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Generation and Application of Type-specific Anti-Heparan Sulfate Antibodies Using Phage Display Technology

FURTHER EVIDENCE FOR HEPARAN SULFATE HETEROGENEITY IN THE KIDNEY*

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Detailed analysis of various heparan sulfate (HS) species is seriously hampered by a lack of appropriate tools, such as antibodies. We adopted phage display technology to generate anti-HS antibodies. A "single pot" semisynthetic human antibody phage display library was subjected to four rounds of selection on HS from bovine kidney using panning methodology. Three different phage clones expressing anti-HS single chain variable fragment antibodies (HS4C3, HS4D10, and HS3G8) were isolated, with an amino acid sequence of the complementarity-determining region 3 of GRRLKD (V_H3 gene, *DP-38*), SLRMNGCGAHQ (V_H3 gene, *DP-42*), and YYHYKVN (V_H1 gene, *DP-8*), *respectively. The antibodies react with HS and heparin, but not with DNA or other glycosaminoglycans.* K_d values for HS are about 0.1 μM. The three antibodies react differently toward various HS preparations and show different staining patterns on rat kidney sections, indicating recognition of different HS molecules. This also holds for two described mouse anti-HS IgMs (JM403 and 10E4; both generated by conventional hybridoma technique) and indicates the presence of at least 5 different HS species in the kidney. *O*- and *N*-sulfation are important for binding of HS to HS4C3 and HS3G8. The three single chain antibodies, but not JM403, block a basic fibroblast growth factor binding site of HS. It is concluded that phage display technology presents a powerful technique to generate antibodies specific for HS epitopes. This is the first time this technique has been successfully applied to obtain directly antibodies to (poly)saccharides.

Heparan sulfate (HS)[1] represents a heterogeneous class of molecules within the group of glycosaminoglycans. It has been implicated in many basic cellular phenomena, such as cell growth, migration, and differentiation (1–4). HS binds and modulates various proteins, including growth factors and cytokines, enzymes, protease inhibitors, and extracellular matrix proteins. Studies involving specific enzymatic or chemical cleavage and subsequent analysis of the resulting oligosaccharides indicate the existence of many HS species and the presence of domain structures within the HS molecule (5–15). There are clues that specific monosaccharide sequences within the molecule dictate the specific features of a given species, *e.g.* a pentasaccharide for the binding of HS/heparin to anti-thrombin III and a preferential sequence for the binding of HS to bFGF (4, 16–19). The appreciation of the structural diversity of HS species and its role in pathological conditions is strongly hampered by the lack of appropriate methodologies. Sequence strategies are not at hand, and specific antibodies, obvious tools for studying diversity, are difficult to raise. HS, and glycosaminoglycans in general, are almost nonimmunogenic, and consequently, only a few specific antibodies have been described (20, 21). To circumvent this, we adopted antibody phage display technology because this system allows one to generate antibodies against "self" antigens. We report here on the generation and application of three specific antibodies against HS species using this technique. We compared these antibodies with two described mouse monoclonal antibodies, with regard to immunostaining on sections of rat kidney, immunoreactivity toward various HS preparations, and reactivity with bFGF sites on HS.

EXPERIMENTAL PROCEDURES

Materials

A "single pot" human semisynthetic phage library (22) (now officially named synthetic scFv library 1) was generously provided by Dr G. Winter, Cambridge University, Cambridge, United Kingdom. This library contains 50 different V_H genes with synthetic random complementarity-determining region 3 segments, which are 4–12 amino acid residues in length. The heavy chains are combined with a single light chain gene (*DPL 16*). The library contains $> 10^8$ different clones.

Two *Escherichia coli* strains were used: the suppressor strain TG1 (K12, D(*lac-pro*), *sup*E, *thi, hsd*D5/F′*tra*D36, *pro*A$^+$B$^+$, *laq*I^q, *lac*ZDM15), and the nonsuppressor strain HB2151 (K12, *ara*, D(*lac-pro*), *thi*/F″*pro*A$^+$B$^+$, *lac*I^{q}ZDM15). Helper phages VCS-M13 were from Stratagene (La Jolla, CA).

Heparan sulfate from bovine kidney, chondroitinase ABC (*Proteus vulgaris*, EC 4.2.2.4), chemically modified heparan sulfate and heparin kits, and mouse anti-heparan sulfate antibody (clone 10E4) were from Seikagaku Kogyo Co. (Tokyo, Japan). Heparin from porcine intestinal mucosa, chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, dermatan sulfate from porcine skin, keratan sulfate from bovine cornea, hyaluronate from human umbilical cord, DNA from calf thymus, dextran sulfate, bovine serum albumin (fraction V), FITC-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-mouse IgG, heparinase I (from *Flavobacterium heparinum*, EC 4.2.2.7), heparinase II (from *F. heparinum*), and heparinase III (heparitinase, from *F. heparinum*, EC 4.2.2.8), were from Sigma. Horseradish peroxidase-conjugated, and alkaline phosphatase-conjugated rabbit anti-mouse IgG were from Dakopatts (Glostrup, Denmark). Recombinant

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[1] The abbreviations used are: HS, heparan sulfate; IdoA, iduronic acid; K_d, dissociation constant; PBS, phosphate-buffered saline; 2xTY/glu/amp, 2xTY medium containing 1% (w/v) glucose and 100 μg ampicillin/ml; bFGF, basic fibroblast growth factor; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.

bovine bFGF (146 amino acids) and mouse anti-c-Myc tag IgG (clone 9E10) were from Boehringer Mannheim. PCR kit was from Promega (Madison, WI), and the DNA sequencing kit (Sequenase version 2.0) and [α-^{35}S]dATP were from Amersham Pharmacia Biotech. Restriction enzyme BstNI was from New England Biolabs (Beverly, MA). Polystyrene Maxisorp Immunotubes were from Nunc (Roskilde, Denmark). Various HS preparations were a generous gift from Dr. U. Lindahl, University of Uppsala (Uppsala, Sweden).

