The Structural Motif in Chondroitin Sulfate for Adhesion of Plasmodium falciparum-infected Erythrocytes Comprises Disaccharide Units of 4-O-Sulfated and Non-sulfated N-Acetylgalactosamine Linked to Glucuronic Acid*

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An important characteristic of malaria parasite Plasmodium falciparum-infected red blood cells (IRBCs) is their ability to adhere to host endothelial cells and accumulate in various organs. Sequestration of IRBCs in the placenta, associated with excess perinatal and maternal mortality, is mediated in part by adhesion of parasites to the glycosaminoglycan chondroitin sulfate A (CSA) present on syncytiotrophoblasts lining the placental blood spaces. To define key structural features for parasite interactions, we isolated from CSA oligosaccharide fractions and established by electrospray mass spectrometry and high performance liquid chromatography disaccharide composition analysis their differing chain length, sulfate content, and sulfation pattern. Testing these defined oligosaccharide fragments for their ability to inhibit IRBC adhesion to immobilized CSA revealed the importance of non-sulfated disaccharide units in combination with 4-O-sulfated disaccharides for interaction with IRBCs. Selective removal of 6-O-sulfates from oligo- and polysaccharides to increase the proportion of non-sulfated disaccharides enhanced activity, indicating that 6-O-sulfation interferes with the interaction of CSA with IRBCs. Dodecasaccharides with four or five 4-O-sulfated and two or one non-sulfated disaccharide units, respectively, comprise the minimum chain length for effective interaction with IRBCs. Comparison of the activities of CSA and CSB oligo- and polysaccharides with a similar sulfation pattern and content achieved from partial desulfation demonstrated that glucuronic acid rather than iduronic acid residues are important for IRBC binding.

An important pathological characteristic of red blood cells infected by the malaria parasite Plasmodium falciparum is their ability to adhere to host endothelial cells and accumulate in various organs. During pregnancy, the accumulation of infected red blood cells (IRBCs) in the placenta is a key feature of infection and is associated with adverse outcomes and excess perinatal and maternal mortality (1, 2). Several studies (3–6) in the past few years suggest that sequestration of IRBCs in the placenta is mediated in part by adhesion of parasites to the glycosaminoglycan chondroitin sulfate A (CSA) present on syncytiotrophoblasts lining the placental blood spaces. Recently we have reported that hyaluronic acid (HA) can also support the adhesion of IRBCs in vitro and appears to be another receptor for parasite sequestration in the placenta (7, 8). HA is non-sulfated and is the simplest member of the glycosaminoglycan family. Because there are thought to be no other modifications in the carbohydrate chain, the structural requirement of HA for interaction with IRBC can be readily deduced, and the major feature is believed to be a minimum chain length of 12 monosaccharide residues (7, 8). However, understanding the molecular interactions involved between CSA and IRBC is more difficult due to the greater complexity of chondroitin sulfate (CS) chains.

CS chains comprise repeating disaccharide units of 4-O-substituted hexuronic acid (HexA) β1-3-linked to N-acetylgalactosamine (GalNAc), i.e. 4HexAβ1–3GalNAc. CS chains show heterogeneity in sulfation patterns and hexuronic acid compositions (glucuronic acid (GlcUA)/iduronic acid (IdoUA)) due to different sulfate content and substitution and differing degrees of isomerization of GlcUA to IdoUA. Typically, GalNAc is mono-O-sulfated at either the 4- or the 6-O-position, and this differentiates the principal CSA and CSC disaccharide units, respectively. CSB (or dermatan sulfate) is similar in sulfation to CSA, but the hexuronic acid is predominantly IdoUA. In addition, other variations in sulfation pattern frequently occur, such as GalNAc being non-sulfated or 4,6-di-O-sulfated, and GlcUA can be 2-O-sulfated. Several studies demonstrate the importance of 4-O-sulfation for binding of IRBC (3, 9–11). Parasite adhesion to purified or cell surface CSA can be effectively inhibited by CSA but not by CSB, CSC, heparin, or desulfated chondroitin (3, 9–11). We previously defined the minimum chain length of CSA required for parasite adhesion to be 12–14 monosaccharide residues and identified that undersulfation was a structural feature of the active CSA, and oversulfation was a feature of the inactive CSC (10). This suggested that non-sulfated disaccharide units may also be important elements of the structural motif required for parasite adhesion.

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† The abbreviations used are: IRBC, P. falciparum-infected red blood cells; CS, chondroitin sulfate; HA, hyaluronic acid; HexA, hexuronic acid; GlcUA, glucuronic acid; IdoUA, iduronic acid; GalNAc, N-acetylgalactosamine; SAX, strong anion exchange; APS, aminopropyl silica; ES-MS, electrospray mass spectrometry; 0S, ΔUA-GalNAc; 4S, ΔUA-GalNAc(4S); 6S, ΔUA-GalNAc(6S); HexA, hexuronic acid; HPLC, high performance liquid chromatography.

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in addition to 4-O-sulfate groups of GalNAc.

