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Potentiation of C1 Inhibitor by Glycosaminoglycans

Dextran Sulfate Species Are Effective Inhibitors of In Vitro Complement Activation in Plasma[1]

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Activation of the complement system may contribute to the pathogenesis of many diseases. Hence, an effective inhibitor of complement might be useful to reduce tissue damage. Some glycosaminoglycans (GAG), such as heparin, are known to inhibit the interaction of C1q with activators and the assembly of the classical and the alternative pathway C3 convertases. Furthermore, they may potentiate C1 inhibitor-mediated inactivation of C1s. To search for potential complement inhibitors, we systematically investigated the complement inhibitory properties of various synthetic and naturally occurring GAG (dextran sulfates 500,000 and 5,000, heparin, *N*-acetylheparin, heparan sulfate, dermatan sulfate, and chondroitin sulfates A and C). First, we assessed the effect of GAG on the second-order rate constant of the inactivation of C1s by C1 inhibitor. This rate constant increased 6- to 130-fold in the presence of the GAG, dextran sulfate being the most effective. Second, all tested GAG were found to reduce deposition of C4 and C3 on immobilized aggregated human IgG (AHG) and to reduce fluid phase formation of C4b/c and C3b/c in recalcified plasma upon incubation with AHG. Dextran sulfate again was found to be most effective. We conclude that GAG modulate complement activation in vitro and that the low molecular weight dextran sulfate (m.w. 5000) may be a candidate for pharmacologic manipulation of complement activation via potentiation of C1 inhibitor. ***The Journal of Immunology,*** **1997, 159: 1953–1960.**

The complement system is composed of up to 20 plasma proteins. The system can be activated via the classical or the alternative pathway, both of which can trigger activation of the common terminal pathway (1–3). Classical pathway activation starts with activation of the first component, C1, a macromolecular complex consisting of C1q, two C1r, and two C1s proteins (4). Upon binding of C1 to immune complexes, C1q induces activation of C1r and C1s by changing the conformation of C1r, which then is proteolytically autoactivated. Finally, each polypeptide chain of C1r or C1s in the C1 complex is cleaved (5). The activated C1 complex then activates the complement components C4 and C2, which together form the bimolecular C4b2a complex (6, 7), which in turn activates the third component of complement C3. Activation of the classical pathway of complement is controlled by C1 inhibitor, which inactivates activated C1 by binding to C1r and C1s, resulting in the dissociation of $C1rC1s(C1Inh)_2$ complexes from C1q (5, 8, 9); at a later stage, this activation is controlled by C4-binding protein and factor I, which inactivate the C4b2a complex (10, 11).

Dextran sulfate, heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfates belong to the so-called glycosaminoglycans (12). Except for dextran sulfate, which is a synthetic polyanion, these are compounds that occur in the human body (13, 14). For example, heparan sulfate is the predominant cell-associated glycosaminoglycan in the vascular bed (15, 16). Several studies report regulation of complement activation by heparin and related glycosaminoglycans: heparin has been shown to potentiate the inhibition of C1s by C1 inhibitor 15- to 35-fold (17–23). Moreover, heparin and other glycosaminoglycans have multiple other inhibitory effects on the complement system, such as inhibiting effects on the binding of C1q to an activator, and on the formation of the classical and the alternative C3 convertases (24–30). These data suggest that glycosaminoglycans potentially may be complement inhibitors for clinical use. Up to now, however, a study comparing the effects of various glycosaminoglycans on the interaction of C1s and C1 inhibitor with those on activation of complement in plasma by various activators has been lacking.

The present study was performed to investigate the inhibitory capacity of various synthetic and naturally occurring glycosaminoglycans on the potentiation of C1 inhibitor-mediated inactivation of C1s and on complement activation in plasma.

Materials and Methods

Reagents

Dextran sulfate, m.w. 500,000 (sulfur content 17%), dextran sulfate, m.w. 5,000, dermatan sulfate (chondroitin sulfate B), chondroitin sulfate A, chondroitin sulfate C, heparan sulfate (from bovine intestinal mucosa), *N*-acetylheparin, and zymosan were obtained from Sigma Chemical Co., St. Louis, MO; unfractionated heparin (1 U/ml corresponding to 7 μg/ml), from Kabi Vitrum, Stockholm, Sweden; hexadimethrine bromide (Polybrene), from Janssen Chimica, Beerse, Belgium; and Tween-20 (Tw),[3] from J. T. Baker Chemical, Phillipsburg, NJ. The chromogenic tripeptide-*p*-nitroanilide substrate S-2314 was from Chromogenix, Mölndal, Sweden.

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[3] Abbreviations used in this paper: Tw, Tween-20; AHG, aggregated human immunoglobulin G; PTG, phosphate-buffered saline (10 mmol/L sodium phosphate, 0.14 mmol/L NaCl, pH 7.4) containing 0.1% Tween-20 (w/v) and 0.2% (w/v) gelatin; IC_{50}, 50% inhibitory concentration.

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Plasma samples and proteins

Citrated blood was obtained from healthy human volunteers by clean venipuncture. Nine volumes of blood was collected in siliconized glass tubes containing 1 volume of trisodium citrate, 3.2% (w/v). Plasma was then obtained by centrifuging blood samples at 4°C for 20 min at 1600 × *g*. Citrated (10 mmol/L, final concentration) plasma was then recalcified by adding 10 mmol/L $CaCl_2$. After 15 min at 37°C, a clot had formed, which was removed by centrifuging the plasma for 10 min at 2000 × *g*; supernatant serum ("recalcified plasma") was collected and stored in aliquots at −70°C.