Growth of Library

5.10^8 bacteria, corresponding to about 10^8 phage clones, were inoculated into 50 ml 2xTY medium containing 1% (w/v) glucose and 100 μg ampicillin/ml (2XTY/glu/amp), and grown at 37 °C while shaking until an absorbance at 600 nm of 0.6–0.8 was reached. To 10 ml of this culture, 5.10^{11} VCS-M13 helper phages were added, and after 30 min at 37 °C (no shaking) cells were spun down for 10 min at 3000 × *g* at 37 °C. Bacteria were resuspended in 10 ml of 2xTY and added to 300 ml of preheated (30 °C) 2xTY/amp containing 25 μg of kanamycin/ml. Incubation was for 16 h at 30 °C with shaking.

Isolation of Phages

After spinning the culture for 10 min at 10,000 × *g* (37 °C), 20% (v/v) of a polyethylene glycol/NaCl solution containing 20% (w/v) polyethylene glycol 6000 and 2.5 M NaCl was added to the supernatant. After mixing, the solution was kept on ice for 1 h, spun (10,000 × *g* for 30 min at 4 °C), and resuspended in 40 ml of ice-cold Milli Q water followed by addition of 8 ml of the polyethylene glycol/NaCl solution. After 20 min on ice and spinning (30 min at 3000 × *g* at 4 °C), the supernatant was carefully removed, and the pellet was respun to aspirate any residual polyethylene glycol/NaCl. The pellet was resuspended in 2.5 ml of ice-cold PBS (4 °C), spun down (10 min at 3000 × *g*), and filtered through a 0.45-μm filter to remove any bacterial debris. This solution, containing the phages, was immediately used for selection.

Selection of Heparan Sulfate Binders by Library Panning

The library was subjected to four rounds of panning. Polystyrene tubes were coated with HS from bovine kidney using a solution of 20 μg/ml for 16 h, 4 °C. Tubes were rinsed three times with PBS, blocked by incubation with 2% (w/v) Marvel (nonfat milk powder) in PBS for 2 h (22 °C), and rinsed again three times with PBS. To the tubes, 2 ml of phage suspension and 2 ml of 4% (w/v) PBS with 2% (w/v) Marvel were added, and incubation was for 30 min under continuous rotating, followed by standing for 90 min (22 °C). Tubes were washed 20 times with PBS containing 0.05% (w/v) Tween-20 and 20 times with PBS. Bound phages were eluted by addition of 1 ml 100 mM triethylamine, which was neutralized with 0.5 ml of 1 M Tris/HCl, pH 7.4. One ml of this phage suspension was added to 9 ml of a suspension of TG1 bacteria ($A_{600\ nm}$, 0.6–0.8) and incubated for 30 min at 37 °C in order to allow for infection. Bacteria were spun down, taken into 1 ml of 2xTY, and grown for 16 h at 37 °C on a 24 × 24-cm TYE plate (TY with bacto-agar) containing 1% (w/v) glucose and 100 μg of ampicillin/ml. Bacteria were scraped from the plate using a glass spreader and 5 ml of ice-cold 2xTY/15% (v/v) glycerol. Of this suspension, 50 μl was inoculated into 50 ml of 2xTY/glu/amp and grown at 37 °C until an absorbance at 600 nm of 0.6–0.8 was reached. Phages were rescued after addition of helper phages as described and used for further rounds of selections. A total of four selections were carried out. In one case, an additional negative selection step was introduced. In this case, after the second round of selection, 1 ml of phages was added to a tube containing 3 ml of PBS containing 30 μg of dermatan sulfate and 30 μg of chondroitin 4-sulfate and incubated for 1 h at 22 °C under continuous rotation. Two ml of this suspension was then subjected to two additional rounds of panning on HS.

Screening for Phages Expressing Heparan Sulfate Binding Antibodies

Bacteria picked from single colonies after the last round of selection were grown in 200-μl 2xTY/0.1% (w/v) glu/amp in 96-well polystyrene round bottom plates for about 3 h at 37 °C until bacterial growth was visible. Antibody production was induced by adding 25 μl of 2xTY containing 9 mM isopropyl-β-D-thiogalactopyranoside. Plates were centrifuged, and the supernatant containing soluble antibodies was applied to wells of polystyrene microtiter plates previously coated with HS and blocked with 2% (w/v) PBS with 2% (w/v) Marvel. Bound antibodies were detected using a mouse monoclonal antibody (9E10) directed against the c-Myc tag, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG. Peroxidase activity was detected using tetramethylbenzidine as a substrate. The enzymatic reaction was stopped after 5 min with 2 M H_2SO_4, and absorbance was measured at 450 nm.

