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Nephrotic Syndrome and Subepithelial Deposits in a Mouse Model of Immune-Mediated Anti-Podocyte Glomerulonephritis

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Subepithelial immune complex deposition in glomerular disease causes local inflammation and proteinuria by podocyte disruption. A rat model of membranous nephropathy, the passive Heymann nephritis, suggests that Abs against specific podocyte Ags cause subepithelial deposit formation and podocyte foot process disruption. In this study, we present a mouse model in which a polyclonal sheep anti-mouse podocyte Ab caused subepithelial immune complex formation. Mice developed a nephrotic syndrome with severe edema, proteinuria, hypoalbuminemia, and elevated cholesterol and triglycerides. Development of proteinuria was biphasic: an initial protein loss was followed by a second massive increase of protein loss beginning at approximately day 10. By histology, podocytes were swollen. Electron microscopy revealed 60–80% podocyte foot process effacement and subepithelial deposits, but no disruption of the glomerular basement membrane. Nephrin and synaptopodin staining was severely disrupted, and podocyte number was reduced in anti-podocyte serum-treated mice, indicating severe podocyte damage. Immunohistochemistry detected the injected anti-podocyte Ab exclusively along the glomerular filtration barrier. Immunoelectron microscopy localized the Ab to podocyte foot processes and the glomerular basement membrane. Similarly, immunohistochemistry localized mouse IgG to the subepithelial space. The third complement component (C3) was detected in a linear staining pattern along the glomerular basement membrane and in the mesangial hinge region. However, C3-deficient mice were not protected from podocyte damage, indicating a complement-independent mechanism. Twenty proteins were identified as possible Ags to the sheep anti-podocyte serum by mass spectrometry. Together, these data establish a reproducible model of immune-mediated podocyte injury in mice with subepithelial immune complex formation. The Journal of Immunology, 2011, 187: 000–000.
cytes, and Ab levels seem to correlate with membranous disease activity (11), but clearly not all patients with membranous nephropathy also have phospholipase A2 receptor Abs in their serum (11). Definite proof of principle is lacking because phospholipase A2 receptor is not expressed on murine podocytes. In summary, the Ag on human podocytes remains at least partly unknown. It has been speculated that the Ag is localized on the basal aspect of the podocyte foot process (14) and that local microinflammation at the site of Ag/Ab recognition contributes to podocyte damage (15). Histologically, the development of subepithelial glomerular basement membrane (GBM) deposits that contain IgG and complement is the hallmark of rat and human disease. Until recently, complement activation and C5b-9 membrane attack complex formation at the podocyte were thought essential to the development of PHN (16, 17). However, PHN can be induced in complement 6-deficient rats (PVG C6−/−) that cannot activate the complement cascade to form C5b-9 membrane attack complex (18). The same rats were used to show that HN is not complement dependent (19).

Recently, we presented a mouse model of immune-mediated podocyte injury in which a polyclonal rabbit anti-podocyte antisera was used to induce massive proteinuria in mice (20). We proposed that differentiated, cultured murine podocytes expressed specific Ags that would lead to the generation of anti-podocyte Abs after injection into rabbit, similar to the development of anti-FX1A Abs in PHN. Rabbit anti-podocyte serum injection into mice induced massive proteinuria in a prolonged fashion, but subepithelial deposits were not observed (20). Because of limited quantities of rabbit anti-podocyte serum, the same approach was now used to generate sheep anti-podocyte antisera. In addition, a murine podocyte cell line from Schiwek et al. (21) was used as Ag for sheep immunization instead of podocytes by Mundel et al. (22) as these cells more stably express podocyte marker proteins such as nephrin in culture upon differentiation. To our surprise, the effect of the sheep antisera on mouse was profoundly different from the effect of rabbit antisera. We find that injection of sheep anti-podocyte serum into mice induced severe nephrotic syndrome with podocyte foot process effacement and formation of subepithelial deposits. Several putative Ags were identified by mass spectrometry. This work summarizes the data obtained with the mouse model and discusses similarities and differences with the rabbit anti-podocyte nephritis (APN) model and the rat model of PHN.

Materials and Methods

Podocyte culture

Murine podocytes (provided by Endlich, Greifswald, Germany) (21) were cultured under permissive conditions (33°C, 5% CO2, RPMI 1640 supplemented with 10% FCS, 1 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μU/ml IFN-γ [PeproTech, London, U.K.], as described (21)). Human podocytes (a gift of Saleem, Bristol, U.K.) were cultured under permissive conditions (33°C, 5% CO2, RPMI 1640, 10% FCS, 1×insulin-transferrin-sodium selenium) and for differentiation under nonpermissive conditions (37°C), as described (23). For differentiation, both podocyte cell lines were cultured for 14 d under nonpermissive conditions (37°C, 7.4% CO2) in respective media, omitting IFN-γ for murine podocytes. Cell density was kept below 80% to allow process formation.

Sheep immunization and polyclonal Ab concentration

Three sheep were immunized with murine podocytes. Prior to immunization, sheep were bled from the ear vein to obtain preimmune serum for control experiments. For immunization, a primary s.c. immunization with 1 ml murine podocyte cell suspension (∼10 × 106 cells) in 1 ml CFA (Sigma-Aldrich, Steinheim, Germany) was followed by four monthly boost immunizations of podocytes emulsified in IFA and serum titer assessments, as described before (20). One week after the final boost and monthly after reboots, sheep serum was collected. Following removal of the blood clot, sheep anti-podocyte serum was centrifuged at 2500 U/min for 20 min. Serum was complement inactivated by heating (30 min at 56°C in a water bath) and concentrated 2-fold by high-pressure membrane filtration technique (Amicon stirred cell 8000 series; Millipore, Eschborn, Germany). IgG purification was achieved with the ImmunoPure IgG purification kit from Pierce (Bonn, Germany). Agarose-coupled protein A was bound to 2.6 ml centrifuged sheep serum in binding buffer in a column, according to the manufacturer’s instructions. After several washes with binding buffer, purified IgG was eluted with elution buffer in three fractions. The pH of all fractions was adjusted with 1 M Tris base to pH 8.0 before dialysis of IgG with PBS to remove elution buffer. IgG concentration was monitored with Gelcode (Pierce)-stained SDS-PAGE gels and Bradford tests.

