C1-inhibitor potentiation by glycosaminoglycans
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Citation for published version (APA):
Chapter 3

The potentiation of human C1-Inhibitor by dextran sulphate is transient \textit{in vivo}; studies in a rat model

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\textit{Int. Immunopharmacol.} (2001), 1: 1583-1595
ABSTRACT

C1-inhibitor (C1-Inh) is an important regulator of inflammatory reactions because it is a potent inhibitor of the contact and complement system. C1-Inh application in inflammatory disease is, however, restricted because of the high doses required.

The glycosaminoglycan-like molecule dextran sulphate enhances C1-Inh function in vitro. Hence, we investigated whether co-administration with dextran sulphate reduces the amount of C1-Inh required, through enhancement in vivo. C1-Inh potentiation was measured in a newly developed C1s-inactivation assay that is based on activation of C4 by purified C1s. Activated C4 in rat plasma was quantified with a newly developed ELISA. Human C1-Inh (2.5 μM) inhibited C1s in rat plasma fifty-five-fold faster in the presence of dextran sulphate (15 kDa, 5 μM).

To study the stability of the complex in vivo, rats were given a mixture of C1-Inh (10 mg/kg) and dextran sulphate (3 mg/kg). C1-Inh activity during 5 hours was analysed ex vivo with the C1s inactivation assay. The non-covalent C1-Inh-Dextran sulphate complex resulted in a transient enhancement of the inhibitory capacity of C1-Inh, lasting for 60 to 90 minutes. Dextran sulphate did not affect plasma clearance of C1-Inh.

We conclude that the enhanced inhibitory capacity of C1-Inh complexed to dextran sulphate is transient in vivo. Hence, co-administration of these compounds seems a feasible approach to achieve short-term inhibition of complement in vivo.
The serine protease inhibitor C1-inhibitor (C1-Inh) is a major inhibitor of the classical pathway of complement and of the contact system. Activation of either system results in the generation of potent inflammatory peptides. Thus C1-Inh is an important regulator of inflammation. The complement system is a so-called plasma cascade system, and consists of at least 20 plasma proteins. The system can be activated via the classical, the alternative or the mannose-binding-lectin (MBL) pathway. C1-Inh can inhibit both the classical and the MBL pathway. Upon classical pathway activation, for example by immune complexes, C1-Inh binds to activated C1r and C1s to generate C1rC1s (C1-Inh) complexes which subsequently dissociate from the C1q molecule. The C1rC1s (C1-Inh) complex is rapidly cleared from the circulation.

In a number of inflammatory diseases C1-Inh administration has been suggested to have a beneficial effect. For example, the vascular leakage syndrome, induced by IL-2, and bone marrow transplantation, are accompanied by activation of complement and contact systems, and can be attenuated by C1-Inh. Inhibition of inflammation by C1-Inh has been demonstrated in various other animal models and clinical situations such as sepsis and acute myocardial infarction. However, in spite of these promising results, the application of C1-Inh in these situations may be limited, amongst others due to the large doses of C1-Inh needed for these effects. To overcome these constraints, we have explored mechanisms to enhance the inhibitory capacity of C1-Inh to inhibit the complement system.

Already in 1976, it was shown that the glycosaminoglycan (GAG) heparin markedly enhances the ability of C1-Inh to inhibit C1. Heparin has, however, strong anticoagulatory effects, which seriously hampers its application as a complement inhibitor in vivo. Recently we have studied several other GAGs with respect to their effects on C1-Inh activity. Dextran sulphate (DXS), a semi-synthetic polyanion, appeared to be very effective in enhancing the inhibitory properties of C1-Inh. Inhibition of the protease factor Xla, the link between the contact and the coagulation systems, was also enhanced by DXS, while inhibition of kallikrein and factor XIIa was unaffected. DXS with a molecular weight of 500 kDa hardly enhanced the inhibitory properties of antithrombin III towards factor Xla. Hence, DXS may be a potential drug to inhibit complement activation in vivo since it may not interfere with antithrombin III function. Administration of GAG in vivo has been shown to have a beneficial effect in ischemia reperfusion but whether this is due to C1-Inh potentiation or other mechanisms is unknown.