Screening Phage Clones for Unique Antibody Inserts Using PCR, Fingerprinting, and Sequencing

Phages displaying anti-HS antibodies were checked for the presence of full-length inserts by 25 cycles of PCR using phagemid DNA as template. *Taq* polymerase was used; LBM3 (5′-CAGGAAACAGCTATGAC) was used as the backward primer, and fd-SEQ1 (5′-GAATTTTCTGTATGAGG) was used as the forward primer (23). The primers span a region containing the V_H, linker, and V_L elements (about 1 kilobase pair). Fingerprinting was performed using *Bst*NI as the restriction enzyme. PCR and fingerprinting analysis were performed using 1% (w/v) and 4% (w/v) agarose gels, respectively. To establish the complementarity-determining region 3 and the germ line V_H gene DNA segments, unique clones were sequenced using the dideoxy method of Sanger *et al.* (24) using FOR LINK SEQ RIC (5′-GCCACCTCCGCCTGAACC) as the primer (located in the linker region between the V_H and the V_L genes). For this purpose, single-stranded DNA was isolated using standard procedures.

Source of Antibodies

To obtain optimal amounts of soluble anti-HS antibodies, phages were allowed to infect the nonsuppressor *E. coli* strain HB2151. Unless stated otherwise, culture medium was used as source of antibodies in ELISAs, whereas the periplasmatic fraction was applied in immunohistochemical experiments. The latter, in which antibodies are more concentrated, was prepared as follows. Bacteria were grown for 22 h at 37 °C in 5 ml 2xTY/glu/amp, subsequently added to 500 ml of the same medium containing 0.1% (w/v) instead of 1% (w/v) glucose, and incubated until an absorbance of 0.5–0.8 was reached. Induction was obtained by addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM). After incubation at 30 °C for 3 h, the culture was left on ice for 20 min, centrifuged (3000 × *g* for 10 min at 4 °C), and the pellet was resuspended in 5 ml of ice-cold 200 mM sodium-borate buffer (pH 8.0) containing 160 mM NaCl and 1 mM EDTA. After centrifugation (3000 × *g* for 10 min at 4 °C) and recentrifugation (48,000 × *g* for 30 min at 4 °C), the supernatant was filtered through a 0.45 μm filter and dialysed *versus* PBS. The preparation thus obtained is the periplasmatic fraction.

Evaluation of Specificity by ELISA

Reactivity of the anti-HS antibodies with various molecules was evaluated in ELISA in two ways: (*a*) by application of antibodies to wells of polystyrene plates coated with the test molecules (1 μg/ml coating solution), and (*b*) by an inhibition assay in which the antibodies were incubated with the test molecule (40 μg/ml, unless stated otherwise) for 16 h at 22 °C in PBS, followed by transfer to wells previously coated with HS (bovine kidney). Test molecules included HS from bovine kidney, aorta, lung, intestine, human aorta, pig intestine, and whale lung; heparin; dermatan sulfate; chondroitin 4- and 6-sulfate; keratan sulfate; dextran sulfate; hyaluronate; BSA; Marvel (the nonfat milk preparation using as a blocking reagent during panning); and DNA. In addition, HS (bovine kidney) digested with heparinase I, II, or III or with HNO_2 at pH 1.5 was evaluated. Furthermore, chemically modified heparan sulfates (bovine kidney) and heparins (porcine intestine) were analyzed, *viz*. preparations that were completely desulfated and *N*-acetylated, preparations that were completely *O*-desulfated and *N*-sulfated, and preparations that were *N*-desulfated and *N*-acetylated. Bound antibodies were detected using anti-c-Myc tag mouse monoclonal antibody 9E10 and alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody. Enzyme activity was detected using *p*-nitrophenyl phosphate as the substrate, and absorbance was read at 405 nm.

Determination of Affinity

K_d values of the antibodies were determined using an indirect competition ELISA, essentially according to Friquet *et al.* (25). Briefly, bacterial culture supernatants containing 0.1 μg of antibodies/ml were incubated for 16 h at 22 °C in wells of microtiter plates with various amounts of HS from bovine kidney. Next, 100 μl was transferred to a corresponding well previously coated with HS and incubated for 1.5 h at 22 °C. Bound antibodies were detected as described for ELISA. By plotting the reciprocal of the fraction of antibodies bound *versus* the reciprocal of the concentration HS used, a Klotz plot can be generated in which the slope represents the K_d. For HS, a molecular mass of 20 kDa was taken.

TABLE I
Reactivity of anti-HS antibodies with various HS preparations and heparin in ELISA

Culture media from bacteria expressing anti-HS antibody HS4C3, HS4D10, and HS3G8 were incubated with various molecules immobilized on the wells of microtiter plates. Bound antibodies were visualized using mouse anti-c-myc IgG, followed by phosphatase-conjugated rabbit anti-mouse IgG. In addition, the mouse anti-HS IgM antibodies JM403 and 10E4 were evaluated and visualized using alkaline phophatase-conjugated goat anti-mouse IgM. The ratio of sulfate groups to disaccharide is indicated in parentheses. Values (in % of reactivity with HS (bovine kidney) represent mean ± S.D. (n = 4).