Characterization of sheep polyclonal Ab against murine podocytes

Generated Abs were tested by immunofluorescence on differentiated murine podocytes cultured on glass coverslips and by immunohistochemistry on paraffin-embedded mouse kidney sections (see below). Cells were fixed with 4% paraformaldehyde in 2% sucrose for 8 min. Specific binding of anti-podocyte serum to the cells was assessed by indirect immunofluorescence. Briefly, anti-podocyte serum was diluted 1:200 in 5% horse serum (Vector, Burlingame, CA) and incubated for 1 h at room temperature. Localization of Ab was visualized using an affinity-purified Cy2 donkey anti-sheep Ab (Jackson ImmunoResearch Laboratories, Sokus, U.K.) diluted 1:200 in 5% horse serum for 30 min. Stainings were evaluated with a confocal microscope (LSM 510; Zeiss, Oberkochen, Germany) using the LSM software.

Animal care

Mice were housed in a controlled animal facility with free access to water and standard animal chow. All experiments were performed according to national and institutional animal care and ethical guidelines and have been approved by the local animal care committee.

Murine model of sheep anti-podocyte nephritis

In a preliminary experiment, all three antisera were injected into C57BL/6N and BALB/c mice to compare the effects of the sera. In the following eight separate experiments with a total of 229 mice (n = 3–5 for each condition and time point), male C57BL/6N mice (25–30 g body weight, 12–14 wk old; Charles River, Sulzfeld, Germany) were treated with 300 μl concentrated sheep anti-podocyte serum injected into the tail vein. Control mice received equal amounts of concentrated sheep preimmune serum. Purified Ab was injected into mice in a 2-d scheme with 1.2 mg injected on day 1 and 1.6 mg injected on day 2. Mice were weighed and sacrificed on day 1–24 by cervical neck dislocation. Following the collection of blood, kidneys were perfused through the aorta with PBS. Kidneys were cut into equal pieces for histology and electron microscopy. Serum creatinine, urea nitrogen, cholesterol, and triglycerides were determined by standard methods in the department of clinical chemistry at the university hospital. Composite dependence was tested in one experimental series with C3-deficient C57BL/6N mice (24) and control C57BL/6N mice. Five C3-deficient and control mice were injected with 300 μl anti-podocyte serum, and four C3-deficient and control mice were injected with preimmune serum. All mice were sacrificed on day 11, and urine, serum, and kidneys were processed, as described above.

Analysis of proteinuria

Urine was collected for proteinuria from all mice on several days along the time course. Proteinuria was assessed by SDS-PAGE of mouse urine collected over 6–8 h in 96-well plates, as described before (20). Briefly, 5 μl urine were loaded in 4% lithium dodecyl sulfate (LDS) sample buffer onto a 4–12% graded Bis-Tris NuPage gel (Invitrogen, Karlsruhe, Germany) and run in 50 mM MOPS buffer for 1 h at 60 mA. Following electrophoteral separation, gels were visualized by Gelcode staining technique, according to the manufacturer’s instructions, and scanned. Urine albumin content was further quantified using a commercially available ELISA system (Bethyl), according to the manufacturer’s instructions, using an ELISA plate reader (BioTek; EL 808), as described (20). The urinary albumin concentration was calculated according to the formula for absorbance = (A492)–(x/C492 + D), where A and D are values from the standard curve. Regression values for the standard curve with r values >0.9950 indicated accurate measurements. Urinary albumin values were standardized against urine creatinine values of the same animals and plotted on a logarithmic scale.

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Serum albumin measurement

Serum albumin was measured with the same mouse albumin ELISA system as detailed above. Whole blood was collected by aortic puncture and centrifuged for 5 min at 4°C at 1000 × g, and serum was separated for further analysis. ELISA plate readings were performed in triplicate.

Immunoblot

Immunoblots against sheep anti-podocyte Ab were performed with HRP-coupled donkey anti-sheep Ab (Jackson ImmunoResearch Laboratories). Lysis of cultured, differentiated murine podocytes was performed in tissue lysis buffer (T-PER; Pierce), followed by denaturation in LDS sample buffer (Invitrogen). Protein lysates were separated on a 4–12% Bis-Tris NuPage gel (Invitrogen) in 50 mM MOPS buffer for 1 h at 60 mA. Protein transfer to an activated polyvinylidene difluoride membrane (Millipore) was performed in transfer buffer (25 mM Tris base, 0.192 mM glycine, 20% methanol in ddH2O) in a Novex Mini Cell (Invitrogen) for 1.5 h at 30 V and 4°C. The polyvinylidene difluoride membranes were blocked in 5% nonfat milk for 60 min prior to incubation with primary Abs diluted in Superblock blocking reagent (Pierce). Primary Ab was removed by washing in TBS-T for 40 min and detected by incubation for 45 min with HRP-coupled secondary Abs (Jackson ImmunoResearch Laboratories) diluted 1:15,000 in 5% nonfat milk. After washing in TBS-T for 40 min, protein was visualized with ECL SuperSignal (Pierce), according to the manufacturer’s instructions, on a Biomax Light Film (Kodak).

Specimen collection

This study was conducted according to the Declaration of Helsinki principles, with approval from the local ethics committee. Tissue samples were obtained from the renal register of the University Hospital Hamburg between 2005 and 2007.

