

Brief Report

ANTENATAL MEMBRANOUS GLOMERULONEPHRITIS DUE TO ANTI-NEUTRAL ENDOPEPTIDASE ANTIBODIES

HANNA DEBIEC, PH.D., VINCENT GUIGONIS, M.D.,
BÉATRICE MOUGENOT, M.D., FABRICE DECOBERT, M.D.,
JEAN-PHILIPPE HAYMANN, M.D., ALBERT BENSMAN, M.D.,
GEORGES DESCHÊNES, M.D., PH.D.,
AND PIERRE M. RONCO, M.D., PH.D.

MEMBRANOUS glomerulonephritis, a major cause of the nephrotic syndrome and chronic renal insufficiency, is associated with a wide spectrum of infections, cancers, autoimmune diseases, and drugs. The condition 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's nephritis, a rat model of membranous glomerulonephritis induced by immunization with an antigenic fraction of the renal brush border.¹ Studies of 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 found in human glomeruli or in immune deposits in patients with membranous glomerulonephritis.⁴ Dipeptidyl-peptidase IV and neutral endopeptidase are two other antigens shared by the brush border and podocytes that are involved in the formation of immune deposits in animal models; these two proteins are expressed on the human podocyte.^{5,6}

In this article, we report that anti-neutral endopeptidase antibodies produced by a pregnant woman were transferred to her fetus, in which a severe form of membranous glomerulonephritis developed prenatally. The mother had a deficiency of neutral endopeptidase and probably had become immunized against the antigen at the time of or after an earlier miscarriage.

CASE REPORT

A male infant born at 38 weeks of gestation (birth weight, 3260 g; length, 50 cm) presented with oligoanuria (urine vol-

From INSERM Unité 489, Tenon Hospital (H.D., B.M., J.-P.H., P.M.R.), and the Department of Pediatric Nephrology, Armand Trousseau Hospital (V.G., F.D., A.B., G.D.), Assistance Publique-Hôpitaux de Paris and University of Paris 6, Paris. Address reprint requests to Dr. Ronco at INSERM Unité 489, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France, or at pierre.ronco@tnn.ap-hop-paris.fr.

Drs. Debiec, Guignonis, and Mougenot contributed equally to the article.

ume, 10 ml per 24 hours), massive proteinuria (Table 1), and respiratory distress on the first day of life. His parents were unrelated, healthy persons without a family history of renal or autoimmune disease. The mother, who was 24 years old, had had a miscarriage at 14 weeks of gestation 2 months before she became pregnant with this child. Her blood pressure, findings on urinalysis, and serum creatinine concentration were normal throughout and after the pregnancy, and she took no medications. However, antenatal ultrasonography showed oligohydramnios and enlarged fetal kidneys from the 34th week of gestation. The mother's level of antineutrophil cytoplasmic antibodies, antinuclear antibodies, anti-DNA antibodies, and complement were normal.

Mechanical ventilation for hypoxemia was necessary from birth to 10 days. The infant's serum creatinine concentration was 1.9 mg per deciliter (170 μ mol per liter) on day 2 and peaked at 2.7 mg per deciliter (240 μ mol per liter) on day 4. Diuresis increased after the administration of intravenous furosemide. The serum creatinine concentration subsequently decreased, and nephrotic-range proteinuria developed (Table 1), as did hypoalbuminemia (1.9 g per deciliter on day 7). Calcium-channel blockers and beta-blockers were needed for blood-pressure control from day 5 until 6 weeks after birth. Urinary protein excretion progressively decreased to 4.2 mg per milligram of creatinine (0.48 g per millimole) on day 52. However, at four months of age, the blood pressure and proteinuria increased, although the serum creatinine concentration remained normal (Table 1). Symptoms of serum sickness were not observed at any time.

A kidney biopsy guided by computed tomography was performed at four weeks of age. Tests for neonatal syphilis, toxoplasmosis, cytomegalovirus, and hepatitis B virus infection were negative. A Coombs' test was negative, and the levels of complement components were normal on day 35 (C3, 0.97 g per liter; C4, 0.25 g per liter). Low levels of circulating immune complexes (4.0 μ g per milliliter) were detected in the serum on day 13, with the use of an enzyme immunoassay kit (CIC-C1q, Quidel). This activity was no longer detected on day 40.

