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A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats

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A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats. After immunization of mice with partially-purified heparan sulfate proteoglycan (HSPG) isolated from rat glomeruli, a monoclonal antibody (mAb JM-403) was obtained, which was directed against heparan sulfate (HS), the glycosaminoglycan side chain of HSPG. In ELISA it reacted with isolated human glomerular basement membrane (GBM) HSPG, HS and hyaluronic acid, but not with the core protein of human GBM HSPG, and not with chondroitin sulfate A and C, dermatan sulfate, keratan sulfate and heparin. Furthermore, it did not bind to laminin, collagen type IV or fibronectin. Specificity of JM-403 for HS was also suggested by results of inhibition studies, which found that intact HSPG and HS, but not the core protein, inhibited the binding of JM-403 to HS. In indirect immunofluorescence on cryostat sections of rat kidney, a fine granular to linear staining of the GBM was observed, along with a variable staining of the other renal basement membranes. Pretreatment of the sections with heparitinase completely prevented the binding of mAb JM-403, whereas pretreatment with chondroitinase ABC or hyaluronidase had no effect. The precise binding site of mAb JM-403 was investigated by indirect immunoelectron microscopy. It revealed a diffuse staining of the whole width of the GBM. One hour after intravenous injection of JM-403 into rats, the mAb was detected along the glomerular capillary wall in a fine granular pattern, which shifted towards a more mesangial localization after 24 hours. No binding was observed anymore by day 15. Intravenous injection induced a dose-dependent, transient and selective proteinuria that was maximal immediately after the injection. Administration of 2 mg of JM-403 increased the urinary albumin excretion within the first 24 hours after injection from (mean ± SD) 177 ± 19 to 20,755 ± 10,310 μg/24 hr ($P < 0.01$); the urinary IgG excretion increased from 5.8 ± 2.9 to 236.1 ± 132.2 μg/24 hr ($P < 0.03$); the selectivity index (clearance IgG/clearance albumin) decreased from 0.33 ± 0.12 to 0.12 ± 0.05 ($P < 0.004$).

Heparan sulfate (HS) is thought to play an important role in the permselective properties of the glomerular basement membrane (GBM) [1–3]. In several clinical and experimental glomerulopathies an increased permeability of the GBM is associated with a diminished HS content of the GBM [4–9]. Moreover, enzymatic removal of HS from the glomerular capillary wall increases the passage of native ferritin and albumin into the urinary space [10, 11]. These data were corroborated by investigations which showed that after intrarenal or intravenous injection of cationic molecules, GBM permeability increased due to the neutralization of HS-associated anionic sites of the glomerular capillary wall [12–16]. However, the significance of HS for the permselectivity of the GBM and for the induction of proteinuria is still not completely understood, and a number of controversies still exist [17–20].

To study the impact of HS for the permselective properties of the GBM in more detail, antibodies against HS can be an important tool. There are some reports with regard to the nephritogenic potential of polyclonal antibodies against the core protein of GBM heparan sulfate proteoglycan (HSPG) [21–24]. However, at present there are no studies which describe the nephritogenic effects of antibodies against HS, the glycosaminoglycan (GAG) side chain of HSPG. This is probably due to the difficulty to raise anti-HS antibodies because of the low immunogenicity of these polysaccharide structures. Kure and Yoshie [25] described a syngeneic monoclonal antibody (mAb) to murine meth-A sarcoma which recognizes HS. The preparation of an mAb against HS of Engelbreth-Holm-Swarm (EHS) tumor proteoglycan was also reported [26]. It was recently applied in studies on cerebral amyloid deposits [27, 28].

In this paper, we describe the production and characterization of an mAb against GBM HS. After intravenous (i.v.) injection in rats an immediate, transient binding of mAb to the GBM occurred with a concommitant development of a selective proteinuria.

Methods

Partial purification of rat glomerular HSPG

All procedures were carried out at 4°C, in the presence of a mixture of proteinase inhibitors (1 mM phenylmethanesulphonyl fluoride, 5 mM benzamidine hydrochloride, 10 mM N-ethylmaleimide, 0.1 M 6-aminohexanoic acid, 5 mM iodocetamide and 10 mM EDTA). Rat glomeruli (9.3 g) were isolated from rat kidney cortices (715 g) by the differential sieving technique according to Meezan et al [29]. The glomerular suspension was maintained for more than 85% of glomeruli and was only contaminated with a few tubular fragments, but not with interstitial tissue. Glomeruli were homogenized using a Sorvall omnimixer, and extracted twice overnight in 150 ml 4 M guanidine HCl/50 mM sodium acetate buffer, pH 5.8. The combined supernatants obtained after centrifugation at 40,000 g for 30 minutes, were concentrated to 35 ml by ultrafiltration on

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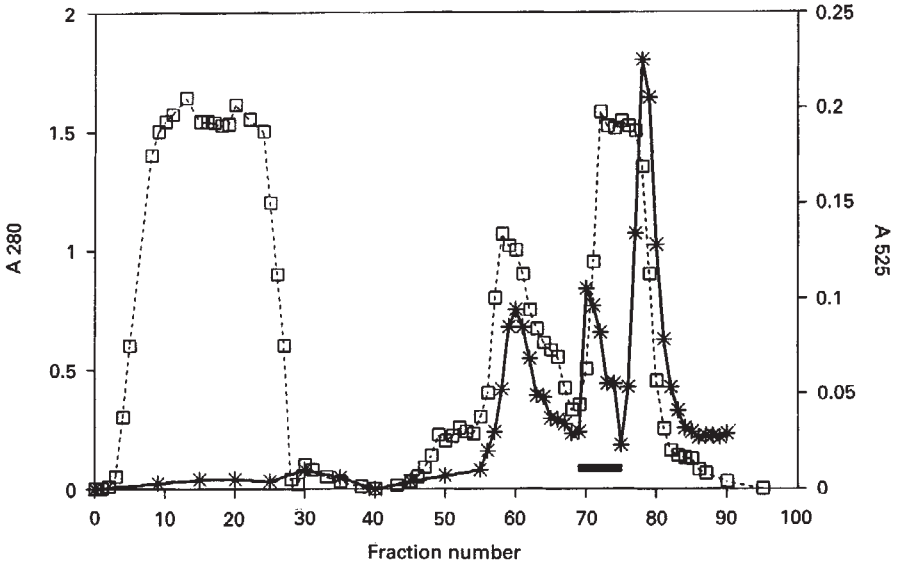

