

The NEW ENGLAND JOURNAL of MEDICINE

ESTABLISHED IN 1812

JULY 2, 2009

VOL. 361 NO. 1

M-Type Phospholipase A₂ Receptor as Target Antigen in Idiopathic Membranous Nephropathy

Laurence H. Beck, Jr., M.D., Ph.D., Ramon G.B. Bonegio, M.D., Gérard Lambeau, Ph.D., David M. Beck, B.A., David W. Powell, Ph.D., Timothy D. Cummins, M.S., Jon B. Klein, M.D., Ph.D., and David J. Salant, M.D.

ABSTRACT

BACKGROUND

Idiopathic membranous nephropathy, a common form of the nephrotic syndrome, is an antibody-mediated autoimmune glomerular disease. Serologic diagnosis has been elusive because the target antigen is unknown.

METHODS

We performed Western blotting of protein extracts from normal human glomeruli with serum samples from patients with idiopathic or secondary membranous nephropathy or other proteinuric or autoimmune diseases and from normal controls. We used mass spectrometry to analyze the reactive protein bands and confirmed the identity and location of the target antigen with a monospecific antibody.

RESULTS

Serum samples from 26 of 37 patients (70%) with idiopathic but not secondary membranous nephropathy specifically identified a 185-kD glycoprotein in non-reduced glomerular extract. Mass spectrometry of the reactive protein band detected the M-type phospholipase A₂ receptor (PLA₂R). Reactive serum specimens recognized recombinant PLA₂R and bound the same 185-kD glomerular protein as did the monospecific anti-PLA₂R antibody. Anti-PLA₂R autoantibodies in serum samples from patients with membranous nephropathy were mainly IgG4, the predominant immunoglobulin subclass in glomerular deposits. PLA₂R was expressed in podocytes in normal human glomeruli and colocalized with IgG4 in immune deposits in glomeruli of patients with membranous nephropathy. IgG eluted from such deposits in patients with idiopathic membranous nephropathy, but not in those with lupus membranous or IgA nephropathy, recognized PLA₂R.

CONCLUSIONS

A majority of patients with idiopathic membranous nephropathy have antibodies against a conformation-dependent epitope in PLA₂R. PLA₂R is present in normal podocytes and in immune deposits in patients with idiopathic membranous nephropathy, indicating that PLA₂R is a major antigen in this disease.

From the Boston University School of Medicine, Boston (L.H.B., R.G.B.B., D.M.B., D.J.S.); Institut de Pharmacologie Moléculaire et Cellulaire, Unité Mixte de Recherche 6097, Centre National de la Recherche Scientifique, and Université de Nice Sophia Antipolis, Valbonne, France (G.L.); and the University of Louisville School of Medicine, Louisville, KY (D.W.P., T.D.C., J.B.K.). Address reprint requests to Dr. Salant at Evans Biomedical Research Center, Rm. 504, 650 Albany St., Boston, MA 02118, or at djsalant@bu.edu.

N Engl J Med 2009;361:11-21.

Copyright © 2009 Massachusetts Medical Society.

IDIOPATHIC MEMBRANOUS NEPHROPATHY, a common cause of the nephrotic syndrome in adults, is an organ-specific autoimmune disease. Despite extensive investigation, a target antigen has been elusive. Studies of membranous nephropathy in a rat model (Heymann's nephritis) established that the subepithelial immune deposits that characterize the disease are formed in situ, as a result of capping and shedding of the target antigen, megalin, from the basal surface of podocytes when it forms a complex with circulating antimegalin antibodies.¹⁻⁸ Although megalin is not expressed on human podocytes, we hypothesized that a similar process, albeit with an unknown antigen, is operative in human membranous nephropathy. Additional evidence from the work of Debiec et al.^{9,10} supports the in situ formation of glomerular immune deposits in patients with membranous nephropathy. They described an alloimmune form of membranous nephropathy in neonates born to mothers with a deficiency of neutral endopeptidase, a protein expressed on podocytes, to which the mothers had been sensitized during previous pregnancies.

To identify the target antigen in patients with idiopathic membranous nephropathy, we used circulating antibodies from adults with this disease to detect normal glomerular proteins by using Western blotting. Subsequent analysis with the use of mass spectrometry and confirmation with the use of protein-specific reagents allowed for the identification and characterization of the predominant protein detected by these circulating antibodies.

METHODS

SERUM AND KIDNEY-TISSUE SAMPLES

We collected and stored serum samples from patients with membranous nephropathy, those with other glomerular or autoimmune disorders, and normal controls (healthy volunteers). The samples were assigned codes to render them anonymous. The institutional review board of the Boston University School of Medicine approved the study, and written informed consent was obtained from all the subjects. In addition, serum specimens from deceased donors with preserved renal function were obtained from the New England Organ Bank, as were kidneys, from deceased donors, that were unsuitable for transplantation. These

samples were exempt from the requirement of informed consent, according to the institutional review board's approved use of organs and tissues from deceased donors for research.

Idiopathic membranous nephropathy was diagnosed by means of renal biopsy in patients who lacked features suggestive of secondary membranous nephropathy, such as antinuclear antibodies or hepatitis B antibodies in the serum (Table 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

Glomeruli were isolated from the kidneys that were unsuitable for transplantation with the use of graded sieving,¹¹ and glomerular proteins were extracted in radioimmunoprecipitation-assay buffer (Boston BioProducts). Contaminating IgG was removed through incubation with Immobilized Protein G Plus (Fisher Scientific). Peptide N-glycosidase F (New England BioLabs) was used to deglycosylate glomerular proteins. Glomerular glycoproteins were partially purified through absorption by immobilized wheat-germ agglutinin (Vector Laboratories) and elution with *N*-acetyl glucosamine.

WESTERN BLOTTING

Human glomerular extract or recombinant PLA₂R expressed by human cells (see the Methods section of the Supplementary Appendix) was electrophoresed under nonreducing conditions and transferred to nitrocellulose membranes, according to standard protocols. Human serum was used as the primary antibody, at a dilution of 1:100, unless otherwise indicated. A guinea pig polyclonal antibody against the M-type phospholipase A₂ receptor (hereafter referred to simply as PLA₂R) was raised against full-length purified rabbit PLA₂R.¹² Sheep antibodies against the four IgG subclasses were used as recommended by the manufacturer (Binding Site). Detecting antibodies were peroxidase-conjugated donkey antibodies against human IgG, guinea pig IgG (Jackson ImmunoResearch Laboratories), or sheep IgG (Sigma). Immunoglobulins were acid-eluted from the remnant cores of kidney-biopsy specimens from patients with membranous nephropathy, lupus membranous nephropathy, or IgA nephropathy (see the Methods section of the Supplementary Appendix). This eluted IgG was used to immunoblot the human glomerular extract or recombinant PLA₂R directly.

