

Hereditary Porcine Membranoproliferative Glomerulonephritis Type II Is Caused by Factor H Deficiency

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Abstract

We have recently described hereditary membranoproliferative glomerulonephritis type II in the pig. All affected animals had excessive complement activation, revealed as low plasma C3, elevated plasma terminal complement complex, and massive deposits of complement in the renal glomeruli, and eventually died of renal failure within 11 wk of birth. The aim of the present study was to investigate the cause of complement activation in this disease. Transfusion of normal porcine plasma to affected piglets inhibited complement activation and increased survival. Plasma was successively fractionated and the complement inhibitory effect of each fraction tested in vivo. A single chain 150-kD protein which showed the same complement inhibitory effect as whole plasma was finally isolated. Immunologic cross-reactivity, functional properties, and NH₂-terminal sequence identified the protein as factor H. By Western blotting and enzyme immunoassay, membranoproliferative glomerulonephritis-affected piglets were demonstrated to be subtotally deficient in factor H. At 1 wk of age, median (range) factor H concentration was 1.6 mg/liter (1.1–2.3) in deficient animals ($n = 13$) and 51 mg/liter (26–98) in healthy littermates ($n = 52$). Our data show that hereditary porcine membranoproliferative glomerulonephritis type II is caused by factor H deficiency. (*J. Clin. Invest.* 1995. 95:1054–1061.) **Key words:** glomerulonephritis • mesangiocapillary • complement activation • complement inactivators • complement pathway, alternative • hereditary diseases

Introduction

Membranoproliferative glomerulonephritis (MPGN),¹ also called mesangiocapillary glomerulonephritis, is a relatively rare

kidney disease characterized by a combination of glomerular capillary wall thickening and a variable degree of mesangial cell proliferation and increased matrix. Regularly heavy deposits of complement (C), in the mesangium and along the capillary wall is found (1). MPGN is divided into three types by electron microscopic evaluation of the glomerulus basement membrane (2, 3). Type I is characterized by subendothelial deposits, type II by intramembranous dense deposits, and type III by both subendothelial and subepithelial deposits. Type I and III are probably variants of the same disease (4), in which glomerular deposition of immune complexes that activate the classical pathway of C seems to play a key role (1). By contrast, deposits of immune complexes are absent in MPGN type II. MPGN type II is, however, associated with alternative pathway C activation, usually caused by C3 nephritic factors, IgG autoantibodies which stabilize the alternative C3 convertase, C3bBb (5). C3 nephritic factors potentially activating the terminal pathway of C have been described (6).

MPGN type II has been reported in two children deficient in factor H (FH) (7). FH is an alternative pathway regulatory protein with a normal serum concentration in humans of ~ 500 mg/liter (8). It has several effects: it competes with factor B for C3b binding, it disassembles C3 convertase by facilitating the dissociation of Bb from C3b, it inhibits C5 convertase by competing with C5 for C3b binding, and it acts as a cofactor for factor-I-mediated inactivation of C3b to iC3b and further into C3c and C3dg (9–12). Deficiency of FH leads to consumption of C3 and factor B (13). The levels of terminal components may be normal (13), but are low in most patients (7, 11, 14, 15).

We have recently described the first animal model for MPGN type II in piglets of the Norwegian Yorkshire breed (16, 17). Affected piglets appeared clinically healthy at birth and for the first few weeks of life. Thereafter they failed to thrive with growth retardation due to rapidly progressive glomerulonephritis and eventually they all died of renal failure within 72 d (median 37 d) after birth ($n = 25$). The kidneys showed extensive glomerular proliferation and profound thickening of the glomerular capillary wall. Large amounts of dense deposits were consistently found within the glomerular basement membrane being up to 1,250 nm thick, compared to 150–200 nm in the normal animals. Excessive C activation was observed in affected pigs, revealed as low plasma C3, elevated plasma terminal complement complex (TCC), and massive deposits of C, but not immunoglobulins, along the capillary walls of renal glomeruli. The disease was hereditary (16), with a simple autosomal recessive pattern and complete penetrance (Jansen, J. H., K. Høgåsen, and A. M. Grøndahl, unpublished results). No disease was detected in heterozygous carriers of the MPGN trait.

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1. *Abbreviations used in this paper:* AU, arbitrary unit; C, complement; EACA, ϵ -amino- n -caproic acid; FH, factor H; HFH, human FH; HRP, horseradish peroxidase; MPGN, membranoproliferative glomerulonephritis; NHS, normal human serum; NPS, normal porcine serum; PEG, polyethylene glycol; PFH, porcine FH; TCC, terminal complement complex.

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We have previously suggested that this porcine MPGN type II may be caused by an inherited deficiency of a C regulatory protein (17). The aim of the present study was to clarify the etiology of the disease.