Fig. 1. *Chromatography of the extract of rat glomeruli on DE-52 cellulose.* Elution took place with 7 M urea/10 mM Tris buffer, pH 7.6, followed by a gradient starting at fraction 40 of 0 to 2 M NaCl in the same buffer. Fractions were analyzed for protein (A280) (– –□– –) and sulfated GAGs (A525, Farndale assay) (–*–). Fractions 69-75 (indicated by the bar) were pooled and dialyzed against PBS.

an Amicon XM-50 filter, and extensively dialyzed against 7 M urea/10 mM Tris-HCl buffer, pH 6.8. After centrifugation (40,000 g for 30 min) the supernatant was purified on a DE-52 cellulose column (2.5 × 20 cm) equilibrated with 7 M urea/10 mM Tris-HCl buffer, pH 6.8. The bound material was eluted by a linear 0 to 2 M sodium chloride gradient. The protein content of the fractions was determined spectrophotometrically at A280 and the glycosaminoglycan (GAG) content by the Farndale assay [30]. During elution, two protein peaks appeared, the first containing one Farndale-positive peak, the second comprising two Farndale-positive peaks (Fig. 1). This elution profile was completely identical to that obtained during purification of human glomerular extracts. From this previous analysis, for which we used human specific anti-HSPG antibodies [31], we knew that HSPG was only present in the middle Farndale-positive peak. For that reason fractions 69 to 75 were pooled and dialyzed extensively against PBS. Comparison of the protein/GAG ratio of the 7 M urea extract before DE-52 chromatography of 51.5 with that of 4.3 of the pooled fractions 69 to 75 after DE-52 chromatography shows an evident enrichment for HSPG, as indicated by this decreased protein/GAG ratio. This ratio is of the same order as for isolated human GBM HSPG [31]. This crude glomerular HSPG preparation was used for immunizations.

Hybridoma production

Female BALB/c mice were immunized intraperitoneally at days 0 and 30 with 50 μg of the glomerular HSPG preparation dissolved in 150 μl PBS and emulsified with an equal volume of complete Freund's adjuvant. At days 60, 61 and 62 the mice received an intravenous booster injection of 15 μg of the same immunogen in 0.15 ml PBS. At day 63 the mice were sacrificed, their spleens were removed, and splenocytes were fused with the non-producing SP2/0 mouse myeloma cell line, according to the principles of Kohler and Millstein [32]. Syngeneic peritoneal macrophages (10,000 to 20,000 cells/well) were used as feeder cells. Wells with growing hybridomas were screened in ELISAs with either the immunogen or HS as coated antigen, and by indirect immunofluorescence (IF) on rat kidney cryostat sections. After HAT selection, screening and expansion, cell cultures were transferred to HEPES-buffered RPMI-1640 culture medium containing 10% fetal calf serum, gentamycin (40 μg/ml), pyruvate (110 μg/ml) and glutamine (292 μg/ml). Selected wells were recloned by limiting dilution until all subclones displayed the same reactivity pattern. All cells were cultured in an humidified incubator at 37°C with 95% air and 5% CO_2. The antibody subclass was determined in ELISA using subclass-specific antibodies conjugated to peroxidase (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA). Ten to twelve weeks old, pristane (2,6,10,14 tetramethylpentadecane, Janssen Chimica, Beerse, Belgium) primed, male BALB/c mice received 3 × 10^6 viable hybridoma cells intraperitoneally. After one to three week ascites was drained, left to clott overnight at 4°C, centrifuged, filtered through 0.2 μm filters, aliquoted and stored at −20°C until use. The IgM concentration of the ascites was determined by the single diffusion technique according to Mancini, Carbonara and Heremans [33], using purified mouse IgM (Bionetics, Kensington, Maryland, USA) as the standard and goat anti-mouse IgM (Sigma Chemical Co., St. Louis, Missouri, USA) as antibody.

Characterization of mAb

ELISA studies. Anti-glycosaminoglycan (GAG) reactivity was measured in ELISA as described [34] using protamine chloride precoating to ensure sufficient binding of the negatively charged GAGs to the plates. The following GAGs were used: HS isolated from bovine kidney (Seikagaku, Tokyo, Japan); chondroitin sulfate A and C isolated from whale cartilage (Sigma); dermatan sulfate isolated from porcine skin (Sigma); keratan sulfate isolated from calf cartilage (from Dr. R. van de Stadt, Jan van Breemen Institute, Amsterdam, The Netherlands), heparin (Thromboliquine, Organon Teknika, Oss, The Netherlands), and hyaluronic acid (HA) isolated from human umbilical cord (Sigma). The ELISA for determination of antibody reactivity against glomerular HSPG (pool 69-75), human GBM HSPG (isolated as described previously [31]), and its core

protein [obtained after treatment of GBM HSPG with trifluoromethane sulphonic acid (TFMS) [31]], laminin (from the mouse EHS tumor; Sigma), collagen type IV (from the mouse EHS tumor, Collaborative Res. Inc., Bedford, Massachusetts, USA) and fibronectin (from rat plasma, Calbiochem, La Jolla, California, USA) was carried out according to Rennard et al [35]. As positive controls in the ELISAs were used: serum of the mouse selected for fusion (positive control for pool 69-75), rabbit anti-EHS laminin (GIBCO Europe, Breda, The Netherlands), rabbit anti-mouse collagen type IV (Collaborative Res. Inc.) and goat anti-rat fibronectin (Calbiochem). The inhibition ELISA was carried out with the different GAGs and with isolated human GBM HSPG or its core protein as inhibitor. For this inhibition ELISA, mAb JM-403 was diluted to a concentration giving 80% of the maximal ELISA signal and preincubated with an equal volume of 60 μl inhibitor at concentrations ranging from 0.2 to 100 μg/ml for one hour at 37°C and subsequently for one hour at 4°C and tested in the ELISA with HS as coated antigen. Results are expressed as % inhibition of the ELISA signal calculated as: [1 − (A450 + inhibitor/A450 − inhibitor)] × 100%.