MASS SPECTROMETRY ANALYSIS AND DATA INTERPRETATION

We excised regions of interest from gels identical to those used for Western blotting and performed in-gel tryptic digestion, as previously described.¹³ We analyzed the resulting peptides by using a modified version of a method that couples liquid chromatography with tandem mass spectrometry¹⁴ and entered the acquired data into existing databases of human proteins to identify relevant proteins in our patients.

IMMUNOPRECIPITATION

Reactive or nonreactive human serum was incubated with human glomerular extract, immunoprecipitated with Immobilized Protein G Plus, electrophoresed in the presence of a reducing agent, and blotted with guinea pig anti-PLA₂R antibodies.

IMMUNOHISTOLOGIC ANALYSIS

Cryosections of normal human kidney and kidney-biopsy specimens obtained from patients with membranous nephropathy and made anonymous were fixed in methanol and acetone and blocked with 10% bovine serum albumin. We detected PLA₂R by using guinea pig anti-PLA₂R antibodies (at a dilution of 1:150), followed by a Cy3-conjugated secondary antibody (see the Supplementary Appendix), and established specificity by preabsorbing the primary antibody with a recombinant fragment of rabbit PLA₂R protein¹⁵ containing C-type lectinlike domains (CTLDs) 4, 5, and 6. We used a commercial rabbit antibody against human agrin at a dilution of 1:25 (Santa Cruz Biotechnology), followed by an Alexa Fluor 488-conjugated secondary antibody (Invitrogen), to delineate the glomerular basement membrane. To detect podocyte nuclei, we used rabbit antibodies against Wilms' tumor 1 antigen at a 1:100 dilution (Santa Cruz Biotechnology). We detected IgG4 in immune deposits from patients with membranous nephropathy by using sheep anti-IgG4 antibodies at a dilution of 1:500 (Binding Site), followed by a fluorescein isothiocyanate-conjugated secondary antibody. Cell nuclei were visualized by including a nuclear stain (Hoechst 33342, Molecular Probes) in the mounting medium. Immunofluorescence images were obtained on a microscope (TE-2000, Nikon) linked to a Peltier-cooled CCD camera (CoolSNAP HQ, Photometrics). Con-

focal microscopy was performed with the use of a spinning-disk confocal instrument (UltraVIEW LCI, PerkinElmer).

STATISTICAL ANALYSIS

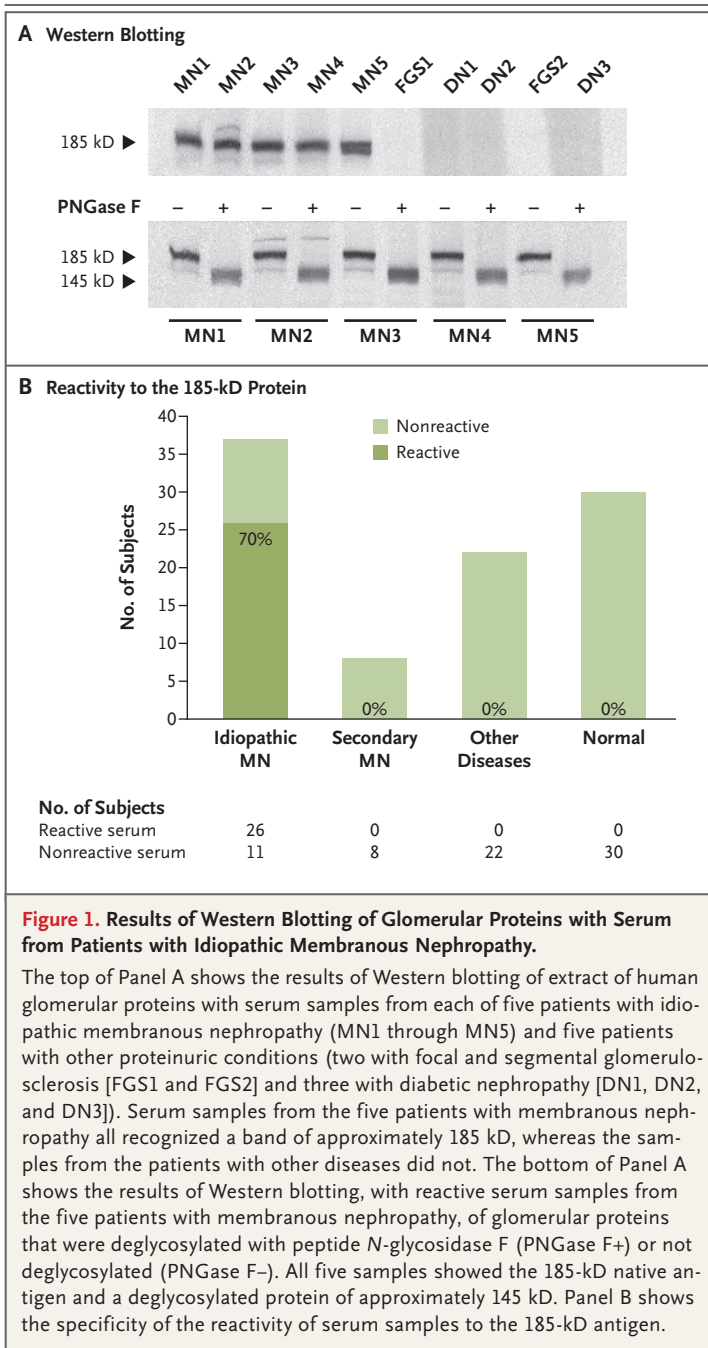
Chi-square analysis was performed to compare observed with expected results. The null hypothesis was that a positive signal on Western blotting would occur in a similar proportion of subjects in each group, on the basis of chance alone.

RESULTS

IDENTIFICATION OF A TARGET PROTEIN IN GLOMERULAR EXTRACTS

We performed Western blotting, under nonreducing conditions, of extracts of normal human glomeruli from donor kidneys with serum samples from 37 patients with idiopathic membranous nephropathy. A 185-kD protein band was detected in samples from 26 patients (70%) (Fig. 1). An identical protein band was seen in glomerular extracts prepared from all individual donor kidneys tested thus far. In contrast, serum from 30 normal controls, 15 patients with proteinuric conditions other than membranous nephropathy (diabetic nephropathy or focal and segmental glomerulosclerosis), and 7 patients with other autoimmune disorders did not react with this antigen when assayed under identical conditions (Fig. 1, and Table 1 in the Supplementary Appendix). Moreover, none of the serum samples from eight patients with secondary membranous nephropathy (six with lupus membranous nephropathy and two with hepatitis B-associated membranous nephropathy) were reactive with the 185-kD antigen (Fig. 1B). Chi-square analysis indicated an extremely low probability of such a distribution resulting from chance alone ($P=2\times 10^{-12}$ for the whole series; $P=0.004$ for secondary vs. idiopathic membranous nephropathy). Treatment with peptide *N*-glycosidase F caused a substantial shift in the mobility of this antigen, to approximately 145 kD, indicating that it is heavily glycosylated. All serum samples that were initially reactive with the native 185-kD band also recognized the smaller, deglycosylated band (Fig. 1A).