To further define key structural features that influence adhesion of IRBCs, we have isolated and characterized a range of variously sized oligosaccharide fragments from CSA polysaccharides, including fully sulfated components and undersulfated minor components, and tested their ability to competitively inhibit parasite adhesion to CSA. Selective desulfation of oligo- and polysaccharides was performed to directly compare the influence of 6-O-sulfated disaccharides and nonsulfated units on adhesion, and undersulfated CSB oligo- and polysaccharides were prepared to evaluate the relative importance of the form of hexuronic acid (GlUA or IdoUA).

**EXPERIMENTAL PROCEDURES**

**Preparation and Fractionation of CS Oligosaccharide Fragments—** Chondroitin sulfate A (from bovine trachea, Sigma; average molecular mass: 45,500 kDa, based on a single angle laser light scattering), B (from porcine intestinal muosus, Sigma), and C (from shark cartilage, Sigma) were partially depolymerized by controlled digestion with chondroitin lyase ABC (EC 4.2.2.4, from Proteus vulgaris, Sigma) essentially as described previously (12, 13). In brief, 1 g of CSA was incubated with 2 units of lyase. Reaction products were desalted on a short G-10 column and fractionated on a Bio-Gel P-6 column (1.6 x 90 cm) with elution by ammonium sulfate (0.1 M) at a flow rate of 1 ml/min. The major fractions were fractionated in a similar manner.

**De-6-O-sulfation of CS Poly- and Oligosaccharides—** De-6-O-sulfation of CS was accomplished with a linear gradient of NaCl (solvent A, 0.2M NaCl; solvent B, 1.5 M NaCl; pH 3.5). The deca-, dodeca-, and tetradecasaccharide fractions were partially depolymerized by controlled digestion with chondroitin lyase ABC (EC 4.2.2.4, from Proteus vulgaris, Sigma) essentially as described previously (12, 13). In brief, 1 g of CSA was incubated with 2 units of lyase. Reaction products were desalted on a short G-10 column and fractionated on a Bio-Gel P-6 column (1.6 x 90 cm) with elution by ammonium sulfate (0.1 M) at a flow rate of 1 ml/min. The major fractions were desalted and detected by electrospary mass spectrometry (ES-MS) to be the di- to eicosasaccharide fragments. CSB oligosaccharide fragments were similarly prepared but on a smaller scale (100 mg of polysaccharide and 0.5 units of chondroitin lyase ABC in 4 ml of solution).

**CSA decarboxylation—** CSA decarboxylation fraction F8C was further fractionated on a short cartrige column (Hitrap Q-Sepharose HP, 1 ml, Amersham Biosciences) of strong anion exchange (SAX) with detection at UV 232 nm. Elution was carried out with a linear gradient of NaCl (solvent A, 0.2 M NaCl; solvent B, 1.5 M NaCl; pH 3.5). The deca-, decade-, and tetradeaccharide fractions were chromatographed on a gradient of 0–40% B in 20 min, whereas the hexadecasaccharide fraction was from 10 to 50% B in 20 min at a flow rate of 1 ml/min. The subfractions were collected and desalted on a short G-10 column before lyophilization. CSB tetradecasaccharides were fractionated in a similar manner.

The undersulfated CSA decodexacaccharide fraction F8C was further fractionated by SAX-HPLC on a Spherisorb SS-SAX column (4.6 x 250 mm, Waters, Milford, MA). A gradient of NaCl (solvent A and B as above) was 60% B in 20 min, was set at a flow rate of 1 ml/min with detection at UV 232 nm. The SAX-HPLC fractions were desalted and freeze-dried before further fractionation by normal phase HPLC on an aminoethyl silica (APS) column (APS-2, 4.6 x 250 mm, Hypersil, Runcorn, England). A linear gradient of NaH2PO4 (solvent A, 0.1 M; and solvent B, 1.0 M; 30–50% B in 40 min) was used to elute SAX-HPLC fractions F8C-2 and F8C-3. Identification of components present in each subfractions was carried out by ES-MS, and quantitation was based on the band area for glucuronic acid content using p-glucuronol-6,3-lactone as a standard (14).

**De-6-O-sulfation of CS Poly- and Oligosaccharides—** De-6-O-sulfation of CS was essentially as described (15) but with some modifications. CS polysaccharides (5 mg) or oligosaccharides (200 µg) were converted into their pyridinium salts by passing through a short column of cation exchange resin (AG50W-X8, H-form) and washing with H2O. The eluents were added to a small amount of pyridine and freeze-dried. To the residue of the ammonium salt of the polysaccharide was added 1 ml of anhydrous pyridine and 200 µl of N,O-bis(trimethylsilyl)acetamide (BSTA). For oligosaccharides, 100 µl of pyridine and 20 µl of BSTA were used. The mixture was heated at 80°C for 4 h. After cooling, the excess reagent and the silyl ester were decomposed by the addition of H2O. The bulk of liquid was evaporated under reduced pressure. The residual trimethylsilyl groups were hydrolyzed in a 2.5% NH3·H2O solution and removed essentially as described (16). 2 De-C6-O-sulfated CSA decasaccharide fraction (dF6) and the subfractions derived from it, dF6A to dF6G containing 0 to 6 sulfates, respectively, were obtained essentially as described above for CSA polysaccharide for CSA oligosaccharides.