Immunofluorescence (IF). Indirect IF was performed on 2 μm cryostat sections of rat kidneys, which were incubated with 25 μl of primary antibody (culture supernatant of hybridoma cells) for 30 minutes at 20°C. Incubation of the sections with a non-relevant mAb served as a negative control. After washing with PBS, binding was visualized by incubating the sections for 30 minutes at 20°C with 25 μl, 1:500 diluted FITC-labeled $F(ab)_2$ fragments of sheep anti-mouse IgG, heavy and light chains specific (Cappel, Organon Teknika, Turnhout, Belgium) or with FITC-labeled goat anti-mouse IgM, Fc specific (Nordic, Tilburg, The Netherlands), 1:50 diluted in PBS containing 1% bovine serum albumin (BSA), 0.05% sodium azide and 5% normal rat serum. Direct IF was performed on 2 μm cryostat sections of rat kidneys snap frozen in liquid nitrogen. Sections were incubated for 30 minutes with FITC-labeled goat anti-mouse IgM, Fc specific, 1:50 diluted in PBS containing 1% BSA, 0.05% sodium azide and 5% normal rat serum. Sections were washed with PBS, embedded in Aqua Mount (pH 7.0) and examined with a Leitz fluorescence microscope equipped with a Ploemopak epi-illuminator. Pretreatment of the sections was performed by incubating unfixed cryostat sections for 30 minutes at 37°C in the appropriate buffer with 1 U/ml heparitinase (Seikagaku, Tokyo, Japan), 0.5 U/ml chondroitinase ABS (Sigma) or 50 U/ml hyaluronidase (Sigma). After washing with PBS, indirect IF with mAb JM-403 was performed. As a positive control, goat antibodies against human GBM HSPG-core protein were used, which cross react with rat GBM HSPG. Indirect IF was performed as described above, using FITC-labeled rabbit anti-goat IgG (Kallestad, Chaska, Minnesota, USA) to detect the bound goat antibodies. Inhibition studies in indirect IF on kidney sections were carried out by incubating mAb JM-403 in the presence of GBM HSPG, its core protein, different GAGs or the monosaccharide components of HS and HA, N-acetyl-glucosamine and glucuronic acid in a concentration of 100 μg/ml.

Immunoelectron microscopy (IEM). For indirect IEM, rat kidneys were perfused for 10 minutes via the retrograde aortic route with PBS, followed by perfusion with a mixture of periodate-lysine-2% paraformaldehyde (PLP) fixative for 10 minutes. Small pieces of renal cortex were further immersed in the same fixative for an additional three hours, cryoprotected in 2.3 M sucrose for 45 minutes, and snap frozen in liquid nitrogen. Twenty-five μm cryostat sections were incubated with mAb JM-403, diluted 1:30 in PBS containing 1% BSA, for 18 hours at 4°C and washed several times with PBS afterwards. For the direct IEM, rats were injected i.v. with one ml JM-403 ascites. After one hour, the kidneys were perfused and fixed, cryoprotected and stored as described for the indirect IEM. Twenty-five μm cryostat sections were cut. Sections for the direct as well as for the indirect IEM were incubated with a peroxidase-labeled rabbit anti-mouse Ig (Dakopatts, Copenhagen, Denmark), diluted 1:20 in PBS-1% BSA for 90 minutes. After several washes in PBS, the sections were preincubated in 0.05% diaminobenzidine (DAB) medium containing 0.6% TRIS for 10 minutes, followed by the same medium with addition of 10 μl of 30% H_2O_2 for 10 minutes. After washing in H_2O for 18 hours, the sections were post-fixed in 0.1 M phosphate buffered 1% OsO_4 pH 7.4 for 30 minutes at room temperature, dehydrated, and embedded in Epon 812. Thin sections were prepared on a LKB Ultratome, and examined in a Philips 300 electron microscope.

Nephritogenicity of mAb JM-403

Animals. The nephritogenic properties of mAb JM-403 were investigated in male Wistar rats of 200 g, bred at our animal laboratory. During the experiments rats were placed in individual metabolic cages, with free access to water and standard chow.

Experimental design. In the first experiment, rats were injected i.v. in the tail vein under ether anaesthesia with one ml of JM-403 ascites (containing 1 mg IgM/ml). Rats were sacrificed one hour, 24 hours and 14 days after injection. Kidneys were removed, snap frozen in liquid nitrogen and used for direct IF as described above. In the second experiment, five groups of five rats were used. Rats were injected i.v. with respectively 0.032, 0.125, 0.5 and 2 mg IgM (JM-403 ascites, in PBS with a final volume of 2 ml). In control experiments rats received 2 ml ascites in PBS containing 2 mg of a non-relevant IgM mAb (anti-human B-cell idiotype). After injection rats were placed in individual metabolic cages and 24-hour urine specimens were sampled during 10 days. Thereafter, a blood sample was taken from each rat. In the third experiment 10 rats received 0.5 ml JM-403 ascites (containing 0.5 mg IgM) i.v. Immediately after injection rats were placed in metabolic cages and urine was sampled every two hours, during 24 hours.