Our objective was to quantify the extent of potentiation in a full plasma system and to study whether DXS could be used to enhance the complement inhibiting capacity of C1-Inh in vivo. For the former purpose, we compared the inhibitory capacity of C1-Inh*DXS in normal rat plasma to that of excessive concentrations of C1-Inh. To study the latter question we studied the stability of complexes of C1-Inh and DXS (C1-Inh*DXS) in vivo. For this purpose rats were injected intravenously with non-covalently linked C1-Inh*DXS. Plasma samples were taken at several time points and analysed ex vivo for their complement inhibiting properties.
MATERIALS AND METHODS

Materials
Normal rat plasma (NRP) was obtained by centrifugation of blood, freshly collected from Wistar rats in 10 mM EDTA (final concentration), for 10 minutes at 10000 g. Normal human EDTA plasma (NHP) was prepared freshly from blood taken from healthy volunteers. Aged normal rat serum (NRA), used as an in house standard for measurements of activated C4, was prepared by incubating normal rat serum for 7 days at 37°C in the presence of sodium azide. Heat-inactivated rat plasma was prepared by incubating plasma for 30 minutes at 56°C.

Biotinylated polyclonal rabbit antibodies against human C1-Inh and the monoclonal antibody (mAb) RII (against human C1-Inh) have been described before \(^{35,36}\). Sheep polyclonal antibodies against human C4 (SHC4) were obtained from the Dept. of Immune Reagents (CLB, Amsterdam, the Netherlands). As described below, these antibodies appeared to cross-react with activated rat C4.

C1s was purified and biotinylated (C1s-BT) as described before \(^{37}\). C1s used for the C1s inactivation assay was purchased from Calbiochem (La Jolla, CA, USA). Human C1-Inh (Cetor) was obtained from the CLB; a concentration of 250 μg/ml equals 1 U/ml and is about 2.5 μM. Dextran sulphate with molecular weight of 5000 or 15000 Da (DXS\(_{5000}\) and DXS\(_{15000}\)) was purchased from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Streptavidin coupled to polymerised horseradish peroxidase (poly-HRP) was obtained from the CLB (Dept. Immune Reagents). Streptavidin coupled to monomeric peroxidase (strep-PO) was purchased from Amersham (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK).

Elisas for activated C4 and for C1-Inh
Activated rat C4 was detected with a newly developed sandwich ELISA: 100 μl of an IgG fraction of polyclonal antibody SHC4 against human C4 which showed cross-reactivity with activated rat C4 (CLB, Amsterdam, the Netherlands) was incubated at a concentration of 2 μg/ml in 0.1 M carbonate/bicarbonate, pH 9.6, in Nunc Maxisorp plates (Nunc Brand Products, Denmark) overnight at room temperature. Plates were washed twice in phosphate buffered saline (PBS) -0.02 % (w/v) Tween 20 (PBS-Tween). Rat plasma samples were appropriately diluted (1:1000, 2000, etc.) in PBS-Tween containing 0.2 % (w/v) gelatin and 10 mM EDTA. Hundred μl of each dilution were incubated for 1 hour at 4°C, the plates being gently shaken. The plates were washed 5 times in PBS-Tween and incubated with biotinylated IgG fraction of SHC4 (SHC4-BT) diluted in PBS-Tween-0.2 % gelatin (PTG) for 1 hour at room temperature. Plates were then washed 5 times in PBS-Tween and incubated with poly-HRP (1 to 10,000 diluted in PBS containing 2% (v/v) cow milk) for 25 minutes at room temperature. Finally, the plates were developed with 3, 3', 5, 5' -tetramethylbenzidine (0.1 mg/ml in 0.11 M NaAc pH 5.5, 0.003% H₂O₂) and stopped by addition of H₂SO₄. Absorption was measured at 450 nm. Levels of activated C4 in the plasma samples tested were compared to those in the in house standard consisting of NRA (see Materials).
Functional human C1-Inh was detected as described before. Briefly, C1-Inh was bound to plates coated with mAb RII against human C1-Inh and detected with Cls-BT. Notably, this assay is an end-stage, and not a kinetic assay. In other words, it measures the number of functional C1-Inh molecules and not the kinetics of the interaction between Cls and C1-Inh. C1-Inh antigen was detected with an ELISA modified from a radioimmunoassay-procedure. Briefly, mAb RII was coated onto Nunc Maxisorp plates (2 μg/ml). Rat plasma samples were appropriately diluted in PBS containing 2% (v/v) cow milk and incubated for 1 hour at room temperature while gently shaking. Bound human C1-Inh was detected by a subsequent incubation with biotinylated polyclonal anti-human C1-Inh antibodies. Results were quantified by comparison with a standard consisting of NRP supplemented with 1U/ml human C1-Inh.