Histology and immunohistochemistry in podocytes and mice

Cultured podocytes were fixed with 4% parafomaldehyde and 2% sucrose. Whole kidneys were perfusion fixed and postfixed in 4% buffered formalin for 24 h and embedded in paraffin (Medin Histotechnologie, Buseck, Germany). Sections were either stained by the periodic acid-Schiff (PAS) reaction or processed for immunohistochemical stainings. Tissue sections (1 μm) were deparaffinized and rehydrated. Ag retrieval was performed by incubation with proteinase type XXIV (5 mg/ml; Sigma-Aldrich, Steinheim, Germany, and St. Louis, MO) for 15 min at 37°C or by microwave Ag retrieval in citrate buffer (pH 6.1) for 25 min. After 30-min block in 5% horse serum (Vector), the tissue or cultured podocytes were incubated with primary Abs in 5% horse serum for 1 h at room temperature or overnight at 4°C. For immunohistochemistry, binding was visualized using affinity-purified secondary Abs (Cy2 or Cy3 conjugated; Jackson ImmunoResearch Laboratories) diluted 1:200 in 5% horse serum for 30 min. For light microscopy, staining was visualized by biotinylated affinity-purified secondary Abs (Jackson ImmunoResearch Laboratories) diluted 1:400 in 5% horse serum for 30 min, followed by the ABC kit from Vector, according to the manufacturer’s instructions. For direct immunohistochemistry of mouse IgG and IgG isotypes, HRP-conjugated primary Abs (goat anti-mouse IgG [1:100; Biozol, Eching, Germany] and goat anti-mouse IgG1 and IgG2b [1:100 from Zymed Immivitrogen]) were visualized using diaminobenzidine as a substrate. For indirect immunohistochemical evaluation of glomerular binding of anti-podocyte serum to naive kidney sections, preimmune sheep or APN sheep serum (7319, 7258, and 7789) was diluted 1:100. Primary Abs used were nephrin, synaptopodin (both 1:50; TBS-L500; donkey anti-sheep IgG, Cy2 donkey anti-mouse IgG (both Jackson ImmunoResearch Laboratories), laminin (1:400, CM6 by bridoma; Sigma-Aldrich), α-actinin-4 (clone Ig701; ImmunoGlobe, Hmelstadt, Germany), FTTC anti-complement 3 (1:50; MP Biomedicals), rabbit anti-human CD3 (1:1000; DakoCytomation, Hamburg, Germany), rat anti-mouse Ly6G (1:50; Hycult, Uden, Netherlands), rat anti–Mac-2 (1:1000; Cedarlane, Biozol), rat anti-F4/80 (1:800, BM8; eBioscience, San Diego, CA), and rabbit anti-human annexin 3 (1:100 immunofluorescence, 1:200 immunohistochemistry; Aviva Systems Biology, San Diego, CA). ToPro3 (Molecular Probes, Invitrogen) was used for nuclear staining in immunofluorescence, and hematoxylin was used for light microscopy. Stainings were evaluated under an Axioskop (Zeiss, Jena, Germany) and photographed with an AxioCam HRc (Zeiss) or by confocal microscopy with a Leica TCS SP5 microscope. Images were analyzed with Photoshop software (Adobe). Overlapping green and red stainings (yellows) were digitally extracted, inverted, and converted to greyscale for visualization.

Quantification of podocytes, T cells, neutrophils, monocytes/macrophages

For quantification of glomerular neutrophil (Ly6G), macrophage (Mac-2), and T cell (CD3) influx, inflammatory cells were counted in 50 glomeruli per mouse per condition in a double-blinded fashion at a 400-fold magnification in the respective specific stains. Macrophage influx (F4/80) in the immediate periglomerular region was quantified in 20 high power fields per mouse per condition in a double-blinded fashion. Values are expressed as mean ± SEM. A p value <0.05 was accepted as statistically significant using the unpaired Student t test.

ELISA against mouse anti-sheep IgG isotypes

Mouse anti-sheep IgG Ab titers were measured, as described (25), by ELISA using sera collected at days 1, 3, 6, 9, and 14 of preimmune and anti-podocyte-serum-injected mice and of untreated control animals. A n = 3 was experimented per time point and condition. In brief, ELISA microtiter plates were coated with 100 μl 100 μg/ml sheep IgG (Sigma-Aldrich, Taufkirchen, Germany) in carbonate–bicarbonate buffer overnight at 4°C. After blocking with 1% BSA in TBS, the plates were incubated with serial dilutions of mouse serum (1:20, 1:100, 1:500, 1:2500) for 1 h at room temperature. Bound mouse IgG was detected using peroxidase-conjugated goat anti-mouse IgG isotype Abs (IgG1, IgG2b; Zymed Invitrogen) or peroxidase-conjugated goat anti-IgG (Biozol) at 1:1000 and tetramethylbenzidine peroxidase substrate. Absorbance readings were performed at 450 nm on a spectrophotometer. Lack of cross-reactivity of the secondary Abs with sheep IgG was demonstrated by omitting the primary Ab.

Quantitative PCR

Glomeruli were isolated using Dynabead perfusion, as described (26). Total RNA from whole kidney slices or isolated glomeruli was prepared, as described previously (27), and reverse transcribed (Superscript II; Invi-trogen) by the use of random hexamer primers (Invitrogen). mRNA expression was quantified with an ABI Prism 7700 (Applied Biosystems) and analyzed using the cycle threshold method.

Electron microscopy

Electron-microscopical analysis was performed on small cortical samples that were perfusion fixed in 4% buffered paraformaldehyde. Tissue was postfixed with 1% osmium in 0.1 M sodium-cacodylate buffer, stained with 1% uranylacetate, and embedded in epoxy-resin (Serva, Heidelberg, Germany). Ultrathin sections were cut (Ultramicrotome; Reichert-Jung) and contrast with uranyl acetate in methanol. Lead citrate Micrographs were generated with a transmission–electron microscope (EM10; Zeiss, Oberkochen, Germany).

Immunoelectron microscopy

Immunoelectron microscopy was performed, as reported before (28). Briefly, paraformaldehyde-fixed cortical tissue was dehydrated and embedded in LR-White resin. Ultrathin sections were stained for 1 h in citrate buffer at pH 9.0 (DakoCytomation) and blocked with donkey serum (Jackson ImmunoResearch Laboratories). Sections were then incubated with 12 nm colloidal gold-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories) overnight at 4°C, followed by contrast with uranyl acetate and lead citrate. Representative micrographs were taken on a Philips electron microscope (EM201; Philips).

Immobilization of sheep IgG to agarose beads

Purified IgG was covalently coupled to agarose beads in a column using the AminoLink Immobilization Kit (Pierce), according to the manufacturer’s recommendations. Cova lent binding of IgG to the beads was chosen to allow stringent washing and elution conditions without IgG loss from the column. Briefly, 7 mg sheep IgG per ml agarose was dialyzed with coupling buffer (0.1 M NaPO4, pH 7.0) to reduce unspecific amines before coupling with coupling reagent (NaCNBrH2 in 10 mM NaOH) and
stacking in gravity-flow columns overnight at 4°C. Residual binding capacity of the agarose was quenched, and the columns were washed with washing buffer, PBS, and elution buffer (100 mM glycine [pH 2.5]) to prepare for binding experiments.