Urine analysis. Urinary albumin concentrations were determined by rocket immunoelectrophoresis according to Laurell [36], using goat anti-rat albumin and rat albumin as the standard (both from Nordic, Tilburg, The Netherlands). Urinary IgG concentrations were determined in a capture ELISA, using rat IgG (Nordic), 2 to 2000 ng/ml as the standard. Polystyrene microtiter plates (Greiner) were coated overnight at room temperature with affinity-purified goat anti-rat IgG, heavy and light chains specific (Cappel), 1:500 in 0.05 M carbonate buffer, pH 9.6, 100 μl/well. After washing with PBS containing 0.05% Tween-20 (PBS-T), urines were diluted 1:4 to 1:4096 in PBS-T containing 0.2% BSA, 100 μl/well. Plates were incubated for one hour at 37°C. After washing with PBS-T, 100 μl peroxidase conjugated, affinity-purified goat anti-rat IgG, (Cappel), 1:500

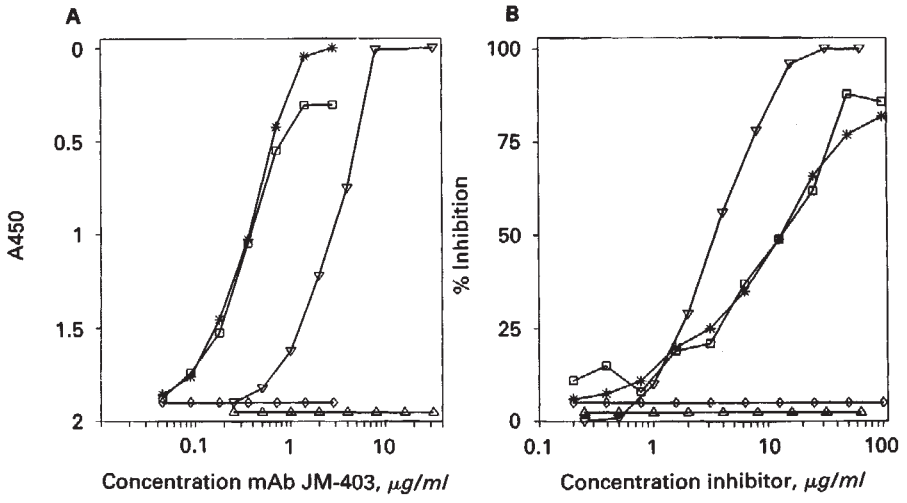

Fig. 2. *Antigen specificity of mAb JM-403.* **A.** Reactivity of mAb JM-403 in ELISA. MAb JM-403 bound to HS (–*–) and HA (–□–), but not to the other GAGs (chondroitin sulfate A and C, dermatan sulfate, keratan sulfate and heparin) (–◇–). Binding also occurred to isolated human GBM HSPG (–▽–), whereas its core protein was not recognized (–△–). **B.** Dose-dependent inhibition of mAb JM-403 in ELISA. HS was used as coated antigen. Inhibition was found with intact human GBM HSPG (–▽–), with HS (–*–) and with HA (–□–), but not with the other GAGs (–◇–) or the HSPG core protein (–△–).

in PBS-T, 0.2% BSA were added to the wells and incubated for another hour at 37°C. Finally, after washing with PBS-T, 100 μl freshly prepared substrate solution (0.8 mg/ml 5-aminosalicylic acid in 50 mM phosphate buffer, pH 6.0, containing 0.8 μl/ml 30% vol/vol H_2O_2) were added to each well. After 30 minutes the absorbance was measured at 450 nm in a Titertek multiscan. Serum albumin and IgG concentrations were determined as described above for urine. The selectivity index was defined as clearance IgG/clearance albumin.

Statistical analysis

For statistical analysis of results with a normal distribution the paired *t*-test was used, for not normally distributed data the Wilcoxon's test. Probability values <0.05 were regarded as significant. All values are expressed as mean ± SD.

Results

Production of monoclonal antibodies

After immunization of female BALB/c mice with the glomerular HSPG preparation and fusion of the splenocytes with the non-producing SP2/0 myeloma cell line, growing hybridomas were screened in indirect IF on rat cryostat kidney sections and in ELISA on the immunogen and HS. After this screening procedure one out of 700 clones was positive. This hybridoma, called JM-403, was subcloned twice until all subclones displayed the same reactivity pattern. MAb JM-403 belongs to the IgM class. Ascites contained 1 mg IgM/ml.

Characteristics of mAb JM-403

ELISA studies. The reactivity pattern of mAb JM-403 in ELISA with different GAGs is shown in Figure 2A. A clear reactivity was observed with HS and hyaluronic acid (HA), but no binding occurred to chondroitin sulfate A and C, dermatan sulfate, keratan sulfate or heparin. Since mAb JM-403 cross reacted with human tissues (not shown), we tested its binding to isolated human GBM HSPG and its core protein in ELISA. The core protein was obtained after treatment with TFMS, a method known to cleave all saccharide units [31]. Figure 2A also shows that mAb JM-403 bound to human GBM HSPG in ELISA, but not to its core protein. The GAG specificity of mAb JM-403 was confirmed by the inhibition ELISA shown in Figure 2B. Both HS and HA were able to inhibit the binding of mAb JM-403 to HS in a dose dependent manner. None of the other GAGs, even at the highest concentration (100 μg/ml), showed any inhibition. Cellulose acetate electrophoresis of HA used in our experiments did not show any contamination of HA with HS, which excluded impurity of the HA preparation as a cause of this binding. Figure 2B also showed that the intact HSPG molecules were able to inhibit the binding of mAb JM-403 to HS in ELISA in a dose-dependent manner, whereas the core protein did not inhibit at all. This lack of inhibition of the core protein was due to the removal of HS-side chains and not to denaturation or degradation of the core protein by the TFMS treatment, since the core protein inhibited equally effectively as intact HSPG for the binding of an anti-HSPG core mAb to HSPG (data not shown). All results corroborated the HS specificity of mAb JM-403. In ELISA mAb JM-403 did not bind to other basement membrane components, that is, laminin, collagen type IV or fibronectin (data not shown).