Preparation	Antibody				
	HS4C3	HS4D10	HS3G8	JM403	10E4
HS, bovine kidney (used for panning)	100 ± 1	100 ± 5	100 ± 2	100 ± 5	100 ± 4
K5, capsular polysaccharide[a] from *E. coli* (0)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
HS, bovine aorta (0.56)	2 ± 0	9 ± 0	3 ± 1	226 ± 8	67 ± 20
HS, human aorta (0.60)	1 ± 0	16 ± 1	0 ± 1	203 ± 0	1039 ± 3
HS, bovine kidney (0.84) (fraction A)[b]	28 ± 3	16 ± 2	31 ± 3	65 ± 2	8 ± 0
HS, bovine lung (0.91)	28 ± 2	6 ± 2	27 ± 3	247 ± 3	2 ± 0
HS, bovine intestine (0.98)	112 ± 1	11 ± 0	66 ± 2	67 ± 3	283 ± 10
HS, bovine kidney (1.01) (fraction B)[b]	79 ± 1	77 ± 3	59 ± 0	13 ± 3	20 ± 2
HS, whale lung (>1)	16 ± 0	56 ± 2	13 ± 3	0 ± 1	10 ± 0
HS, porcine mucosa (1.64)	26 ± 1	35 ± 1	22 ± 1	1 ± 1	7 ± 0
Heparin, porcine mucosa	120 ± 5	19 ± 2	53 ± 10	0 ± 0	19 ± 4

[a] Similar to the HS precursor polysaccharide.
[b] Fraction A elutes from an anion exchange column between 0.7 and 1.1:M NaCl; fraction B between 1.1 and 1.25:M (14).

Immunohistochemistry

Immunofluorescence—Specimens from rat kidney (Wistar, male, 3 months old) were snap-frozen in liquid isopentane cooled with liquid nitrogen and stored at −70 °C. Cryosections (6 μm) were rehydrated for 10 min in PBS, blocked with PBS containing 1% (w/v) BSA (PBS/BSA), and incubated with anti-HS antibodies for 90 min at 22 °C. As a control, antibodies (single chain variable fragments) against filaggrin were used. Filaggrin is a nonrelated protein not present in kidney (26). Phage display antibodies were detected by incubation with mouse anti-c-Myc tag antibodies (9E10, culture supernatant), followed by FITC-labeled goat anti-mouse IgG (1:100 in PBS/BSA), both for 90 min at 22 °C. After each incubation, sections were washed in PBS (3 × 10 min). Additional controls were the omission of primary or conjugated antibody. In addition to the phage display-derived antibodies, two mouse anti-HS IgMs, JM403 (21) and 10E4 (20), were used, both obtained by conventional hybridoma techniques. They were detected using FITC-conjugated goat anti-mouse IgM antibodies (1:100 in PBS/BSA).

Enzyme Immunohistochemistry—Samples from human kidney and uterine myometrium (rich in heparin-containing mast cells) were fixed in 4% (w/v) paraformaldehyde in PBS and embedded in paraffin. Sections were deparaffinized and treated for 30 min with 1% (v/v) hydrogen peroxide in methanol to remove intrinsic peroxidase activity. After rehydration, sections were incubated with anti-HS antibodies, which were detected by incubation with mouse anti-c-Myc tag antibodies (9E10, culture supernatant), followed by peroxidase-conjugated rabbit anti-mouse IgG (1:100 in PBS containing 1% (w/v) BSA) for 90 min. Sites of peroxidase activity were revealed using diaminobenzidine as a substrate. Sections were counterstained with Harris's hematoxylin. The same controls were used as described under immunofluorescence.

Evaluation of Specificity by Immunofluorescence

Cryosections were pretreated with heparinase III (digests HS; 0.02 units/ml; 50 mM NaAc/50 mM $Ca(Ac)_2$, pH 7.0), and with chondroitinase ABC (digests dermatan and chondroitin sulfate; 1 unit/ml; 25 mM Tris/HCl, pH 8.0) for 30 min at 37 °C. As control, sections were incubated in the reaction buffer without enzyme. After rinsing three times with PBS and blocking with PBS/1% BSA, sections were incubated with antibodies and processed for immunofluorescence as described. The efficacy of chondroitinase ABC treatment was evaluated by incubation of sections with antibodies against chondroitin sulfate "stubs," generated by chondroitinase ABC (Ab 2B6 from Seikagaku Kogyo Co., Tokyo, Japan)

Specificity of the anti-HS antibodies was further tested by preincubation of the antibodies with various molecules, *viz*. HS (bovine kidney), heparin, dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate, dextran sulfate, hyaluronate, and DNA. Incubation was for 16 h at 22 °C at concentrations of 0.5–1.0 mg/ml periplasmatic fraction. Processing for immunofluorescence on sections was as above.

Inhibition of Antibody Binding by bFGF

To study whether the HS epitope recognized by the antibodies is involved in the binding of bFGF, 100 μl of a solution containing bFGF (1.7 μg/ml TBS containing 0.1% (w/v) Tween-20 and 1% (w/v) BSA) was applied for 8 h at 22 °C to wells of polystyrene microtiter plates previously coated with HS from bovine kidney. After washing, anti-HS antibodies (0.04 μg of protein/ml) were applied for 16 h at 22 °C, and bound antibodies were detected as described for ELISA. Alternatively, cryosections of rat kidney were incubated for 16 h with bFGF (20 μg/ml PBS/1% (w/v) BSA) and, after washing, incubated for 90 min with anti-HS antibodies. Detection of bound antibodies was as described for immunofluorescence.

