Antenatal Membranous Glomerulonephritis with Vascular Injury Induced by Anti-Neutral Endopeptidase Antibodies: Toward New Concepts in the Pathogenesis of Glomerular Diseases

HANNA DEBIEC,* VINCENT GUIGONIS,† BEATRICE MOUGENOT,* JEAN-PHILIPPE HAYMANN,* ALBERT BENSMA†, GEORGES DESCHENES†, and PIERRE M. RONCO*
*INSERM Unit 489 Tenon Hospital, Paris, France; and †Department of Pediatric Nephrology, Armand Trousseau Hospital, Assistance Publique-Hôpitaux de Paris and University of Paris 6, Paris, France.

Membranous glomerulonephritis is a major cause of nephrotic syndrome and chronic renal insufficiency. This condition may be associated with a wide spectrum of infections, cancers, autoimmune diseases, and drugs, although primary forms of the disease remain the most common. Histologically, membranous glomerulonephritis is characterized by an accumulation of immune deposits on the outer aspect of the glomerular basement membrane, but the target antigens have not been identified.

Major contributions to our current understanding of the disease come from Heymann nephritis, a rat model of membranous glomerulonephritis induced by an antigenic fraction of the renal brush border (1). This experimental rat model led to the identification of megalin, a unique constitutive antigen expressed on the podocyte (2,3). Although megalin has been found in human proximal tubules, it has not been detected in human glomeruli or in immune deposits in patients with membranous glomerulonephritis (4). The antigens responsible for human membranous glomerulonephritis have eluded identification. Hepatitis B, hepatitis C, and Helicobacter pylori antigens; tumor antigens; and thyroglobulin have been detected in the subepithelial deposits, but there is no real proof that these antigens are pathogenic (5–7). Some similarities, such as glomerular deposition of renal tubular epithelial antigens, have been found between experimental Heymann nephritis and individual cases of membranous glomerulonephritis, but the antigens could not be characterized at the molecular level (8–10).

We first showed that antibodies directed against two enzymatic antigens, dipeptidyl-peptidase IV and neutral endopeptidase (NEP), could be involved in the formation of epimembranous deposits when injected into rodents (11–13). These antigens are shared by the brush border and podocytes and are also expressed on the human podocyte (13,14). We therefore suggested that they might play some role in the pathogenesis of membranous glomerulonephritis in humans (13). We recently demonstrated that anti-NEP antibodies produced by a pregnant woman were transferred to her fetus, in which a severe form of membranous glomerulonephritis developed prenatally (15). The mother had a deficiency of NEP and had become immunized against the antigen at the time of or after an earlier miscarriage. This unusual case opens up new avenues of research in the pathogenesis of membranous glomerulonephritis.

Case Report
A male infant who was born at 38 wk of gestation (birth weight, 3260 g; length, 50 cm) presented with oligoanuria (urine volume, 10 ml per 24 h), massive proteinuria (Table 1), and respiratory distress on the first day of life. His parents were unrelated, healthy individuals without a family history of renal or autoimmune disease. The mother, aged 24, had had a miscarriage at 14 wk of gestation 2 mo before this pregnancy. Her BP, urinalysis, and serum creatinine concentration were normal throughout and after the pregnancy, and she took no medications. However, antenatal echography showed oligohydramnios and enlarged fetal kidneys from the 34th week of gestation. Her levels of antineutrophil cytoplasmic antibodies, antinuclear and anti-DNA antibodies, and complement were normal.

Mechanic ventilation was necessary from birth to 10 d. The infant’s serum creatinine concentration was 1.9 mg/dl (170 μmol/L) on day 2 and peaked at 2.7 mg/dl (240 μmol/L) on day 4. Diuresis increased after intravenous furosemide. The serum creatinine concentration subsequently decreased, during which time nephrotic-range proteinuria (Table 1) with hypoaalbuminemia developed (1.9 g/dl on day 7). Calcium channel blockers and beta-blockers were needed for BP control from day 5 until 6 wk. Urinary protein excretion progressively decreased to 4.2 mg/mg creatinine (0.48 g/mmol) at day 52. However, at 4 mo of age, the BP and proteinuria increased.