Clα inactivation assay
This assay is based on the activation of C4 in plasma by purified Clα. At higher concentrations of functional C1-Inh, purified Clα is more rapidly inactivated and thus less C4 can be activated. The procedure of the assay was as follows: 10 μl of fresh NHP or NRP, 10 μl of C1-Inh*DXS solution in Veronal Buffered Saline (VBS) pH 7.4 and 10 μl VBS were mixed and incubated in round-bottom wells of a microtiter plate for 10 minutes at 37°C. Ten μl of Clα solution, prewarmed to 37°C, was then added. Samples were mixed and incubated for 12.5 minutes at 37°C. The mixtures were put on ice and supplemented with benzamidine (final concentration 100 mM, Acros Organics, New Jersey, USA) and EDTA (final concentration 10 mM), to block further activation of C4. Samples were analysed for C4 activation in the ELISAs for activated human or rat C4 as described above. The Clα concentrations used yielded (sub-)maximal C4 activation in the plasma samples, which allowed accurate measurement of C1-Inh activity. The concentrations of C1-Inh, DXS, and Clα mentioned, refer to the protein concentration in the 10 μl of solution mentioned above. In all experiments the capacity of C1-Inh*DXS to inhibit Clα was compared with the capacity of C1-Inh alone (which was tested at higher concentrations).

Preparation of C1-Inh*DXS complexes
Non-covalent complexes between human C1-Inh and DXS or DXS were prepared freshly before use: C1-Inh (1U/ml =2.5 μM, final concentration) and DXS (5 μM) were incubated in VBS, for 10 minutes at 37°C. Complex formation was checked with protein electrophoresis on 1% agarose-gel. Complex purification is not possible because the affinity of the interaction is not high enough and thus the mixture was used without further purification.

Protein electrophoresis
Protein electrophoresis on 1% agarose-gel in tris-barbital buffer pH 8.6 was performed under standard conditions on the Sebia Hydragel. Migration was carried out under 14 W constant at 20°C controlled by Peltier effect until 75 Vh have accumulated for about 16
minutes. The gel was stained with acid violet according to the protocol of the manufacturer.

**Calculation of the potentiation factor of C1-Inh by DXS**

Addition of C1-Inh·DXS (1 U/ml C1-Inh and 5 μM DXS) was compared to addition of increasing concentrations of C1-Inh in the range between 5 and 80 U/ml. The ratio of the concentration of C1-Inh alone that caused the same C1s inactivation as C1-Inh·DXS and the C1-Inh used in the C1-Inh·DXS complex was called the potentiation factor. The potentiation factor was determined in 4 separate experiments and the standard deviation was determined.

**Stability of C1-Inh·DXS in vivo**

Rats were injected at t=0 with a solution of C1-Inh either or not pre-incubated with DXS in PBS. EDTA-plasma samples were taken at various time points, immediately frozen in liquid nitrogen, and stored at -80°C until tested. Samples were analysed in the C1s inactivation assay as described above, to measure the activity of the C1-Inh administered. The amount of activated C4 at all time points was expressed as the percentage of the maximal C4 activation observed in a plasma sample taken at t = -5 minutes in that particular rat. C1s in a concentration of 92 nM was used to assess maximal activation of C4 in NRP, and C4 activation was measured. Levels of antigenic and of functional C1-Inh were determined with the assays described above.

**Experimental groups**

The following 5 experimental groups were studied to investigate potentiation of C1-Inh and stability of C1-Inh·DXS in vivo. C1-Inh 10 mg/kg (n=4), C1-Inh 10 mg/kg complexed with DXS 3 mg/kg (n=4), DXS 3 mg/kg (n=3), C1-Inh 10 mg/kg and DXS 3 mg/kg successively administered within 2 minutes (n=3), and C1-Inh 300 mg/kg (n=3).