Human IgG was purified as described (31) and aggregated (aggregated human IgG (AGH)) by heating at 63°C for 20 min. C1s was purified from a Cohn-I fraction of human plasma (obtained from the Department of Plasma Fractionation, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (32)). Briefly, fibrinogen was converted into fibrin by incubation with thrombin and then removed by centrifugation. The supernatant was supplemented with EDTA (10 mmol/L, final concentration), incubated for 60 min at 37°C to generate active C1s (as monitored with the chromogenic substrate S-2314), and fractionated by anion exchange chromatography on a DEAE-Sephacel column. C1s eluted from the column as the final protein fraction. Upon SDS-PAGE, this preparation migrated as a homogeneous band with a M_r of 88,000 under nonreducing conditions and a M_r of 55,000 and 33,000 under reducing conditions. Active C1s was titrated with a C1 inhibitor preparation of known concentration (33). C1 inhibitor, obtained from Behringwerke AG (Marburg, Germany), was a single band on SDS-PAGE with a molecular mass of approximately 105 kDa. Its specific activity was determined by the manufacturer using Berichrom C1 inhibitor assay (Behringwerke) and was found to be 7.2 U/mg.

Kinetic studies of the inhibition of C1s by C1 inhibitor

Inhibition of C1s by C1 inhibitor was studied in a purified protein system. Inactivation of the enzyme by the inhibitor is according to the following reaction (34): C1 inhibitor + C1s→C1 inhibitor-C1s.

Since preliminary experiments showed the rate of the interaction of C1s and C1 inhibitor in the presence of glycosaminoglycans to be $\geq 10^5$ $M^{-1}s^{-1}$, kinetic analysis was done using second-order conditions (35). All incubations were performed at 37°C in PBS (10 mmol/L sodium phosphate, 0.14 mmol/L NaCl, pH 7.4) containing 0.05% (w/v) Tween-20 (PBS-Tw). Prewarmed (5 min 37°C) C1s and prewarmed C1 inhibitor were combined at equimolar concentrations (usually 25 nmol/L) in the absence of glycosaminoglycans or in the presence of dextran sulfate 500,000 (100 μg/ml, final concentration), dextran sulfate 5,000 (100 μg/ml), heparin (350 μg/ml), *N*-acetylheparin (1 mg/ml), heparan sulfate (1 mg/ml), dermatan sulfate (1 mg/ml), chondroitin sulfate A (1 mg/ml), and chondroitin sulfate C (1 mg/ml). The reaction was started by adding C1s to mixtures containing C1 inhibitor and glycosaminoglycans at the indicated concentrations. After incubation for various times, the reaction was stopped by at least a 10-fold dilution in PBS-Tw containing S-2314. The residual amidolytic activity of C1s was determined using the chromogenic substrate S-2314 at a final concentration of 0.8 mmol/L in PBS-Tw. The initial change in absorbance at 405 nm (ΔA), which was constant during the time of measurement, was recorded in a Lambda 5 spectrophotometer (Perkin-Elmer, Norwalk, CT) at 37°C in a total volume of 300 to 500 μl. The second-order rate constant, k_{ass}, was then calculated according to the equation $1/E = 1/E_0 + k_{ass}t$ and was deduced from a plot of $1/E$ (reciprocal residual enzyme concentration) against t (time of sampling), which has a slope of k_{ass} and a y-intercept of $1/E_0$ (initial enzyme concentration) (35). Each rate constant was determined at least twice; the variation between the different determinations was 12.4 ± 2.3% (mean ± SEM).

Assays for the assessment of complement activation in plasma

Activation of C1 was assessed by measuring C1-C1 inhibitor complexes using a RIA as described (36). Activation of C4 and C3 was assessed by measuring the activation products C4b/C4bi/C4c and C3b/C3bi/C3c, respectively, using ELISA techniques (37). Serial dilutions of serum in which a maximal amount of C1-C1 inhibitor complexes was generated by incubation with AHG were used as standards in the C1-C1 inhibitor assay (31). Serial dilutions of normal serum that had been incubated at 37°C for 7 days in the presence of 0.02% (w/v) NaN_3 (normal serum, aged) were used as in-house standards in the C3 and C4 activation ELISA (37). The amount of C4b/C4bi/C4c and C3b/C3bi/C3c in this standard was assessed by comparison with purified C4b and C3b, respectively. No influence of the glycosaminoglycans on these assays has been observed, as shown in experiments in which samples containing various amounts of complement activation products were tested in the presence or absence of glycosaminoglycans.

In vitro activation of the complement system in recalcified plasma

To determine solid phase complement activation, 1 μg/ml of AHG in 0.1 ml of carbonate buffer, 0.1 mol/L, pH 9.6, were incubated in 96-well microtiter plates (Dynatech, Plochingen, Germany) at room temperature overnight. Free binding places on the plate were then blocked with PBS containing 2% milk. Fifty microliters of various glycosaminoglycans, diluted in veronal-buffered saline, pH 7.4, containing 5 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ (veronal buffer) were added to the plate. The reaction was started by adding 50 μl of recalcified plasma (diluted 1000-fold in veronal buffer) to the wells containing either glycosaminoglycans, veronal buffer without any glycosaminoglycans (= 100% deposition), or 10 mmol/L EDTA (= 0% deposition). The microtiter plate was then incubated 30 min at room temperature on a shaker at 200 rpm. C4 and C3 deposition on immobilized AHG were determined with biotinylated polyclonal Abs against C4c and C3c, respectively. The glycosaminoglycans tested were dextran sulfate 500,000 (3 ng-10 μg/ml, final concentration), dextran sulfate 5,000 (80 ng-250 μg/ml), heparin (0.25–772 μg/ml), *N*-acetylheparin (2.6–8000 μg/ml), heparan sulfate (0.8–2500 μg/ml), dermatan sulfate (0.8–2500 μg/ml), chondroitin sulfate C (8 μg-25 mg/ml). No C4 or C3 deposition was detected when veronal buffer contained 0.5 mol/L NaCl nor when the plasma was incubated at 56°C for 30 min.