Methods

Animals. 13 piglets affected by MPGN type II were produced by test matings of parental animals associated with the disease as previously described (16). Affected animals were identified at 1 wk of age (4–10 d) by demonstration of low plasma C3 (median 4.2% of a pool, range 2.4–8.7%) and elevated plasma TCC (median 13.9 arbitrary unit (AU)/ml, range 8.0–25.3 AU/ml) as described previously (17). Their healthy littermates ($n = 52$) had a median C3 concentration of 51.8% (range 24.4–150.4%), and a median TCC concentration of 1.0 AU/ml (range 0.4–2.4 AU/ml). Healthy 3-wk-old control piglets not associated with the disease ($n = 15$) had a median C3 concentration of 89.4% (range 53.6–107.4%), and a median TCC concentration of 0.8 AU/ml (range 0.6–1.0 AU/ml) (17). The aberrant C profile of the MPGN-affected piglets persisted until death. In all MPGN piglets the diagnosis was confirmed by autopsy with the following findings: (a) glomerular hypercellularity and thickened glomerular capillary wall; (b) dense deposits within the glomerular basement membrane; and (c) heavy deposits of C3 and TCC along the capillary wall. All nonaffected littermates remained healthy until they were slaughtered ~ 6 mo old. All passed the routine meat inspection at the slaughterhouse performed by an experienced veterinarian. MPGN is definitely a rare finding in slaughter pigs, since no macroscopic or light microscopic renal changes compatible with that diagnosis were detected among 668 pigs examined at the same slaughterhouse (18).

Ethics. The use of animals in this study was approved by the committee for animal experiments.

Materials. Benzamidine HCl, zymosan A, Coomassie brilliant blue R, PMSF, 3,3'-diaminobenzidine, ϵ -amino-*n*-caproic acid (EACA), and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Purified human C3 and FH, mAb against human C3c and C6, and goat antiserum against human C3 and FH were purchased from Quidel Corp. (San Diego, CA). Rabbit antiserum against porcine C3 was purchased from Organon Teknika BCA/Cappel (West Chester, PA). Horseradish peroxidase (HRP)-linked donkey anti-rabbit Ig, HRP-linked sheep anti-mouse Ig, and rainbow mol wt markers were purchased from Amersham International (Little Chalfont, United Kingdom). HRP-linked mouse anti-goat Ig was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Q Sepharose Fast Flow, Lysine Sepharose 4B, Sepharose CL-6B, Protein G Sepharose 4 Fast Flow, Protein A Sepharose CL-4B, Resource Q, and low mol wt standards were purchased from Pharmacia LKB Biotechnology Inc. (Sollentuna, Sweden). Mol wt protein standards for SDS gel electrophoresis were purchased from Bio Rad Laboratories (Richmond, CA). Polyethylene glycol (PEG) 4000 was purchased from Prolabo (Paris, France). An mAb isotyping kit (SAB clonotyping system II) was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Enzyme immunoassays for C3 and TCC. Enzyme immunoassays for porcine C3 and TCC were performed as described (17). Briefly, in the C3 assay, a rabbit antiserum against porcine C3 was used as capture antibody. A pool of porcine plasma from 3-wk-old healthy Yorkshire piglets ($n = 15$) from a population spared for MPGN was used as standard, defining 100%. An mAb against human C3c was used as secondary antibody. In the TCC assay, our own mAb aE11 (anti-C9 neoepitope) was used as capture antibody (19), zymosan-activated human serum was used as standard, defining 1,000 AU/ml, and an mAb against human C6 was used as detecting antibody.

Nephritic factor test. A test for nephritic factor, or any other form of C-activating factor in serum was performed as follows: 1 vol of serum from MPGN-affected piglets ($n = 7$, median age 1 wk), or from healthy, age-matched littermates ($n = 7$) were mixed with 4 vol normal porcine serum (NPS) and frozen immediately, or incubated for 1, 4, or

24 h at 37°C. PBS was used as a negative control and PBS with zymosan (50 g/liter, final dilution 10 g/liter) was used as a positive control. The resulting C activation was evaluated by measuring the TCC concentration of the samples. This approach was feasible since increased TCC formation was a characteristic finding in MPGN-affected piglets.

Preparation of plasma and erythrocytes. Blood was obtained from carbon dioxide-anesthetized slaughter pigs by aortapuncture, collected into bottles containing 1/10 vol of 0.1 M sodium citrate, pH 7.4, cooled on ice, and centrifuged within 8 h. Plasma was stored at -20°C and used within 3 mo. Erythrocytes were washed in saline and stored at 4°C and transfused within 24 h. EDTA-plasma or serum from 20 pigs (age 6 mo) were pooled and designated normal porcine plasma and NPS, respectively.