**Partial De-4-O-Sulfation of CSB Polysaccharides—** CSA polysaccharide was converted into the pyridinium salt as described above. The pyridinium salt was dissolved in 90% Me2SO in H2O and heated to 80°C (17). The reaction was stopped at 40, 70-, and 100-min intervals (products designated as CSB dE-1, -2, and -3, respectively) by cooling to room temperature and neutralized with dilute NaOH solution. The mixture was freeze-dried and co-evaporated with H2O three times. HPLC disaccharide composition analysis found that 30.1, 45.6, and 50% of sulfates were removed for CSB dE-1, -2, and -3, respectively.

**Electrospray Mass Spectrometry—** Electrospray mass spectrometry was carried out on a Micromass Q-Tof mass spectrometer (Micromass UK Ltd, Manchester, England) in the negative-ion mode (18). A cone voltage of 20 volts was used, and the capillary voltage was kept at 4000 volts. The solvent, acetonitrile, 0.5 mm NH4HCO3 1:1, was delivered into the electrospray source by a syringe pump at a flow rate of 5 µl/min. Nitrogen was used as the nebulizing and desolvation gas. Source temperature was maintained at 80°C, and desolvation temperature was maintained at 150°C. Sample solution (5 µl) typically at a concentration of 10–20 pmol/µl, was injected using a flow injector. The raw data were processed and transformed into mass values using the MassLynx data system (Micromass UK Ltd) to derive the compositions of oligosaccharide fragments in terms of GalNAc, HexA, and sulfate.

**Disaccharide Composition Analysis—** Analysis for disaccharide composition was essentially as described previously (13). Typically, polysaccharide (50 µg) or oligosaccharide (10 µg) was dissolved in 5 mM sodium phosphate, pH 7.0, containing 0.2 M NaCl and digested exhaustively at 37°C overnight with chondroitin ABC lyase (2 µl, 5 milliunits in the same phosphate buffer). The disaccharides were collected by SAX-HPLC. A gradient of NaCl (solvent A and B as above), 0–80% B in 45 min was used for the elution of disaccharides at a flow rate of 1 ml/min with detection at UV 232 nm.

**Parasitized Erythrocytes—** P. falciparum was cultured in group O red blood cells suspended in RPMI-HEPES medium at pH 7.4 supplemented with hypoxanthine (50 µg/ml), NaHCO3 (25 mM), gentamicin (25 µg/ml), and 10% human serum (v/v) in an atmosphere of 1% O2, 4% CO2, and 95% N2, as previously described (5). Parasite cultures were synchronised weekly by sorbitol lysis (19).

**Cytodherence Assays—** Assays were performed using P. falciparum trophozoite-infected erythrocytes at a parasitemia of 3–7% and hematocrit of 1% in 150-mm diameter plastic Petri dishes (Falcon 1058; BD PharMingen) as described (5, 10). Receptors used were CSA from bovine trachea (Sigma) and CD36 purified from platelets (a gift of M. Berndt, Baker Medical Research Institute, Melbourne, Australia). Parasites from culture, suspended in RPMI-HEPES containing 10% pooled human serum, pH 6.9, were incubated with inhibitors or control (phosphate-buffered saline) for 10 min at room temperature before testing adhesion. Samples were randomized and coded, and cytodherence assays were carried out blinded.

**RESULTS**

**Minor Subfractions of CSA** 12-mer, 14-mer, and 16-mer Fragments Are the Most Effective Inhibitors of IRBC Adhesion to CSA—Subfractions derived from the decar-, dedeca-, tetra-, and hexadecasaccharide fractions (Fig. 1) were tested at a concentration of 50 µg/ml for inhibition of IRBC adhesion to CSA. None of the decasaccharide subfractions (F5A to F5D) effectively inhibited adhesion (Fig. 2e). Dodecasaccharide subfractions F6C to F6F (Fig. 2b), tetradecasaccharide subfractions F7B to F7E (Fig. 2c), and hexadecasaccharide subfractions (F8B to F8G) (Fig. 2d) substantially inhibited adhesion at this concentration.

To identify differences in inhibitory activity among these active subfractions, they were further tested at reduced concentrations. At 10 µg/ml (ranging from 3.1 to 4.9 nmol/ml for F6A to F6F), the minor subfraction F6C was clearly the most effective inhibitor of adhesion among the dodecasaccharide subfractions (Fig. 2b), being more active than the parent fraction F6. A similar pattern of inhibitory activity was observed among larger oligosaccharides, with the subfractions F7C,
F7D, and F7E derived from tetradecasaccharides and F8C, F8D, and F8E from hexadecasaccharides the most inhibitory when re-tested at 50 μg/ml (ranging from 1.4 to 2.3 nmol/ml for F7B to F7G) and 2 μg/ml (ranging from 0.5 to 0.6 nmol/ml for F8B to F8G), respectively. In both cases, minor oligosaccharide subfractions were the most active and inhibited adhesion to a greater degree than the parent oligosaccharide fraction or CSA polysaccharide. The inhibitory activity of the subfractions increased with increasing chain length; the minimum concentration of hexadecasaccharide subfraction F8C required for near complete inhibition of adhesion was severalfold lower than that for the corresponding dodecasaccharide subfraction F6C.