Both the native and *N*-deglycosylated forms of the antigen could be bound and substantially enriched through absorption by wheat-germ agglutinin (data not shown). The binding of the de-



glycosylated form may reflect the presence of residual *N*-linked carbohydrates that are inaccessible to peptide *N*-glycosidase F under nonreducing conditions. Native and deglycosylated glomerular proteins were eluted from wheat-germ agglutinin and separated by means of gel electrophoresis. The regions corresponding to the 185-kD and 145-kD antigen bands on the Western blots

were excised, subjected to in-gel tryptic digestion, and analyzed by means of mass spectrometry. On the basis of the assumption that peptide sequences corresponding to the putative target antigen would be identified in both the 185-kD and 145-kD samples, we generated a working list of candidate proteins (see both Table 2 and Fig. 1 in the Supplementary Appendix). We used available antibodies and recombinant proteins to test candidate antigens and found that antibodies against PLA₂R identified a glomerular-protein band of the same size as that recognized by the serum from a patient with membranous nephropathy (Fig. 2A).

ANTI-PLA₂R ANTIBODIES IN PATIENTS WITH MEMBRANOUS NEPHROPATHY

Cell-expressed recombinant human PLA₂R was immunoblotted with serum from a patient with membranous nephropathy. A distinct band was detected that was slightly smaller than the corresponding band from extracts of normal human glomeruli (Fig. 2A). When both types of samples were deglycosylated, however, they migrated to the same position, suggesting a minor difference in overall glycosylation between the native protein and the recombinant form. Western-blot analysis of the same samples with the use of a monospecific polyclonal antibody against PLA₂R revealed an identical pattern (Fig. 2A). The specificity of serum samples from patients with membranous nephropathy for recombinant PLA₂R was established by demonstrating that they had no reactivity with extracts of cells transfected with an irrelevant plasmid (Fig. 2A in the Supplementary Appendix). All serum samples from patients with membranous nephropathy that reacted with the 185-kD glycoprotein from human glomeruli also recognized recombinant PLA₂R, further suggesting that the 185-kD band from human glomeruli is indeed PLA₂R.

We next confirmed that the serum samples from patients with membranous nephropathy and the anti-PLA₂R antibody identified the same protein. As shown in Figure 2B (and Fig. 2B in the Supplementary Appendix), immunoprecipitates of human glomerular extract incubated with reactive serum samples from patients with membranous nephropathy yielded a 185-kD protein that was detected by anti-PLA₂R antibodies on Western blotting.

None of the positive serum specimens retained reactivity to the 185-kD protein when the glomerular extract was electrophoresed under reducing conditions (Fig. 3A and data not shown). Moreover, recombinant PLA₂R and the native glomerular protein share the same reduction-sensitive epitope (Fig. 3A). Both the monospecific anti-PLA₂R antibodies and the serum samples from patients with membranous nephropathy strongly detected nonreduced recombinant and native glomerular PLA₂R. Under reducing conditions, the polyclonal antibodies still detected both recombinant and native PLA₂R (albeit less robustly), but the serum samples from the patients with membranous nephropathy did not. This suggests that autoantibodies in patients with membranous nephropathy uniquely recognize a conformation-dependent epitope in both the native and recombinant antigen.

The immunoglobulin profile in patients with membranous nephropathy is typical of the type 2 helper T-cell response, with the glomerular immune deposits containing predominantly IgG4-subclass antibodies.^{16,17} Similarly, serum samples from patients with membranous nephropathy are highly enriched in IgG4 autoantibodies that react with recombinant PLA₂R or the native protein in glomerular extract. Although IgG4 is the predominant subclass of anti-PLA₂R IgG antibodies, other subclasses are also present in smaller amounts (Fig. 3B).

GLOMERULAR LOCATION OF PLA₂R

Although we hypothesized that the target antigen resides on the surface of podocytes, we first discounted the possible formation of circulating immune complexes with soluble PLA₂R. Neither serum samples from patients with membranous nephropathy nor serum samples from controls had any detectable PLA₂R, even after enrichment by means of lectin binding (Fig. 3A in the Supplementary Appendix). Nor could we detect circulating immune complexes of PLA₂R-IgG through either precipitation with polyethylene glycol 6000¹⁸ or protein G immunoprecipitation of IgG in serum samples from either group (Fig. 3B and 3C in Supplementary Appendix). Conversely, we detected PLA₂R in human glomeruli (Fig. 4A) and, more specifically, in podocytes through immunofluorescence microscopy with the monospecific anti-PLA₂R antibody (Fig. 4D through 4G). PLA₂R

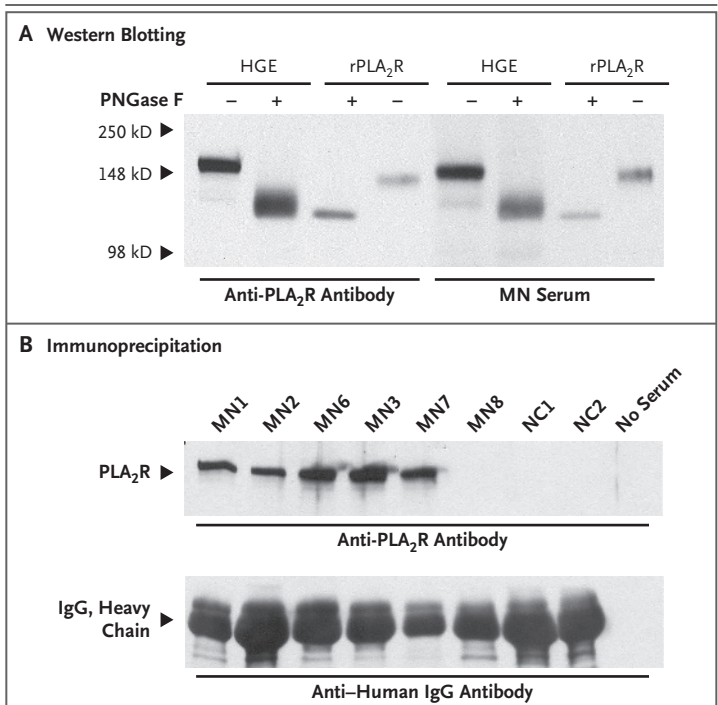
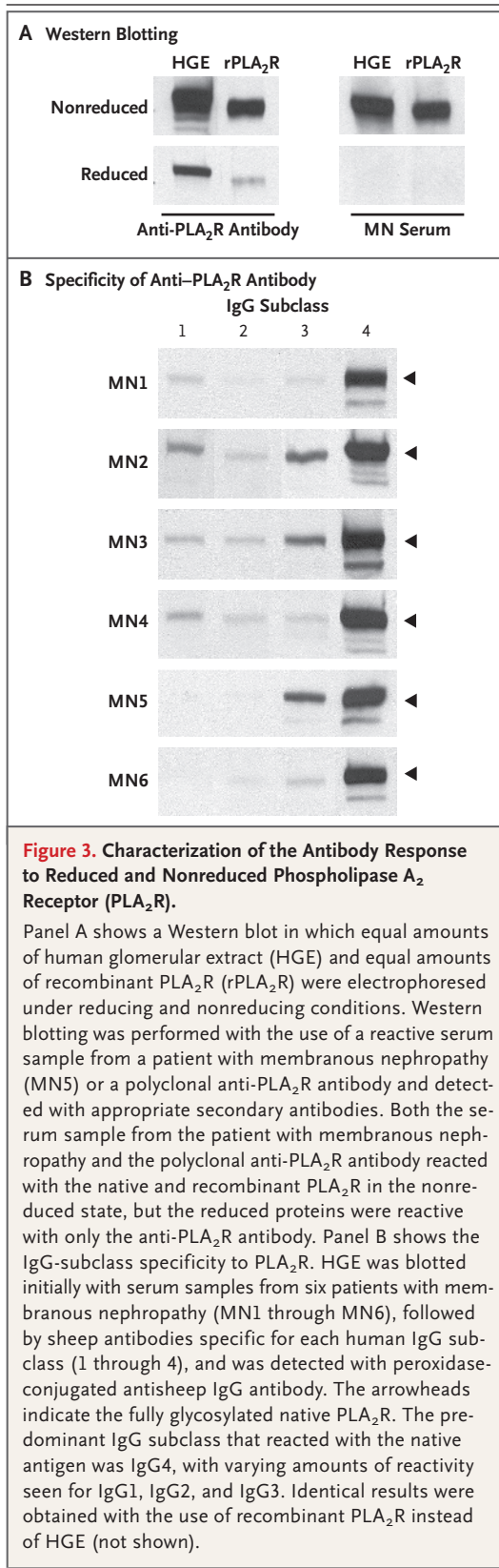