**Animals**

All experiments were approved by the institutional ethical committee for animal experiments according to the national regulations. Any discomfort was avoided by use of anaesthesia throughout the whole experiment. Female Wistar HsbCpb: WU rats (Harlan/CPB, Zeist, The Netherlands) weighing 200-300 g were anaesthetised by intramuscular injection of a mixture of 10 mg fluanisone and 0.2 mg fentanyl base per ml (Hypnorm, Janssen Pharmaceutica, Belgium), 0.5 ml/kg body weight and intraperitoneal injection of pentobarbital 10 mg/kg and atropine 0.05 mg/kg subcutaneously (s.c.). Anaesthesia was maintained throughout the whole experiment by s.c. injection of pentobarbital if required. The rat was fixed (back downward) on a heated support (38°C) to prevent decrease in body temperature. After opening the skin, a cannula (Silastic®) was introduced into the left carotid artery. Saline was infused at a rate of 1 ml/hour in the cannula to ensure patency. The arterial cannula was connected to a pressure transducer for continuous recording of the mean arterial blood pressure.
**Statistical analysis**

All experiments in plasma were performed at least 3 times, representative examples of each series are shown. For statistical analysis of the data the program GraphPad Prism 2.01 for Windows (GraphPad Software Inc, San Diego CA, www.graphpad.com) was used. Values in the text, figures 1, 6, and 7, and table I are expressed as the mean ± s.e.m. of n observations. Serial data were analysed with Bonferroni's Multiple Comparison test. The half-life of clearance from the circulation of active C1-Inh was analysed with an unpaired students' t-test. P-values (two-sided) of <0.05 were considered to represent a statistically significant difference.

**RESULTS**

**ELISA for activated rat C4**

Upon screening of a number of poly- and monoclonal antibodies against human C4, we noticed that sheep polyclonal anti-human C4 antibodies (SHC4; CLB) cross-reacted with rat C4. Moreover, as is illustrated in figure 1a, dilutions of rat plasma incubated with heat aggregated immunoglobulin (AHG) or C1s yielded considerably stronger responses in the assay than those of recalcified normal rat plasma (NRP++) not incubated with complement activators, indicating that the antibodies preferentially bound activated rat C4. EDTA or heat-inactivation at 56°C abrogated the increased response of NRP++ incubated with AHG in the ELISA (figure 1b). This supports the presumed specificity of the antibodies for activated rat C4. Titration curves of various samples yielded parallel responses, and, therefore, we could use the polyclonal antibodies to develop an assay for activated rat C4. Aged normal rat serum (NRA) was used as a convenient in-house standard for activated C4 because it is a stable preparation and can be prepared easily and with high reproducibility. Results were expressed as percentage of the amount of activated C4 in the standard. The intra-assay variation of the ELISA was ± 5% (n = 18) and the inter-assay variation 12% (n = 6).

**Complexes of C1-Inh*DXS**

Complexes were analysed with protein electrophoresis on 1% agarose to show that C1-Inh and DXS interact with each other. This is a non-covalent interaction. A band shift of C1-Inh occurred upon incubation with DXS. This is shown in figure 2. C1-inh complexed with DXS shows retarded migration.

**C1s inactivation assay**

A major function of C1-Inh is to inhibit C1s activity in plasma. We, therefore, decided to develop a C1s inactivation assay to monitor C1-Inh function of the C1-Inh*DXS complex. In this assay the activation of C4 in plasma upon addition of purified C1s is measured. In pilot experiments we established the amount of C1s needed by incubating NHP or NRP with increasing concentrations of C1s. As is shown in figure 3, C1s dose-
dependent generated activated C4 in NRP. This C4 activation was reduced by addition of exogenous C1-Inh. For example, at 92 and 184 nM of C1s, C4 activation by C1s was dose-dependently inhibited by exogenous human C1-Inh at concentrations of 1 to 20 U per ml (figure 3).

In further experiments the activity of C1-Inh<sup>DXS</sup> (at 1 U of C1-Inh per ml) was compared with a dose-response curve of native human C1-Inh to calculate a "potentiation" factor.