The Most Inhibitory Subfractions Are Undersulfated—Analysis of oligosaccharide subfractions by negative-ion ES-MS (Table I) showed that the major subfractions of each Bio-Gel P-6 fractions (F5D, F6E, F7F, and F8F) were mainly the fully sulfated CSA oligosaccharides comprising one sulfate per disaccharide unit. The subfractions eluting after the major subfractions (F6F, F7G, and F8G) were mainly the fully sulfated higher oligomers (Table I) that resulted from incomplete size fractionation on size exclusion chromatography. The earlier-eluting subfractions (Fig. 1) were the undersulfated analogues.
Homogeneous fractions were used for inhibition, the CSA tetradecasaccharide fraction was found to contain the minimum chain length able to almost completely inhibit binding, whereas the decasaccharide fraction gave only minor inhibition. Dodecasaccharides exhibited intermediate inhibition. As shown above, inhibition of IRBC binding to CSA is not only influenced by oligosaccharide chain length but also by sulfate content. It is important to compare activities of oligosaccharides of different chain lengths with the same degree of sulfation. At 10 μg/ml, the dodecasaccharide fraction only gave minor inhibition (~30%), but the subfraction F6C containing mainly two-sulfated 12-mer (Table I) showed significant inhibition (>80%, Fig. 2b). Similarly, 14-mer and 16-mer subfractions F7D and F8D, which contained mainly two-undersulfated components, showed significant inhibition even at a concentration of 5 and 2 μg/ml (Fig. 2, c and d), respectively. However, with the same degree of undersulfation, the 10-mer subfraction F5B did not show any inhibitory activity even at the higher concentration of 50 μg/ml (Fig. 1a).

A similar pattern of activity was observed with one-undersulfated subfractions. The 14- and 16-mer subfractions F7E and F8E effectively inhibited adhesion (Fig. 2, c and d), whereas the 12-mer subfraction F6D showed moderate activity (Fig. 2b). The 10-mer subfraction F5C lacked any inhibitory effect. These data clearly demonstrated that CSA fragments with 12 monosaccharide residues and an appropriate sulfation pattern comprise the minimum chain length required for activity.

6-O-Sulfate Interferes with Interaction—CSA and CSC polysaccharides were selectively desulfated at the 6-O-position of GalNAc residues. Upon re-testing for inhibition of parasite adhesion to CSA, de-6-O-sulfated CSA polysaccharide showed an increase in its inhibitory activity (Fig. 3, a and c), whereas that of de-6-O-sulfated CSC increased to a lesser extent (Fig. 3a). The reason for the difference in the increases of activity may be attributed to the difference in the contents of the dodecasaccharide units 0S and 4S in these desulfated polysaccharides and/or in the sequence arrangement of the 0S and 4S disaccharides. The de-6-O-sulfated CSA contained 45.3% 0S and 54.7% 4S, whereas the de-6-O-sulfated contained CSC 74.5% 0S and 15.9% 4S (Table II). In addition, the presence of a significant amount (7.8%) of dodecasaccharide unit 2S (ΔUA(2S)1–3GalNAc) in the de-6-O-sulfated CSC may also have a negative effect on inhibition.

Two CSA 14-mer subfractions, the more inhibitory F7D and the less active F7F, were also submitted to regioselective removal of 6-O-sulfates (Table II). De-6-O-sulfation substantially increased the inhibitory activity of the less active subfraction F7F but only slightly increased the activity of F7D (Fig. 3, b and c), suggesting that further removal of 6-O-sulfate groups does not increase the abundance of inhibitory motifs in this fraction. Interestingly, de-6-O-sulfated F7F was more inhibitory than the de-6-O-sulfated F7D, which again may be accounted for by the higher ratio of dodecasaccharide units 4S (55.0%) to 0S (44.6%) in the de-6-O-sulfated F7F subfraction.

Dodecasaccharides with Four or Five 4-O-Sulfates Are the Most Active—To gain further information on the effect of sulfation pattern without the influence of 6-O-sulfate, de-6-O-sulfated CSA was partially depolymerized, and the dodecasaccharide fraction was isolated. Further group separation by anion-exchange chromatography gave seven subfractions, dF6A to dF6G, containing mainly dodecasaccharides (except dF6A) with zero to six 4-O-sulfates, respectively, as judged by ES-MS analysis (Table III). Inhibition of IRBC adhesion to CSA by the subfractions was tested at various concentrations (Fig.