Findings on clinical examination at 11 months were unremarkable, although nicardipine (2 mg per kilogram of body weight per

TABLE 1. SERUM CREATININE CONCENTRATION AND PROTEINURIA IN THE INFANT OVER TIME.*

AGE	SERUM CREATININE CONCENTRATION	URINARY PROTEIN EXCRETION
	mg/dl	mg/mg of creatinine
1 day	ND	14.2
2 days	1.9	15.2
4 days	2.7	ND
5 days	2.2	ND
6 days	1.6	14.0
22 days	1.4	28.3
31 days	1.3	16.2
40 days	0.8	8.4
52 days	0.6	4.2
4 mo	0.5	7.9
9 mo	0.6	3.9
11 mo	0.6	1.3

*To convert values for serum creatinine to micromoles per liter, multiply by 88.4. To convert values for urinary protein excretion to grams per millimole of urinary creatinine, multiply by 0.113. ND denotes not determined.

day) was required to control blood pressure. The serum creatinine concentration was normal, and urinary protein excretion had markedly decreased (Table 1). The parents gave written informed consent for the studies described below.

METHODS

Analysis of Renal-Biopsy Specimen and Immunohistochemical Studies

The infant's renal-biopsy specimen was prepared for light, immunofluorescence, and electron microscopy by standard techniques. Serum samples from both the mother and the infant were assayed by indirect immunofluorescence after incubation with cryostat sections of kidney. Serum samples had been obtained from the mother seven months before her earlier miscarriage and at three months of gestation during the pregnancy with this child; we obtained additional samples five weeks and seven months after delivery. Serum samples were obtained from the infant 13 and 40 days after birth. We also examined biopsy specimens containing normal glomeruli from seven different patients ranging in age from 4 to 70 years, as well as normal rabbit and rat kidneys.

Western Blotting, Immunoprecipitation, and Enzymatic-Activity Analyses

Fractions of membrane were prepared from rat renal brush border and rabbit kidney cortex, as previously described.^{7,8} Fractions of this membrane, cultured human podocytes,⁹ and granulocytes from the mother and father were lysed, separated by 7 percent sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) under reducing conditions, and analyzed by Western blotting with serum from the mother or control serum and with monoclonal (Novocastra) or rabbit polyclonal (Santa Cruz Biotechnology) anti-neutral endopeptidase antibodies. Circulating immune complexes were isolated by precipitation of the serum sample obtained from the infant on day 13 with polyethylene glycol-6000 and were analyzed by Western blotting with anti-neutral endopeptidase antibodies.¹⁰

For immunoprecipitation experiments, rat renal brush-border membranes were lysed in immunoprecipitation buffer and centrifuged. The supernatant was incubated with serum from the mother or control serum. The antigen–antibody complexes were isolated by an immunoprecipitation system (Immuno-catcher, Cytosignal) with the use of protein A–G resin. The bound complexes and unbound material were analyzed by SDS-PAGE and, after blotting, were incubated with anti-neutral endopeptidase antibody.

To measure the enzymatic activity of the antigen identified with the use of maternal antibodies, IgG fractions from the mother's serum or control serum were bound to CNBr-activated Sepharose 4B (Pharmacia Biotech) as recommended by the manufacturer, incubated with rat renal brush-border lysates, and washed, and then the antigen was eluted with 0.05 M diethylamine at pH 11.0 and immediately neutralized.¹¹ The enzymatic activity of neutral endopeptidase was measured by a coupled assay with the use of Suc-Ala-Ala-Phe-pNA (Bachem Bioscience) and aminopeptidase N (Roche Diagnostics).¹²

Transfer of Disease to Rabbits

Two female New Zealand white rabbits were injected intravenously with 10 mg of IgG from the infant's mother or father prepared on a Sepharose 4B–coupled protein A column (Pharmacia). Four days later, the animals were killed, and their kidneys were processed as described above. Three other rabbits were injected with 5 mg of IgG from the infant's mother or father and followed for up to six weeks.

Analysis of the Composition of the Glomerular Immune Deposits by Confocal Microscopy

Cryosections of the biopsy specimen from the infant and of the kidneys from the injected rabbits were first incubated with fluo-

rescein-isothiocyanate–conjugated antihuman IgG antibodies, then with goat polyclonal anti-neutral endopeptidase antibodies (Santa Cruz), followed by rhodamine-conjugated anti-goat IgG antibodies (Chemicon). After being washed, sections were examined under a confocal microscope.

Flow Cytometric Analysis of Neutral Endopeptidase Expression on Granulocytes

After lysis of red cells, granulocytes from each parent were incubated with a monoclonal antibody against neutral endopeptidase or with serum from both parents, then incubated with fluorescein-isothiocyanate–conjugated secondary antibodies. Results were analyzed on a flow cytometer (Elite, Beckman Coulter).