**Figure 1.** ELISA for activated rat C4. Plates coated with sheep anti-human C4 antibodies were incubated with samples. Activated C4 was detected with biotinylated anti-C4 antibodies. a) Absorption at 450 nm actually measured when plasma samples were tested; b) Amount of activated C4 in samples expressed as a percentage of the NRA standard. Samples: aged normal rat plasma (NRA), normal rat plasma (NRP-EDTA); recalcified NRP (NRP++); heat inactivated NRP (NRP++, 30°C); heat inactivated NRP incubated with aggregated human IgG (NRP++, 30°C, AHG); fresh NRP incubated with AHG in presence of EDTA (NRP, AHG); recalcified NRP incubated with AHG (NRP++, AHG); NRP incubated with 20 nM active C1s (NRP 20 nM C1s). Data represent mean and S.E.M. of 3 experiments. * P< 0.05 when compared to NRP++.
Figure 2. Protein electrophoresis on 1% agarose of C1-Inh and C1-Inh*DXS. C1-Inh complexed with DXS shows retarded migration. Lane 1, C1-Inh; lane 2, C1-Inh*DXS; lane 3, DXS.

Figure 3. C1s inactivation assay. Varying concentrations of C1s were added to NRP supplemented with 1, 10 and 20 U/ml of human C1-Inh. After incubation for 12 minutes at 37°C C4 activation in the mixtures was measured as described in Materials and Methods. This figure is a representative of 5 similar experiments.

Potentiation of C1-Inh*DXS in normal rat plasma

In initial experiments we studied the potential of both DXS and DXS to enhance the inhibitory capacity of C1-Inh, as measured in the C1s inactivation assay. The lower molecular weight DXS, tested at varying concentrations, enhanced the activity of C1-Inh up to 10 times compared to [native] human C1-Inh (data not shown). DXS enhanced C1-Inh function more strongly. At a molar ratio of 1:2 (C1-Inh: DXS) the C1s inactivating capacity of C1-Inh*DXS was comparable to that of 60 U/ml of C1-Inh (figure 4). As the C1-Inh*DXS complex was tested at a C1-Inh concentration of 1 U/ml, the calculated potentiation factor for C1-Inh*DXS was about 60. Four experiments yielded essentially similar results and the average potentiation factor was 55 with a standard deviation of 5. As the potentiation of C1-Inh by DXS in a plasma environment was 6 times higher than that by DXS, all further experiments were done with DXS.
Figure 4. Potentiation of C1-Inh by DXS<sub>15000</sub>: Human C1-Inh (either as a complex with DXS<sub>15000</sub> or without DXS) was added to NRP after which 46 nM C1s was added to activate C4. Note that the activity of 1 U/ml C1-Inh in the presence of DXS is comparable to that of 60 U/ml C1-Inh without DXS. This figure is a representative of 5 similar experiments. The standard deviation of the calculated potentiation factor was 5.

Stability of C1-Inh<sup>DXS<sub>15000</sub></sup> in NRP
To study the stability of the complex of C1-Inh<sup>DXS<sub>15000</sub></sup> in normal rat plasma we followed the course of the C1s inactivating capacity of the complex in time. The C1-Inh<sup>DXS<sub>15000</sub></sup> complex was incubated for 10 or 90 minutes in NRP and analysed in the C1s inactivation assay. Potentiation was similar after 10 and 90 minutes of incubation (figure 5). This indicates that during this period of time DXS<sub>15000</sub> did not dissociate from human C1-Inh, for example to bind to other proteins in plasma. In some pilot experiments we also tested whether the presence of blood cells could influence the stability of the complex. Also in the presence of blood cells the C1-Inh<sup>DXS<sub>15000</sub></sup> appeared to be stable (data not shown).

A similar experiment was performed to investigate the effect of preincubation of C1-Inh<sup>DXS<sub>15000</sub></sup>. C1s inactivation by preincubated C1-Inh<sup>DXS<sub>15000</sub></sup> or by separately added C1-Inh and DXS<sub>15000</sub> in either order were compared and no difference was observed (data not shown). Thus, under conditions that may mimic the in vivo situations better than NRP alone (in plasma environment in the presence of peripheral blood cells) C1-Inh<sup>DXS<sub>15000</sub></sup> complexes appeared to be stable.
Clearance of C1-Inh*DXS\textsubscript{15000} in rats

The clearance from the circulation of C1-Inh given as a complex with DXS\textsubscript{15000} by intravenous injection was similar to that of C1-Inh alone in the rats. Figure 6 shows the clearance of active C1-Inh (as measured with an end-stage assay, see Methods), whereas Table I shows the half-life of active C1-Inh and total C1-Inh antigen levels. Half-life was calculated in GraphPad Prism fitting the curve from figure 6 to a one-phase exponential decay. There was no significant difference between the half-life of C1-Inh with or without DXS\textsubscript{15000} neither for active C1-Inh nor for total antigen levels as illustrated by the high P-values in table I.