### Table I

| Subfractions | Activity  | Found | Calculated | Assignment |
|--------------|-----------|-------|------------|------------|
| F5 (10-mer)  | A         | 1676.8| 1676.4     | 8-mer 2S   |
|              | B         | 1852.8| 1852.4     | 9-mer 2S   |
|              | C         | 1756.8| 1756.3     | 8-mer 3S   |
|              | D         | 2215.5| 2215.4     | 10-mer 4S  |
|              | E         | 2012.4| 2012.3     | 9-mer 4S   |
|              | F         | 2295.6| 2295.3     | 10-mer 5S  |
| F6 (12-mer)  | A         | 2055.8| 2055.4     | 10-mer 2S  |
|              | B         | 1852.6| 1852.4     | 9-mer 2S   |
|              | C         | 1922.5| 1923.2     | 10-mer 3S  |
|              | D         | 2595.0| 2594.5     | 12-mer 4S  |
|              | E         | 2216.0| 2215.4     | 10-mer 4S  |
|              | F         | 2675.0| 2674.5     | 12-mer 5S  |
|              | G         | 2254.8| 2254.4     | 12-mer 6S  |
| F7 (14-mer)  | B         | 2135.9| 2135.4     | 10-mer 3S  |
|              | C         | 1922.9| 1932.3     | 9-mer 3S   |
|              | D         | 3054.9| 3053.6     | 14-mer 5S  |
|              | E         | 2754.8| 2754.4     | 12-mer 6S  |
|              | F         | 3123.9| 3123.5     | 14-mer 7S  |
|              | G         | 3672.8| 3672.5     | 16-mer 8S  |
| F8 (16-mer)  | B         | 3351.8| 3352.7     | 16-mer 4S  |
|              | C         | 3150.9| 3149.6     | 15-mer 4S  |
|              | D         | 3564.9| 3563.6     | 16-mer 5S  |
|              | E         | 3213.5| 3213.5     | 14-mer 7S  |
|              | F         | 3214.0| 3213.5     | 14-mer 7S  |
|              | G         | 3672.8| 3672.5     | 16-mer 8S  |

- Inhibitory activity measured at different concentrations for each group of samples 50 μg/ml for the 10-mer, 10 μg/ml for the 12-mer, 5 μg/ml for the 14-mer and 2 μg/ml for the 16-mer subfractions). A + sign indicates >50%, ++ indicates >80%, whereas – indicates <40% inhibitory effect.
- Main subfractions.

Together with some lower oligomers. The most active dodecasaccharide subfraction F6C contained mainly 12-mer with 4 sulfates and also 10-mer with 4 sulfates at a lower concentration (Table I). The most effective inhibitors in the tetradecasaccharide subfractions F7E and F7D were 14-mer with 6 and 5 sulfates, respectively, whereas F7C was analogous to F6C, and its major component was a 12-mer with 4 sulfates. Similarly, the most active hexadecasaccharide subfractions F8E, F8D, and F8C all contained undersulfated 16-mers with 7, 6, and 5 sulfates, respectively. Clearly, undersulfation is a major structural feature of the active CSA oligosaccharide fragments in addition to 4-O-sulfation.

HPLC disaccharide composition analysis (Table II) of selected poly- and oligosaccharides is in agreement with this conclusion. The difference in composition between CSA and CSC polysaccharides was not only the content of 4-O-sulfate and 6-O-sulfate but also the degree of sulfation. CSA contained more than 5% non-sulfated disaccharide units (0S) compared with less than 1% in CSC. For CSA tetradecasaccharides, the more active subfraction F7D contained 13.3% 0S, and the less active F7F contained only 1.3% 0S, although their 8S contents were similar.

Twelve Monosaccharide Residues Comprise the Minimum Chain Length Required for Activity—When each of the size
was shown to contain no IdoUA residues when analyzed by 1H NMR (Ref. 23; data not shown). CSB, which contains IdoUA rather than GlcUA, did not substantially inhibit parasite adhesion to CSA (Fig. 5) despite being predominantly 4-O-sulfated (Table II) as is CSA. However, a lower concentration of disaccharide unit 0S was present in CSB than CSA (Table II), and it was therefore not clear if either the presence of IdoUA or the lower degree of undersulfation accounted for the lower inhibitory activity of CSB.

To examine the possible influence of uronic acid on parasite adhesion, CSB tetradecasaccharide fraction F7 was fractionated according to sulfate content, and the inhibitory activities of subfractions F7C, F7D, and F7E were compared with the parent polysaccharide and CSA (Fig. 5a). All the subfractions were relatively non-inhibitory at 5 μg/ml, whereas the undersulfated CSA 14-mers were strongly inhibitory at the same concentration. At a higher concentration of 50 μg/ml CSB, subfraction F7C, which contained 5.3% of 0S, was significantly more inhibitory than the corresponding tetradecasaccharide fraction F7F, which contained 1.3% of 0S (Table II), showed considerable inhibition (82.6%), whereas CSA inhibited adhesion by 90%.