Transfusion of plasma or erythrocytes. Plasma transfusions were started at 2 wk age (10–25 d) and repeated once a week, if possible, until the pigs died. Citrated plasma was administered via a venaefin in an auricular vein. For pigs of body wt < 40 kg the dose was 30 ml/kg, for larger pigs the dose was reduced to 20 ml/kg. The transfusions were finished in 10–90 min depending on how well they were tolerated (e.g., signs of hypocalcemia, shivering, ticks, and cramps). Transfusion of plasma fractions showing similar C inhibitory effect as whole plasma were counted as plasma transfusions. The dose of transfused washed erythrocytes (hematocrit 0.88) was 30 ml/kg (pigs of wt 10–13 kg).

Purification of porcine factor H (PFH). For the plasma fractionation, porcine blood was collected into bottles containing 1/30 vol of 0.2 M sodium EDTA, pH 7.4, then cooled on ice, centrifuged, and plasma stored at -20°C . All purification procedures were performed at 4°C .

Precipitation. Frozen plasma was thawed in microwave oven or in cold water and the cryoprecipitate was removed by centrifugation (1,500 g for 30 min). The supernatant was diluted with 1/20 vol of an enzyme inhibitor solution containing 0.2 M benzamidine HCl in 1 M KH_2PO_4 , pH 7.4, and 1/100 vol 0.1 M PMSF in anhydrous isopropanol essentially as described by Hammer et al. (20). The solution was then precipitated stepwise with PEG 4000. First the solution was made 5% (wt/vol) in PEG 4000 by slow addition, with stirring, of the powder and allowed to equilibrate for 1 h. The precipitate (5% PEG precipitate) was removed by centrifugation at 4,000 g for 1 h. The supernatant was thereafter made 16% in PEG 4,000, equilibrated for another 1 h, and the 5–16% PEG precipitate was removed by centrifugation. New precipitates were made by repeating the procedure using final PEG 4,000 concentrations of 20 and 45%. In a second purification, more narrow precipitations were performed with PEG concentrations of 1, 3, and 5%, respectively. Generally protein concentrations were calculated by measuring absorbance at 280 nm, using $E_{1\text{ mg/ml}} = 1.0$ for simplicity. However, for the purified protein, $E_{1\text{ mg/ml}} = 1.42$ as determined for human FH (HFH) was used (21).

Lysine Sepharose chromatography. The 3–5% PEG precipitate was dissolved in a buffer containing 30 mM Tris, 100 mM NaCl, 10 mM Na_2EDTA , and 5 mM benzamidine HCl, pH 8.7. The solution was cleared by centrifugation (27,500 g for 1 h) and filtered through a 0.8- μm filter before application onto a lysine Sepharose column which was equilibrated with the same buffer (5×5 cm, linear flow rate 6 cm/h). Bound protein was eluted with the same buffer adjusted to 200 mM EACA and discarded.

Q Sepharose chromatography (anion-exchange). The effluent from the lysine Sepharose column was collected and made 30 mM in EACA by adding solid powder. The solution was applied to a Q Sepharose Fast Flow column (5×28 cm, linear flow rate 6 cm/h) which had been equilibrated with the same buffer. The column was washed with the buffer until baseline was reestablished. Bound protein was then eluted with a linear gradient (0–100% over 16.7 h) of the buffer adjusted to 1 M NaCl.

Gel filtration (Sepharose CL-6B). Appropriate fractions from the ion-exchange chromatography were dialyzed against a buffer containing 100 mM KH_2PO_4 , 150 mM NaCl, 5 mM Na_2EDTA , and 50 mM EACA, pH 7.4, and 50 ml was applied on a Sepharose CL-6B column (5×84

cm, linear flow rate 2 cm/h) which had been equilibrated with the same buffer.

Resource Q chromatography (anion exchange). A fraction from gel filtration was dialyzed against a 30-mM Tris HCl buffer, pH 8.7, and applied on a Resource Q HPLC column (1.6 × 3 cm, linear flow rate 90 cm/h). Bound protein was eluted by a linear gradient (0–100% over 15 min) of the same buffer adjusted to 0.5 M NaCl.

Protein G Sepharose chromatography. A fraction from Resource Q was applied on a Protein G Sepharose column (1 × 5 cm, linear flow rate 40 cm/h). The effluent was dialyzed against 30 mM Tris HCl pH 8.7 and reapplied on the Resource Q column.