H.D., V.G., and B.M. contributed equally to this work.
Correspondence to Dr. Pierre Ronco, INSERM Unit 489, Hôpital Tenon, 4 rue de la Chine, 75020-Paris, France. Phone: +33-1-56-01-66-39; Fax: +33-1-56-01-69-99; E-mail: pierre.ronco@tnn.ap-hop-paris.fr
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Table 1. Time course of serum creatinine and proteinuria

<table>
<thead>
<tr>
<th>Age</th>
<th>Serum Creatinine (mg/dl)</th>
<th>Urinary Protein Excretion (mg/mg creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 d</td>
<td>ND</td>
<td>14.2</td>
</tr>
<tr>
<td>2 d</td>
<td>1.9</td>
<td>15.2</td>
</tr>
<tr>
<td>4 d</td>
<td>2.7</td>
<td>ND</td>
</tr>
<tr>
<td>5 d</td>
<td>2.2</td>
<td>ND</td>
</tr>
<tr>
<td>6 d</td>
<td>1.6</td>
<td>14.0</td>
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<td>22 d</td>
<td>1.4</td>
<td>28.3</td>
</tr>
<tr>
<td>31 d</td>
<td>1.3</td>
<td>16.2</td>
</tr>
<tr>
<td>40 d</td>
<td>0.8</td>
<td>8.4</td>
</tr>
<tr>
<td>52 d</td>
<td>0.6</td>
<td>4.2</td>
</tr>
<tr>
<td>4 mo</td>
<td>0.5</td>
<td>7.9</td>
</tr>
<tr>
<td>9 mo</td>
<td>0.6</td>
<td>3.9</td>
</tr>
<tr>
<td>11 mo</td>
<td>0.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

ND, not determined.

a To convert values for serum creatinine to micromoles per liter, multiply by 88.4.

b To convert values for urinary protein excretion to grams per millimole urinary creatinine, multiply by 0.113.

although the serum creatinine concentration remained normal (Table 1). Symptoms of serum sickness were not observed at any time.

A computed tomography–guided kidney biopsy was performed at 4 wk of age (Figure 1). The biopsy specimen showed a severe, unusual form of membranous glomerulonephritis. Capillary tufts were collapsed in the majority of the 40 glomeruli. Most Bowman’s spaces were also distended. In all glomeruli, there was a thickening of the capillary walls; such thickening was most apparent in noncollapsed glomerular tufts in which capillary loops showed spikes. Marked tubular atrophy and severe lesions of the interlobular arteries and arterioles were also observed. Immunofluorescence studies showed marked subepithelial deposits of IgG, C3, and C5b-9 in all glomeruli. No immune deposits were seen in proximal tubules and vessels. Electron microscopy examination revealed diffuse alterations of glomerular capillary walls and a marked atrophy of the brush border. Abundant, electron-dense deposits were seen on the outer aspect of the glomerular basement membrane. These deposits contained annular formations, often overlaid by an expansion of the lamina densa. There were neither subendothelial nor mesangial deposits.

Tests for neonatal syphilis, toxoplasmosis, cytomegalovirus, and hepatitis B virus infection were negative. The Coombs’ test was negative, and complement components were normal at day 35 (C3, 0.97 g/L; C4, 0.25 g/L). Low levels of circulating immune complexes (4.0 μg/ml) were detected in the serum at day 13, using an enzyme immunoassay kit. This activity was no longer detected at day 40.

Clinical examination at 11 mo was unremarkable, although nicardipine (2 mg/kg body wt per d) was required to control BP. The serum creatinine concentration was normal, and urinary protein excretion had markedly decreased (Table 1).

Identification of NEP as the Target Antigen of Nephritogenic Antibodies

Because of the early development of membranous glomerulonephritis in this infant, we suspected pregnancy-induced immunization of the mother with transplacental passage of nephritogenic antibodies. This hypothesis was first tested by indirect immunofluorescence examination of normal human kidneys. A serum sample obtained 9 mo before pregnancy (7 mo before she had a miscarriage) was negative. Serum samples obtained at 3 mo of gestation and after delivery showed reactivity on the glomerular capillary walls and the brush border on all kidney biopsy specimens, as did the serum obtained from the infant 13 d after birth. No reactivity was detected in the infant’s serum 40 d after birth.