RESULTS

Analysis of the Renal-Biopsy Specimen from the Infant

The renal-biopsy specimen from the infant showed a severe, unusual form of membranous glomerulonephritis. Capillary tufts were collapsed in the majority of the 40 glomeruli (Fig. 1A). 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 (Fig. 1B), in which capillary loops showed spikes. Marked tubular atrophy (Fig. 1A) and severe lesions of the interlobular arteries and arterioles (Fig. 1B) were also observed.

Immunofluorescence studies showed marked subepithelial deposits of IgG (Fig. 1C) and C3 (not shown) in all glomeruli. No immune deposits were seen in proximal tubules and vessels. Examination by electron microscopy 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 (Fig. 1D), often overlaid by an expansion of the lamina densa. There were neither subendothelial nor mesangial deposits.

Analysis of Antibodies in Serum Samples from the Mother and the Infant

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 (Fig. 2A, 2B, 2C, and 2D). A serum sample obtained from the mother nine months before she became pregnant with this child (seven months before she had a miscarriage) was negative (Fig. 2A). Serum samples obtained at 3 months of gestation (not shown) and at 5 weeks (Fig. 2B) and 7 months (not shown) 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 days after

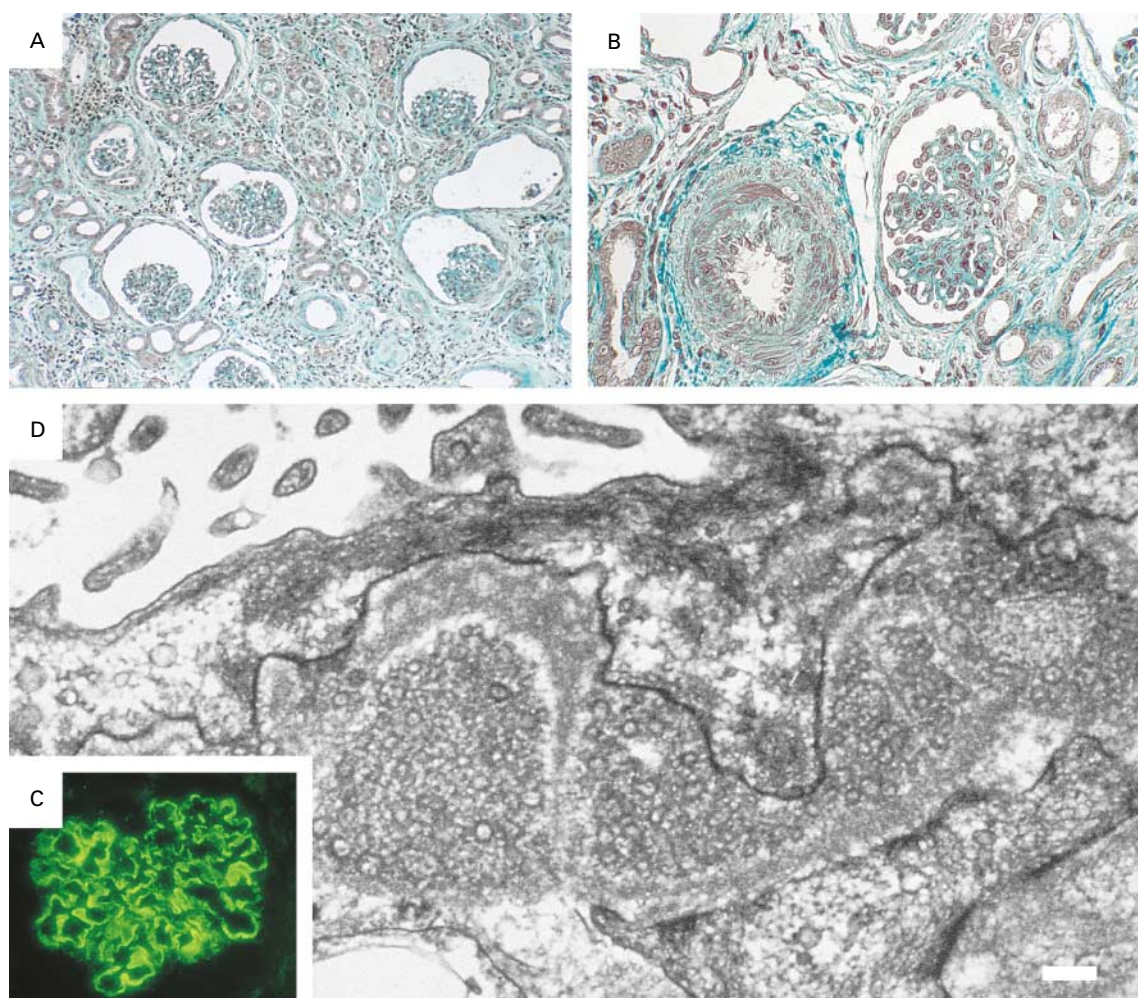


Figure 1. Renal-Biopsy Specimen Obtained from the Infant at Four Weeks.