**Affinity chromatography of podocyte lysates**

Differentiated murine podocytes (6 × 10^6 cells) were lysed in non-denaturing T-PER (Pierce) lysis buffer and spun at 14,000 × g for 10 min at 4°C. The column was equilibrated with T-PER before lysis incubation at 4°C overnight. The column was then washed six times with lysis buffer and three times with PBS before fractionated elution of the bound proteins (100 mM glycine [pH 2.5]), followed by pH adjustment with TRIS base. The eluted proteins were further analyzed by one-dimensional or two-dimensional (2D) SDS-PAGE and Gelcode stain or Western blot analysis.

**Two-dimensional gel electrophoresis**

Podocyte lysate or column eluate was dialyzed (Zebra Desalt Columns; Pierce) against ZOOM 2D protein solubilizer 1 (Invitrogen) to eliminate salt and detergents. In the first dimension, isoelectric focusing of 150 μg protein from a total podocyte cell lysate was achieved with pH 4–8 ampholytes at voltage gradient of 200–500 V in a ZOOM IPG Runner (Invitrogen). After isoelectric focusing, the IPG strips were equilibrated (0.375 M Tris-HCl [pH 8.8], 6 M urea, 20% glycerol, 2% SDS, and 130 mM DTT). For the second dimension, the strip was equilibrated with LDS and fit onto a ZOOM Gel (Invitrogen) for SDS-PAGE with 60 mA per gel using a PROTEAN II system (Bio-Rad). After electrophoresis, the gels were stained with Gelcode (Pierce), as described above. Molecular weights were determined by using a SDS-PAGE standard (Invitrogen). Stained gels were digitalized at 1200 dots per inch using a Canon Scanner and Photoshop software (Adobe).

**In-gel digestion and mass spectrometry protein identification**

Protein identification was performed, as described recently (29). Protein spots from Gelcode-stained 2D gels were selected and excised manually using a clean razor blade. Digestion with trypsin and subsequent spotting of peptide solutions onto the MALDI targets were performed automatically in an Ettan Spot Handling Workstation (GE Healthcare, Uppsala, Sweden). For preparation of MALDI-TOF-mass spectrometry analysis, protein spots were destained and digested in the gel with trypsin (Promega) and extracted, as described (29). The MALDI-TOF measurement of spotted peptide solutions was carried out on a 4800 MALDI TOF/TOF analyzer (Applied Biosystems, Foster City, CA). The spectra were recorded in reflector mode in a mass range from 800 to 3700 Da with an internal one-point calibration on the autolytic fragment of trypsin. Additionally, MALDI-TOF/TOF analysis was performed for the five strongest peaks of the TOF spectrum after subtraction of peaks corresponding to background or trypsin fragments. After calibration, a combined database search of MS and MS/MS measurements was performed using GPS Explorer software v3.5 (Applied Biosystems). Peak lists were compared with the SwissProt database rel.54.6 reserved to mouse taxonomy using the Mascot search engine 2.0 (Matrix Science, London, U.K.). Peptide mixtures that yielded at least twice a Mowse score of at least 54 for SwissProt results were regarded as positive identifications.

**Statistics**

Values are means ± SEM; n refers to the number of animals or individual measurements in separate samples. Differences between multiple experimental groups were compared by Kruskal-Wallis analysis with post hoc analysis by Mann-Whitney U test. Paired Student t test was used to compare mean values within one experimental series. A p value <0.05 was accepted as statistically significant.

**Results**

**Sheep anti-podocyte Ab induces proteinuria and nephrotic syndrome in a biphasic pattern**

Three sheep were immunized with whole podocyte preparation. One serum (7319) was chosen on the basis of several criteria, as follows: 7319 serum induced severe proteinuria and podocyte effacement in all treated mice in a preliminary experiment with C57BL/6N and BALB/c mice; immunohistochemistry revealed exclusive staining of the glomerulus in treated mice (Supplemental Fig. 1); cultured podocytes showed specific staining with the Ab; and immunoblots with podocyte lysate revealed a limited number of proteins identified by the Ab. Characteristic findings of the three antisera in treated mice are shown in Supplemental Table 1A. Proteinuria was heavier in BALB/c mice induced with 300 μl anti-podocyte antisera 7319 compared with C57BL/6N mice, whereas characteristic morphologic findings of APN were similar in both strains (Supplemental Table 1B).

In the following eight individual experiments, 229 mice were treated with 7319 sheep anti-podocyte serum or purified Ab from this serum. Urine of serum-treated and purified Ab-treated and control C57BL/6N mice was examined for proteinuria on SDS-PAGE gels (Fig. 1A, 1B). Mice injected with 300 μl on day 0 developed proteinuria on day 2. In these mice, proteinuria was further increased on day 8 with severe albuminuria at approximately day 11–14. Mice injected with 1.2 and 1.6 mg purified Ab on days –1 and 0, respectively, developed initial proteinuria on day 4. Thereafter, proteinuria remained stable until day 10 and increased further to maximal values on day 18. Albumin content was quantified using an ELISA for mouse albumin (Fig. 1C) and corrected for urine creatinine. Urine albumin/creatinine ratio did not increase in control mice and mice treated with preimmune serum of the same sheep that later produced the anti-podocyte Ab, whereas in mice treated with anti-podocyte antisera, urine albumin/creatinine significantly increased by 2 log ranks. These mice also developed clinical signs of nephrotic syndrome with edema and ascites. Mice treated with anti-podocyte Ab were significantly heavier (31.2 ± 0.65 g) than mice treated with preimmune serum (28.9 ± 0.64 g) p < 0.05; Fig. 1D) on day 14. Serum albumin concentration was significantly lower in anti-podocyte serum-treated mice on day 14 (9.56 ± 3.51 g/l) compared with preimmune serum-treated mice (24.7 ± 2.8 g/l, p < 0.05; Fig. 1D). Serum cholesterol and triglycerides were also quantified along the time course and showed a significant increase in anti-podocyte serum-treated mice 9 and 14 d after injection, whereas preimmune serum-treated mice remained stable (Fig. 1E). Serum creatinine and serum urea nitrogen, measured in proteinuric mice on day 24, were not elevated in any of the treated groups (Fig. 1F). However, some severely ill mice that had received 300 μl anti-podocyte serum developed acute renal failure with maximal serum urea nitrogen at 61.5 mg/dl on day 14 (data not shown).