Table II

|              | 0S  | 2S  | 4S  | 6S  | 26DiS | 46DiS | 24DiS |
|--------------|-----|-----|-----|-----|-------|-------|-------|
| CSA          | 5.3 | -   | 55.0| 39.7| -     | -     | -     |
| CSB          | 0.5 | -   | 85.6| 4.8 | -     | 6.4   | 2.3   |
| CSC          | 0.8 | -   | 15.5| 76.7| 7.0   | 0.5   | -     |
| CSA de6S     | 45.3| -   | 54.7| <0.5| -     | -     | -     |
| CSA de6S     | 74.5| 7.8 | 15.9| 1.8 | -     | -     | -     |
| CSB deS-1    | 30.1| 2.1 | 64.7| 3.1 | -     | <0.5  | -     |
| CSB deS-2    | 45.6| 2.2 | 50.1| 2.2 | -     | <0.5  | -     |
| CSB deS-3    | 50.8| 2.0 | 45.2| 1.9 | -     | -     | -     |
| CSA F7F      | 1.3 | -   | 54.9| 43.7| -     | -     | -     |
| CSA F7F de6S | 44.6| -   | 55.0| 0.4 | -     | -     | -     |
| CSA F7D      | 13.3| -   | 45.6| 41.1| -     | -     | -     |
| CSA F7D de6S | 53.5| -   | 44.9| 1.6 | -     | -     | -     |
| CSB F7C      | 5.3 | -   | 88.4| 6.2 | -     | -     | -     |
| CSB F7D      | 1.9 | -   | 93.1| 4.9 | -     | -     | -     |
| CSA F7E      | 0.3 | -   | 94.1| 4.9 | -     | 0.4   | -     |
| CSA F7C-3c   | 11.6| -   | 41.6| 46.8| -     | -     | -     |
| CSA F7C-3d   | 14.3| -   | 43.4| 42.3| -     | -     | -     |
| CSA F7C-3e   | 17.0| -   | 45.5| 37.5| -     | -     | -     |

4) and clearly demonstrated that CS 12-mers with four or five 4-O-sulfates (subfractions dF6E and dF6F, respectively) were the most effective inhibitors. The non-sulfated subfraction (dF6A) and those with one or two 4-O-sulfates (dF6B and dF6C, respectively) were only partially inhibitory at the maximum concentration tested (50 μg/ml). The fully sulfated and the trisulfated subfractions (dF6G and dF6D, respectively) were intermediate in effect. The major component of subfraction dF6G was fully sulfated 12-mer; however, 1-undersulfated 12-mers were also present, and these would be expected to have contributed to its inhibitory activity.

GlcUA Is Required for Inhibitory Activity—The oligosaccharide inhibitors were prepared from CSA polysaccharide that was shown to contain no IdoUA residues when analyzed by 1H NMR (Ref. 23; data not shown). CSB, which contains IdoUA rather than GlcUA, did not substantially inhibit parasite adhesion to CSA (Fig. 5) despite being predominantly 4-O-sulfated (Table II) as is CSA. However, a lower concentration of disaccharide unit 0S was present in CSB than CSA (Table II), and it was therefore not clear if either the presence of IdoUA or the lower degree of undersulfation accounted for the lower inhibitory activity of CSB.

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De-6-O-sulfated CSA (0S, 45.3%; 4S, 54.7%) almost completely inhibited parasite adhesion and had a very similar composition to CSB deS-2 and deS-3, which were not inhibitory. These experiments clearly demonstrate that GlcUA is required for...
maximum inhibitory activity.

HPLC-fractionated Under sulfated 12-mer Fragments Differ in Activity—To investigate if a specific undersulfated sequence is required for adhesion to IRBCs, the most active CSA dodecasaccharide subfraction F6C, containing mainly 2-undersulfated 12-mers, was further fractionated by HPLC. Incomplete separation was obtained with a SAX column (Fig. 6a), and further chromatography of fractions F6C-2 and F6C-3 was then carried out on an APS column (Fig. 6b and c). Due to the high degree of complexity and despite two different HPLC columns being used sequentially, the final fractions still contained mixed components. Fractions with sufficient amounts of oligosaccharides were then tested for inhibitory activity at 10 and 20 μg/ml. F6C-3e was clearly the most inhibitory at both concentrations, with the other fractions demonstrating only a modest inhibitory effect (Fig. 7). Disaccharide composition analysis (Table II) revealed a similar content of 4S disaccharide unit among the different fractions, but the most active subfraction F6C-3e had the highest proportion of non-sulfated OS and a correspondingly lower proportion of 6S units. This reaffirmed that undersulfation together with 4-O-sulfation has an important influence on IRBC adhesion. However, it has not yet been possible to determine the sequence order of disaccharide units in each oligosaccharide fragment in fractions to derive a key sequence determinant(s) for activity.

Discussion

The importance of 4-O-sulfation of CS for the adhesive interaction with P. falciparum-IRBCs has been previously demonstrated by comparing the activity of CSA, CSB, and CSC polysaccharides, CSA and CSC oligosaccharide mixtures, and desulfated chondroitin (3, 9–11). However, because of the heterogeneous composition of CS chains, particularly regarding the sulfate content and sulfation pattern, it remained possible that other structural features could influence the interaction. The present study indicates that the minimum motif of CSA for interaction with IRBCs is a sequence of 12 monosaccharide residues formed by 4-O-sulfated and non-sulfated disaccharide units, that 6-O-sulfation interferes with the interaction, and that the form of the uronic acid residues is important, with GlcUA being required for high inhibitory activity.