Panel A shows collapsed capillary tufts, with prominent tubular atrophy and mild interstitial cellular infiltration and fibrosis (trichrome stain, $\times 170$). Panel B shows thickening of capillary walls in a noncollapsed glomerulus, conspicuous lesions of an interlobular artery, and severe alterations of the epithelium of the proximal tubule (trichrome stain, $\times 430$). Panel C shows a frozen section incubated with fluorescein-isothiocyanate-labeled antihuman IgG antibody, revealing heavy epimembranous granular deposits ($\times 400$). Panel D shows a representative segment of the capillary wall analyzed by electron microscopy (bar, 200 nm).

birth (Fig. 2C). No reactivity was detected in the infant's serum 40 days after birth (Fig. 2D).

To identify the target antigen, two sets of experiments were performed. First, positive serum samples were studied by indirect immunofluorescence microscopy on sections from rabbit and rat kidneys. The same pattern observed in the sections from human kidneys was found in the rabbit kidney (Fig. 2E), whereas in the rat kidney, staining was restricted to cells of Bowman's capsule and to the brush border of

deep cortical segments of the proximal tubule (Fig. 2F). We had previously observed identical interspecies differences with anti-neutral endopeptidase antibodies, whereas the distribution of dipeptidyl-peptidase IV is not species-dependent.⁶ Second, antibody specificity was analyzed by immunochemical and enzymatic techniques. The mother's IgG antibody (Fig. 2G) and the infant's IgG antibody (at 13 days, not shown) recognized a single antigen of approximately 90 kD in protein extracts from rat brush bor-

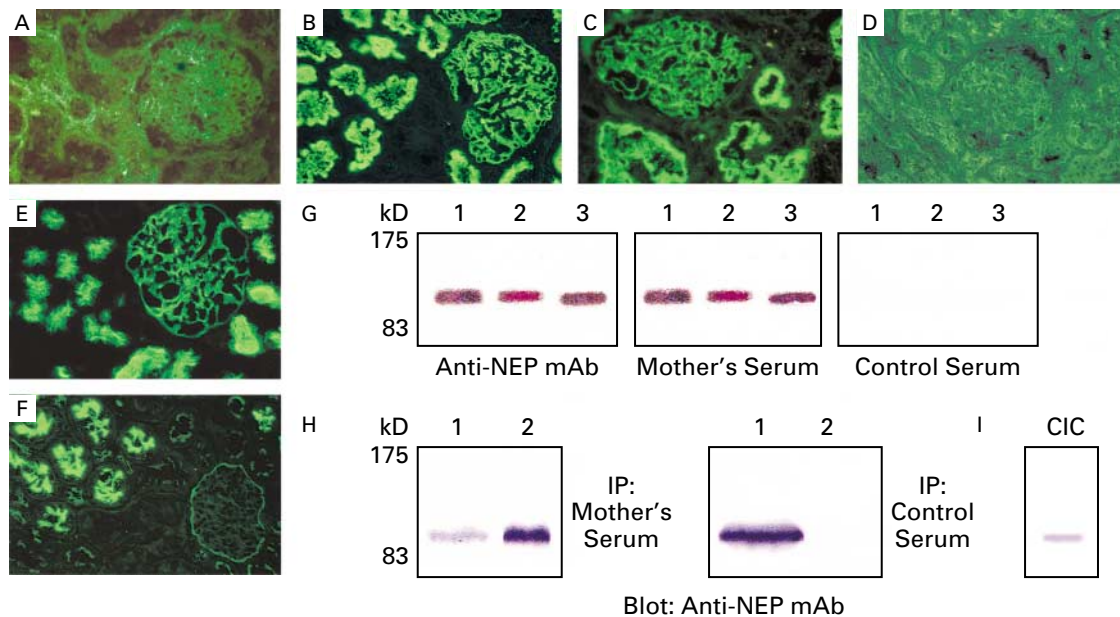


Figure 2. Immunohistochemical and Immunochemical Analyses of the Target Antigen Recognized by the Mother's Antibodies.