**Histological changes in Ab-treated mice**

Histology and immunohistochemistry, mice were sacrificed on day 11 (300 μl 7319 antiserum) or day 24 following administration of anti-podocyte or the same volume of preimmune serum or Ab, respectively. PAS staining showed predominantly glomerular disease in anti-podocyte serum- or Ab-treated mice (Fig. 2Ab, 2Ac) but not in control mice treated with the same amount of preimmune serum (Fig. 2Aa). In all anti-podocyte serum-treated mice, glomerular pathological changes showed a diffuse distribution. Glomeruli were enlarged without mesangial hypercellularity. Capillary diameters were normal, except for some heavily proteinuric mice treated with 300 μl antiserum that displayed some endothelial swelling. Podocytes were swollen (Fig. 2Ab, 2Ac insets), and the glomerular basement membrane was thickened. In heavily proteinuric mice, some tubules were obstructed by proteinaceous casts. Within the obstructed tubules, tubular epithelial cells were swollen and vacuolated, possibly due to endocytosis of protein (data not shown).

Immunofluorescence of the podocyte-specific proteins nephrin and synaptopodin showed a linear staining pattern along the GBM in preimmune serum-treated mice (Fig. 2Ba, 2Bd, respectively). In anti-podocyte serum-treated mice, both proteins were found in a granulated pattern along the GBM and internalized into the
cytosol of podocytes (Fig. 2Bb, 2Be). The linear staining pattern was completely abolished (Fig. 2Bc, 2Bf).

**Increased glomerular size and reduced podocyte number in anti-podocyte serum-treated mice**

Glomerular size was measured on day 24 in 50 glomeruli of three mice that were treated with either anti-podocyte serum or preimmune serum. Glomerular size increased significantly in anti-podocyte serum-treated mice (3770.5 ± 114.1 μm²) when compared with preimmune serum-treated mice (3062.8 ± 18.3 μm²), p < 0.05 (Fig. 2C, left panel). Podocyte number per glomerular section was calculated from 50 glomerular sections per mouse; podocyte nuclei were stained with Abs against WT-1, as described before (20). Because in sheep serum APN glomerular area was significantly different in anti-podocyte serum- and preimmune serum-treated mice (in contrast to rabbit anti-podocyte serum nephritis), podocyte number was expressed as podocytes per glomerular section, not as podocytes per glomerular area. Quantification of podocytes per glomerular section revealed a significant decrease in podocyte number in Ab-treated mice compared with control mice (6.24 ± 0.18 and 7.72 ± 0.12, respectively; Fig. 2C, right panel). Together, these data suggest a loss of podocytes from the glomerular tuft in sheep serum APN.

**Podocyte foot process effacement and subepithelial deposits in anti-podocyte serum-treated mice**

Transmission electron microscopy of proteinuric mice revealed swollen podocytes with segmental foot process effacement of 60–80% of all foot processes (Fig. 2Da, 2Db). Interestingly, subepithelial deposits were present in most areas with foot process effacement. These pathological changes were observed in mice with massive (Fig. 2De, 2Df) as well as mild proteinuria (Fig. 2Dc, 2Dh). Similar deposits were present in the mesangial hinge region (Fig. 2Dc, 2De). In all mice, the GBM was well preserved. Some endothelial cell swelling was observed in mice with strong proteinuria.

**Sheep polyclonal anti-podocyte Ab partially colocalized with laminin along the GBM**

The in vivo binding pattern of the sheep anti-podocyte Ab after i.v. injection was highly specific on all days examined (Fig. 3A). Staining of mouse kidney sections with anti-sheep IgG Ab detected the injected anti-podocyte Abs only in the glomeruli at all time points analyzed. Within the glomerulus, immunostaining was detected in a linear pattern along the glomerular filtration barrier (Fig. 3Ac) and partly colocalized with the GBM protein laminin (Fig. 3Ab, 3Ac). Sheep preimmune serum was not detected within the glomerulus (Fig. 3Ad–f). Glomerular endothelial cells, podocyte cell bodies, or tubular cells were not stained.

**Immunogold electron microscopy detected sheep anti-podocyte Ab on podocyte foot processes and along the GBM**

By immunogold labeling, sheep IgG was detected on and next to podocyte foot processes and in all layers of the GBM (Fig. 3B). Weak labeling was also detected in the primary podocyte processes on day 7. Endothelial cells, mesangial cells, mesangial matrix, tubular epithelial cells, and all other cortical basement membranes were negative.
Mouse IgG deposits in glomeruli of Ab-treated mice

Immunostaining of sections from anti-podocyte Ab-treated mice with Abs against mouse IgG demonstrated glomerular mouse IgG deposition in a linear and granular pattern along the GBM in partial overlap with laminin (Fig. 4A). In addition, some IgG clusters were observed within the mesangium. Confocal microscopy showed colocalization of mouse IgG with laminin and substantial amounts of mouse IgG outside the GBM, most likely in the subepithelial space (Fig. 4Ag, 4Ah). Mice injected with pre-immune serum showed minimal mouse IgG reactivity (Fig. 4Ai–I). To further localize mouse IgG, costainings with the podocyte foot process protein α–actinin-4 were performed (Fig. 4B). Again, partial overlap of mouse IgG and α–actinin-4 was observed, with additional mouse IgG staining inside of α–actinin-4, toward the GBM. Together, these stainings indicate that mouse IgG is deposited in the subepithelial space in a linear pattern together with small granules along the GBM. Mouse IgG subclasses directed against sheep IgG were further studied by immunostaining and ELISA of serum samples along the time course (Supplemental Fig. 2). By ELISA, serum total mouse anti-sheep IgG, IgG1, and IgG2b increased to a maximum at day 6 or 9 and decreased at day
However, the observed increase was identical in anti-podocyte serum- or preimmune serum-treated mice. In contrast, immunohistochemistry showed significant mouse anti-sheep IgG, IgG1, and IgG2b deposition only in the glomeruli of anti-podocyte serum-treated mice and not in preimmune-treated mice. Therefore, serum mouse IgG reaction was unspecific to the sheep IgG load, whereas glomerular deposition was specific for anti-podocyte serum.