Histological studies. By indirect IF on 2 μm cryostat sections of a rat kidney, mAb JM-403 bound strongly to the GBM and Bowman's capsule in a fine granular to linear pattern (Fig. 3A). Also the basement membranes of endothelial cells and of smooth muscle cells in arteries and arterioles were positive, as well as some tubular basement membranes (TBMs), although this staining was much weaker and had a variable intensity (Fig. 3B). The staining pattern of the glomerulus corresponded very well to that of antibodies directed against the core protein of GBM HSPG (Fig. 3D). The HS specificity of mAb JM-403 was further substantiated by the finding that binding was completely prevented by pretreatment of the sections with heparitinase, an enzyme which degrades HS (Fig. 3C). This pretreatment did not degrade the core protein of HSPG, since antibodies against the HSPG core protein still bound (Fig. 3D). No alterations in staining pattern were observed after pretreatment of the sections with chondroitinase ABC or hyaluronidase. Binding of mAb JM-403 to the sections could also be inhibited by the addition of 100 μg/ml human GBM HSPG, HS or HA to the mAb before incubation of the sections, but not by the addition of the other GAGs or the core protein of human GBM HSPG,

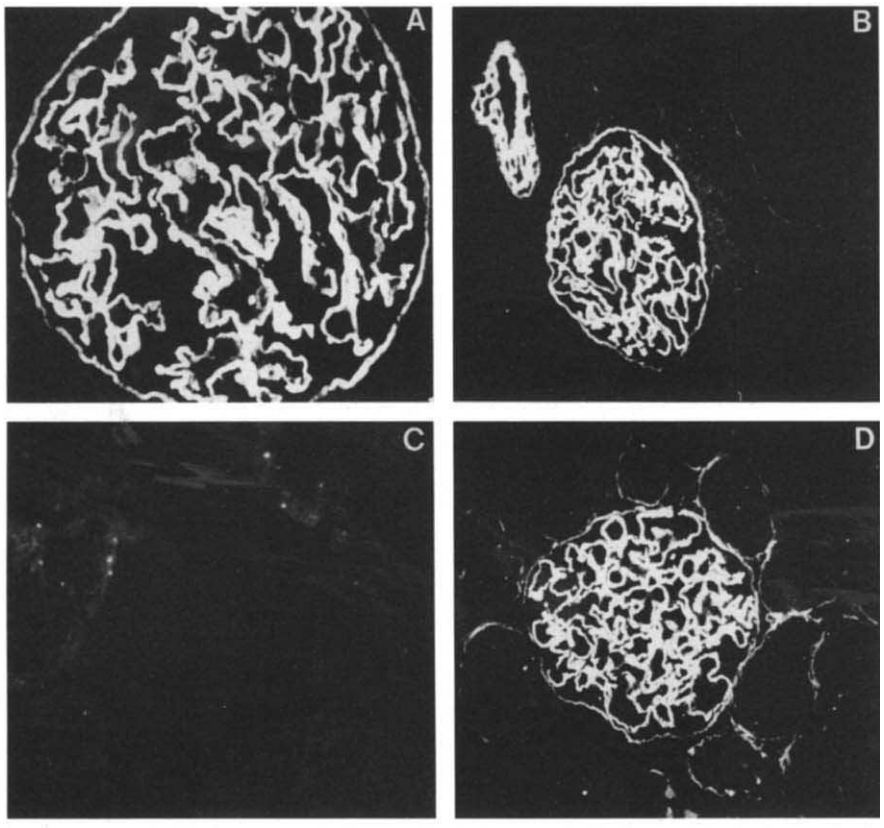

Fig. 3. *Indirect IF of a rat kidney using mAb JM-403* **(A, B and C)** *and a polyclonal antibody against the GBM HSPG-core protein* **(D)**. Sections shown in photomicrographs C and D were pretreated with heparitinase. Note the absence of staining with mAb JM-403 after heparitinase pretreatment (C), whereas binding of the anti-HSPG-core antibodies was unaltered (D) (A × 508; B × 254; C × 422; D × 264).

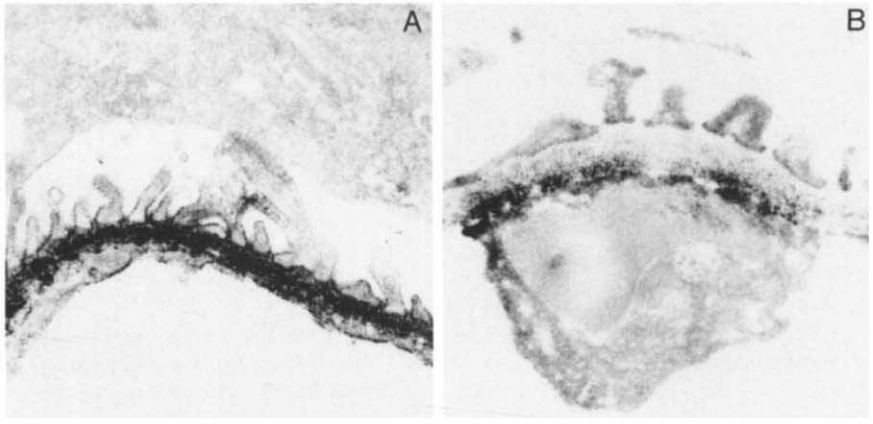

Fig. 4. *IEM photomicrographs illustrating the in vitro and in vivo binding of mAb JM-403.* **A.** Indirect IEM showed a clear staining of the whole GBM. **B.** Direct IEM of a capillary wall prepared one hour after i.v. injection of 1 ml JM-403 ascites (1 mg IgM/ml). Reaction product was located in the lamina rara interna of the GBM and its border adjacent to the endothelium. In some but not all glomeruli, a faint staining was observed of the endothelial cell membranes (A × 13,000; B × 18,850).

nor by the addition of the monosaccharide components of HS and HA, N-acetyl-glucosamine and glucuronic acid. Tissues known to contain large amounts of HA (skin and umbilical vein) only revealed a basement membrane staining in indirect IF. Moreover, this staining was completely abolished after pretreatment of the sections with heparitinase, whereas hyaluronidase did not alter the staining pattern, indicating that mAb JM-403 did not recognize HA in situ. In indirect IEM, mAb JM-403 bound diffusely to the whole width of the GBM (Fig. 4A). Occasionally staining was observed of the cell membranes of endothelial cells and the foot processes of epithelial cells. It is difficult to distinguish whether this is due to binding to the cell membrane or to a diffusion artifact.