The reactivity of serum samples was analyzed by indirect immunofluorescence staining of biopsy specimens from human kidneys (Panels A, B, C, and D) and from normal rabbit (Panel E) and rat (Panel F) kidneys ($\times 312$). Serum samples were obtained from the mother nine months before she became pregnant with this child (Panel A) and five weeks after delivery (Panels B, E, and F). Serum samples were obtained from the infant 13 days after birth (Panel C) and 40 days after birth (Panel D). Serum dilutions are 1:50 in Panels A and D, 1:1000 in Panels B and F, and 1:100 in Panels C and E. Panel G shows immunoblots of protein extracts from rat brush border, rabbit cortex, and human podocytes (lanes 1, 2, and 3, respectively) incubated with anti-neutral endopeptidase monoclonal antibody (anti-NEP mAb), serum obtained from the mother five weeks after delivery (dilution, 1:4000), and control serum (dilution, 1:250). The same band of approximately 90 kD was detected in all tissue extracts incubated either with anti-neutral endopeptidase antibody or with the mother's serum. Panel H shows the results of immunoprecipitation (IP) studies. Rat renal brush border membranes were lysed and incubated with the mother's serum obtained five weeks after delivery or control human serum. Antigen-antibody complexes were immunoprecipitated and were analyzed by Western blotting with anti-neutral endopeptidase monoclonal antibody. A 90-kD band was detected primarily in the immunoprecipitate obtained with the mother's serum (lane 2), whereas in the control, all anti-neutral endopeptidase immunoreactivity rested in the flow-through fraction (lane 1). Panel I shows an immunoblot of circulating immune complexes (CIC) isolated from the infant's serum on day 13, incubated with anti-neutral endopeptidase antibody.

der, rabbit kidney cortex, and human podocytes. This antigen had the same electrophoretic mobility as neutral endopeptidase (Fig. 2G).

To confirm that neutral endopeptidase was the reactive antigen, immunoprecipitation experiments were performed by incubating rat brush border with either the mother's serum or control serum, and the bound and unbound fractions were incubated after blotting with anti-neutral endopeptidase antibody. Neutral endopeptidase was identified primarily in the antigenic fraction bound to maternal IgG, whereas it was detected only in the unbound fraction of the control immunoprecipitation (Fig. 2H). Furthermore, enzymatic activity of neutral endopeptidase was detected in the fraction eluted from material bound to maternal IgG (0.20 μmol per milligram of protein per minute) but not in the one eluted from control

IgG. More than 95 percent of the enzymatic activity was blocked by 2 μM of phosphoramidon or 50 μM of thiorphan — two specific inhibitors of neutral endopeptidase. Together, these results demonstrate that neutral endopeptidase is the target antigen of circulating antibodies. Moreover, the presence of neutral endopeptidase in the circulating immune complexes isolated from the serum sample obtained from the infant on day 13 was demonstrated by Western blotting (Fig. 2I).

To evaluate a potential effect of anti-neutral endopeptidase antibodies on enzymatic activity, lysates of human podocytes were preincubated with IgG antibodies from the mother or the father. The endopeptidase-24.11 activity of podocyte lysates was blocked by its specific inhibitors, thiorphan and phosphoramidon, and was also inhibited in a dose-dependent

manner by IgG from the mother but not by IgG from the father (Table 2).

Colocalization of Neutral Endopeptidase and IgG in Immune Deposits

To demonstrate that neutral endopeptidase was localized in subepithelial immune deposits, sections of the renal-biopsy specimen from the infant were incubated with antihuman IgG antibody (Fig. 3A) and with polyclonal anti-neutral endopeptidase antibody (Fig. 3B). IgG antibodies and neutral endopeptidase were colocalized in many areas of the outer aspect of the capillary wall (Fig. 3C).

Induction of Renal Disease in Rabbit by the IgG Fraction from the Mother

Kidneys from three rabbits injected with the IgG fraction from the mother showed glomerular deposits of this IgG along capillary walls (Fig. 3D). When the same section was incubated with anti-neutral endopeptidase antibody (Fig. 3E), we observed a clear colocalization of the injected IgG with neutral endopeptidase (Fig. 3F). The rabbit that received the higher dose of IgG showed respiratory distress and was killed when death was imminent. No deposits were seen in glomeruli of two control rabbits injected with IgG from the father (Fig. 3G). Tests conducted between four days and six weeks after injection revealed that proteinuria (5.8 to 7.6 mg of protein per milligram of creatinine [0.65 to 0.86 g per millimole]) had developed in all three rabbits that were injected with IgG from the mother, whereas the rabbits that were injected with IgG from the father had urinary protein excretion of 1.1 to 1.8 mg per milligram of creatinine (0.12 to 0.20 g per millimole).