Development of anti-podocyte nephritis was independent of complement activity

To investigate the role of complement activation in the sheep antisem-induced APN, C3-deficient mice were treated with anti-podocyte serum or preimmune serum. The C3-deficient mice cannot activate the complement cascade beyond the initiation of the classical pathway and are unable to activate the alternative pathway of complement activation. We have found that the C3-deficient mice treated with anti-podocyte Ab were not protected from glomerular injury (Fig. 5). PAS staining showed similar histology with swollen podocytes and APN in C3-deficient and control mice (Fig. 5Ab, 5Ad, respectively). Immunostaining with Ab against mouse C3 showed C3 deposits along the GBM in control anti-podocyte serum-treated mice and background staining in preimmune-treated mice (Fig. 5Ag, 5Af). In contrast, C3 was completely absent in kidneys of C3-deficient animals (Fig. 5Ac, 5Ad). Similar destruction of nephrin staining was observed in C3-deficient and control mice after treatment with sheep anti-podocyte Ab (Fig. 5Ah, 5Al, respectively), indicating that complement activation was not necessary for podocyte disruption.

Proteinuria was evaluated by albumin ELISA after 11 d and showed a significant increase in anti-podocyte serum-injected control and C3-deficient mice compared with preimmune serum-treated mice (Fig. 5B). However, proteinuria in C3-deficient mice was not statistically different from control mice.

Inflammatory cells were increased in the periglomerular and glomerular space

In PAS histology, mononuclear cells were observed in the periglomerular space after injection of sheep anti-podocyte Ab (data not shown). In PHN, an increase in glomerular, periglomerular, and tubulointerstitial T cell number has been reported (18, 30). Therefore, we determined the number of inflammatory cells in the glomerulus and in the periglomerular space by immunostaining with Abs against F4/80 (monocytes/macrophages), MAC-2 (monocytes/macrophages in the glomerulus), CD3 (T cells), and Ly6G (neutrophils) in anti-podocyte and preimmune serum-treated mice at day 14 (Fig. 6A).

Sheep polyclonal anti-podocyte Abs bound specifically to in vitro cultured mouse podocytes

Binding of the sheep anti-mouse podocyte polyclonal Ab to paraformaldehyde-fixed, cultured, differentiated podocytes was evaluated by immunofluorescence. Specific staining of podocyte structures was detected along the cell membrane (Fig. 7Aa) with
a perinuclear, granular staining pattern (Fig. 7Ab). Podocytes stained with preimmune serum or negative controls without primary Ab did not exhibit any specific staining for sheep IgG (Fig. 7Ac, 7Ad).

Identification of 20 specific anti-podocyte serum Ags
Sheep IgG was purified from anti-podocyte sheep serum or preimmune serum, linked covalently to Sepharose beads, and stacked in a column. After incubation with murine podocyte lysate, the

**FIGURE 5.** APN is independent of C3. A, Histology on day 11 of complement C3-deficient (C3−/−) and control (C3+/+) mice treated with preimmune (PI) or anti-podocyte serum (APN), as indicated. Upper panels, Light micrographs of PAS (a–d) demonstrate equally diseased glomeruli in anti-podocyte serum-treated C3−/− (b) and wild-type (d) mice. Middle panels, Confocal micrographs of glomerular complement C3 deposition (e–h) in wild-type mouse treated with preimmune serum (g, background) and in a linear pattern in anti-podocyte serum-treated wild-type mouse (h). C3−/− mice demonstrate no C3 immunoreactivity (e, f). Lower panels, Confocal micrographs of nephrin immunofluorescence (i–l) demonstrate equally disrupted nephrin staining in anti-podocyte serum-treated C3−/− (i, j) and C3+/+ (k, l) mice. Original magnification ×1000. B, Quantification of albuminuria by albumin ELISA in C3−/− and wild-type mice treated, as indicated, with preimmune (PI, n = 4) or anti-podocyte serum (APN, n = 5) on day 11. Values were normalized against urine creatinine values and are expressed as log × μg albumin/mg creatinine ± SEM. n.s., nonsignificant.

**FIGURE 6.** Anti-podocyte serum increases periglomerular and glomerular inflammatory cells. A, Representative immunohistochemistry against F4/80-positive periglomerular monocytes/macrophages (a, b), MAC-2–positive intraglomerular monocytes/macrophages (c, d), CD3-positive intraglomerular T cells (e, f), and Ly6G-positive intraglomerular neutrophils (g, h). The arrows denote positive cells. Scale bar, 10 μm. B, Quantification of F4/80-positive cells (left panel), glomerular MAC-2–positive cells (middle left), CD3-positive cells (middle right), and Ly6G-positive cells in preimmune (PI, ●) or anti-podocyte serum (APN, ○)-treated mice on day 14. Fifty consecutive visual fields at original magnification ×400; n = 3 for each condition and staining. Values are expressed as positive cells/visual field ± SEM. *p < 0.05.
column was washed several times, and the bound proteins were eluted in fractions and separated on one-dimensional and 2D SDS-PAGE gels (Fig. 7B, 7C). The number of specific bands that were detected by anti-podocyte Ab was limited, indicating that possible Ags can be trapped by this method (Fig. 7B). Similarly, protein staining of 2D gels also detected only a few proteins eluted from the column (Fig. 7Cb) when compared with whole podocyte lysate (Fig. 7Ca). The identified spots were excised and subjected to fingerprint mass spectrometry (MS) methods, followed by partial peptide sequence digest. Protein identification used the Mascot search algorithm, and proteins were sorted by their respective Mowse score. By using this method, a total of 20 proteins was identified (Fig. 7Cc) in four separate column experiments.

Annexin-3 expression in APN and human membranous nephropathy

The expression of annexin-3 was further studied in quantitative PCR experiments from isolated glomeruli and whole kidney lysates in APN. Annexin-3 was significantly increased in glomeruli with immune complex nephritis (Fig. 8A). We therefore investigated annexin-3 expression in human podocytes and human biopsy samples from normal kidney and patients with membranous nephropathy. Annexin-3 immunostaining was strongest along the cell membrane of cultured human podocytes and concentrated in podocyte processes (Fig. 8B). In human biopsies from patients with membranous nephropathy, podocytes were more intensely stained than in normal kidney biopsies (Fig. 8C). Together, these data indicate increased annexin-3 expression during immune complex nephritis in mice and human kidneys.