To determine fluid phase complement activation, 50 μl of recalcified plasma was incubated with 25 μl of AHG (0–2 mg/ml, final concentration) in veronal buffer and with 25 μl of veronal buffer containing glycosaminoglycans. The glycosaminoglycans tested were dextran sulfate 500,000 (2–1000 μg/ml, final concentration), dextran sulfate 5,000 (2–1000 μg/ml), heparin (2–1000 μg/ml), *N*-acetylheparin (2–1000 μg/ml), heparan sulfate (10 μg-10 mg/ml), dermatan sulfate (10 μg-10 mg/ml), chondroitin sulfate A (10 μg-10 mg/ml), and chondroitin sulfate C (10 μg-10 mg/ml). Immediately after thawing, recalcified plasma and all other reagents were kept on ice in a 96-well mirotiter plate. Reaction was started by adding recalcified plasma to the veronal buffer containing AHG with or without glycosaminoglycans, and the mirotiter plate was incubated at 37°C for 1, 2, 5, 7.5, 10, 15, 20, and 30 min, respectively. Reaction was stopped by diluting aliquots of the reaction mixture 20-fold in precooled (4°C) PBS containing Tw 0.1% (w/v) and 0.2% (w/v) gelatin (PTG), with or without 100 mmol/L benzamidine. Diluted samples were then stored at −20°C until assayed for C3 and C4 activation (PTG without benzamidine), or for C1s-C1 inhibitor complexes (PTG with benzamidine). Experiments were done in triplicate using different plasma donors. In similar experiments, zymosan (1 mg/ml, final concentration) or *Escherichia coli* bacteria (10^9/ml, final concentration), isolated as described previously (38), were used as activators, and dextran sulfate 5,000 (100 μg/ml or 10 μg/ml, respectively, final concentration) as glycosaminoglycan.

Inhibition of complement activation by dextran sulfate 5,000 in whole blood

Citrated blood was separated in cells and plasma by centrifugation. The plasma was recalcified, and the cells were washed once with veronal buffer. One volume of cells was then added to 1 volume of recalcified citrated plasma, 1 volume of veronal buffer containing dextran sulfate 5,000 (20, 200, or 500 μg/ml, final concentration), and 1 volume of veronal buffer containing AHG (1 mg/ml, final concentration). After a 30-min incubation at 37°C, the reaction was stopped as described above, and C3 and C4 activation products generated in the mixtures were measured. Results obtained with the mixtures containing dextran sulfate were calculated as percentage of inhibition, with the mixtures incubated with veronal buffer containing no dextran sulfate as reference value.

Effect of dextran sulfate on the hemolytic activity of plasma

Mixtures containing 25 μl of recalcified plasma (diluted 1:9 in veronal buffer), 25 μl of either dextran sulfate 5,000 (1–400 μg/ml in veronal buffer) or veronal buffer, and 50 μl of either C1 inhibitor (2.5–250 μmol/l in veronal buffer) or veronal buffer were prepared. The hemolytic activity of the mixtures was then assessed by adding 100 μl of Ab-sensibilized sheep erythrocytes (5 × 10^8 erythrocytes/ml). After a 1-h incubation at 37°C, the mixture was centrifuged for 10 min and the absorbance at 415 nm was measured. The absorbance values of the mixtures without dextran sulfate and/or without C1 inhibitor were taken as 100% hemolysis.

Table I. *Influence of various glycosaminoglycans on the second-order rate constant for the inhibition of C1s by C1-inhibitor*

GAG[a]	Final Concentration	10^5 $M^{-1}s^{-1}$ [b]	Potentiation Factor
No GAG		0.45	1
DXS 500,000	0.1 mg/ml	58.8	130
DXS 5,000	0.1 mg/ml	34.1	76
Heparin	0.35 mg/ml	26.2	58
N-acetylheparin	1 mg/ml	4.9	11
Heparan sulfate	1 mg/ml	8.8	20
Dermatan sulfate	1 mg/ml	13.4	30
CSA	1 mg/ml	2.5	6
CSC	1 mg/ml	3.6	8

[a] GAG, glycosaminoglycan; DXS, dextran sulfate; CSA/C, chondroitin sulfate A/C.
[b] Each rate constant was determined at least twice; mean values are given.

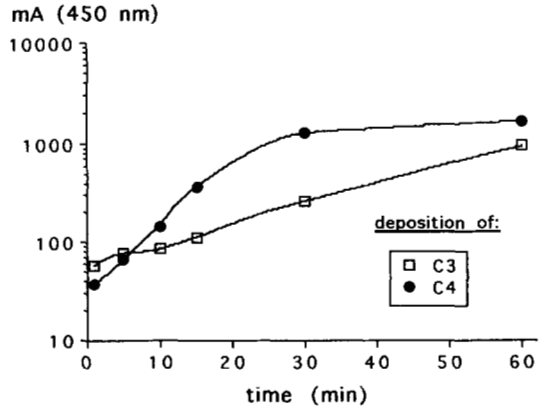

FIGURE 1. Deposition of C4 and C3 activation products onto AHG immobilized on a microtiter plate. Plates were coated with AHG (1 μg/ml in 0.1 mol/L carbonate buffer, pH 9.6) and incubated with 100 μl of veronal buffer containing 1 to 2000 diluted recalcified plasma. Fixation of C3 and C4 activation products onto AHG was measured by subsequent incubation with biotinylated anti-C3 and anti-C4 activation product Abs. Results are given as absorbance values (mA) and represent means of three different experiments.