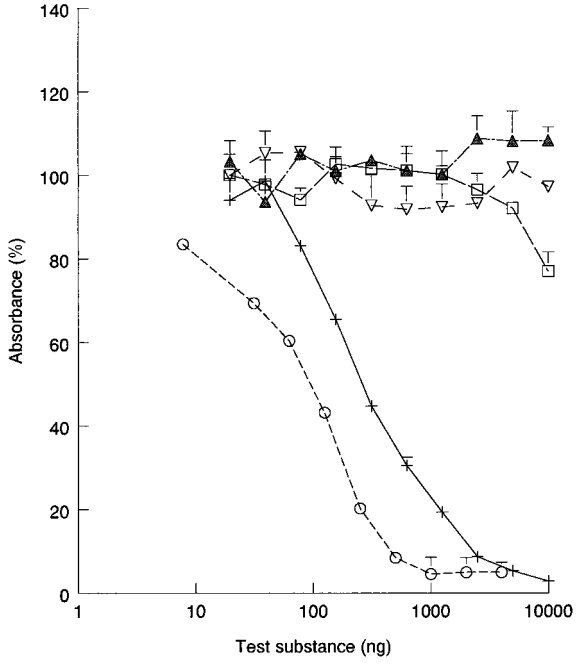

FIG. 1. **Inhibition immunoassay to identify certain chemical modifications influencing the epitope recognized by antibody HS4C3.** Antibodies were incubated with the test molecule and then transferred to wells previously coated with HS (bovine kidney). Bound antibodies were detected using anti-c-Myc tag mouse monoclonal antibody 9E10 and alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody. Enzyme activity was detected using *p*-nitrophenyl phosphate as the substrate, and absorbance was read at 405 nm. Test molecules were heparin (○), desulfated/*N*-acetylated heparin (▲), *O*-desulfated/*N*-sulfated heparin (▽), *N*-desulfated/N-acetylated heparin (□), and heparan sulfate (from bovine kidney) (+). Similarly modified HS preparations reacted similarly to the modified heparins (data not shown). Antibody HS3G8 reacted similarly to HS4C3. *Bars* indicate S.D. (n = 3).

RESULTS

Selection of Anti-heparan Sulfate Antibodies

After four rounds of panning, 19 clones expressing anti-HS antibodies and containing full-length DNA inserts were iso-

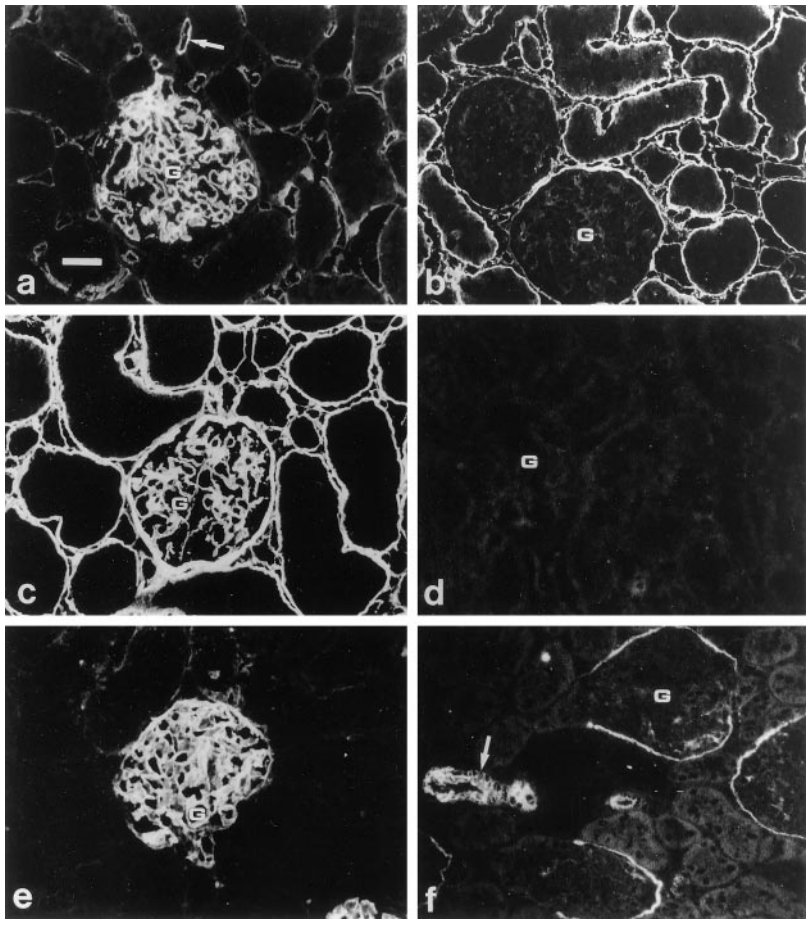

FIG. 2. **Immunostaining of rat kidney with anti-HS antibodies.** Cryosections were incubated with periplasmatic fractions of bacteria expressing anti-HS antibody HS4C3 (*a*), HS4D10 (*b*), HS3G8 (*c*), and anti-filaggrin (control) (*d*). Bound antibodies were visualized using mouse anti-c-Myc IgG followed by FITC-conjugated goat anti-mouse IgG. In addition, the mouse anti-HS IgM JM403 (*e*) and 10E4 (*f*) were evaluated and visualized using FITC-conjugated goat anti-mouse IgM. *Bar*, 25 μm. *G*, glomerulus. *Arrow* in *a*, peritubular capillary; *arrow* in *f*, smooth muscle cells.

lated. The 19 clones included 3 unique clones expressing antibody HS4C3, HS4D10, and HS3G8. The clone expressing antibody HS3G8 was obtained after additional negative selection on chondroitin sulfate and dermatan sulfate. Sequencing analysis revealed an amino acid sequence of the V_H complementarity-determining region 3 of GRRLKD for antibody HS4C3, of SLRMNGCGAHQ for HS4D10, and of YYHYKVN for HS3G8. The V_H family and germ line segments are V_H3 and *DP-38* for HS4C3, V_H3 and *DP-42* for HS4D10, and V_H1 and *DP-8* for HS3G8 (nomenclature according to Ref. 27).