The nature of the target antigen was suspected by indirect immunofluorescence examination of rabbit and rat kidney sections incubated with the mother’s or the infant’s antibody. The same pattern as in human kidneys was observed in the rabbit, whereas in the rat, staining was restricted to the cells of Bowman’s capsule and to the brush border of deep cortical segments of the proximal tubule. We had previously observed identical interspecies differences with anti-NEP antibodies, whereas distribution of dipeptidyl peptidase IV is not species dependent (14). The mother’s IgG antibody and the infant’s IgG antibody recognized by Western blotting a single antigen of approximately 90 kD in protein extracts from rat brush border, rabbit kidney cortex, and cultured human podocytes. This antigen had the same electrophoretic mobility as NEP that was specifically immunoprecipitated from rat brush border with the mother’s IgG, whereas it was detected only in the unbound fraction of the control immunoprecipitation. Furthermore, NEP activity was found in the eluted fraction from material bound to the mother’s IgG but not in the one eluted from control IgG.

The anti-NEP antibodies produced by the mother, which were found in the infant’s serum 13 d after birth, were most likely responsible for the infant’s membranous glomerulonephritis, given that the injection of rabbits with the serum IgG fraction from the mother induced intraglomerular deposits and proteinuria, whereas injection with the IgG fraction from the father did not (Figure 2, D and G). Furthermore, NEP was localized by confocal microscopy in immune deposits both in the infant and in the rabbits injected with the mother’s IgG (Figure 2).

A similar case of transplacental induction of membranous nephropathy in a neonate was reported in 1990 (16). Recent studies from our laboratory indicate that the mother’s serum, provided by Dr. De Heer (Leyden, The Netherlands), contain anti-NEP antibodies (Debiec et al., unpublished data).

Mechanisms of the Deposition of Immune Complexes in the Infant’s Glomeruli

Membranous glomerulonephritis has long been considered as a model of immune glomerulopathy because of the deposition of preformed circulating immune complexes. Initial studies of the active model of Heymann nephritis suggested that the subepithelial deposits resulted from glomerular trapping of
Circulating immune complexes formed by circulating brush border–related antigens and the corresponding antibodies. This hypothesis was based on the observation that the glomerular disease was induced by fractions of membrane prepared from rat renal brush border, not from glomerular extracts. Subsequently, the development of passive Heymann nephritis led to the suggestion that subepithelial Ig deposits could be formed without the intervention of circulating immune complexes. The latter hypothesis was confirmed by the demonstration of megalin expression in the coated pits of rat podocytes, particularly at the sole of foot processes facing the glomerular capillary wall (2).

Transient low levels of circulating immune complexes were detected in the infant’s serum at day 13. The immune complexes isolated from the serum sample contained NEP, as demonstrated by Western blotting (15). However, their contribution to the formation of subepithelial immune deposits is uncertain, because levels of circulating immune complexes were low, manifestations of serum sickness were absent, and subendothelial and mesangial immune deposits were not seen. Immune complexes could also be formed in situ at the sole of podocyte foot processes where NEP is expressed (17). The two mechanisms are not mutually exclusive.

Contrary to megalin, NEP is expressed in a diffuse pattern on the membrane of podocytes, as is angiotensin-converting enzyme on the plasma membrane of mature oocytes (18). In vivo interaction of angiotensin-converting enzyme with divalent antibodies induces the formation of granular immune deposits through a mechanism of “patching” and “shedding” of immune complexes (18). A similar mechanism may be implicated in the formation of immune deposits in the infant’s glomeruli. One can speculate that the immune complexes that are shed from the foot processes are sequestered between the lamina rara externa of the glomerular capillary wall and the podocytes’ slit diaphragms, whereas those that are shed from the podocyte cell bodies are excreted in the infant’s urine.