Nephritogenicity of anti-HS mAb JM-403

After intravenous injection mAb JM-403 immediately localized predominantly along the GBM, as indicated by direct IF one hour after injection (Fig. 5A). A fine granular staining of the GBM was observed along with some staining of the mesangium. Twenty-four hours after injection, the staining pattern had shifted towards a more mesangial localization, while the staining in the GBM had decreased considerably (Fig. 5B). Two

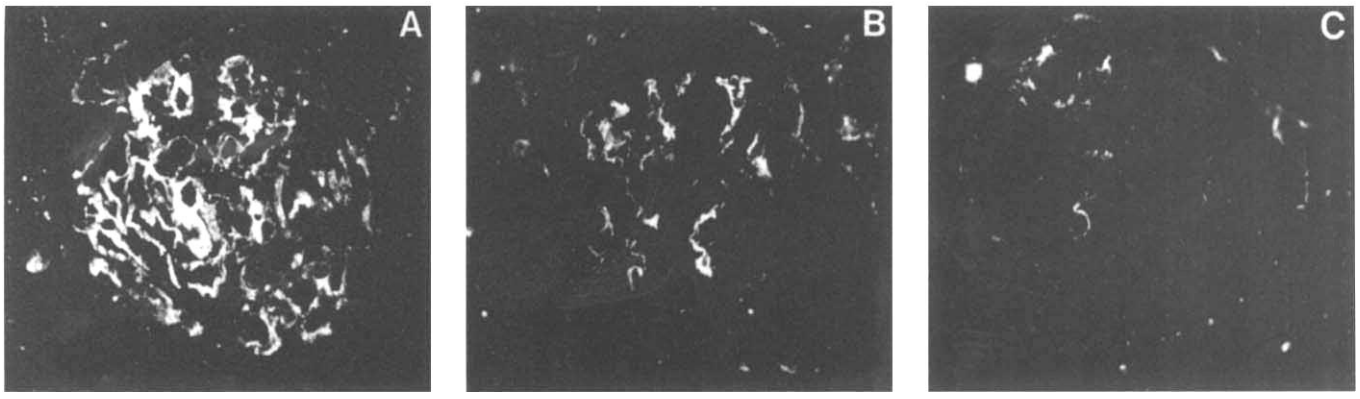

Fig. 5. *Direct IF of rat kidneys obtained one hour* (A), *twenty-four hours* (B) *or two weeks* (C) *after i.v. injection of 500 μg mAb JM-403.* There was a shift from GBM binding at one hour (A) to a more mesangial localization at twenty-four hours (B). After two weeks no mAb was detectable in the glomeruli (C) (A, B and C × 380).

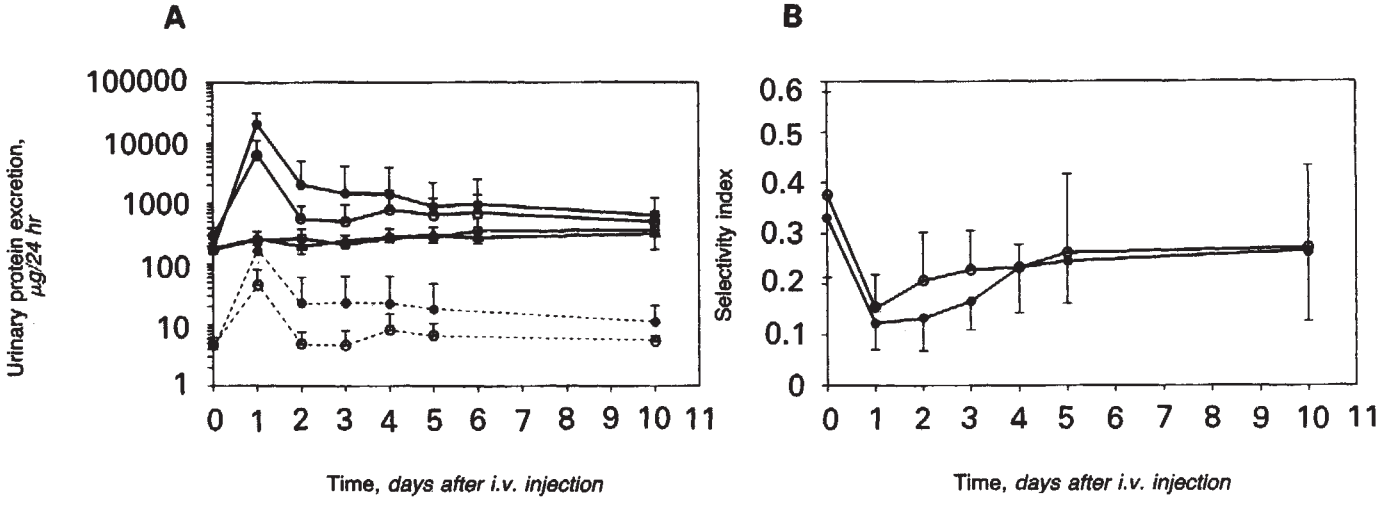

Fig. 6. *Urinary protein excretion per 24 hours after i.v. injection of 0.032 (–□–), 0.125 (–△–), 0.5 (–○–) and 2 (–●–) mg mAb JM-403.* Each group consisted out of 5 rats. Day 0 gives baseline levels before injection of mAb. Graph A shows the course of the albuminuria (solid lines) and the urinary IgG excretion (dashed lines), both expressed as μg/24 hours. Graph B shows the course of the selectivity index of the proteinuria (clearance IgG/clearance albumin). Urinary IgG excretion and selectivity indexes are given for the rats that received 0.5 and 2 mg mAb.

weeks after injection, hardly any antibody could be detected in the glomerulus, however, a weak staining of Bowman's capsule remained (Fig. 5C). These immunohistological data indicate a direct, transient binding of anti-HS mAb JM-403 along the GBM.