Test for C inhibitory activity. Products from the blood and plasma fractionation were administered intravenously to MPGN-affected piglets. Precipitates and chromatographic fractions containing toxic substances were thoroughly dialyzed against PBS before administration to the animals. Blood was sampled before, immediately after, 1 and 8 h after the treatment, or more frequently. EDTA plasma was then assayed for the content of C3 and TCC. A decreasing TCC level and/or an increasing C3 level observed within the first 8 h after the transfusion was finished, was regarded as a C inhibitory effect.

SDS-PAGE and Western blotting. SDS-PAGE was performed using precasted 8–18% gradient gels (Excel Gel SDS) and a Multiphor II electrophoresis unit (Pharmacia LKB Biotechnology Inc.). Gels were stained with Coomassie brilliant blue R (2.5 g/liter), or blotted onto 0.45-μm pore nitrocellulose using a Multiphor II NovaBlot electrophoretic transfer unit (Pharmacia LKB Biotechnology Inc.). The nitrocellulose was blocked with 3% BSA over night. The blots were stained with primary antibodies and horseradish peroxidase-linked secondary antibodies, all diluted in PBS containing 1% BSA. Incubations were for minimum 1 h at room temperature, and the blots were washed three times for 15 min in PBS between each incubation. The blots were developed with 3,3'-diaminobenzidine and H₂O₂.

Production of rabbit antiserum. Two rabbits were immunized with 50 μg each of the purified protein (PFH) in Freund's complete adjuvant and then boosted with the same amount of protein after 4 wk. An IgG fraction from the antiserum was obtained by Protein A affinity chromatography.

Production of mAb. Balb/c mice were immunized with purified factor H. Spleen cells were fused with the myeloma cell line X63–Ag 8.653 according to the method described by Galfre and Milstein (22). Screening and selection of clones were performed in microtiterplates which had been coated with the immunisate. One clone (designated 7C3) was selected, expanded, and ascites was produced after intraperitoneal injection in mice. The mAb was purified by PEG precipitation (15% PEG 4,000 at 4°C for 1 h), followed by ion-exchange chromatography using a Resource Q HPLC column. Isotyping of heavy and light chains was performed using a commercial kit. An immunosorbent was made by coupling 29 mg mAb to 9 ml CNBr-activated Sepharose 4B.

In vitro tests for C3c formation. NPS or MPGN-serum were mixed with 10 g/liter zymosan and incubated under continuous rotation at 37°C for 24 h. Conversion of C3 to C3c was evaluated by Western blotting using antiserum against porcine C3.

C3c formation in porcine serum was also tested by the following experiment: Purified human C3 (0.1 g/liter) and zymosan (10 g/liter) were mixed with NPS or MPGN-serum. Aliquots of MPGN-serum were in addition, mixed with 0.1 g/liter of purified HFH or purified PFH. The mixtures were incubated for 24 h at 37°C. Conversion of human C3 to C3c was evaluated by Western blotting using an antiserum against human C3 which showed insignificant cross-reactivity against porcine C3 by this method.

Double immunodiffusion, agarose gel electrophoresis, and crossed immunoelectrophoresis. Ouchterlony double diffusion and agarose gel electrophoresis were performed with conventional techniques. Crossed immunoelectrophoresis was performed using antisera against human or porcine FH in the top gel.

NH₂-terminal sequencing. The sequencing was done by Professor K. Sletten at the Biotechnology Center, University of Oslo, Norway. 75 μg purified PFH (500 pmol) was analyzed by automatic Edman

Table I. Test for Nephritic Factor

Sample	Median TCC content (AU/ml) after			
	0 h	1 h	4 h	24 h
NPS + MPGN	5.9	8.8	12.9	18.5
NPS + healthy	2.8	7.6	11.7	21.6
NPS + PBS	2.0	6.2	9.3	26.2
NPS + zymosan		183.1	197.3	171.4

4 vol NPS were mixed with 1 vol serum from individual MPGN piglets ($n = 7$) or from healthy littermates ($n = 7$) and incubated for 0, 1, 4, or 24 h at 37°C. The resulting C activation was evaluated by measuring the TCC concentration of the mixtures. As negative and positive controls, NPS was mixed with PBS ($n = 1$), or zymosan (50 g/liter in PBS, $n = 1$), respectively.

degradation on a 477A Protein Sequencer (Applied Biosystems, Foster City, CA) with an on-line 120A phenylthiohydantoin amino acid derivatives analyzer.