Figure 1. Infant’s kidney biopsy specimen obtained at 4 wk. (A) Collapsed capillary tufts, with prominent tubular atrophy and mild interstitial cellular infiltration and fibrosis. (B) Thickening of capillary walls in a noncollapsed glomerulus, conspicuous lesions of an interlobular artery, and severe alterations of the proximal tubule epithelium. (C) Frozen section incubated with FITC-labeled anti-human IgG antibody revealing heavy epimembranous granular deposits. (D) Representative segment of the capillary wall analyzed by electron microscopy (bar, 200 nm). From Debiec et al., N Engl J Med 346: 2053–2060, 2002 (Copyright 2002 Massachusetts Medical Society. All rights reserved). Magnification: ×170 in A (trichrome), ×430 in B (trichrome), ×400 in C.
Mechanisms of the Immunization against NEP in the Infant’s Mother

Because the mother had no apparent renal abnormalities despite high serum titers of anti-NEP antibody, we hypothesized that she might be deficient in NEP and analyzed NEP expression in granulocytes from both parents. Fluorescence-activated cell sorter analysis of the mother’s granulocytes incubated with either anti-NEP monoclonal antibody or the mother’s serum sampled 5 wk after delivery showed no NEP at the cell membrane. Cell extracts prepared from maternal granulocytes failed to react with either monoclonal or polyclonal antibodies against NEP after Western blotting. Moreover, the mother’s serum reacted with the father’s granulocytes but not with her own granulocytes, suggesting an alloimmunization process. Alloimmunization in the mother most likely occurred at the time of her miscarriage, given that a plasma sample obtained earlier did not show anti-NEP antibodies. Despite the absence of NEP, the mother was healthy, as were mice with a targeted disruption of the NEP gene, suggesting enzymatic redundancy (19).

Renal injury mediated by alloimmune responses to major renal antigens was first described in tubular basement membrane in rat (20). A previously reported case of neonatal membranous glomerulonephritis might also involve NEP deficiency and alloimmunization because the mother had no renal findings (16). It is likely that additional individuals who lack NEP will be identified and that additional cases of acute renal failure and membranous glomerulonephritis in the neonate may be ascribed to anti-NEP alloantibodies.

Potential Role of the Blockade of the Enzymatic Activity of NEP by Nephritogenic Antibodies

NEP (nephrilysin, enkephalinase, CD10, EC 3.4.24.11) is a 90- to 110-kD zinc-dependent metallopeptidase, identical to the common acute lymphoblastic leukemia antigen (21,22). It is expressed in brain tissue; on polymorphonuclear leukocytes and lymphoid progenitor cells; and on epithelial cells within nonlymphoid organs such as kidney, liver, breast, and lung (23,24). It is also found in the serum and the urine (25,26). This enzyme is involved in the metabolism of a number of regulatory peptides and plays an important role in turning off peptide signaling events at the cell surface (27). In the human kidney, NEP is found on brush border, podocyte, and vascular smooth muscle cells (14,28).

For evaluating a potential effect of anti-NEP antibodies on enzymatic activity, lysates of human podocytes were preincubated with maternal or paternal IgG. The endopeptidase 24.11 activity of podocyte lysates was blocked by its specific inhibitors thiorphan and phosphoramidon and was also dose-dependently inhibited by maternal but not paternal IgG.

Figure 2. Colocalization of neutral endopeptidase (NEP) and IgG in immune deposits and induction of renal disease in rabbit by maternal IgG. (A to C) Immunofluorescence staining of kidney sections from the infant’s biopsy specimen after double labeling with anti-human IgG antibodies (A) and polyclonal anti-NEP antibody (B). (C) The merged image. The insets show the colocalization of NEP and IgG on the outer aspect of the capillary wall. (D to G) Immunofluorescence staining of kidney sections from rabbits injected 4 d earlier with mother’s (D to F) or father’s (G) IgG fractions, respectively. The sections in D to F were double labeled with anti-human IgG antibodies (D) and with polyclonal anti-NEP antibody (E); the merged image is shown in F. The section in G was labeled with anti-human IgG antibodies. From Debiec et al., N Engl J Med 346: 2053–2060, 2002 (Copyright 2002 Massachusetts Medical Society. All rights reserved). Magnification: ×600 in A–G, ×2000 in insets.
The infant’s kidney biopsy specimen showed unusually severe arterial lesions without immune deposits and a collapsing of glomerular capillary tufts that was suggestive of major renal ischemia during prenatal development. These lesions may result from the enzymatic activity of NEP as it cleaves vasoactive mediators, including bradykinin, atriopeptin, and endothelins, and thus may modify local blood flows (27,28). Because the mother’s antibodies inhibited NEP activity, their transplacental passage might increase concentrations of vasoconstrictor peptides in the vascular wall and thus induce the proliferation of vascular smooth muscle cells.