Table I

<table>
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<th>C1-Inh (n = 4)</th>
<th>C1-Inh*DXS\textsubscript{15000} (n = 4)</th>
<th>P value C1-Inh vs. C1-Inh*DXS</th>
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<tr>
<td>C1-Inh antigen</td>
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<td>195.1 ± 21.7</td>
<td>0.7031</td>
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<tr>
<td>Active C1-Inh</td>
<td>273.4 ± 29.2</td>
<td>299.2 ± 21.3</td>
<td>0.5026</td>
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Figure 6. Clearance of active human C1-Inh in Wistar rats. Wistar rats were injected with 10 mg/kg (yielding a plasma concentration of about 1 U/ml) C1-Inh with or without 3 mg/kg (≈ 5 μM) DXS_{15000}. Plasma samples were taken after various time intervals, and active C1-Inh was measured in these samples with an end-stage assay for active C1-Inh. The error bars represent the S.E.M.

Stability of C1-Inh*DXS in rats
The use of an end-stage assay for functional C1-Inh does not allow conclusions regarding the course of the enhanced activity of the C1-Inh*DXS_{15000} in the rats. Therefore, plasma samples obtained from the rats injected with the complex C1-Inh*DXS_{15000} or with C1-Inh alone, were analysed in the C1s inactivation assay. This is shown in figure 7. An enhanced activity of C1-Inh was detectable in the rats only during the first 90 minutes after administration of the complexes of C1-Inh*DXS_{15000}. In the control experiments, when only C1-Inh 10 mg/kg or only DXS_{15000} 3 mg/kg was injected, no enhanced C1s inactivation was detected in the plasma samples. C1-Inh*DXS_{15000} showed significantly enhanced C1s inactivation during the first 60 minutes (p < 0.001 compared to 10 mg/kg). At 90 minutes still some enhanced activity of C1-Inh*DXS was observed (p ~ 0.4). After 120 minutes the effect was completely abolished. The enhanced activity was observed longer when the C1-Inh*DXS_{15000} complexes were pre-incubated for 10 minutes at 37°C. When the two components were administered separately, the activity of C1-Inh was much less enhanced, and lasted not longer then 30 minutes. In another control experiment where 300 mg/kg C1-Inh was administered (~ 30 U/ml), C1s inactivation at 5 minutes after injection was comparable to that of the C1-Inh*DXS_{15000} complex, and remained more or less constant during the observation period of 3 hours. The half-life of the enhanced activity of C1-Inh*DXS_{15000} was calculated in GraphPad Prism. The C1s inactivating capacity of C1-Inh*DXS was compared to that of C1-Inh alone and assumed to be cleared according to a one-phase exponential decay. The half-life was about 60 minutes.
**Figure 7.** Activity of C1-Inh\(\times\)DXS\(\text{15000}\) in vivo in rats. Rats were injected at \(t = 0\) with C1-Inh (10 mg/kg; \(n = 4\)), preincubated C1-Inh\(\times\)DXS\(\text{15000}\) (10 mg/kg and 3 mg/kg, respectively; \(n = 4\)), DXS\(\text{15000}\) (3 mg/kg; \(n = 3\)), C1-Inh (300 mg/kg; \(n = 3\)), and C1-Inh and DXS\(\text{15000}\) (10 mg/kg and 3 mg/kg, respectively) administered separately (\(n = 3\)). The course of C1-Inh activity was assessed by measuring C1-Inh activity in the C1s inactivation assay using 92 nM of activated C1s. Results are expressed as inhibition of C4 activation, related to the activation of C4 by C1s observed in each rat in a plasma sample obtained at \(t = -5\). The error bars represent the S.E.M.

* \(P < 0.05\) compared to C1-Inh 10 mg/kg.