Characterization of Anti-heparan Sulfate Antibodies

Evaluation of Specificity and Affinity Using ELISA—Antibodies from all three clones reacted with HS from bovine kidney (the "antigen") (Table I). No reactivity was observed with chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, dextran sulfate, hyaluronate, DNA, BSA, Marvel, or polystyrene (data not shown). Major cross-reactivity with heparin was observed for HS4C3 and HS3G8. All three antibodies reacted differently with HS preparations isolated from different organs, each displaying its own pattern (Table I). Antibody HS4C3 preferentially reacted with HS isolated from bovine intestine and with fraction B of HS from bovine kidney, which eluted from an anion-exchange column between 1.1 and 1.25 M NaCl. HS4D10 preferentially reacted with HS isolated from whale lung and with fraction B of HS from bovine kidney. HS3G8 has a reactivity toward different HS preparations that is comparable, but not identical, to that of HS4C3. There appears to be no correlation between antibody reactivity and the ratio of sulfate groups/disaccharide (Table I).

To elucidate some chemical aspects of the epitope, reactivity of the antibodies was evaluated with chemically/enzymatically treated HS and heparin preparations using an inhibition ELISA. Reactivity with antibodies HS4C3 and HS3G8 was abolished when HS or heparin was completely desulfated and *N*-acetylated, completely desulfated and *N*-sulfated, or *N*-desulfated and *N*-acetylated (Fig. 1). Treatment of HS from bovine kidney with heparinase I, II, or III or with nitrous acid at pH 1.5 also destroys reactivity with these antibodies (data not shown). Antibody HS4D10 could not be analyzed in the inhibition ELISA, probably due to the low amount of HS species containing the epitope in the kidney HS preparation used.

Mouse monoclonal IgM JM403 reacted primarily with HS from bovine and human aorta and from bovine lung. Chemical modification of HS or heparin destroyed immunoreactivity, as did heparinase II and III. Heparinase I, however, did not eliminate the reactivity of HS with JM403. These data are in accordance with published data (28). Mouse monoclonal 10E4 reacted strongly with HS from human aorta and bovine intestine.

The dissociation constants (K_d) of antibody HS4C3 and HS3G8 for bovine kidney HS, as deduced from Klotz plots, are 0.12 and 0.15 μM, respectively. No value could be obtained for HS4D10.

Evaluation of Specificity Using Immunohistochemistry—Anti-HS antibodies stained basement membranes in rat kidney with different specificity (Fig. 2, Table II). Antibody HS4C3 predominantly stained basement membranes of the glomerulus and of peritubular capillaries, whereas HS4D10 reacted mainly with HS present in basement membranes of tubules and of smooth muscle cells. HS3G8 had a rather promiscuous staining behavior: it reacted with most basement membranes in kidney. Mouse monoclonal antibody JM403 preferentially stained basement membranes of the glomerulus and of smooth muscle cells, whereas mouse monoclonal antibody 10E4 reacted mainly with Bowman's capsule and basement membranes of smooth muscle

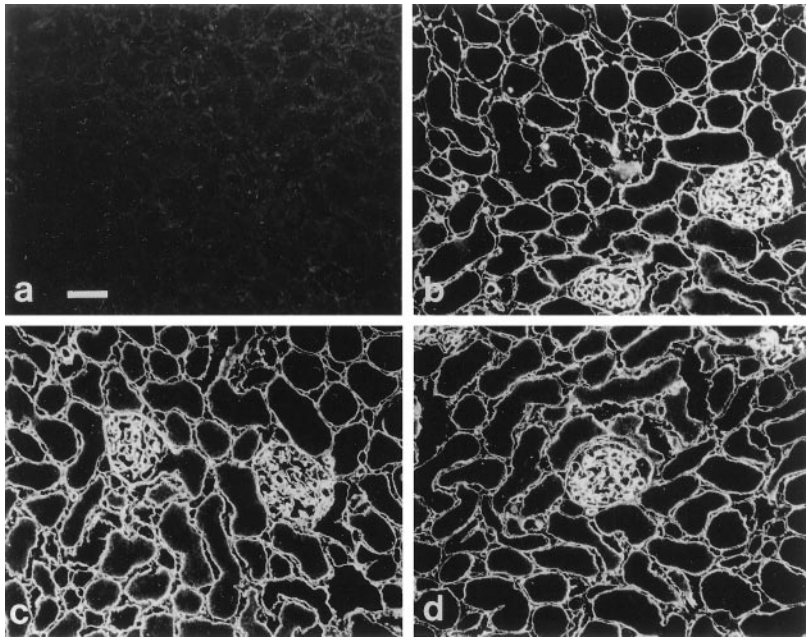

FIG. 3. **Specificity of anti-HS antibody HS3G8.** Prior to incubation with HS3G8 (periplasmatic fraction), rat kidney cryosections were treated with heparitinase (*a*), heparitinase incubation buffer (*b*), chondroitinase ABC (*c*), and chondroitinase ABC incubation buffer (*d*). Bound antibodies were visualized using mouse anti-c-Myc IgG followed by FITC-conjugated goat anti-mouse IgG. *Bar*, 50 μm.