Analysis of Neutral Endopeptidase Expression in the Parents

Because the mother had no apparent renal abnormalities despite high serum titers of anti-neutral endopeptidase antibody, we hypothesized that she might be deficient in neutral endopeptidase, and we therefore analyzed neutral endopeptidase expression in granulocytes from both parents. Fluorescence-activated cell-sorter analysis of the mother's granulocytes incubated with either anti-neutral endopeptidase monoclonal antibody or with the serum obtained from the mother five weeks after delivery showed no neutral endopeptidase at the cell membrane (Fig. 4A and 4B). Cell extracts prepared from the mother's granulocytes did not react with either monoclonal or polyclonal antibodies against neutral endopeptidase on Western blotting (Fig. 4C). Moreover, the mother's serum reacted with the father's granulocytes but not with her own granulocytes, suggesting an alloimmunization process (Fig. 4B and 4C).

TABLE 2. EFFECT OF MATERNAL IgG ON ENDOPEPTIDASE 24.11 ACTIVITY.*

EXPERIMENTAL CONDITIONS	ENDOPEPTIDASE ACTIVITY nmol/mg of protein/min
Control	42.1±3.8
Inhibitors	
Phosphoramidon (2 μM)	3.2±0.5
Thiorphan (10 μM)	3.5±0.3
Mother's IgG	
150 μg/ml	31.5±2.7
300 μg/ml	13.4±0.9
600 μg/ml	8.2±0.6
Father's IgG	
150 μg/ml	43.0±2.6
300 μg/ml	42.5±3.5
600 μg/ml	41.8±4.2

*Human podocyte lysates were preincubated for 30 minutes at 37°C with phosphoramidon, thiorphan, or various concentrations of IgG from either the mother or the father. Endopeptidase 24.11 activity was then determined with the use of Suc-Ala-Ala-Phe-pNA as a substrate. Results are the means (±SD) of triplicate determinations from two separate experiments.

DISCUSSION

In this infant born with severe membranous glomerulonephritis, nephropathy appears to have been due to anti-neutral endopeptidase antibodies from the mother. These antibodies were found in the infant's serum 13 days after birth but disappeared thereafter, suggesting passive transplacental immunization. They were most likely responsible for the infant's membranous glomerulonephritis, given that the injection of rabbits with the IgG fraction of serum from the mother induced intraglomerular immune deposits and proteinuria, whereas injection with the IgG fraction of serum from the father did not. Furthermore, neutral endopeptidase was localized in immune deposits both in the infant and in the rabbits injected with the mother's IgG.

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.¹³⁻¹⁵ Some similarities, such as glomerular deposition of renal tubular epithelial antigens, have been found between experimental Heymann's nephritis and individual cases of membranous glomerulonephritis, but the antigens could not be characterized at the molecular level.¹⁶⁻¹⁸ Neutral endopeptidase is thus the first podocytic antigen that has been found to be responsible for human membranous glomerulonephritis. Neutral en-