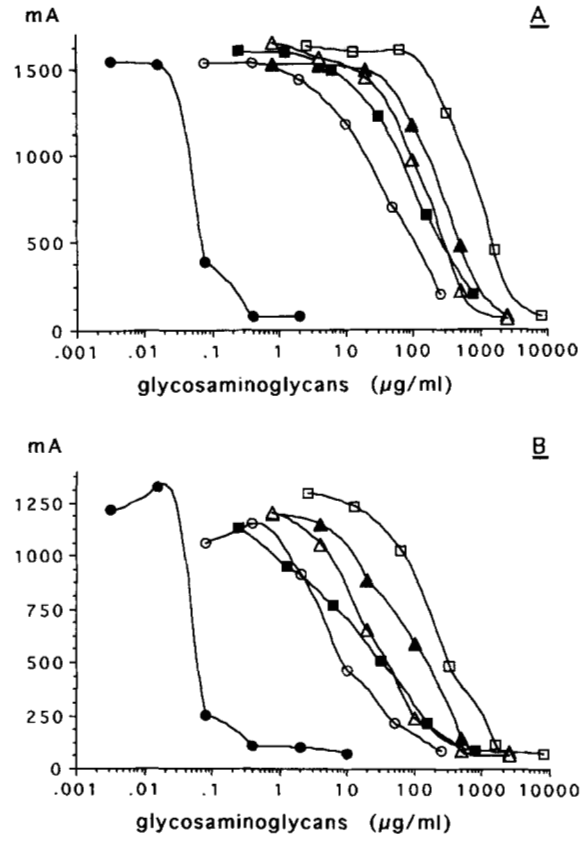

FIGURE 2. Inhibition by various glycosaminoglycans of C4 (*A*) and C3 (*B*) deposition on AHG immobilized on microtiter plates. ●, Indicates dextran sulfate 500,000; ○, dextran sulfate 5,000; ■, heparin; □, *N*-acetylheparin; ▲, heparan sulfate; △, dermatan sulfate. C4 and C3 deposition onto immobilized AHG was determined with biotinylated mAbs against C4c and C3c, respectively. Absorbance values (mA) from representative experiments are given.

Results

Kinetics of the inhibition of C1s by C1 inhibitor

In separate experiments, it was established that the glycosaminoglycans tested had no effect on the amidolytic activity of C1s against S-2314. Inactivation of C1s by C1 inhibitor was studied by measuring the disappearance of the amidolytic activity of C1s using second-order conditions with equimolar concentrations of enzyme and inhibitor. The second-order rate constant found for the inhibition of C1s by C1 inhibitor was 0.45×10^5 $M^{-1}s^{-1}$ (Table I). The second-order rate constant was increased in the presence of all tested glycosaminoglycans (Table I), showing the highest values with dextran sulfate 500,000, dextran sulfate 5,000, and heparin, respectively.

Complement deposition on immobilized AHG and its inhibition by glycosaminoglycans

AHG immobilized onto microtiter plates was used to study the influence of the various glycosaminoglycans on C4 and C3 deposition after solid phase activation of the complement system. An incubation time of 30 min appeared to be sufficient to allow substantial amounts of C4 and C3 to be deposited (Fig. 1) and was used to assess the effect of glycosaminoglycans on the deposition. The results from experiments without glycosaminoglycans were taken as 100% deposition of C3 or C4, respectively, whereas the results from experiments with EDTA were taken as 0% deposition. All glycosaminoglycans inhibited C4 and C3 deposition on immobilized AHG (Fig. 2). The concentration of each glycosaminoglycan at which deposition of C4 and C3, respectively, was inhibited for 50% (IC_{50}) was calculated (Table II); it varied from 0.05 μg/ml (dextran sulfate 500,000) to >2500 μg/ml (chondroitin sulfate C) and for the inhibition of C3 deposition, from 0.03 μg/ml (dextran sulfate 500,000) to >2500 μg/ml (chondroitin sulfate C), respectively. The correlation between the second-order rate constant and the IC_{50} for C3 and C4 deposition, respectively, shown in Figure 3, was found to be $r_s = 0.83$ ($p = 0.04$) and $r_s = 0.83$ ($p = 0.04$), respectively.

Activation of the complement system in recalcified plasma by AHG

AHG in solution, as a convenient model for immune complexes, was used as the fluid-phase activator of the complement system in recalcified plasma. In preliminary experiments, we found activation of C4 and C3 to be maximal with AHG concentrations of 0.5 mg/ml or more. Therefore, we used AHG at a final concentration of 0.5 mg/ml for additional experiments. Next, we investigated the time course of AHG-mediated complement activation. Increasing amounts of C1 , C4, and C3 activation products, respectively, were generated for an incubation time of up to 30 min (Fig. 4). After a

Table II. *Inhibitory activity of various glycosaminoglycans on the deposition and on the activation of C4 and C3 after solid phase and fluid phase activation of the complement system in recalcified plasma*

	Solid Phase IC_{50}[b]		Fluid Phase IC_{50}[b]	
GAG[a]	C4	C3	C4	C3
DXS 500,000	0.05 ± 0.01	0.03 ± 0.02	40 ± 8	90 ± 9
DXS 5,000	7 ± 0.6	0.9 ± 0.5	25 ± 5	100 ± 12
Heparin	29 ± 16	6.7 ± 2.8	50 ± 8	150 ± 14
N-acetylheparin	366 ± 245	108 ± 55	500 ± 45	>1000
Heparan sulfate	158 ± 61	350 ± 197	1500 ± 160	2000 ± 135
Dermatan sulfate	1084 ± 149	217 ± 100	1500 ± 125	>2500
CSA	ND	ND	>2500	>2500
CSC	>2500	>2500	>2500	>2500

[a] GAG, glycosaminoglycan; DXS, dextran sulfate; CSA/C, chondroitin sulfate A/C.