Figure 2. Identification of the 185-kD Antigen in Human Glomeruli.

Panel A shows the results of Western blotting of samples of human glomerular extract (HGE) and recombinant phospholipase A₂ receptor (rPLA₂R), deglycosylated with peptide N-glycosidase F (PNGase F+) or not deglycosylated (PNGase F-), with either a reactive serum sample from a patient with membranous nephropathy (MN) or a polyclonal antibody raised against the M-type phospholipase A₂ receptor (PLA₂R). The recombinant protein migrated to a slightly lower position than the native glomerular protein, although deglycosylation with peptide N-glycosidase F caused both the recombinant and native isoforms to migrate to the same position. Panel B shows that immunoprecipitation of native PLA₂R occurred in a reactive serum sample from each of five patients with membranous nephropathy (MN1, MN2, MN3, MN6, and MN7) but not in a nonreactive sample (MN8) and not in nonreactive serum samples from normal controls (NC1 and NC2) or in the absence of serum. The immunoprecipitates were then electrophoresed under reducing conditions and subjected to Western blotting with antibodies against PLA₂R (top) or against human IgG (bottom). In the reactive samples from all five patients with membranous nephropathy, PLA₂R was immunoprecipitated from human glomerular extract, whereas there was no immunoprecipitation in nonreactive samples from one patient and from the controls. No PLA₂R was detected when human serum was omitted from the reaction. The bottom image shows that the immunoprecipitates of samples from controls and patients with membranous nephropathy contained at least equivalent amounts of IgG.

expression in podocytes was further confirmed by positive staining with Wilms' tumor 1 antigen of PLA₂R-positive cells in normal human glomeruli (Fig. 4G), as well as by the detection of PLA₂R in cultured immortalized human podocytes¹⁹ through the polymerase-chain-reaction assay and Western blotting (Fig. 4 in the Supple-



mentary Appendix). The specificity of PLA₂R staining was shown by the near-complete inhibition of the glomerular signal after preincubation of anti-PLA₂R antibody with a recombinant fragment of PLA₂R containing CTLDs 4, 5, and 6 (Fig. 4B, 4E, and 4H). In contrast to podocytes, mesangial cells in normal human kidney tissue did not show staining for PLA₂R (Fig. 5 in the Supplementary Appendix).

Experimental models of membranous nephropathy have shown capping and shedding of megalin from the surface of podocytes and accumulation of megalin–antimegalin antibody complexes in subepithelial immune deposits. On the basis of these data, we sought evidence that PLA₂R and IgG colocalize in the glomerular immune deposits in patients with membranous nephropathy. As shown through confocal analysis of cryosections of kidney-biopsy specimens from patients with membranous nephropathy (Fig. 5A through 5F, with control samples shown in Fig. 6 and 7 in the Supplementary Appendix), PLA₂R is present and colocalizes with IgG4 in a fine granular pattern that is typical of membranous nephropathy. As in the normal kidney, the staining of PLA₂R could be blocked by the recombinant PLA₂R fragment (Fig. 5G). Although the intensity of staining varied among the specimens from eight patients with membranous nephropathy, all revealed the same granular pattern of PLA₂R (data not shown). In contrast, PLA₂R and IgG4 did not colocalize in a biopsy specimen from a patient with lupus membranous nephropathy, examined under identical conditions (Fig. 5I).

To confirm that the IgG in the glomeruli from patients with membranous nephropathy was reactive with PLA₂R, we eluted IgG from biopsy specimens and used it in Western blotting with native and recombinant PLA₂R. IgG was successfully eluted from four biopsy samples from patients with idiopathic membranous nephropathy, one from patients with lupus membranous nephropathy, and two from patients with IgA nephropathy (Fig. 8 in the Supplementary Appendix). The IgG eluted from the samples from patients with idiopathic membranous nephropathy specifically detected the appropriately sized PLA₂R bands in human glomerular extract and in cell lysates that were positive for recombinant PLA₂R, whereas the IgG eluted from the three other samples from patients with immune-complex glomerular disease did not (Fig. 5J and 5K).