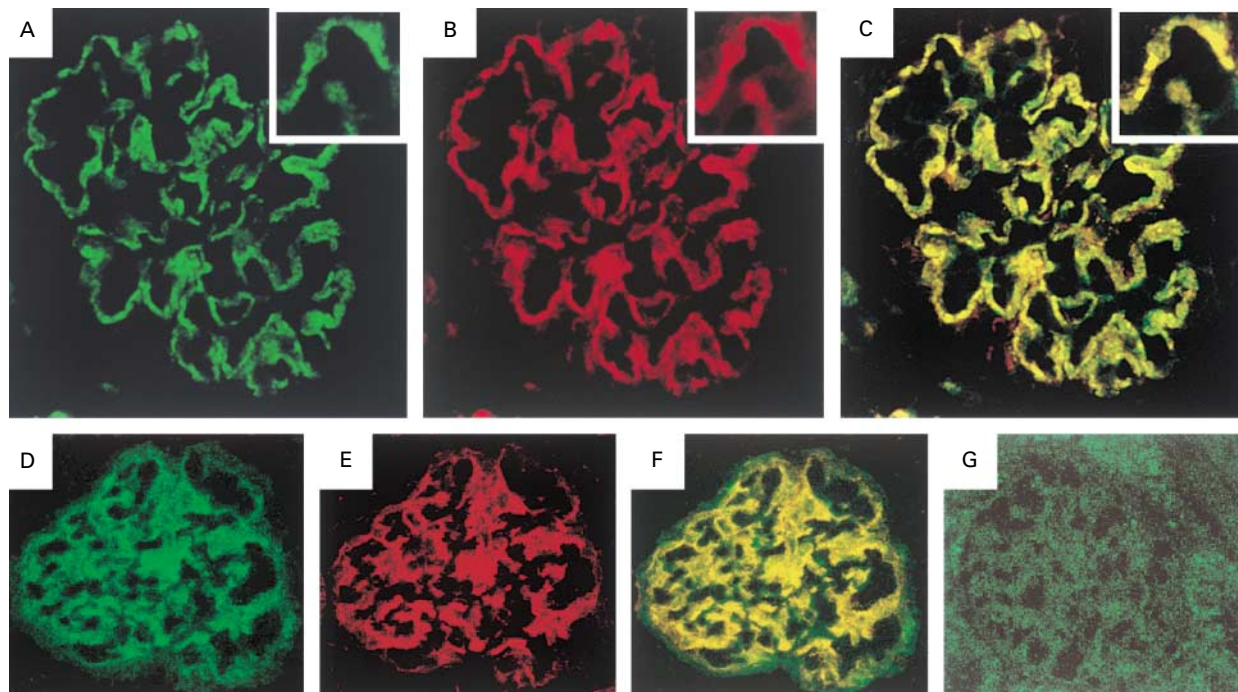


Figure 3. Colocalization of Neutral Endopeptidase and IgG in Immune Deposits and Induction of Renal Disease in Rabbits by IgG from the Mother.

Panels A, B, and C ($\times 600$) show immunofluorescence staining of kidney-biopsy specimens from the infant that have been double-labeled with antihuman IgG antibodies (Panel A) and polyclonal anti-neutral endopeptidase antibody (Panel B). Panel C shows the merged image. The insets ($\times 2000$) show the colocalization of neutral endopeptidase and IgG on the outer aspect of the capillary wall. Panels D, E, F, and G ($\times 600$) show immunofluorescence staining of kidney sections from rabbits injected four days earlier with IgG fractions from the mother (Panels D, E, and F) or the father (Panel G). The sections shown in Panels D, E, and F were double-labeled with antihuman IgG antibodies (Panel D) and with polyclonal anti-neutral endopeptidase antibody (Panel E); the merged image is shown in Panel F.

dopeptidase (also called neprilysin, enkephalinase, CD10, or EC 3.4.24.11) is a 90-to-110-kD zinc-dependent metallopeptidase, identical to the common acute lymphoblastic leukemia antigen.^{19,20} It is expressed in brain tissue, on polymorphonuclear leukocytes and lymphoid progenitor cells, and on epithelial cells within nonlymphoid organs, such as the kidneys, the liver, the breasts, and the lungs.^{21,22} It is also found in the serum and the urine.^{23,24} This enzyme is involved in the metabolism of a number of regulatory peptides, and plays an important role in turning off peptide signaling at the cell surface.²⁵ In the human kidney, neutral endopeptidase is found on the brush border, podocytes, and vascular smooth-muscle cells.^{6,26}

Circulating immune complexes containing neutral endopeptidase were found in the infant's serum on day 13. However, their contribution to the formation of subepithelial immune deposits is uncertain,

because the levels of circulating immune complexes were low, there were no manifestations of serum sickness, and no subendothelial and mesangial immune deposits were seen. Immune complexes could also be formed in situ at the "sole" of podocyte foot processes where neutral endopeptidase is expressed.²⁷ The two mechanisms are not mutually exclusive.