TABLE II
Immunofluorescence pattern of rat kidney by anti-HS antibodies

Periplasmatic fractions of bacteria expressing anti-HS antibodies HS4C3, HS4D10, and HS3G8 were applied to cryosections of rat kidney. Bound antibodies were visualized by incubation with mouse anti-c-myc IgG followed by FITC-conjugated goat anti-mouse IgG. Staining: ++, strong; +, moderate; +/−, weak; −, absent.

Basement membrane	HS4C3	HS4D10	HS3G8
Glomerulus	++	−	++
Bowman's capsule	+/−	+/−	+
Peritubular capillaries	++	+/−	+
Proximal tubules	−	++	++
Distal tubules	−	++	++
Collecting tubules	−	++	+
Smooth muscle cells	−	++	−
Large blood vessel endothelium	+	++	+

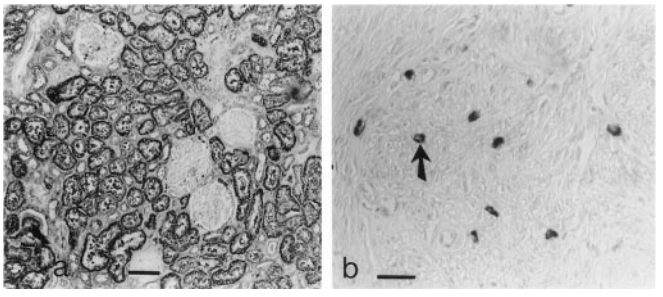

FIG. 4. **Application of anti-HS antibodies on paraffin sections.** *a*, section of human kidney incubated with antibody HS4D10 (periplasmatic fraction) (*bar*, 100 μm). *b*, section of human uterine myometrium incubated with antibody HS4C3 (periplasmatic fraction) (*bar*, 50 μm). Note the strong immunoreactivity of mast cells due to the cross reactivity of the antibody with heparin. Bound antibodies were visualized using mouse anti-c-Myc IgG, followed by peroxidase-conjugated rabbit anti-mouse IgG.

cells. Staining with HS3G8 (Fig. 3), HS4C3, and HS4D10 (not shown) was abolished by treatment of sections with heparinase III but not with chondroitinase ABC. Staining could be precluded by preincubation of antibodies with HS and heparin but not with hyaluronate, dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate, dextran sulfate, and DNA. Antibodies could also be used on paraffin sections (Fig. 4). Antibody HS4C3 reacted strongly with heparin-containing granules in mast cells (Fig. 4*b*).

Inhibition of Antibody Binding by bFGF

Preincubation of HS-coated wells with bFGF partially inhibits the binding of all anti-HS antibodies obtained by phage display. Binding of antibody HS4C3 was blocked by 59 ± 2% (mean and range) (n = 2), of antibody HS3G8 by 64 ± 1%, and of HS4D10 by 92 ± 2%. bFGF had no influence on the binding of mouse JM403 antibody to HS (0 ± 2%). Preincubation of rat kidney sections with bFGF largely precluded binding to HS by the three antibodies obtained by phage display (Fig. 5). No effect was observed for mouse monoclonal antibody JM403.

DISCUSSION

We describe the generation of three anti-HS antibodies from a semisynthetic phage display library. To our knowledge, this is the first time this technique has been successfully used for the direct generation of antibodies against (poly)saccharides. All three antibodies recognize different types of HS molecules, as indicated by their different staining pattern and different reactivity toward various HS preparations. HS biosynthesis starts with the formation of a precursor polysaccharide heparosan, consisting of a glucuronic acid-*N*-acetylglucosamine polymer, which is then subjected to a number of modifications (4). All three antibodies generated by phage display technology did not react with the bacterial K5 polysaccharide, which has the same structure as heparosan. This indicates that the epitopes involved represent additional modifications. Modification of the HS precursor polysaccharide starts with *N*-deacetylation and *N*-sulfation of *N*-acetylglucosamine residues, followed by epimerization of glucuronic acid to L-iduronic acid (IdoA) residues and *O*-sulfation at various positions, including C-2 of the IdoA residues and C6 of GlcN residues (4). The modification reactions are incomplete, so not all disaccharides are similarly modified. In addition, less frequent modifications have been reported, including sulfation at C2 and C3 of glucuronic acid residues, sulfation of C3 of GlcN residues, and the presence of an unsubstituted amino group on GlcN. At least 19 different hexuronic acid-GlcN and 12 GlcN-hexuronic acid disaccharides have been identified in HS/heparin (29). Because an HS chain may be composed of over a 100 disaccharides, a large potential of different HS species can be generated. This may form the