**DISCUSSION**

The glycosaminoglycans heparin, heparan sulphate, dermatan sulphate and the synthetic molecule dextran sulphate are able to enhance the inhibitory capacity of the serpin C1-Inh. The aim of the present study was: 1) to quantify the extent of potentiation of C1-Inh by DXS in a plasma system; and 2) to study whether C1-Inh potentiated with DXS retains its enhanced complement inhibiting capacity in vivo. To answer these questions we developed an ELISA specific for activated C4 in rats and a C1s inactivation assay to study the inhibitory capacity of C1-Inh.

Activated rat C4 was detected in a novel ELISA, in which polyclonal antibodies against human C4 were used. We found that our polyclonal antibodies mainly recognised activated C4. Presumably the native conformation of C4 (as well as C3) is dependent on an intact internal thioester bond that frequently becomes hydrolysed during purification \(^{38,39}\). Apparently such neoepitope-specific antibodies cross-react with activated rat C4 whereas most antibodies against native C4 did not. Although we do not know exactly the epitopes that are recognised, it is clear that this ELISA discriminates between native and activated C4 (figure 1a). We have used aged rat serum as a standard. In this serum probably not all of the C4 was activated, as can be seen in figure 1a and 3. Activation with high concentrations of C1s gives about 400% C4 activation compared to the NRA standard (figure 3). Aging serum is a reproducible and convenient method to produce an in-house standard that has been used before for human complement activation
The reason for the C4 conversion in NRA is probably spontaneous hydrolysis or autoactivation of C1. Considering the submaximal activation of C4 in NRA it can be extrapolated that the background activation in NRP-EDTA in our assays is in fact only 2.5% of the levels that can be generated maximally. For comparison, background activation in humans is about 1% of the total amount of C4 generated.

In the Cls inactivation assay, we were able to determine the kinetics of the inhibition by C1-Inh and C1-Inh*DXS. This new assay facilitates kinetic studies of C1-Inh in rat plasma. This is in contrast to the previously developed ELISA where biotinylated C1s is used for detection. In this ELISA active C1-Inh is measured in the same amounts both for C1-Inh and C1-Inh*DXS (figure 6). The difference between these assays is that in the ELISA for active C1-Inh complex formation is measured after a relatively long period of time. Thus the ELISA is an end-stage assay, in contrast with the Cls inactivation assay where the velocity of Cls inactivation is a major determinant of the amount of C4 activated in the short period of time. Hence, this assay represents a kinetic assay for C1-Inh activity. DXS alone at a concentration of 5 μM has in NRP only an effect that is comparable to 1 U/ml C1-Inh (data not shown). This is negligible compared to the effect of C1-Inh*DXS and for that reason we used this assay as a direct way to measure activity of human C1-Inh*DXS in rat plasma.

We determined that the Cls inhibitory capacity of 1U/ml of C1-Inh*DXS was equal to the inhibitory capacity of ~55 U/ml of C1-Inh. For DXS we observed a lower potentiation factor of ~10 times (data not shown). A 76-fold increase in the second-order rate constant of C1-Inh and Cls has been previously described in the presence of DXS concentrations in the same range as we used. This analysis was performed in a system with purified proteins whereas we used a plasma system. We assume that interaction of DXS with other plasma proteins accounted for the observed difference between the purified system and the plasma system. Although the inhibitory capacity in plasma was enhanced less than described for a purified system, a potentiation factor of 55 times still implies that ~55 times less C1-Inh would be required to obtain the same inhibition of classical pathway complement activation when C1-Inh*DXS is used in stead of C1-Inh alone.

To investigate whether C1-Inh*DXS retained its enhanced complement inhibiting capacity in vivo we studied the stability of C1-Inh*DXS. Injection of C1-Inh*DXS showed significantly enhanced complement inhibiting capacity only for 60 minutes after injection (figure 7) when compared to C1-Inh alone. Since no difference in the clearance of C1-Inh with and without DXS could be observed, we infer that the non-covalently linked complex is unstable in vivo. Since C1-Inh*DXS was stable in a plasma system and in a whole-blood system, we assume that DXS binds to components outside the plasma compartment or is rapidly cleared from the circulation. The latter is very likely because an initial plasma half-life of 30 minutes has been described before for 3H DXS in rats. The fact that preincubation enhanced and extended the potentiation of C1-Inh in vivo (figure 7) supports the hypothesis that the complex is unstable in vivo. The half-life of dissociation of preformed complexes was estimated to be ~60 minutes. This correlates with the observed enhanced inhibitory capacity of about 60 minutes. Several studies report on the beneficial effect of GAG administration in ischemia...
We show that the possible C1-Inh potentiating effects in these conditions are only effective during a short period of time. This could mean that in ischemia reperfusion complement activation occurs in a short period of time or that the positive effect of GAG is due to another mechanism.