The infant's nephropathy had several unusual features. The deposits contained annular formations. Similar structures were previously found in a case of neonatal membranous glomerulonephritis that was associated with the transplacental transfer of maternal antibodies of undefined specificity,²⁸ as well as in the zona pellucida of rabbit oocytes after the injection of antibodies against angiotensin-converting enzyme.²⁹ These circular particles may contain fragments of cells or basement membrane or the membrane-attack complex C5b-9. We also observed unusual alterations of the glomerular basement membrane that

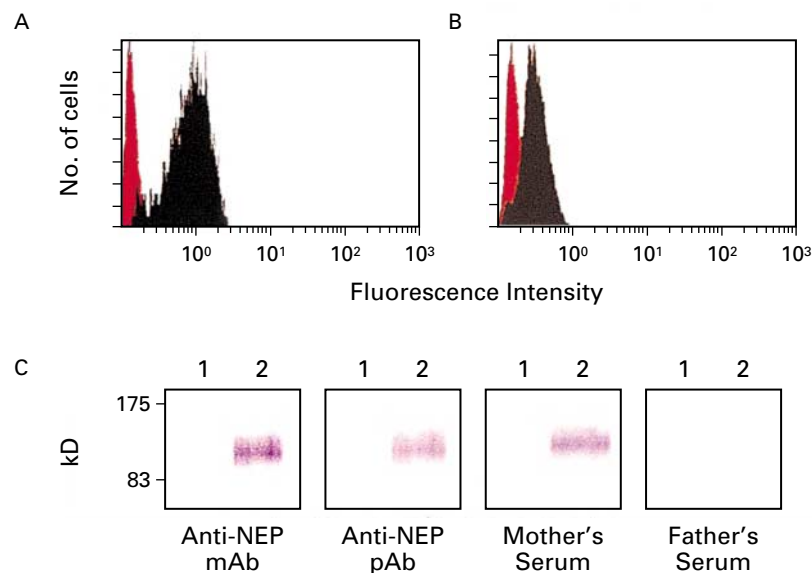


Figure 4. Analysis of Neutral Endopeptidase Expression in the Parents.

Fluorescence-activated cell-sorter analysis (Panels A and B) and immunoblotting (Panel C) show a lack of expression of neutral endopeptidase in the mother's granulocytes. The mother's granulocytes were incubated with anti-neutral endopeptidase monoclonal antibody (anti-NEP mAb) (Panel A) or with positive serum from the mother (Panel B). Panel A shows the lack of neutral endopeptidase staining in the mother's granulocytes (red), whereas the father's granulocytes (black) are positive. Panel B shows that the mother's serum reacted with the father's granulocytes (black) but not with the mother's granulocytes (red). In Panel C, the reactivity of the mother's granulocyte extract (lane 1) is compared with that of the father's granulocyte extract (lane 2) with the use of monoclonal antibodies or polyclonal antibodies (pAb) against neutral endopeptidase and with the mother's or father's serum.

may have resulted from an early insult by antibodies to the embryonic kidney, during a time when intensive remodeling of the basement membrane was occurring. Even more striking were the findings of severe arterial lesions without immune deposits and of the collapse of glomerular capillary tufts, suggestive of major renal ischemia during prenatal development. These lesions may result from the enzymatic activity of neutral endopeptidase as it cleaves vasoactive mediators, including bradykinin, atriopeptin, and endothelins, and thus may modify local blood flow.^{25,26} Because maternal antibodies inhibited neutral endopeptidase activity, their transplacental passage might increase concentrations of vasoconstrictor peptides in the vascular wall. Furthermore, binding of antibodies to granulocytes might trigger the activation of granulocytes and the release of vasoactive mediators, as suggested by the poor tolerance of rabbits for higher doses of the mother's IgG.

The mother's neutral endopeptidase deficiency was confirmed by fluorescence-activated cell sorting and Western blotting of granulocytes. Despite the absence of neutral endopeptidase, the mother was healthy, as were mice with a targeted disruption of the

neutral endopeptidase gene, suggesting enzymatic redundancy.³⁰ Neutral endopeptidase deficiency caused the alloimmunization in the mother that most likely occurred at the time of her miscarriage, since a plasma sample obtained earlier did not show anti-neutral endopeptidase antibodies. Renal injury mediated by alloimmune responses to major renal antigens was first described in the tubular basement membrane of rats.³¹ A previously reported case of neonatal membranous glomerulonephritis may also have involved neutral endopeptidase deficiency and alloimmunization, because there were no renal abnormalities in the mother.²⁸ It is likely that additional persons with neutral endopeptidase deficiency will be identified and that additional cases of acute renal failure and membranous glomerulonephritis in neonates may be ascribed to anti-neutral endopeptidase antibodies.

Supported by grants from INSERM and the University of Paris 6.

We are indebted to the members of the family for their participation in the study; to Philippe Fontanges for assistance with confocal microscopy; to Marie-Christine Verpont for assistance with electron microscopy; to Madeleine Delauche and Béatrice Baudouin for technical assistance; and to Catherine Bazaud for assistance in the preparation of the manuscript.

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