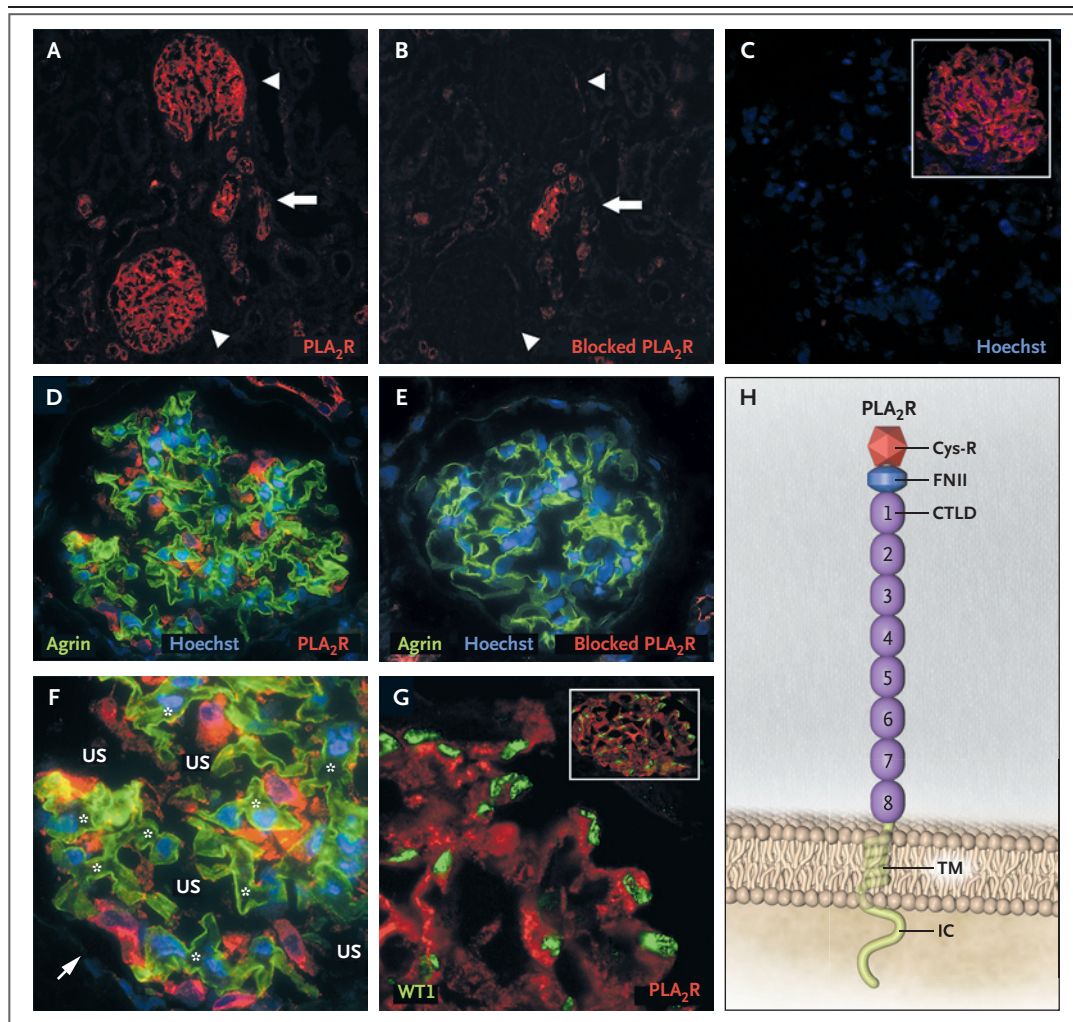
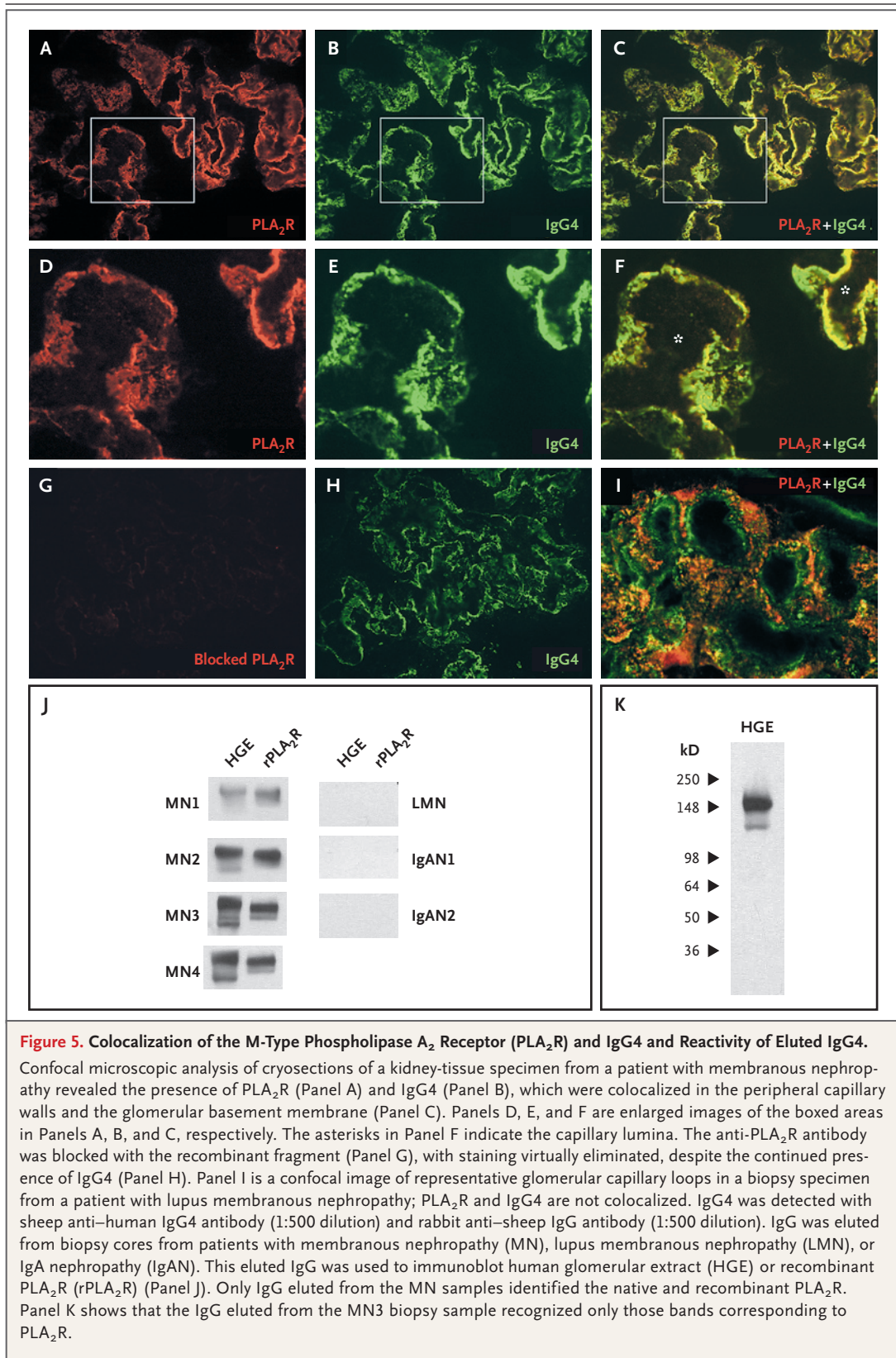


Figure 4. Expression of the M-Type Phospholipase A₂ Receptor (PLA₂R) in Normal Kidney Tissue and Glomeruli.