Initial testing of size-homogenous fractions isolated from CSA digests indicated the presence of inhibitory sequences among the 12-mer and higher oligosaccharide fractions (10). Fractionation, on the basis of sulfate content, of different-sized oligosaccharide fragments together with determination of sulfate content and sulfation pattern by ES-MS and HPLC disaccharide-composition analysis revealed the importance of non-sulfated disaccharide units for the interaction with IRBCs. Among the subfractions tested for inhibitory activity, clearly 1- and 2-undersulfated oligosaccharides of 12 monosaccharide residues or larger exhibited substantial inhibitory activity. Higher degrees of undersulfation were not detected in oligosaccharide fragments from bovine trachea CSA.

The importance of non-sulfated disaccharides in forming a part of the parasite adhesive motif was clearly confirmed by selective removal of 6-O-sulfates to increase the proportion of 0S disaccharide units. This increased the inhibitory activity of CSA polysaccharides and oligosaccharides. Inhibition experiments of dodecasaccharide subfractions derived from de-6-O-sulfated CSA further supported the conclusion that a combination of 4-O-sulfated and non-sulfated disaccharide units is important, the most active oligosaccharides being among the one- and two-undersulfated oligosaccharide sequences.

![Fig. 4. Comparison of inhibitory activities of dodecasaccharide subfractions derived from de-6-O-sulfated CSA.](http://www.jbc.org/)

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**Table III**

| Subfractions | Found    | Calculated | Assignment |
|--------------|----------|------------|------------|
| dF6A         | 2117.4   | 2116.6     | 11-mer/0S  |
| dF6B         | 1896.1   | 1895.6     | 10-mer/0S  |
| dF6C         | 2275.4   | 2274.7     | 12-mer/0S  |
| dF6D         | 2355.4   | 2354.6     | 12-mer/1S  |
| dF6E         | 2277.3   | 2276.6     | 11-mer/2S  |
| dF6F         | 2152.3   | 2151.5     | 11-mer/1S  |
| dF6G         | 2435.3   | 2434.6     | 12-mer/2S  |
|              | 2321.2   | 2320.5     | 11-mer/2S  |
|              | 2277.3   | 2276.6     | 11-mer/2S  |
|              | 2515.1   | 2514.5     | 12-mer/3S  |
|              | 2312.0   | 2311.5     | 11-mer/3S  |
|              | 2691.1   | 2690.6     | 13-mer/3S  |
|              | 2595.0   | 2594.5     | 12-mer/4S  |
|              | 2391.9   | 2391.4     | 11-mer/4S  |
|              | 2770.0   | 2770.5     | 13-mer/4S  |
|              | 2674.9   | 2674.5     | 12-mer/5S  |
|              | 3054.1   | 3053.6     | 14-mer/5S  |
|              | 2850.0   | 2850.5     | 13-mer/5S  |
|              | 2754.8   | 2754.4     | 12-mer/6S  |
|              | 2674.9   | 2674.5     | 12-mer/6S  |

**Note:** Relative abundance is shown in parentheses.

**a** Oligosaccharide fragment of odd-numbered residues with GaINAc at both termini.

**b** Oligosaccharide fragment of odd-numbered residues with GlcUA at the non-reducing and UA at the reducing terminus.

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**Fig. 4. Comparison of inhibitory activities of dodecasaccharide subfractions derived from de-6-O-sulfated CSA.** Seven dodecasaccharide subfractions were tested at various concentrations for competitive inhibition of P. falciparum-IRBCs binding to immobilized CSA. All values represent the mean ± S.E. from two experiments performed in duplicate.
Our data show that the minimum chain length required for inhibitory activity is 12 monosaccharide residues, but only when combined with an appropriate sulfate content and sulfation pattern. None of the undersulfated CSA 10-mer subfractions showed any inhibition, confirming the importance of size. Reflecting the significance of size and sulfation, fully sulfated 12-, 14-, and 16-mer fractions derived from bovine trachea CSA were relatively non-inhibitory when compared with their undersulfated equivalents. The inhibition activities of oligosaccharide subfractions increased with increasing chain length when measured using mass concentrations (Fig. 2). If molar concentrations are taken into account, this trend is still apparent and indicates that additional recognition elements are present in extended sequences. CSA polysaccharide has an average molecular mass of 45,500 Da, equivalent to 200 monosaccharide residues in length. It is not surprising that, on a molar basis, the polysaccharide is 13–17 times more active in inhibition than the 12-, 14-, and 16-mer fractions (average molecular masses 2675, 3135, and 3594 Da, respectively, Table I) due to the presence of multiple recognition motifs and possible polyvalent interactions.

In an attempt to isolate specific sequences present in the active CSA dodecasaccharide subfraction F6C, sequential SAX- and APS-HPLC was used (13). Due to the highly heterogeneous characteristics of CSA, the HPLC fractions obtained were still mixtures. Despite small differences in composition, the 12-mer HPLC fractions demonstrated significantly different inhibitory activities. From these results it is not possible to determine whether a specific sequence(s) is required for interaction with P. falciparum IRBCs or to establish the recognition epitope(s).