Enzyme immunoassay for PFH. Microtiterplates (Maxisorp; Nunc, Roskilde, Denmark) were coated with rabbit anti-PFH overnight at 4°C (IgG fraction, 2 mg/liter in PBS). Normal porcine plasma was used as standard (twofold dilution 1/500–1/16,000). Plasma samples were assayed diluted 1/2,000 or less. The detection antibodies were mAb 7C3 (2 mg/liter), and peroxidase-linked anti-mouse Ig (diluted 1/2,000). The plates were developed using 2,2'-azino-di-[3-ethylbenzthiazolinesulphonate(6)] (Boehringer Mannheim GmbH, Mannheim, Germany) and H₂O₂. The standard and samples were diluted in PBS containing 10 mM Na₂EDTA and 0.2% Tween 20. Triplicate determinations were done. The antibodies were diluted in PBS containing 0.2% Tween 20. Incubations were done for 45 min at 37°C. Purified PFH was used for calibration of the assay. This PFH was purified with conventional technique as described followed by affinity purification on the 7C3 immunosorbent and was 100% pure evaluated from SDS-PAGE. The intraassay and interassay coefficients of variation, CV(x), were estimated from 12 or 44 measurements, respectively, using the formula $CV(x) = SD/\bar{x}$, where SD is the standard deviation and \bar{x} is the mean value of the measurements.

Results

Nephritic factor test. No C-activating factor was detected in the sera from MPGN-affected piglets (Table I). The difference in TCC content at 0 h was due to the increased amount of preformed TCC in MPGN-sera (Fig. 1).

Transfusion of plasma or erythrocytes. Transfusion of citrated plasma (30 ml/kg) resulted in a clear decline of circulating TCC (Fig. 1). There was an initial decline during the transfusion which corresponded well to the hemodilution (the TCC concentration of transfused plasma was ~ 1 AU/ml). An additional decline of TCC was observed during the first 8 h after the transfusion was finished (median decline 56%, range 30–76% in 11 experiments). 24 h after the transfusion, the TCC level had started to incline again and reached the pretransfusional level after 3 d.

The C3 level increased during transfusion since the C3 concentration of transfused plasma was ~ 85%. No significant further increase of C3 was observed after the transfusion was finished. Instead the C3 level gradually declined, but after 3 d it still had not reached down to the pretransfusional level.

In young piglets transfusions were typically finished in 10–

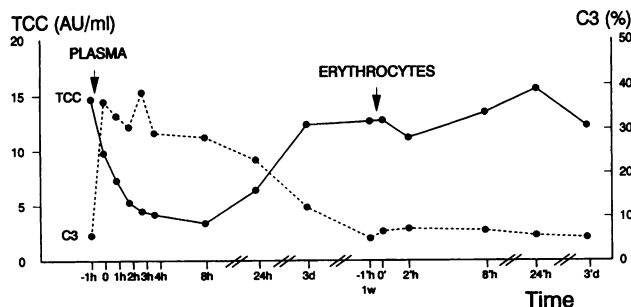


Figure 1. Test for C inhibitory effect of plasma and erythrocytes in vivo. Citrated plasma (30 ml/kg) was administered intravenously to a MPGN-piglet (wt, 10 kg). Samples of EDTA-plasma were obtained before the transfusion (-1 h), immediately after (0), and at several time points after the transfusion. After 1 wk (1 wk = -1 h) the piglet received a transfusion with washed erythrocytes (30 ml/kg, hematocrit 0.88), which was finished at time 0'. The effect was evaluated by assay of C3 (broken line, in percentage of normal) and TCC (solid line, in AU/ml). Similar profiles were observed after plasma transfusion in all 13 MPGN-piglets, also when repeated. One more piglet received erythrocyte transfusion, with a similar result.

20 min. Because of poorer tolerance, transfusions were performed slower in older pigs (30–60 min). These conditions given, most pigs tolerated weekly plasma transfusions well. However, individual differences were seen. Some piglets showed repeated adverse reactions, which always were dose dependent and usually abated when the speed of transfusion was reduced. Generally, close to one transfusion was given per week until the piglets died.

After transfusion of packed erythrocytes (30 ml/kg) no significant changes of C3 and TCC were seen (Fig. 1).

Survival. In the 13 transfused MPGN piglets, median and maximal survival was 82 and 375 d, respectively (Fig. 2). This was significantly longer than previously observed in nontransfused MPGN-affected piglets also surviving the first week ($n = 24$), having median and maximal survival of 37 and 72 d,

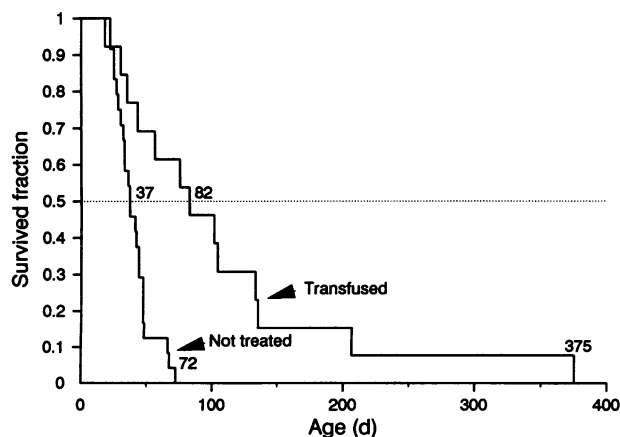


Figure 2. Survival of MPGN-piglets. Kaplan-Meier plot showing the survival of 24 MPGN-piglets that did not receive any treatment (Not treated) as described previously (16), and of the 13 MPGN-piglets that were treated with plasma transfusions (Transfused). Transfusions were started at ~ 2 wk old and generally continued once every week until the piglets died.