Discussion

Subepithelial immune deposit formation induces local inflammation, GBM disruption, podocyte foot process effacement, and nephrotic syndrome in systemic lupus nephritis, membranoproliferative glomerulonephritis, and membranous nephropathy. In membranous nephropathy, the pathogenic mechanism is thought to be a specific Ig–podocyte interaction. The rat model of PHN shows most of the hallmarks of human membranous nephropathy (3, 5). The causative Ab is produced by immunization of sheep with rat-proximal tubule brush border extract (FX1A). The sheep FX1A antiserum then reacts with podocyte foot process...
proteins such as megalin when injected in rats. However, the mouse or human Ag(s) on the podocyte of idiopathic membranous glomerulopathy remains partly elusive.

For this work, we took advantage of the development of murine podocyte cell line cultures (21, 22). We hypothesized that these podocytes express Ags in vitro against which immunized sheep or mouse would produce Abs. Administration of these sheep anti-murine podocyte Abs could therefore result in the induction of membranous nephropathy in mice. Indeed, all three sheep anti-podocyte Abs could therefore result in the induction of membrane proteins nephrin and synaptopodin. Electron microscopy revealed podocyte foot process effacement in areas of subepithelial and hinge region immune complex deposits but found the GBM normal. Similar foot process effacement with immune deposit formation preceding the onset of heavy proteinuria is seen in both rat PHN (5) and our murine model. Immunostaining detected the sheep Ab deposited in a linear pattern along the GBM shortly after disease induction. Immunogold electron microscopy localized the anti-podocyte sheep Ab, but not preimmune serum Abs, to podocyte foot processes and to the GBM. Mouse IgG was distributed in a linear and granular pattern in the subepithelial space. Together, these data suggest that the podocyte foot process was the main target of the anti-podocyte Ab, causing the development of mouse IgG deposition and podocyte disruption similar to PHN in the rat. Subepithelial IgG deposition in mice has been reported previously after active immunization with apoeritin (33) or cationic BSA or γ-globulin (34, 35). However, the pathophysiological relevance of these model systems remains unclear. In our model, the presence of mouse IgG clusters in the mesangial space and endothelial swelling in severely nephritic mice indicates that the model mouse contains additional features that are not typical for membranous nephropathy in rats or humans. The contribution of these histological changes to the induction of renal disease is unclear at present. However, these additional changes coincided with severe podocyte damage and may therefore be secondary in nature.

Previously, we have established a similar model with rabbit antiserum against mouse podocytes (20). However, important differences exist between the two models. Preimmunization was necessary for the induction of rabbit- but not sheep serum-induced APN, possibly reflecting the need for further stimulation of the immune response. The biphasic proteinuria occurred only in sheep serum APN, but not in rabbit serum APN. Serum urea nitrogen was initially (day 5) elevated in rabbit serum APN and later decreased, whereas sheep Ab did not elevate serum urea nitrogen despite massive proteinuria. Clinically, sheep serum APN caused complete nephrotic syndrome, whereas rabbit serum APN mice did not increase weight or exhibit edema. Both rabbit and sheep polyclonal Abs induced proteinuria, but only 10–20% of podocyte foot processes were effaced in rabbit serum APN (20). In contrast, sheep serum APN induced 60–80% foot process effacement. Importantly, subepithelial deposits visualized by transmission electron microscopy did not form in rabbit serum APN. The reason for these differences is not clear. However, the three sheep anti-podocyte antisera that were produced for this work induced slightly different pathologies in mice and reacted with different sets of podocyte proteins even though the same podocyte

Sheep anti-podocyte serum and purified Ab induced proteinuria in a biphasic pattern without the need for preimmunization, indicating that Ab–Ag recognition plays a major role in the pathophysiology of APN in mice (31). The observed time course was similar to the autologous and heterologous phases of PHN (18, 32). Mice treated with anti-podocyte Ab did not develop elevated serum urea nitrogen or serum creatinine values despite nephrotic syndrome. However, some anti-podocyte serum-treated mice had to be sacrificed before day 14 because of massive ascites. In these mice, serum urea nitrogen was elevated, most likely reflecting acute renal failure secondary to nephrotic syndrome.

Upon histopathological examination, the mouse APN showed similar features to those published for rat PHN. Glomeruli in APN were enlarged without crescents, and podocytes appeared swollen. Mesangial expansion occurs in PHN and is thought to result from an increase in cell size rather than cell proliferation (5). Immunostainings showed severe disruption of the slit membrane proteins nephrin and synaptopodin. Electron microscopy revealed podocyte foot process effacement in areas of subepithelial and hinge region immune complex deposits but found the GBM normal. Similar foot process effacement with immune deposit formation preceding the onset of heavy proteinuria is seen in both rat PHN (5) and our murine model. Immunostaining detected the sheep Ab deposited in a linear pattern along the GBM shortly after disease induction. Immunogold electron microscopy localized the anti-podocyte sheep Ab, but not preimmune serum Abs, to podocyte foot processes and to the GBM. Mouse IgG was distributed in a linear and granular pattern in the subepithelial space. Together, these data suggest that the podocyte foot process was the main target of the anti-podocyte Ab, causing the development of mouse IgG deposition and podocyte disruption similar to PHN in the rat. Subepithelial IgG deposition in mice has been reported previously after active immunization with apoeritin (33) or cationic BSA or γ-globulin (34, 35). However, the pathophysiological relevance of these model systems remains unclear. In our model, the presence of mouse IgG clusters in the mesangial space and endothelial swelling in severely nephritic mice indicates that the model mouse contains additional features that are not typical for membranous nephropathy in rats or humans. The contribution of these histological changes to the induction of renal disease is unclear at present. However, these additional changes coincided with severe podocyte damage and may therefore be secondary in nature.