Serial cryosections of the cortex of normal human kidneys were immunostained with anti-PLA₂R antibody (1:150 dilution), followed by Cy3-conjugated anti-guinea pig IgG antibody (1:500 dilution). Panel A shows two positively stained glomeruli (arrowheads) and scattered tubular staining (arrow). Panel B shows an adjacent section in which the anti-PLA₂R antiserum was preabsorbed with a recombinant fragment of PLA₂R containing C-type lectinlike domains (CTLDs) 4, 5, and 6 (Panel H). The glomerular PLA₂R staining is completely blocked by the recombinant fragment (arrowheads), whereas the scattered tubular staining (arrow) is not blocked and is therefore nonspecific. Panel C shows a human kidney-tissue specimen treated with nonimmune guinea pig serum (1:200 dilution), followed by Cy3-conjugated anti-guinea pig IgG antibody, and stained with Hoechst 33342 (a nuclear stain); the inset shows a glomerulus exposed instead to anti-PLA₂R antibody (1:200 dilution) but otherwise assessed under identical conditions. Next, cryosections of normal human kidney cortex were stained with antiaggrin antibody, followed by an Alexa Fluor 488–conjugated antirabbit secondary antibody, to label the glomerular basement membrane (Panels D and E); in addition, PLA₂R staining with guinea pig anti-PLA₂R antibody was either left unblocked (Panel D) or was blocked (Panel E), as described above. The PLA₂R signal is clearly present outside the glomerular basement membrane and localizes to both the cell body and the processes of the podocyte. The PLA₂R staining is markedly reduced when the antibody is preabsorbed with the recombinant PLA₂R fragment (Panel E). Panel F shows an enlargement of the left lower portion of the glomerulus shown in Panel D, with demarcation of the capillary lumina (asterisks) and urinary space (US) to highlight the location of the PLA₂R-stained podocytes. Hoechst-stained nuclei of parietal epithelial cells in the Bowman’s capsule are indicated by the arrow. Panel G shows a portion of a human glomerulus stained with anti-PLA₂R antibody (as described above), anti-Wilms’ tumor 1 antibody (WT1) (1:100 dilution), and Alexa Fluor 488–conjugated anti-rabbit IgG antibody (1:500 dilution) to label podocyte nuclei. The inset shows the image of the entire glomerulus from which the enlarged view was taken. Panel H illustrates the domain structure of PLA₂R, which is composed of an N-terminal cysteine-rich domain (Cys-R), a fibronectin type II domain (FNII), eight CTLDs, a transmembrane domain (TM), and a short intracellular C-terminal tail (IC). The blocking fragment used in the experiments described above consists of CTLDs 4, 5, and 6 of a recombinant rabbit PLA₂R.



ASSOCIATION WITH DISEASE ACTIVITY

We analyzed serum specimens collected serially from several patients in whom treatment-induced or spontaneous remission occurred. We found that, in general, autoantibodies against PLA₂R were present when there was clinically significant disease activity, as measured by urinary protein and serum albumin levels (Fig. 6, and Fig. 9 in the Supplementary Appendix). In the patients with remission, there was a decline or disappearance of anti-PLA₂R antibodies before the proteinuria fully resolved.

DISCUSSION

Our findings show that PLA₂R is a major target antigen in idiopathic membranous nephropathy. Seventy percent of our patients with biopsy-proven idiopathic membranous nephropathy had IgG antibodies (predominantly of the IgG4 subclass) that reacted with a reduction-sensitive epitope present on PLA₂R, a glycoprotein constituent of normal human glomeruli. The distribution of PLA₂R in normal glomeruli suggests that it is located on podocytes, as are megalin in rat models of membranous nephropathy and neutral endopeptidase in patients with alloimmune neonatal membranous nephropathy. PLA₂R colocalizes with IgG4 in immune deposits of kidney-biopsy specimens from patients with idiopathic membranous nephropathy, and IgG eluted from such biopsy specimens is reactive with PLA₂R. Moreover, serial studies in a limited number of patients suggest that the presence of circulating anti-PLA₂R antibodies corresponds to disease activity. Finally, the specificity of the reactivity of anti-PLA₂R antibodies in patients with idiopathic membranous nephropathy, as opposed to secondary forms of membranous nephropathy or other proteinuric diseases, suggests that the antibodies are most likely the cause, rather than a consequence, of podocyte injury and proteinuria.

There are several possible reasons why anti-PLA₂R antibodies are not detected in all patients with idiopathic membranous nephropathy. First, there may be target antigens other than PLA₂R; however, screening by means of Western blotting revealed no consistent alternative candidates. Second, there may be cryptic epitopes that were not revealed under our extraction and electrophoretic conditions. Third, even though all our patients had some degree of proteinuria, a proportion may

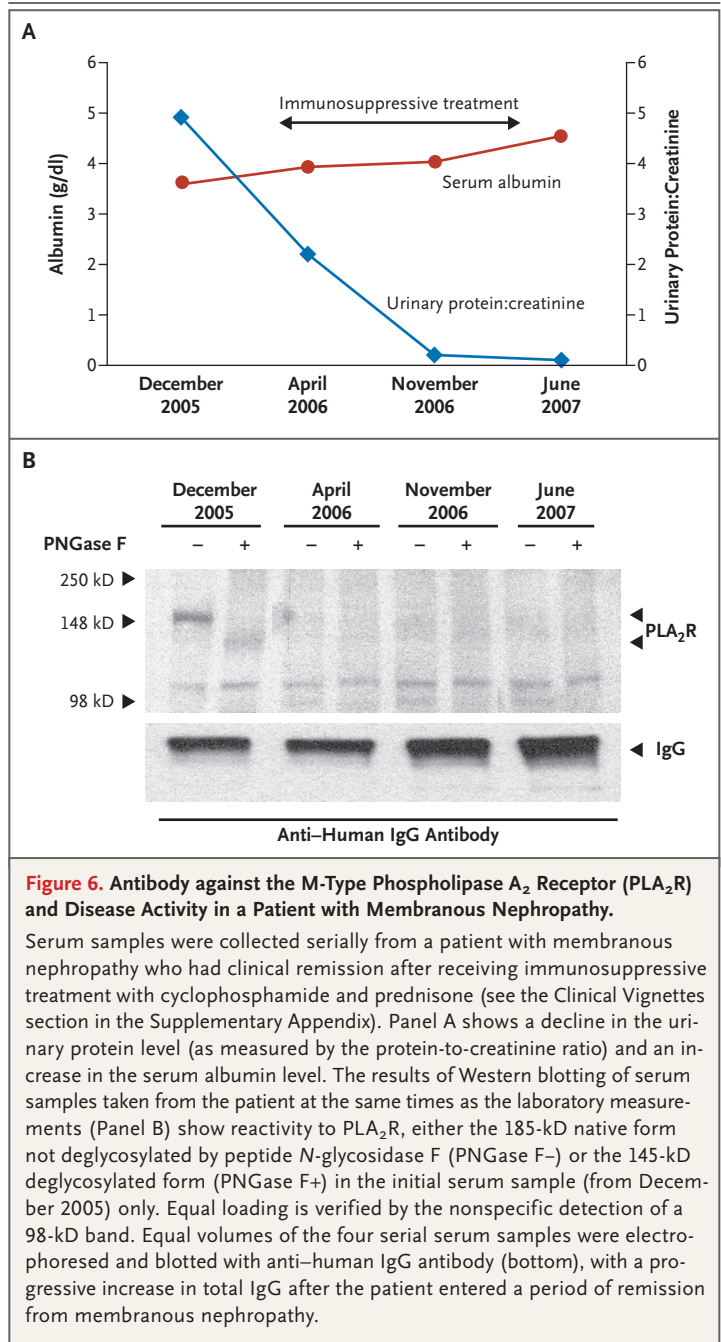


Figure 6. Antibody against the M-Type Phospholipase A₂ Receptor (PLA₂R) and Disease Activity in a Patient with Membranous Nephropathy.