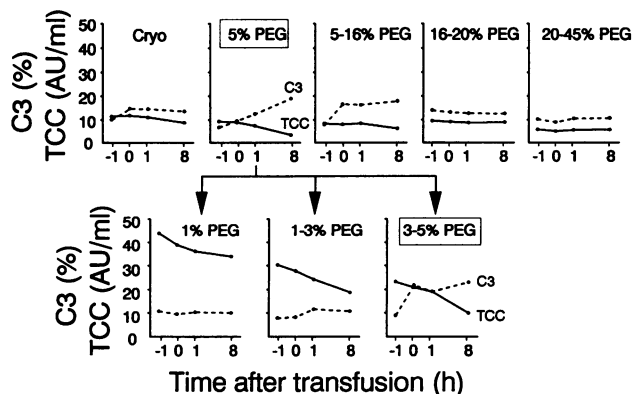


Figure 3. The ability of plasma fractions (precipitates) to inhibit in vivo C activation in MPGN-piglets. Different plasma fractions (dissolved cryoprecipitate or PEG precipitates) were administered intravenously to MPGN-piglets (weight 3–18 kg). Plasma samples were drawn before the infusion (-1), immediately after the infusion (0), after 1 h (1), and after 8 h (8). The effect was evaluated by assay of C3 (broken line, in percentage of normal) and TCC (solid line, in AU/ml) in these samples. From the first fractionation experiment (top), five fractions were tested. Since the 5% PEG precipitate showed a superior effect, a narrower precipitation in this range was performed in the second fractionation experiment (bottom).

respectively, (16) ($P = 0.005$, Mann-Whitney two sample test). All the nontransfused piglets had died of renal failure. In contrast, only five (38%) of the transfused piglets died of renal failure. The remaining eight pigs did not have renal failure and died of miscellaneous causes such as hypocalcemia ($n = 3$), complication to blood sampling ($n = 1$), surgical complications ($n = 3$), or unknown ($n = 1$). Postmortem examination of the kidneys from all transfused animals fulfilled the criteria for MPGN type II, but the picture was less proliferative compared to untreated MPGN animals.

Purification of PFH. EDTA plasma was initially fractionated by cryoprecipitation and by sequential PEG precipitation. The C inhibitory effect of the dissolved precipitates was tested in vivo after intravenous administration to MPGN piglets (Fig. 3). The dissolved 3–5% PEG precipitate showed strongest C inhibitory effect and was regarded to contain the bulk of active protein. This precipitate was therefore further fractionated by ion-exchange chromatography on Q Sepharose, from which the pooled fractions 48–51 showed the strongest C inhibitory activity (Fig. 4). A considerable activity was also found in the effluent, probably due to overloading of the column or to the relatively high salt concentration of the sample buffer which probably impaired binding (100 mM NaCl). The remaining protein of the pooled fractions 48–51 were further fractionated by gel filtration using Sepharose CL-6B (Fig. 4). The pooled gel filtration fractions 68–75 showed strongest activity and were therefore further fractionated using Resource Q, an anion exchange HPLC column. From Resource Q, only fraction 9 showed significant activity (Fig. 4). The activity of this fraction was very high, since transfusion of 5 mg/kg showed an effect comparable to the transfusion of 30 ml/kg citrated plasma, a calculated concentration effect of 370:1 per mg protein. SDS-PAGE of this fraction showed a single band at ~ 150 kD under nonreducing conditions. Under reducing conditions, two additional weaker bands of 50 and 25 kD appeared, indicating contamination of IgG. The fraction was therefore adsorbed on Pro-