[b] IC_{50}, final concentration in μg/ml (mean ± SD of three different experiments) at which deposition/activation is inhibited 50%.

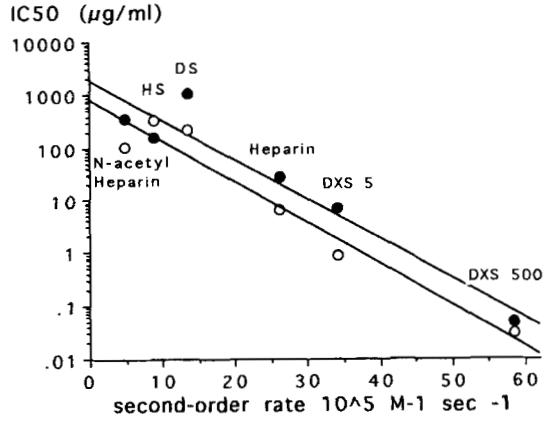

FIGURE 3. Correlation of the second-order rate constant for the inhibition of C1s by C1 inhibitor for the various glycosaminoglycans and the corresponding IC_{50} for C3 deposition (○; $r_s = 0.83$, $p = 0.04$) and C4 deposition (●; $r_s = 0.83$, $p = 0.04$) on immobilized AHG. The IC_{50} was calculated as the concentration of each glycosaminoglycan at which deposition of C4 and C3, respectively, was inhibited by 50%. DXS 500, dextran sulfate 500,000; DXS 5, dextran sulfate 5,000; *N*-acetyl-Heparin, *N*-acetylheparin; HS, heparan sulfate; DS, dermatan sulfate.

15-min incubation period, the amount of C1-C1 inhibitor complexes generated in plasma samples of three different donors was 270 ± 32, 284 ± 11, and 277 ± 40 nmol/L (mean ± SD of three different experiments). The respective amounts for the C4 and C3 activation products were 2840 ± 248, 2000 ± 480, and 1696 ± 96 nmol/L and 3306 ± 456, 3705 ± 570, and 2736 ± 342 nmol/L, respectively.

Inhibition of fluid-phase complement activation in plasma by glycosaminoglycans

In initial experiments, it was established that incubation of plasma with glycosaminoglycans alone did not induce the generation of complement activation products. Recalcified plasma was incubated for 15 min with either AHG alone (0.5 mg/ml, final concentration) or buffer containing AHG and glycosaminoglycan at varying concentrations as indicated in *Materials and Methods*. The generation of C1, C4, and C3 activation products, respectively, was measured. Then, the percentage of glycosaminoglycan-mediated inhibition of complement activation was calculated taking for 100% inhibition

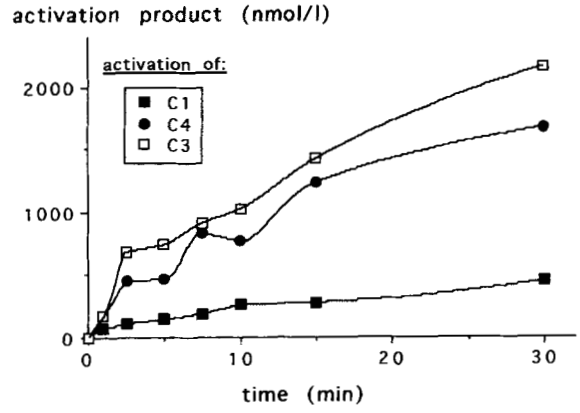

FIGURE 4. Generation of C1 , C4, and C3 activation products in recalcified plasma by AHG. One volume of recalcified plasma was incubated with 1 volume of veronal buffer containing AHG to yield a final concentration of 0.5 mg/ml. Samples were taken at the indicated times, diluted in cooled buffer, and assessed for C1 , C4, and C3 activation products (C1-C1 inhibitor complexes, C4b/C4bi/C4c, or C3b/C3bi/C3c, respectively) as described in *Materials and Methods*.

the amount of complement activation products generated in the absence of AHG, and for 0% inhibition the amount of complement activation products generated by AHG in the absence of glycosaminoglycans. Figure 5 shows the results obtained with various concentrations of dextran sulfate 500,000 and dextran sulfate 5,000, respectively. Approximately 100% inhibition of C4 and C3 activation was achieved with dextran sulfate concentrations of 200 μg/ml or above. High m.w. dextran sulfate also inhibited generation of C1 activation products (Fig. 5*A*), whereas formation of these products was not affected by low m.w. dextran sulfate (Fig. 5*B*). Heparin at concentrations of ≥50 μg/ml inhibited C4 and C3 activation without affecting formation of C1-C1 inhibitor complexes (Fig. 6*A*). Similar, though less pronounced, effects were observed with *N*-acetylheparin (Fig. 6*B*), heparan sulfate, and dermatan sulfate, respectively. In contrast, chondroitin sulfate A and C had only a minimal inhibitory capacity at concentrations of 1 mg/ml or more (data not shown). The concentration of each glycosaminoglycan at which activation of C4 and C3, respectively, was inhibited for 50% (IC_{50}) was calculated (Table II). The correlation between the IC_{50} for inhibition of C4 activation and the IC_{50} for inhibition of C3 activation for the various glycosaminoglycans was $r_s = 0.929$ ($p = 0.04$). The results show dextran sulfate to be the most effective in inhibiting C4 and C3 activation, followed by heparin and *N*-acetylheparin.