Some pilot studies in the rats were also performed using DXS_{5000} in stead of DXS_{15000}. Plasma samples were taken after 5, 60, 90 and 120 minutes and analysed in the C1s inactivation assay. Enhanced inhibitory capacity of pre-incubated complexes was observed ex vivo only in the plasma samples taken after 5 minutes and completely abolished after 60 minutes (data not shown). This indicates that a complex of C1-Inh^DXS_{5000} is less stable than a complex with DXS_{15000}. We suppose that the lower molecular weight of DXS_{5000} and hence the smaller chance of several negatively charged groups to interact effectively with C1-Inh account for this difference. The most likely solution to increase the stability of a complex of C1-Inh and DXS in vivo would be the production of a covalent complex of C1-Inh and DXS. However, this approach has the disadvantage that it requires a complete analysis of the toxicity of this covalent complex. Besides, the clearance of the covalent complex might be enhanced because the plasma half-life of a covalent complex of low molecular weight heparin and antithrombin III (ATIII) in rabbits was shorter than of ATIII alone.

An important issue in possible application of a complex of C1-Inh and DXS is the toxicity of dextran sulphate. Low molecular weight DXS (DXS_{5000} and DXS_{10000}) did not induce contact activation in plasma, nor induce auto activation of factor XII, whereas high molecular weight DXS (DXS_{15000} and DXS_{20000}) did induce both. It is known that doses of 4.8 mg/kg/day of DXS_{15000-40000} administered to cats are subtoxic while 24 mg/kg/day is toxic. Toxicity studies in humans were performed with DXS_{10000} in HIV infected patients that were given 45 mg/h for 10-12 days. Patients who received DXS for more than 3 days developed severe thrombocytopenia whereas side-effects after a short DXS treatment were not especially investigated but also not detected in 2 investigated patients. Taken together this indicates that the DXS doses that are required for C1-Inh potentiation might be non-toxic.

It is known for human C1-Inh that addition of DXS in plasma enhances the activity of C1-Inh. We investigated the enhancement of rat C1-Inh in NRP in a control experiment (figure 3). DXS alone at the concentrations used in this study was able to enhance human C1-Inh strongly but rat C1-Inh only slightly and comparable to the effect of 1 U/ml C1-Inh added. Higher concentrations of DXS, however, could enhance the activity of rat C1-Inh which suggests that rat C1-Inh has a lower affinity for DXS. We took advantage of this property; since administration of DXS alone hardly affected rat C1-Inh function, the observed effects of DXS in combination with human C1-Inh were attributed to interaction with the latter. The reason for this difference in affinity of rat and human C1-Inh for DXS remains to be elucidated. It might implicate that in humans administration of DXS alone can already enhance complement inhibition. We have shown here however, that preincubation of C1-Inh and DXS enhanced the effect and increased the stability of C1-Inh^DXS in vivo. Therefore administration of the complex is probably a more powerful method to inhibit complement activation than administration of DXS alone.
We conclude that DXS is the preferable agent to enhance inhibitory capacity of C1-Inh in plasma and in vivo. It enhances the inhibitory capacity of C1-Inh 55 times in NRP and about 30 times in vivo. The complex is, however, unstable in vivo, probably due to fast clearance of DXS. This results in a short-term enhancement of the inhibitory capacity of C1-Inh. The combination of C1-Inh-DXS may well reduce the pathophysiological effects in diseases with excessive activation of the classical pathway of complement such as sepsis, acute myocardial infarction, after bone marrow transplantation, and even in acute attacks of hereditary angio-edema. This remains to be investigated. Especially the short duration of the effect of DXS on human C1-Inh may be beneficial since it will only lead only temporarily to impaired defence against microorganisms.

ACKNOWLEDGEMENTS
We thank Frits van Oost for technical assistance.

REFERENCE LIST


