comparison. Means of duplicate determinations are shown for each curve.

Comparative analysis of glycosaminoglycans and other polyanions for their effects on factor H binding to ZC3b. The specificity of the interaction of polyanions with factor H was investigated by testing a variety of negatively charged molecules for their ability to increase the affinity of factor H for ZC3b. As shown in Fig. 4 many, but not all,

polyions were capable of enhancing factor H binding to ZC3b in a dose-dependent fashion. The strongest enhancement of factor H binding was seen with dextran sulphate, heparin, DNA, chondroitin sulphate A and carrageenan type III. No enhancement was observed with unsulphated dextran (B) or in binding of H to plain zymosan (A). Notably, free sodium sulphate, sialic acid and larger molecules bearing sialic acid (fetuin, fetuin carbohydrate [not shown] and colominic acid, a polymer of N-acetyl neuraminic acid from *E.coli*) did not affect H binding to ZC3b except at the highest concentrations, where inhibition of H binding occurred possibly due to increases in ionic strength. Anionic polypeptides or dipeptides L-Asp-L-Asp and L-Asp-L-Glu had no enhancing effect on the C3b-factor H interaction. Poly-L-aspartic acid (dp 93, m.w. 12,760) inhibited ^{125}I-H binding at high concentrations (50% inhibition at 3 mg/ml). The effect of dextran sulphate appeared specific for factor H binding, because it did not enhance binding of the ^{125}I-4C2 mAb (Fab) to ZC3b, despite of the partially shared binding sites of H and 4C2 (22) on the C3d portion of C3b. Binding of ^{125}I-C5 to ZC3b was inhibited rather than enhanced by dextran sulphate. The inhibition of ^{125}I-4C2 and ^{125}I-C5 binding to ZC3b began at dextran sulphate concentrations of 1 mg/ml and 50% inhibition was reached at approximately 10 mg/ml of dextran sulphate (data not shown).

DISCUSSION

In the present study we have examined the specificity of the polyanion binding site on factor H which provides the AP with the ability to recognize host markers that suppress C activation. The polyanions include clusters of terminal sialic acids on branched glycolipids or glycoproteins and sulphated glycoconjugates on human cells and tissues. The reduced rate of inactivation exhibited by C3b attached to AP activators (Fig. 1) can be related to changes in the effectiveness of factor H cofactor function. Attachment of C3b to maltoheptaose caused a 3-fold decrease in the restriction index, but this effect was reversed by addition of heparin (Fig. 1) or DNA (Fig. 3). Attachment to dextran yielded restriction equal to that found on the strongest activators of the AP (RI = 0.1). This effect was not completely overcome by soluble polyanions. The effectiveness of heparin was dependent on the length and degree of N-substitution of the molecule (Fig. 2). The degree of N-substitution was critical since removal of approximately one third of the sulphate groups by N-desulphation caused an almost complete loss of activity. Interestingly, inactivation of *free* C3b by factors H and I in the fluid phase was not enhanced by heparin. Thus, the effect of heparin on the interaction between C3b and factor H varies depending on whether C3b is in solution, bound to an activator or to a nonactivator.

The mechanism whereby polyanions augment factor H cofactor function is still unknown. Polyanions could cause a conformational change in factor H. A change in the arrangement of the CCP domains in the long and flexible factor H molecule, e.g. by unfolding of a "bent" or "twisted" structure of H (27, 28), could allow a high affinity interaction with surface-associated C3b. Alternatively, the polyanions could form a "bridge" between activator-bound C3b and factor H. This possibility would necessitate the existence of a polyanion binding site on both factor H and C3b. So far it is not known whether there is a polyanion site on C3b. If such a site exists on C3b, it is either of considerably lower affinity than that on factor H, since

C3b showed no affinity for heparin-Sepharose (4), or it would be expressed only on target associated-C3b.

The biological role of polyanions in regulating C activation is well established. Heparin inhibits formation of the AP C3 convertase (1, 29-31) and C56 initiated reactive lysis (32). The present study together with the earlier analysis of cell surface associated heparin (7) have shown that heparin augments factor H cofactor activity in cleaving activator-bound C3b. Sialic acid on sheep erythrocytes prevents activation of the human AP on their surface and activation by zymosan and synthetic biomaterials is prevented or reduced by attachment of heparin to their surfaces (3, 5-7). Most human cells bear high densities of sialic acid on glycolipids and glycoproteins and many cells such as endothelial cells and glomerular podocytes produce surface-bound glycosaminoglycans like heparan sulphate and chondroitin sulphate (33, 34). While regulatory membrane proteins on human cells play a major role in controlling C damage to cells, many tissues lack these proteins (basement membranes, connective tissues, and other non-cellular surfaces). Recognition of polyanions by factor H appears to provide another system which is additive with the actions of DAF and MCP. Processes leading to destruction or neutralization of the protective glycosaminoglycans could result in local AP activation with pathological consequences such as glomerulonephritis. In general, restriction of C activation by glycosaminoglycans on basement membranes and on the surfaces of normal and malignant cells may play an important protective role against C attack.

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