In analogous experiments, we investigated the influence of dextran sulfate 5,000 on AHG-mediated complement activation in recalcified plasma containing blood cells (whole blood). As shown in Table III, dextran sulfate was a potent inhibitor of complement activation, not only in recalcified plasma but also in the presence of peripheral blood cells.

Effect of dextran sulfate 5,000 on complement activation by other activators

In previous experiments (39), we have shown that *E. coli* bacteria are able to activate complement in serum via the classical and alternative pathway. To extend the effects of dextran sulfate 5,000 observed in recalcified plasma activated with AHG, we did similar experiments with recalcified plasma incubated with *E. coli* bacteria or zymosan, a known alternative pathway activator (1) tested as a control (Table IV). Activation of C4 by *E. coli* was inhibited for

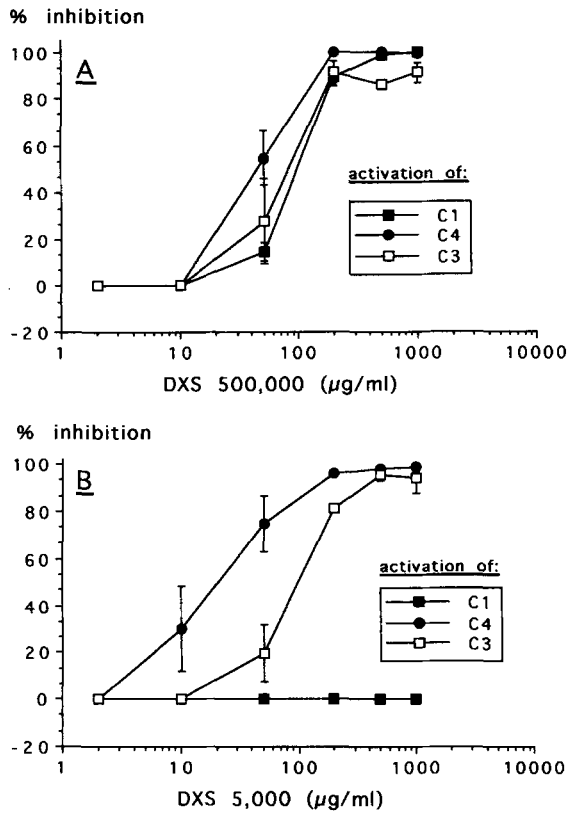

FIGURE 5. Inhibition of complement activation by dextran sulfate (DXS) in recalcified plasma induced by AHG. Fifty microliters of recalcified plasma was incubated for 15 min at 37°C with 25 μl of veronal buffer containing 2 mg/ml AHG and 25 μl of veronal buffer containing DXS 500,000 (*A*) or DXS 5,000 (*B*) to yield the indicated final concentrations. Then C1 , C4, and C3 activation in the mixtures was assessed by measuring C1-C1 inhibitor complexes, using an RIA, and by measuring the activation products C4b/C4bi/C4c and C3b/C3bi/C3c, respectively, using ELISA techniques (see *Materials and Methods*). Results (mean ± SD of three different experiments) are expressed as percentage inhibition, with 0% inhibition being the activation observed when the mixture only contained AHG and no DXS and 100% inhibition being the activation in the plasma sample incubated with veronal buffer alone.

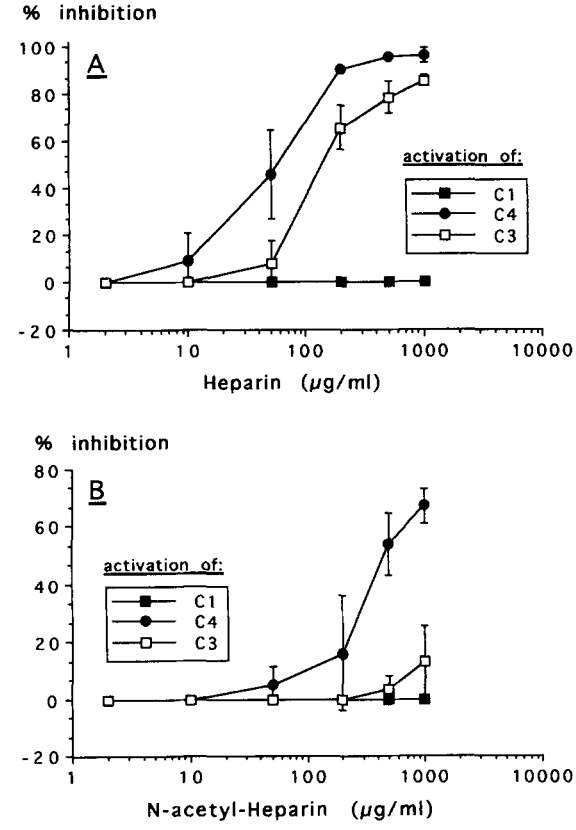

FIGURE 6. Inhibition of complement activation by heparin (*A*) or by *N*-acetylheparin (*B*) induced in recalcified plasma by AHG. For further explanation, see the legend in Figure 5.

Table III. *Percentage inhibition of C4 and C3 activation by dextran sulfate 5,000 after activation of recalcified plasma or recalcified plasma containing blood cells (whole blood) by AHG (1 mg/ml, final concentration)*

Dextran Sulfate 5,000	% Inhibition C3 Activation		% Inhibition C4 Activation	
	Recalcified plasma	Whole blood	Recalcified plasma	Whole blood
20 μg/ml[a]	22	37	36	20
100 μg/ml	44	76	86	87
500 μg/ml	88	100	98	100

[a] Final concentration. Values are given as means of two separate experiments.

about 70% by dextran sulfate 5,000 at a concentration of 100 μg/ml, whereas the effect on C3 activation was much less, presumably because the majority of C3 was activated via the alternative pathway. In our hands, zymosan consistently induced some activation of C4 in recalcified plasma, which was inhibited nearly 80% by dextran sulfate 5,000. Dextran sulfate 5,000 at a concentration of 100 μg/ml also inhibited activation of C3 by zymosan (Table IV). In these experiments, dextran sulfate 5,000 was more effective than 30 μmol/L (12× normal plasma concentration) of C1 inhibitor (Table IV).