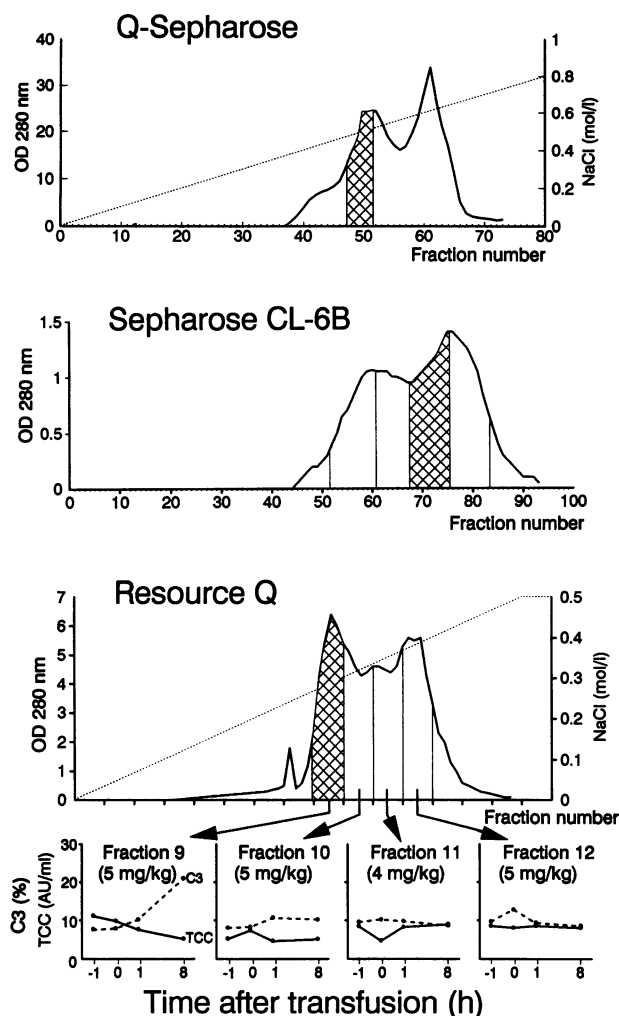


Figure 4. Chromatographic fractionation of plasma. The 3–5% PEG precipitate (see Fig. 3) was fractionated successively in three chromatographic steps using Q-Sepharose (ion-exchange), Sepharose CL-6B (gel filtration), and Resource Q (HPLC ion-exchange). The ability of each fraction to inhibit *in vivo* C activation in MPGN-piglets was tested as described in the legend to Fig. 3. The pool of active fractions from Q-Sepharose was fractionated on Sepharose CL-6B, and the pool of active fractions from Sepharose CL-6B was finally fractionated on Resource Q. The active fractions are cross-hatched, and the results from *in vivo* activity testing of Resource Q fractions are shown in the lower part of the figure.

tein G Sepharose and after another run on Resource Q, it eluted as a single peak. Evaluated by SDS-PAGE the peak contained a pure single chain 150-kD protein (reducing conditions), indistinguishable from HFH (Fig. 5). From these observations, and from the results following, we conclude that the protein isolated was PFH.

Western blotting. An antiserum against PFH was made by immunizing rabbits. The antiserum reacted strongly with NPS and purified PFH under nonreducing conditions (Fig. 5). The 150-kD PFH was detected as a faint band in sera from all MPGN piglets. The staining of 0.1 μ l MPGN-serum and 0.1 ng purified PFH was similar, corresponding to an FH content in MPGN-serum of ~ 1 mg/liter, whereas NPS contained at least 100 times more. In addition to the 150-kD band, the antise-

rum also stained a 31-kD band quite strongly, in addition to three barely visible bands of 46, 52, and 74 kD. All these light bands were equally strong in MPGN-sera and in normal sera. The 31-kD band was definitely not present in the purified PFH (which was the immunisate), but bands similar to the faint ones were seen (Fig. 5). Under reducing conditions only a faint 150-kD band was seen (not shown). The antiserum did not stain 1 μ g porcine IgG, purified by protein A chromatography, which probably corresponded to more IgG than was the content of the serum samples.

The anti-PFH showed some reaction against normal human serum (NHS) and purified HFH (Fig. 5). Similarly, the antiserum against HFH showed reaction against both NPS and purified PFH, revealed as 150-kD bands. Anti-HFH also detected three weak bands of 37, 40, and 43 kD in NHS, probably corresponding to the gene products of an FH-related gene (23, 24).

The mAb 7C3 against PFH was isotypized to be IgG1-kappa. Its specificity was tested in Western blotting against NPS; it only reacted against the 150-kD protein (not shown).

C3c formation *in vivo* and *in vitro*. Low amounts of C3 were detected in MPGN plasma by Western blotting. C3c was clearly present both in healthy and MPGN plasma, indicating *in vivo* C3c formation, excluding a primary factor I deficiency in the MPGN piglets (not shown). *In vitro* C3c formation was evaluated by incubating NPS and MPGN serum with zymosan (10 mg/ml) at 37°C for 24 h. In NPS large amounts of C3c appeared by this treatment, but since MPGN serum contained only small amounts of C3, the degree of C3c formation induced by zymosan activation was difficult to evaluate by this method (not shown). This problem was evaded by mixing the sera with purified human C3 before zymosan activation, using an antiserum specific for human C3 in the detection system. NPS converted almost all human C3 to C3c during zymosan activation, in contrast to the MPGN-serum where C3c formation was hardly detectable (Fig. 6). However, when MPGN-serum was reconstituted with purified HFH or purified PFH before zymosan activation, large amounts of C3c were formed (Fig. 6).