FIGURE 8. Annexin-3 is upregulated in glomeruli of APN mice and in human biopsies of patients with membranous nephropathy. A, Left panel, Quantitative PCR of annexin-3 mRNA from isolated mouse glomeruli. Similar numbers of glomeruli were isolated using Dynabeads at day 14 after application of anti-podocyte serum or preimmune serum (n = 4 mice each). Asterisk indicates significance. Right panel, Quantitative PCR of whole kidney mRNA from anti-podocyte serum- or preimmune serum-treated mice at day 14 (n = 4 mice each). B, Annexin-3 immunofluorescence on differentiated human podocytes in culture (a) and negative control omitting annexin-3 Ab (b). Scale bar, 10 μm. Arrows indicate podocyte membrane protrusions; n indicates the nucleus. C, Annexin-3 immunohistochemistry of representative human biopsies of a normal kidney (normal, a, b) or a patient with membranous nephropathy (MN, c, d). Original magnification ×400 (a, c) and ×1000 (b, d). Scale bar, 10 μm. n.s., nonsignificant.
preparations were used for immunization. It is therefore possible that singular Ags are responsible for immune deposit formation and disease initiation.

Whereas the presence of infiltrating mononuclear cells is not typical for human membranous glomerulopathy, cytotoxic CD8-positive T cells infiltrating the glomerulus were described in both HN and PHN (18). Furthermore, depletion of this T cell population prevented proteinuric disease in both models, indicating that T cells are necessary for podocyte injury (18, 36). Interestingly, we found increased glomerular T cell numbers as well as monocytes/macrophages and neutrophils in APN mice, as has been reported for PHN (30). It is therefore possible that T cells contribute to APN pathology in a similar way. An interesting report by Bao et al. (37) supports these findings. This group applied anti-podocyte serum into mice that induced focal and segmental glomerulosclerosis (FSGS) in decay-accelerating factor-deficient mice, but not in control BALB/c mice in a T cell-dependent manner. Decay-accelerating factor inhibits complement activation, and therefore their work suggested that reduced complement activation was thought to enhance T cell response to the anti-podocyte serum, resulting in FSGS (37).

However, their FSGS mouse model was clearly different from our APN. Bao et al. (37) deliberately chose anti-podocyte serum that would not induce proteinuria in wild-type animals. In our hands, rabbit (20) and sheep anti-podocyte Abs would generally induce heavy proteinuria in BALB/c and C57BL/6N mice. Sclerosis was not observed in any mice. Also, our sheep serum APN was not complement dependent. We acknowledge that complement dependency of PHN in rats had been established in the literature for many years (4, 16, 17). Those studies were carried out with only partial complement depletion by application of cobra venom factor or anti-complement 6 Abs (4, 38) and demonstrated the importance of the formation of the terminal membrane attack complex C5b9 for the development of PHN. However, these studies are now challenged by recent experiments using complement C6-deficient rats that cannot form the C5b9 membrane attack complex (18). C6-deficient rats developed proteinuria and subepithelial deposits in both active and PHN indistinguishable from normal rats (18, 19). Therefore, our results from experiments with C3-deficient mice, which also lack the capability of membrane attack complex formation, are in line with these recent reports. Partial complement depletion with cobra venom factor or anti-C6 Abs might have induced secondary changes that interfered with the inflammatory process at the podocyte.

We further investigated whether specific mouse anti-IgG subgroups were induced in APN mice. Mouse anti-IgG1 and mouse anti-IgG2b were increased similarly in both subgroups in the serum, but deposition of mouse anti-IgG occurred only in the glomerulus of APN mice. These findings argue against unspecific mouse anti-IgG deposition, but rather for specific glomerular deposition of anti-podocyte sheep IgG, but not preimmune serum IgG. This specific deposition at the podocyte most likely induced immune complex formation with mouse anti-IgG similar to immune complex deposition in human disease. Differentiation between IgG1-dependent T cell activation and IgG2-dependent complement activation cannot be derived from these data.

Twenty proteins were identified in four affinity chromatography experiments, followed by MS, suggesting that multiple Ags rather than a single Ag act to initiate APN. Regrettably, for many of the identified proteins, gene-deficient mice or mAbs are not available for the assessment of their individual contributions to the pathogenic process. However, it is interesting to note that known proteins of the podocyte foot process or the slit membrane have not been found in our analysis. Also, the presently known proteins that can induce subepithelial deposits in rodents or humans such as NEP, aminopeptidase A, or phospholipase A2 receptor (9–11) were not detected. Many of the identified proteins such as annexin-3 and spire-1 belong to the cytosolic or nuclear compartment, with only limited exposure to circulating Abs. Annexin-3 belongs to the annexin family of proteins that bind to phospholipids and plasma membranes in a Ca2+-dependent manner (39, 40). The function of annexin-3 remains largely unknown. The protein is abundant in neutrophils, where it promotes Ca2+-dependent aggregation of phagosome granules (41). Annexin-3 has also been implicated in the regulation of cytoskeletal rearrangement under pathologic conditions and binds f-actin (42). Recently, Endlich and colleagues (43) and our group identified upregulated annexin-3 in podocytes in human diabetic nephropathy. We found upregulated annexin-3 expression also in glomeruli of anti-podocyte serum-treated mice. In human biopsy samples from patients with membranous nephropathy, annexin-3 immunostaining was localized to podocytes and appeared more intense compared with normal kidney biopsies. Together, these data indicate increased glomerular annexin-3 expression during immune complex nephritis in mice and membranous nephropathy in humans. Therefore, annexin-3 could be a new target for Ab-mediated podocyte damage. Spire-1, in contrast, is a neuronal protein with actin-nucleating activity that has also been reported to interact with microtubules (44). Spire-1 was localized to endosomal vesicles, where it may control intracellular vesicular trafficking (45). This function of spire-1 could be of importance in podocytes in proteinuric states such as membranous nephropathy, in which an increase in podocyte vesicular transport has been reported (5). However, spire-1 has not been localized to podocytes yet. It is also possible that the responsible Ag(s) was missed by our current approach. Future use of our model may therefore bring about other candidates for Ab-mediated podocyte injury.

In summary, sheep serum APN features potent induction of proteinuria in a biphasic pattern with formation of subepithelial deposits, severe disruption of podocyte slit membrane ultrastructure, and nephrotic syndrome in mice.

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Disclosures
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