Serum samples were collected serially from a patient with membranous nephropathy who had clinical remission after receiving immunosuppressive treatment with cyclophosphamide and prednisone (see the Clinical Vignettes section in the Supplementary Appendix). Panel A shows a decline in the urinary protein level (as measured by the protein-to-creatinine ratio) and an increase in the serum albumin level. The results of Western blotting of serum samples taken from the patient at the same times as the laboratory measurements (Panel B) show reactivity to PLA₂R, either the 185-kD native form not deglycosylated by peptide N-glycosidase F (PNGase F-) or the 145-kD deglycosylated form (PNGase F+) in the initial serum sample (from December 2005) only. Equal loading is verified by the nonspecific detection of a 98-kD band. Equal volumes of the four serial serum samples were electrophoresed and blotted with anti-human IgG antibody (bottom), with a progressive increase in total IgG after the patient entered a period of remission from membranous nephropathy.

have no longer had immunologically active disease at the time of sample collection. Residual proteinuria in patients with membranous nephropathy is most likely due to altered podocyte architecture that results from remodeling of the glomerular basement membrane in the advanced disease state, as shown by the transplantation of kidneys from rats with active Heymann's nephritis into healthy rats.²⁰ Although proteinuria from

the transplanted kidneys lessened in the absence of antimegalin antibodies, a moderate level of residual proteinuria persisted indefinitely in the recipient rats.

Although anti-PLA₂R antibodies are predominantly of the IgG4 isotype, which corresponds to the isotype found in immune deposits in patients with idiopathic membranous nephropathy,^{16,21} the other three IgG subclasses were variably present in serum but in smaller amounts. The presence of anti-neutral endopeptidase IgG1 and IgG4 antibodies, but not IgG4 alone, corresponds to the development of proteinuria in patients with alloimmune membranous nephropathy¹⁰; however, we could not correlate the presence or absence of anti-PLA₂R IgG1 antibodies with the severity or duration of disease.

PLA₂R is a type I transmembrane receptor and one of four mammalian members of the mannose-receptor family.^{22,23} All four have a conserved domain structure (Fig. 4H) and undergo endocytic recycling,¹² which may provide a constant source of accessible PLA₂R at the podocyte membrane for immune-complex formation. PLA₂R was initially identified as a binding protein for secreted phospholipase A₂ (PLA₂) and has a high binding affinity for several types of secreted PLA₂ in certain species.²⁴ However, human PLA₂R has a different affinity for the more common types of secreted PLA₂,²⁵ and its exact physiological role remains unclear.²⁴ Human PLA₂R was originally cloned from kidney tissue, in which it is expressed at high levels²⁵; our studies suggest that its expression in human kidneys is largely confined to glomerular podocytes. In contrast, PLA₂R is weakly expressed in the glomerulus in normal rats and is up-regulated in experimental models of mesangial proliferative glomerulonephritis.²⁶ In addition, we observed that human anti-PLA₂R autoantibodies do not detect PLA₂R in rodent or rabbit glomerular extract (data not shown).

Anti-PLA₂R autoantibodies from unrelated patients react with a similar reduction-sensitive epitope. This commonality implies that the autoantigen exists only when PLA₂R is in a configuration dependent on intramolecular disulfide bonds and that the autoantibody response is restricted to this conformation. A similarly restricted immune response to a reduction-sensitive epitope has been described for Goodpasture's epitope in the noncollagenous domain NC1 in

type IV collagen α 3.^{27,28} The finding of such a restricted response in patients with membranous nephropathy has interesting implications for the proximal events that initiate the immune response and limit epitope spreading to the rest of the PLA₂R molecule. Since members of the mannose-receptor family exist in both extended and bent conformations that confer distinct ligand binding and multimerization capacities,²⁹ these different conformations may also play a role in exposure of the epitope in PLA₂R.

The role of PLA₂R in the pathogenesis of membranous nephropathy is currently unknown. The antigen may simply be a target for antibody binding and complement-mediated podocyte injury, as in experimental models of membranous nephropathy.^{30,31} Alternatively, the antibodies may act as receptor agonists or antagonists, altering podocyte architecture and barrier function. Because PLA₂R is also present in the lungs and on leukocytes,^{32,33} it will be of interest to determine why the pathologic features are limited to the kidney.

Although there is a shorter, alternatively spliced PLA₂R transcript that is predicted to encode a soluble form of the receptor,²⁵ we could not detect such a form in the serum samples from either normal controls or patients with membranous nephropathy. We also found no evidence of circulating complexes of soluble or shed PLA₂R and anti-PLA₂R antibodies. These findings are consistent with many earlier studies in which circulating immune complexes were not detected in patients with idiopathic membranous nephropathy³ and further support the concept that immune deposits form in situ in those with the disease.

In conclusion, we identified circulating autoantibodies against PLA₂R in a large proportion of patients with idiopathic membranous nephropathy whom we examined. This protein is expressed on normal glomerular podocytes and is present in the glomerular immune deposits in patients with the disease. These findings and the characteristics of PLA₂R strongly suggest that it is a major target antigen in patients with idiopathic membranous nephropathy. Moreover, the specificity of these findings for idiopathic membranous nephropathy, in contrast to secondary forms of membranous nephropathy or other proteinuric kidney diseases, may have implications for the diagnosis and treatment of the disease.

Supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK067658 and DK30932, to Dr. Salant, and DK076743, to Dr. Powell), Amgen (to Dr. Beck), the Halpin Foundation (to Dr. Beck), Centre National de la Recherche Scientifique and Association pour la Recherche sur le Cancer (to Dr. Lambeau), and the Department of Veterans Affairs (to Dr. Klein).

Dr. Beck reports receiving grant support from Amgen and having a patent pending for a diagnostic immunoassay to detect anti-PLA₂R antibodies in membranous nephropathy; Dr. Lambeau, holding patents related to the therapeutic use of secretory PLA₂ proteins and their inhibitors; and Dr. Salant, receiving consult-

ing fees from Questcor Pharmaceuticals, Cormedix, and DiObix and having a patent pending for a diagnostic immunoassay to detect anti-PLA₂R antibodies in membranous nephropathy. No other potential conflict of interest relevant to this article was reported.