Effect of dextran sulfate on the hemolytic activity of plasma

Various concentrations (1–400 μg/ml) of dextran sulfate 5,000, either preincubated or not with 2.5 μmol/L C1 inhibitor, were added to recalcified plasma. The inhibition of the hemolytic activity was then determined (Table V). Dextran sulfate 5,000 inhibited the hemolytic activity of plasma in a dose-dependent manner. The effect of dextran sulfate (400 μg/ml) was comparable to that of C1 inhibitor at concentrations higher than 125 μmol/L, i.e., 50 times the C1 inhibitor level in normal plasma.

Discussion

The aim of the present study was: 1) to systematically investigate the inhibitory capacity of various synthetic and naturally occurring glycosaminoglycans on complement activation, particularly on activation of C1, C4, and C3 in recalcified plasma; and 2) to determine the influence of these glycosaminoglycans on the second-order rate constant of the inactivation of C1s by C1 inhibitor.

We determined the second-order rate constant for the inhibition of C1s by C1 inhibitor to be 0.45×10^5 $M^{-1}s^{-1}$ (Table I), in agreement with our previous observation (0.42×10^5 $M^{-1}s^{-1}$) (33) as well as with other studies (0.125 to 4.7×10^5 $M^{-1}s^{-1}$) (18, 21, 22). We found all tested glycosaminoglycans to potentiate the rate of the reaction of C1s with C1 inhibitor by a factor of 6- to 130-fold (Table I). Others found heparin to stimulate the rate constant 14- to 35-fold (18, 21, 22). Our results indicate that among

Table IV. *Percentage inhibition of C4 and C3 activation by dextran sulfate 5,000 (DXS) or C1 inhibitor (C1 Inh) after activation of recalcified plasma by various activators*

Activator	Inhibitor	C4 Activation, % Inhibition	C3 Activation, % Inhibition
AHG (0.5 mg/ml)[a]	DXS (10 μg/ml)[a]	41 ± 11	23 ± 13
	DXS (100 μg/ml)	86 ± 3	58 ± 11
	C1 Inh (15 μmol/L)	40 ± 6	31 ± 7
	C1 Inh (30 μmol/L)	56 ± 7	51 ± 9
E. coli (10^9/ml)[a]	DXS (10 μg/ml)	30 ± 6	6 ± 4
	DXS (100 μg/ml)	72 ± 2	25 ± 3
	C1 Inh (15 μmol/L)	43 ± 4	18 ± 2
	C1 Inh (30 μmol/L)	47 ± 2	24 ± 1
Zymosan (1 mg/ml)[a]	DXS (10 μg/ml)	47 ± 7	11 ± 4
	DXS (100 μg/ml)	82 ± 5	68 ± 9
	C1 Inh (15 μmol/L)	47 ± 4	23 ± 6
	C1 Inh (30 μmol/L)	54 ± 6	52 ± 8

[a] Final concentration. Total amount of C4 activation (C3 activation) was 892 nmol/L (1448 nmol/L), 425 nmol/L (2131 nmol/L), and 88 nmol/L (1632 nmol/L) for activatin by AHG, *E. coli*, or zymosan, respectively. Values of % inhibition are given as mean ± SD of three separate experiments.

Table V. *Effect of dextran sulfate (DXS) and C1 inhibitor (C1 Inh) on the hemolytic activity of recalcified plasma*[a]

	Final Concentration	% Inhibition
DXS 5,000	0 μg/ml	0
	1 μg/ml	2 ± 3
	10 μg/ml	19 ± 5
	100 μg/ml	73 ± 5
	400 μg/ml	87 ± 2
C1 inhibitor, 2.5 μmol/L, and DXS 5,000	1 μg/ml	14 ± 4
	10 μg/ml	56 ± 1
	10 μg/ml	90 ± 1
	400 μg/ml	92 ± 1
C1 inhibitor	2.5 μmol/L	6 ± 2
	5.0 μmol/L	12 ± 5
	15.6 μmol/L	51 ± 5
	31.3 μmol/L	71 ± 2
	62.5 μmol/L	79 ± 3
	125 μmol/L	85 ± 1
	250 μmol/L	90 ± 0

[a] The reaction mixtures tested in the hemolytic assay (see *Materials and Methods* for details) were composed of 25 μl of recalcified plasma (diluted 1:9 in veronal buffer), 25 μl of either dextran sulfate 5,000 (1–400 μg/ml in veronal buffer) or veronal buffer, and 50 μl of either C1 inhibitor (2.5–250 μmol/L in veronal buffer) or veronal buffer were prepared. Results represent mean ± SD of three different experiments.

the naturally occurring glycosaminoglycans heparin is the most potent enhancer of C1 inhibitor function.