Direct IEM visualized the bound mAb JM-403 mainly at the inner side of the GBM one hour after i.v. injection (Fig. 4B). Sometimes, reaction products were also observed on endothelial cells, but not in every glomerulus. No binding occurred to epithelial cells. Additional staining was observed in the paramesangial areas. This staining pattern corresponded well to the IF staining depicted in Figure 5A.

To test whether this GBM binding of JM-403 could alter the permeability of the GBM, groups of five rats were injected i.v. with different doses of mAb, respectively 0.032, 0.125, 0.5 and 2 mg. A dose-dependent albuminuria was induced after 0.5 and 2 mg mAb, which was maximal at day 1 and declined thereafter (Fig. 6A). To investigate whether the induced proteinuria was selective, urinary excretion of IgG was also measured. The urinary IgG excretion followed the same pattern as the albumin excretion, although at a lower level (Fig. 6A). If we calculated the selectivity index (clearance IgG/clearance albumin) we found that the proteinuria was selective (Fig. 6B). The values for the mean (± SD) of the urinary albumin and IgG excretion in the first 24 hours after the i.v. injection of either 0.5 or 2 mg mAb JM-403 and the corresponding selectivity indexes, together with the baseline values for the different parameters are given in Table 1. Control studies in five rats, receiving ascites containing 2 mg of a non-relevant IgM mAb (anti-human B-cell idiotype) i.v., did neither show any glomerular localization of mouse IgM, nor development of albuminuria. Since the albuminuria was maximal at day 1, we analyzed in 10 rats the kinetics of the albumin excretion in two-hour urine samples during the first 24-hours after injection of 0.5 mg mAb JM-403. Figure 7 shows that the albumin excretion is maximal in the first two hours after injection with a gradual decline thereafter. The magnitude of the albuminuria paralleled the glomerular binding of the mAb as seen by direct IF (Fig. 5).

Table 1. Mean (± SD) urinary albumin and IgG excretion after i.v. injection of a 2 ml dilution of JM-403 ascites containing either 0.5 or 2 mg IgM in the first 24 hours after injection (day 1) compared with the normal excretion (baseline)

	0.5 mg i.v.			2 mg i.v.		
	baseline	day 1	*P* value	baseline	day 1	*P* value
Albumin excretion *μg/24 hr*	284 ± 120	6,486 ± 4,640	< 0.04	177 ± 19	20,755 ± 10,310	< 0.01
IgG excretion *μg/24 hr*	5.7 ± 1.5	58.6 ± 42.7	< 0.05	5.8 ± 2.9	236.1 ± 132.2	< 0.03
Selectivity index	0.37 ± 0.20	0.15 ± 0.07	< 0.03	0.33 ± 0.12	0.12 ± 0.05	< 0.004

The selectivity index of the proteinuria (clearance IgG/clearance albumin) is also given.

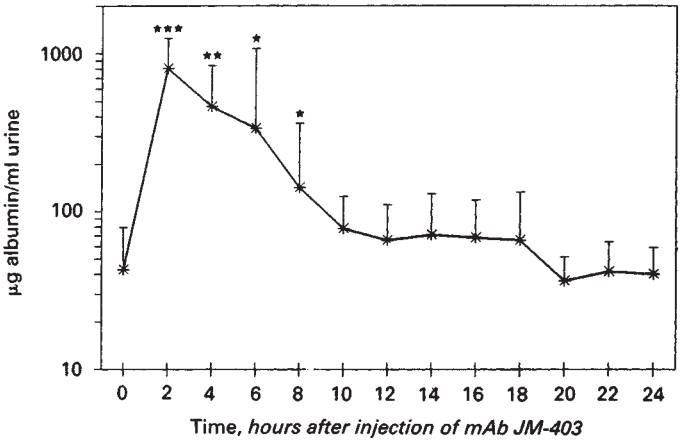

Fig. 7. *Albumin excretion of two-hour urine specimens, sampled during the first 24 hours after i.v. injection of 500 μg mAb JM-403.* Note that the increase of albumin excretion occurred immediately after injection of mAb. Statistical significance between urinary albumin excretion before and in the two-hour urine specimens after injection of mAb is indicated by ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Discussion

In this report we describe the production of a nephritogenic mAb against GBM HS. The HS-specificity of mAb JM-403 can be concluded from the following experimental data: (a) in ELISA a strong reactivity was observed with HS, but not with chondroitin sulfate A or C, dermatan sulfate, keratin sulfate or heparin; (b) mAb JM-403 bound in ELISA to isolated human GBM HSPG, but not to its core protein without HS-chains; (c) pretreatment of kidney sections with heparitinase prevented the binding of JM-403, whereas the binding of polyclonal antibodies against the core protein of GBM HSPG was unaffected. Pretreatment with chondroitinase ABC and hyaluronidase did not influence the binding of JM-403; (d) the binding of mAb JM-403 to HS in ELISA as well as the binding to the kidney sections in indirect IF could be inhibited by human GBM HSPG and HS, but not by other GAGs or the HSPG core protein.