We thank Kathleen Dashner for help with tissue processing for immunofluorescence microscopy, Dr. Eric Boilard for help in preparing the human recombinant PLA₂R and rabbit PLA₂R fragment consisting of CTLDs 4 through 6, the New England Organ Bank for providing human kidneys for research purposes, the families of the organ donors, and the patients and volunteers who provided us with their serum for these studies.

REFERENCES

1. Van Damme BJ, Fleuren GJ, Bakker WW, Vernier RL, Hoedemaeker PJ. Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. *Lab Invest* 1978;38:502-10.
2. Couser WG, Steinmuller DR, Stilmant MM, Salant DJ, Lowenstein LM. Experimental glomerulonephritis in the isolated perfused rat kidney. *J Clin Invest* 1978;62:1275-87.
3. Couser WG, Salant DJ. In situ immune complex formation and glomerular injury. *Kidney Int* 1980;17:1-13.
4. Kerjaszki D, Farquhar MG. The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. *Proc Natl Acad Sci U S A* 1982;79:5557-61.
5. *Idem*. Immunocytochemical localization of the Heymann nephritis antigen (GP330) in glomerular epithelial cells of normal Lewis rats. *J Exp Med* 1983;157:667-86.
6. Makker SP, Singh AK. Characterization of the antigen (gp600) of Heymann nephritis. *Lab Invest* 1984;50:287-93.
7. Raychowdhury R, Niles JL, McCluskey RT, Smith JA. Autoimmune target in Heymann nephritis is a glycoprotein with homology to the LDL receptor. *Science* 1989;244:1163-5.
8. Saito A, Pietromonaco S, Loo AK, Farquhar MG. Complete cloning and sequencing of rat gp330/“megalin,” a distinctive member of the low density lipoprotein receptor gene family. *Proc Natl Acad Sci U S A* 1994;91:9725-9.
9. Debiec H, Guignon V, Mougnot B, et al. Antenatal membranous glomerulonephritis due to anti-neutral endopeptidase antibodies. *N Engl J Med* 2002;346:2053-60.
10. Debiec H, Nauta J, Coulet F, et al. Role of truncating mutations in MME gene in fetomaternal alloimmunisation and antenatal glomerulopathies. *Lancet* 2004;364:1252-9.
11. Salant DJ, Cybulsky AV. Experimental glomerulonephritis. *Methods Enzymol* 1988;162:421-61.
12. Zvaritch E, Lambeau G, Lazdunski M. Endocytic properties of the M-type 180-kDa receptor for secretory phospholipases A₂. *J Biol Chem* 1996;271:250-7.
13. Powell DW, Rane MJ, Joughin BA, et al. Proteomic identification of 14-3-3zeta as a mitogen-activated protein kinase-activated protein kinase 2 substrate: role in dimer formation and ligand binding. *Mol Cell Biol* 2003;23:5376-87.
14. Powell DW, Weaver CM, Jennings JL, et al. Cluster analysis of mass spectrometry data reveals a novel component of SAGA. *Mol Cell Biol* 2004;24:7249-59.
15. Lambeau G, Ancian P, Barhanin J, Lazdunski M. Cloning and expression of a membrane receptor for secretory phospholipases A₂. *J Biol Chem* 1994;269:1575-8.
16. Doi T, Mayumi M, Kanatsu K, Suehiro F, Hamashima Y. Distribution of IgG subclasses in membranous nephropathy. *Clin Exp Immunol* 1984;58:57-62.
17. Kuroki A, Iyoda M, Shibata T, Sugisaki T. Th2 cytokines increase and stimulate B cells to produce IgG4 in idiopathic membranous nephropathy. *Kidney Int* 2005;68:302-10.
18. Brandslund I, Siersted HC, Jensenius JC, Svehag SE. Detection and quantitation of immune complexes with a rapid polyethylene glycol precipitation complement consumption method (PEG-CC). *Methods Enzymol* 1981;74:551-71.
19. Saleem MA, O'Hare MJ, Reiser J, et al. A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 2002;13:630-8.
20. Makker SP, Kanalas JJ. Course of transplanted Heymann nephritis kidney in normal host: implications for mechanism of proteinuria in membranous glomerulonephropathy. *J Immunol* 1989;142:3406-10.
21. Haas M. IgG subclass deposits in glomeruli of lupus and nonlupus membranous nephropathies. *Am J Kidney Dis* 1994;23:358-64.
22. East L, Isacke CM. The mannose receptor family. *Biochim Biophys Acta* 2002;1572:364-86.
23. Lambeau G, Lazdunski M. Receptors for a growing family of secreted phospholipases A₂. *Trends Pharmacol Sci* 1999;20:162-70.
24. Rouault M, Le Calvez C, Boilard E, et al. Recombinant production and properties of binding of the full set of mouse secreted phospholipases A₂ to the mouse M-type receptor. *Biochemistry* 2007;46:1647-62.
25. Ancian P, Lambeau G, Mattéi MG, Lazdunski M. The human 180-kDa receptor for secretory phospholipases A₂: molecular cloning, identification of a secreted soluble form, expression, and chromosomal localization. *J Biol Chem* 1995;270:8963-70.
26. Beck S, Beck G, Ostendorf T, et al. Up-regulation of group IB secreted phospholipase A₂ and its M-type receptor in rat ANTI-THY-1 glomerulonephritis. *Kidney Int* 2006;70:1251-60.
27. Hellmark T, Burkhardt H, Wieslander J. Goodpasture disease: characterization of a single conformational epitope as the target of pathogenic autoantibodies. *J Biol Chem* 1999;274:25862-8.
28. Hudson BG. The molecular basis of Goodpasture and Alport syndromes: beacons for the discovery of the collagen IV family. *J Am Soc Nephrol* 2004;15:2514-27.
29. Llorca O. Extended and bent conformations of the mannose receptor family. *Cell Mol Life Sci* 2008;65:1302-10.
30. Nangaku M, Shankland SJ, Couser WG. Cellular response to injury in membranous nephropathy. *J Am Soc Nephrol* 2005;16:1195-204.
31. Cybulsky AV, Quigg RJ, Salant DJ. Experimental membranous nephropathy redux. *Am J Physiol Renal Physiol* 2005;289:F660-F671.
32. Granata F, Petraroli A, Boilard E, et al. Activation of cytokine production by secreted phospholipase A₂ in human lung macrophages expressing the M-type receptor. *J Immunol* 2005;174:464-74.
33. Silliman CC, Moore EE, Zallen G, et al. Presence of the M-type sPLA₂ receptor on neutrophils and its role in elastase release and adhesion. *Am J Physiol Cell Physiol* 2002;283:C1102-C1113.

Copyright © 2009 Massachusetts Medical Society.