We found all glycosaminoglycans tested (dextran sulfates 500,000 and 5,000, heparin, *N*-acetylheparin, heparan sulfate, dermatan sulfate, and chondroitin sulfates A and C) to dose dependently inhibit AHG-induced fluid-phase activation of C4 and C3 in recalcified plasma (Table II and Figs. 5 and 6) as well as deposition of C4 and C3 after activation of recalcified plasma with surface-bound AHG (Table II and Fig. 2). The influence of glycosaminoglycans on the latter is particularly interesting, since complement activation in vivo may preferentially take place on a surface (3). The observed discrepancy in the IC_{50} between solid phase and fluid phase C3/C4 deposition and activation (Table II), respectively, may be explained by 1) differences in plasma concentrations used in either system and 2) interactions of glycosaminoglycans with complement proteins other than the interaction with C1 inhibitor; e.g., glycosaminoglycans that bind activated C4 and C3 (with exposed thioesters) will more efficiently inhibit deposition. On a weight basis, dextran sulfate was most effective (Figs. 2, 3, and 5 and Table II). The sulfate groups seems to be essential for the observed effect, since unmodified dextran had no anticomplementary action (40). Recently, dextran bearing benzylamide sulfonate groups was found to inhibit complement activation (40). However, this dextran derivative seems to be about 1000 times less active on a weight basis compared with the dextran sulfate preparations used in the present study.

The present work is an extension and further characterization of previous studies showing the capacity of heparin and other glycosaminoglycans to inhibit complement activation in vitro and in vivo (41, 42). It has been suggested that heparin's most effective inhibition is mediated through potentiation of C1 inhibitor (19). In agreement herewith, the capacity of glycosaminoglycans to inhibit activation and deposition of C4 and C3 in recalcified plasma correlated with their potency to increase the second-order rate constant for the inhibition of C1s by C1 inhibitor (Fig. 3).

The mechanism by which glycosaminoglycans potentiate C1 inhibitor toward inhibition of C1s is not known. However, a simple template mechanism seems to be unlikely, since heparin, at least, was found not to bind C1s (43), and low m.w. dextran sulfate 5,000 was as active as the other glycosaminoglycans with a higher m.w.

C1 inhibitor is not only the most important inhibitor of C1, but also the predominant inactivator of the coagulation enzyme factor XIa (44) and the contact system emzymes, factor XIIa (45) and plasma kallikrein (46). We, therefore, recently studied the effects of glycosaminoglycans on the kinetics of the inhibition of these enzymes. We found glycosaminoglycans to enhance C1 inhibitor-mediated inactivation of factor XIa up to 117-fold, whereas they had no effect on the inhibition of factor XIIa or kallikrein (47). This means that glycosaminoglycans have the potential to profoundly modulate the activity of plasma cascade systems: they enhance inhibition of the classical pathway of complement and enhance inhibition of the intrinsic pathway of coagulation (factor XIa), whereas inhibition of the contact system enzymes, factor XIIa and kallikrein, is not affected.

Given the evidence for the role of complement in a variety of pathophysiologic processes (48, 49), enhancing C1 inhibitor activity may be a convincing strategy to reduce complement-mediated morbidity and tissue damage. Indeed, administration of C1 inhibitor has been found to prevent endotoxin-induced pulmonary dysfunction in dogs (50), to have a cardioprotective effect in myocardial ischemia and reperfusion in cats (51), and to attenuate complement and contact activation in patients with septic shock (52). Since, upon infusion of C1 inhibitor, only two- to threefold normal plasma C1 inhibitor levels can be reached (50, 52), glycosaminoglycans may be candidates for pharmacologic potentiation of C1 inhibitor. However, although the complement inhibitory capacity of heparin-like compounds has been known for more than 65 years (53), they are still not used as therapeutic complement inhibitors. The reason for this is the well-known anticoagulant effect of heparin, a result of the strong potentiation of antithrombin-mediated inhibition of thrombin, factor Xa, and other coagulation enzymes (54). The complement-inhibiting effects of heparin are observed at concentrations of 50 μg/ml or higher (Tables I and II), which are at least one order higher than those used for in vivo anticoagulation with heparin (approximately 0.5–2 μg/ml). Using anticomplementary active doses of heparin in vivo would result in severe bleeding. Since the anticomplementary sites of heparin were found to be independent of the anticoagulant sites (30), attempts have been made to find heparin-like molecules with reduced anti-coagulant properties. A *N*-desulfated, *N*-acetylated form of heparin has been

developed for this purpose and found to have anticomplementary activity in vitro and in vivo (41, 42). However, we found *N*-acetylheparin to have considerably less anticomplementary activity than heparin or dextran sulfate (Tables I and II).

Although dextran sulfate 500,000 was the most effective in enhancing C1 inhibitor function and in inhibiting complement activation, this substance is potentially harmful, because it is well capable of activating the contact system (55–57); this property, however, is not shared by dextran sulfate 5,000 (55–57). Therefore, we suggest low m.w. dextran sulfate 5,000 to be the best candidate for therapeutic intervention as an anticomplementary drug. We found it to be highly effective in potentiating C1 inhibitor-mediated inactivation of C1s (Table I) and in inhibiting complement activation in recalcified plasma (Figs. 2 and 5*B*, and Table II). Furthermore, the affinity of dextran sulfate 5,000 to antithrombin was found to be three orders smaller than the affinity of heparin (58), and it lacks the enhancing effect on antithrombin-mediated inhibition of thrombin and factor Xa (59). Moreover, it stimulates C1 inhibitor-mediated inactivation of factor XIa (our unpublished observation). To act as an inhibitor of complement, in vivo dextran sulfate should be able to inhibit complement activation in the presence of peripheral blood cells. This ability, indeed, was found (Table III). However, further studies will be needed to demonstrate the anticomplementary activity of dextran sulfate 5,000 in vivo and to evaluate possible side effects of this synthetic glycosaminoglycan.

In summary, we found that various glycosaminoglycans enhance the second-order rate constant of the inactivation of C1s by C1 inhibitor and inhibit complement activation in vitro in recalcified plasma. We conclude that the synthetic low m.w. dextran sulfate (m.w. 5,000) may be a candidate compound to further develop drugs for pharmacologic manipulation of complement activation via potentiation of C1 inhibitor.

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