An alternative strategy is needed to achieve the separation and preparation of different homogeneous sequences of undersulfated CSA dodecasaccharides.

The present studies have demonstrated the relevance of different forms of hexuronic acid residue (GlcUA/IdoUA) in parasite adhesive interactions. Undersulfated CSB 14-mers were isolated and shown to have a slightly higher inhibitory activity than fully sulfated analogues; however, the extent of undersulfation was less than observed with the more active CSA 14-mers. Partial de-4-O-sulfation of CSB and de-6-O-sulfation of CSA polysaccharides was carried out to produce CSB and CSA with a similar proportion of 0S and 4S disaccharide elements. Significantly higher inhibitory activity was shown from partially desulfated CSA. Because the major difference between the partially desulfated CSA and CSB preparations was the sole presence of GlcUA in CSA and IdoUA in CSB, our results strongly point to specific requirement for GlcUA.

Our experiments unambiguously demonstrate the impor-
three experiments performed in duplicate. Comparing variously sulfated CS polysaccharides, Alkhalil et al. (24) recently reported the importance of 4-O-non-sulfated disaccharides in determining parasite-CS interactions but suggested that 6-O-sulfation neither interacts nor interferes with IRBC binding and that the parasite adhesive motif could be formed by combinations of 4-O-sulfated disaccharides with 0S or 6S disaccharides. This view contrasts with earlier findings suggesting that 4-O-sulfation is required for binding and that 6-O-sulfation of GalNAc interferes with binding (3, 10, 11). Consistent with our findings, Alkhalil et al. (24) conclude that 12-mer was the minimum length for full inhibitory activity, although lower oligomers also showed substantial inhibitory activity.

The importance of 4S and 0S disaccharide units for interactions between CSA and IRBCs is consistent with what is known about the likely CS receptors for parasite sequestration that occurs in the vasculature in vivo. CS proteoglycans isolated from placental blood and tissue support parasite adhesion in vitro and appear to comprise predominantly non-sulfated and 4-O-sulfated disaccharide units (25). The proteoglycan thrombomodulin contains CS chains comprised of 4S disaccharides with GlcUA (26), and is present in placental and other vascular beds (27) where parasite sequestration occurs, supports parasite adhesion in vitro (22), and may be an important receptor in vivo. Undersulfated chondroitin sulfate is also prominent in normal human plasma (28) and may influence parasite sequestration in vivo.

In our studies, CSA from bovine trachea was used as the immobilized receptor, and the derived oligosaccharide fragments were used as inhibitors in adherence assays as they contain predominantly GlcUA with little or no IdooUA (data not shown) and can support high levels of parasite adhesion (7). Although this CSA is different from that of human placenta, it is highly effective at inhibiting parasite adhesion to placental CS proteoglycans (24), placental tissue sections (4), and CSA present on endothelial cells (29). Chondroitin lyase ABC, used in our studies to generate different sized fragments, resulted in oligosaccharides with modified hexuronic acid ΔUA at the non-reducing terminal. Because oligosaccharides prepared from testicular hyaluronidase digestion of CSA (24) have unmodified uronic acid at the terminal also showed that 12-mers were required for inhibitory activity, the form of non-reducing terminal hexuronic acid is not a crucial element in the interaction. Caution should be applied in using hyaluronidase, an endo-type glycosidase (30), because its relatively high transglycosylation activity can result in artificial sequences generated by reconstruction of CS chains from the cleaved oligosaccharide fragments (31).

P. falciparum erythrocyte membrane protein 1 (PfEMP1) has been identified as the parasite protein mediating adhesion of IRBCs to the glycosaminoglycans CSA (32) and heparan sulfate (33, 34) and other host molecules (35). It is clear that dodecasaccharide is the minimum chain length of CSA to effectively interact with P. falciparum IRBCs. The minimum chain length requirement for interaction between HA or HS and P. falciparum IRBCs is also a dodecamer sequence (8, 34). Carbohydrate-protein interactions are generally weak and are enhanced by the cooperative effect of multivalent binding sites, which may explain the apparent requirement for longer chain structures of glycosaminoglycans for parasite binding. Alternatively, it may be that longer CS chains are required because important basic amino acid residues for CS binding occur in discontinuous segments of the PfEMP1 protein. The key amino acid residues could be present in either α-helices or β-strands, and the folding of the protein brings together two or more sequences required for binding and activity. Definition of the structural motifs on host-cell chondroitin sulfate involved in the adhesion of malaria-infected erythrocytes will increase our understanding of the pathogenesis of placental infection at the molecular level and facilitate the development of novel therapeutical approaches to inhibit this adhesion.

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Interactions of P. falciparum-infected Erythrocytes with CSA

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The Structural Motif in Chondroitin Sulfate for Adhesion of *Plasmodium falciparum*-infected Erythrocytes Comprises Disaccharide Units of 4-O-Sulfated and Non-sulfated *N*-Acetylgalactosamine Linked to Glucuronic Acid

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