Besides binding to HS, mAb JM-403 also reacted with HA. Interestingly, Fillit et al described two mAbs against HA, from which one bound to HS [37]. They also demonstrated in sera of patients with post-streptococcal glomerulonephritis the presence of antibodies against HS, which cross reacted with HA [38]. Their and our findings indicate that HS and HA contain cross reactive epitopes. Both HA and HS contain the same basic structure of repeating disaccharide units of N-acetyl-glucosamine and glucuronic acid. Apparently the presence of O- and N-linked sulfate is not important for JM-403 binding since HA does not contain these components. On the other hand, mAb JM-403 exhibits no affinity for heparin that has more iduronic acid and sulfate groups than HS. The binding of mAb JM-403 to HS could not be inhibited by glucuronic acid or N-acetyl-glucosamine. These findings suggest that the epitope may be located within a cluster of unsulfated glucuronic acid—N-acetyl-glucosamine disaccharide units. The fact that HA present in cryostat sections of skin and umbilical vein (tissues known to contain large amounts of HA) did not stain with mAb JM-403 in indirect IF and the absence of an effect of pretreatment of these sections with hyaluronidase suggests that the epitope on HA, which is recognized by mAb JM-403, is hidden in vivo. This could be due to the fact that in situ, HA is bound to proteoglycans and proteins, thereby forming large molecular complexes, which may result in the masking of the JM-403 recognized epitope, which is set free after isolation of HA.

Comparison of the characteristics of mAb JM-403 with those of previously described mAbs against HS [25, 27] indicates differences in epitope specificity. Kure and Yoshie [25] described an anti-HS mAb directed against an epitope which is related to O-sulfated and N-acetylated glucosamine present in cell surface-associated HS. Snow et al [27] recently described an mAb against a glucosamine sulfate $\alpha 1 \rightarrow 4$ glucuronic acid in HS of mouse EHS HSPG. Both mAbs did not bind to other GAGs, including HA and heparin.

In indirect IF on cryostat sections of rat kidneys, the GBM, Bowman's capsule and the basement membranes of endothelial cells and of smooth muscle cells in arteries and arterioles stained much more intensely than the TBMs. The latter staining was variable and much weaker; some TBMs were even negative, which seemed to be related to the different segments of the nephron. In indirect IEM, we observed that the whole width of the GBM was stained by mAb JM-403. At first glance this seems to be in contrast with the findings of Stow, Sawada and Farquhar [39], who found that the HSPG molecule was concentrated in both laminae rarae of the GBM. However, their results were obtained after a short (2 to 4 hr) incubation of the sections with the anti-HSPG antibodies. In accordance with our results, they also found a diffuse staining of the whole GBM after an overnight incubation. Alternatively, differences in staining patterns can also be explained by differences in epitope specificity, since their antiserum was directed against the core protein of GBM HSPG, whereas our mAb recognizes the HS chain.

The i.v. administration of mAb-403 induced an acute selective proteinuria. This shortlasting albuminuria was paralleled by a transient binding of anti-HS mAb JM-403 along the GBM.

Similar transient binding kinetics were found after i.v. administration of exogenous or endogenous cationic molecules [6, 12, 13, 40, 41]. Their binding to the anionic sites of the GBM is also accompanied by a short bout of albuminuria. Furthermore, injection of polycations led to a preferential leakage of albumin compared to IgG [12, 13], like we found with our mAb. These similarities suggest that, like polycations, mAb JM-403 neutralizes the HS-associated anionic sites of the GBM and decreases the charge-selective properties of the GBM, favoring the urinary excretion of negatively-charged serum proteins like albumin. Whether binding of mAb JM-403 to GBM HS can induce a local inflammatory response in addition remains to be investigated.

These in vivo experiments were done with unpurified ascites containing mAb JM-403, since the procedures we used to purify the IgM mAb [42, 43] had a poor recovery and a substantial loss of anti-HS specificity. We performed control studies with ascites containing 2 mg of a nonrelevant IgM mAb. In these rats neither glomerular binding nor albuminuria was detected.

It is tempting to speculate that the quick disappearance of the mAb from the GBM and the short period of albuminuria is related to the IgM isotype of the mAb. The large molecular mass of the IgM molecules (900 kDa) probably prevents penetration into the GBM, and thereby restricts binding to HS present in the lamina rara interna of the GBM. This is exactly the localization of mAb JM-403 which we found with direct IEM after i.v. injection (Fig. 4B). In other models of glomerular injury caused by subendothelial immune deposits, like the Con A-ferritin/anti-ferritin model [44], the immune complexes disappear within 24 hours, in contrast to subepithelial immune deposits which persist for a long time. The final proof has to come from the kinetics of intraglomerular binding and the course of albuminuria after administration of anti-HS mAbs of the IgG isotype, since one can expect that this isotype penetrates the GBM more easily and can induce subepithelial immune deposits in addition to subendothelial localization, as was observed for antibodies against the core protein of HSPG [22, 23]. The two other mAbs against HS that have been described [25, 27] were not tested for their nephritogenic potential. Two other studies have claimed a role for anti-HS antibodies in nephritis. Fillit et al [38] detected anti-HS antibodies in sera of patients with acute post-streptococcal nephritis. Whether these antibodies have a pathogenic significance for the glomerulonephritis is difficult to prove. In another study [45] Abrass and Cohen immunized rats with commercially available HS and investigated the role of anti-HS antibodies in the development of glomerular injury. Binding of anti-HS antibodies in the glomerulus induced a mild inflammatory reaction and a reduction of glomerular filtration rate, but no abnormal proteinuria. Unfortunately however, their HS preparation was contaminated with a 60 kDa protein, and the HS-specificity of the antibodies was not proven unequivocally. Antibodies against the core protein of HSPG are nephritogenic after intravenous injection. A mild form of anti-GBM-like nephritis occurred with [23] or without [22] the development of albuminuria.

In summary, our results show that antibodies to HS are nephritogenic and can induce a selective proteinuria, probably secondary to the neutralization of HS-associated anionic sites in the GBM.

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