Several enzymatic antigens diffusely expressed on podocytes have been implicated in the development of membranous glomerulonephritis in rodents: NEP, dipeptidyl-peptidase IV, and aminopeptidase A (11–13,29). A single injection of a relatively large amount of an anti-aminopeptidase A monoclonal antibody that was able to block the enzyme activity in vitro induced an acute albuminuria that lasted for at least 16 d and subepithelial Ig deposits (29). Because aminopeptidase A is a key enzyme in the intrarenal degradation of angiotensin II, it was hypothesized that angiotensin II mediated the induction of this acute albuminuria. This assumption was supported by the observations that non–enzyme-inhibiting monoclonal antibodies never induced an acute albuminuria and that enalapril and losartan reduced the acute albuminuria by >90%. However, angiotensinogen-deficient mice also developed albuminuria upon injection of a combination of enzyme-inhibiting anti-aminopeptidase A monoclonal antibodies (30), thus demonstrating that angiotensin II is not required for the induction of albuminuria in this model. However, intrarenal angiotensin II levels were significantly increased in BALB/c mice that received the nephritogenic combinations of anti-aminopeptidase A antibodies, which led the authors to suggest that the increased intrarenal angiotensin II levels might be the consequence of albuminuria (30). Initially elevated angiotensin II levels might contribute to late development of glomerular injury and proteinuria. In our model, blockade of NEP activity by nephritogenic antibodies at the podocyte surface may locally change concentrations of important vasoactive mediators and thus alter the permeability of glomerular capillary walls.

**Phenotypic Alterations of Podocytes Induced by NEP–Anti-NEP Complexes**

Heavy deposits of the membrane attack complex of the complement C5b-9 were found on the outer aspect of glomerular capillary walls in the infant’s kidney biopsy specimen (15). These deposits might account for the unusual presence of annular formations within the electron-dense deposits. They raise the question as to the functional consequences of complement attack on podocytes. Sublethal C5b-9 attack in cultured glomerular epithelial cells was found to trigger metabolic changes and induction of de novo synthesis of prostaglandins and proteases (31,32). Podocytes also become “activated” in vivo (33), as they initiate synthesis of several presumably functionally relevant proteins that are not produced at all or only in low concentration in their resting state. Examples are matrix metalloprotease 9 (34) and cytochrome b558, an integral component of the NAPDH oxidoreductase complex (35). In addition, major changes in the distribution of nephrin was observed in patients with primary acquired nephrotic syndrome (36). In glomeruli of patients with membranous glomerulonephritis, a more granular pattern or a loss of staining of nephrin was observed. In cultures of human podocytes, aggregated but not disaggregated human IgG4, plasmaemmal insertion of the membrane attack complex of complement, TNF-α, and puromycin, induced the shedding of nephrin with a loss of surface expression. This phenomenon was abrogated by cytoskeleton inhibitors.

We suggest that the binding of the nephritogenic anti-NEP antibodies may result in alterations of the podocytic phenotype, such as changes in the cytoskeleton, allowing shedding of important podocytic proteins from plasma membrane to an extracellular site and production of toxic mediators including collagenases and oxygen radicals. Further research in this area should help elucidate the pathogenic mechanisms involved in the induction of albuminuria.

In conclusion, we have identified the first target antigen of nephritogenic antibodies in human membranous glomerulonephritis. This antigen exhibits with an enzymatic activity that could play some role in the pathogenesis of the nephropathy. We have also identified the first person with NEP deficiency.

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**References**