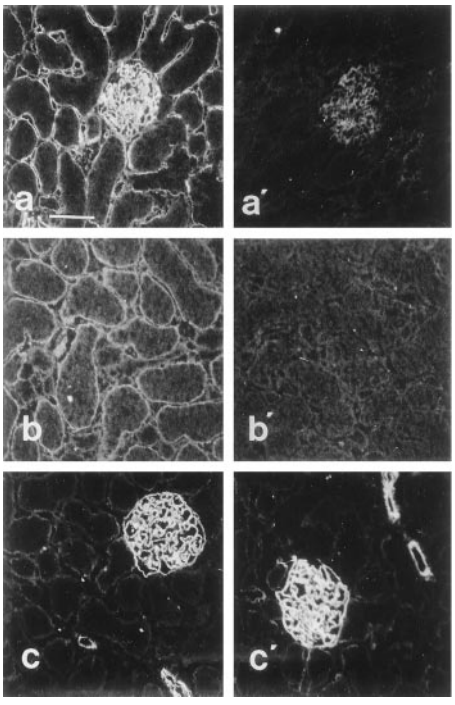

FIG. 5. **Inhibition of HS staining of kidney sections by preincubation with bFGF.** Rat kidney cryosections were preincubated with 1% BSA in PBS without (*a–c*) or with (*a′–c′*) bFGF, followed by incubation with periplasmatic fractions of bacteria expressing anti-HS antibody HS4C3 (*a* and *a′*), HS4D10 (*b* and *b′*), or with mouse anti-HS IgM JM403 (*c* and *c′*). Phage display-derived antibodies were visualized using mouse anti-c-Myc IgG followed by FITC-conjugated goat anti-mouse IgG. JM403 was detected using FITC-conjugated goat anti-mouse IgM antibodies. *Bar*, 50 μm.

basis of the specific reactivity of HS toward a vast array of proteins, including cytokines and growth factors.

Sulfation as such is not sufficient to create the epitope recognized by the phage display antibodies, because dextran, chondroitin, and dermatan sulfate are not immunoreactive. Clearly, some specific modification(s) give rise to epitope formation. Antibody HS4C3 reacted well with HS from bovine intestine and with heparin but poorly with HS from bovine aorta. The former molecules are characterized by a relatively high amount of *N*-sulfation and a high number of contiguous but a low number of spaced *N*-sulfated disaccharides (14). HS from bovine aorta has the opposite characteristics. This indicates that in the HS4C3 epitope heparin-like sequences, characterized by disaccharides formed by 6-*O*-sulfated $GlcNSO_3$ and 2-*O*-sulfated IdoA, are of importance. The importance of sulfation is also indicated by the loss of epitope caused by chemical modifications of HS and heparin preparations. Presence of *O*-sulfation, as well as *N*-sulfation, seems to be necessary for antibody-HS/heparin interaction. However, because heparinase III, which cleaves HS at sites of glucuronic acid rather than IdoA residues, abolishes immunoreactivity, other modifications are involved. It should be stressed here that the HS preparations used herein were not pure, but were mixtures of various HS species.

Antibody HS3G8 has a reactivity toward HS preparations, including chemically/enzymatically modified HS/heparin, that resembles that of HS4C3. It shows, however, a more promiscuous staining behavior. For instance, HS3G8 reacted with HS from the renal peritubular basement membranes, which is unreactive toward HS4C3. This indicates that the epitopes for both antibodies are different, but share similarity. Thus, the antibodies may discriminate between minor structural differences, not easily detected by other means. Antibody HS4D10 is quite different from HS4C3 both in immunostaining and in reactivity toward HS preparations. It reacted well with HS from bovine kidney and with HS from whale lung. It reacted poorly with HS from bovine intestine and with heparin, both of which were highly reactive with HS4C3. HS4D10 could not be analyzed in an inhibition ELISA, precluding identification of chemical modifications in HS important in epitope formation. In general, epitope mapping of glycosaminoglycans poses a major challenge.

The antibodies were different with respect to their DP number (27) and the sequence of the V_H complementarity-determining region 3. The sequence GRRLKD of antibody HS4C3 fits into the glycosaminoglycan binding site *X*BB*X*B*X* (B, basic amino acid residue; *X*, any amino acid residue), which has been found in a subset of heparin-binding proteins (30). The sequence SLRMNGCGAHQ for HS4D10 resembles another motif observed in a number of heparin-binding proteins, in which two basic amino acid residues are at the extreme end of the motif (31). However, the sequence YYHYKVN for HS3G8 does not fit into any of the proposed sequences for heparin binding, and clearly, a linear motif is not a prerequisite for heparin binding.

All three antibodies reacted differently from the described monoclonal IgM antibodies JM403 and 10E4. All five antibodies have their own reactivity toward various HS preparations and display an unique staining pattern in the kidney. This indicates the presence of at least five different HS species in kidney.

Binding and modulation of bFGF is a well defined characteristic of HS, including kidney HS (4, 32–34). On the part of HS, 2-*O*-sulfated IdoA residues promote binding, whereas 6-*O*-sulfation of the GlcN residue seems inhibitory. This would be in line with the almost complete blockage by bFGF of the binding between HS and antibody HS4D10, which reacts poorly with heparin in which 6-*O*-sulfated GlcN residues are prominent. The other two phage display antibodies are much more reactive toward heparin and are less efficiently blocked by bFGF. The epitope recognized by mouse monoclonal antibody JM403, obtained by conventional hybridoma techniques, does not bind bFGF. All epitopes recognized by the phage display antibodies, however, do bind bFGF. Because binding of bFGF to HS seems to be a general characteristic of HS, this indicates that the antibody phage display technique may generate antibodies directed to more common epitopes on the HS molecule, whereas those obtained by hybridoma techniques may be directed to "rare" epitopes. The more restricted staining of kidney basement membranes by the mouse monoclonal antibodies JM403 and 10E4 support this idea.

In conclusion, phage display technology presents a powerful technique to generate antibodies specific for HS epitopes. The availability of specific antibodies and their coding DNAs may be instrumental to further explore the realm of HS and of glycosaminoglycans in general, as is indicated for kidney in this study.

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Generation and Application of Type-specific Anti-Heparan Sulfate Antibodies Using Phage Display Technology: FURTHER EVIDENCE FOR HEPARAN SULFATE HETEROGENEITY IN THE KIDNEY

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