Double immunodiffusion, agarose gel electrophoresis, and crossed immunoelectrophoresis. In Ouchterlony double diffusion goat antiserum to HFH gave a faint precipitate with a reaction of identity between NPS and purified PFH. The rabbit antiserum to PFH showed a strong reaction of identity with NPS and purified PFH, but no precipitate line was observed against MPGN-serum, NHS, or purified HFH. In agarose gel electrophoresis, purified PFH migrated corresponding to the β 2-region showing lower electrophoretic mobility than purified HFH that migrated corresponding to the β 1-region. To rule out a change in mobility of PFH or HFH due to the purification procedures, crossed immunoelectrophoresis was performed, using NPS or NHS as sample and anti-PFH or anti-HFH in the top gel. This experiment confirmed the lower electrophoretic mobility of the porcine protein.

NH₂-terminal sequence. The specific signal was weak and the background level comparatively high in the first batch of purified PFH tested. This problem might be caused by contaminants, extensive protein polymorphism, or degradation. We therefore also purified PFH basically as described, but included affinity chromatography on the 7C3-immunosorbent as a final step. We also purified PFH rapidly from one pig using the 7C3-immunosorbent to avoid protein polymorphism and degradation. No contaminants of these new PFH batches could be detected in SDS-PAGE, but the modifications did not solve the

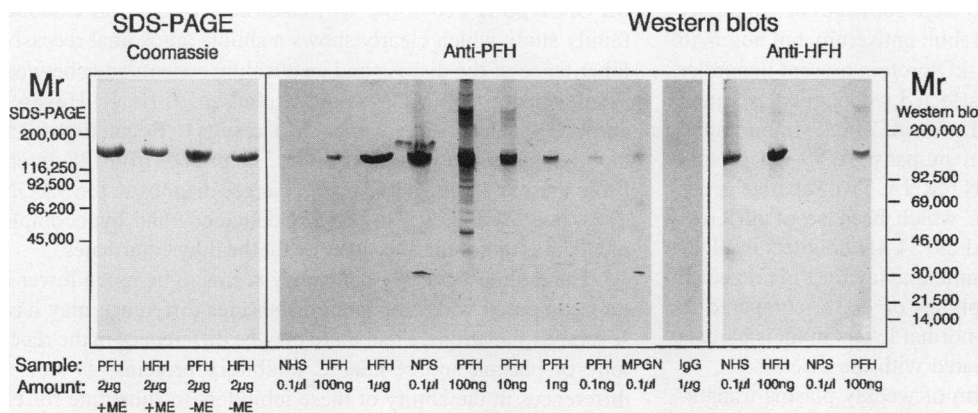


Figure 5. SDS-PAGE and western blots of PFH and HFH. In SDS-PAGE (first four lanes), 2 μ g of purified protein was run per lane and subsequently stained with Coomassie brilliant blue R. The proteins were reduced with mercaptoethanol (+ME), or not reduced (-ME). All remaining samples (sera or purified proteins) were run under nonreducing conditions, blotted onto nitrocellulose, and stained either with rabbit anti-PFH or goat anti-HFH. MPGN, serum from MPGN piglet; IgG, porcine IgG.

problems. However, in the initial 17 steps, the residue was determined with certainty in nine positions. Three positions were uncertain and five residues could not be determined, including the initial four positions. The NH₂-terminal sequence determined was compared with the sequences for human and murine FH as determined by cDNA cloning (25, 26), and the sequence of rabbit FH as determined by automated Edman degradation (Fig. 7) (27). In the 12 positions determined there was 58% homology with human, murine, and rabbit FH.

Enzyme immunoassay for PFH. The intraassay and interassay coefficients of variation were 1.7 and 5.2%, respectively. Normal porcine plasma was determined to contain 215 mg/liter FH. In adult Landrace pigs ($n = 20$), which were not associated with hereditary MPGN type II, median FH concentration was 227 mg/liter (range 200–255 mg/liter). The parents of the FH-deficient piglets ($n = 8$) had a median FH concentration of 98 mg/liter (range 64–135 mg/liter). Plasma samples of 4–10-d-old piglets were analyzed. The deficient piglets (n

= 13) had a median FH concentration of 1.6 mg/liter (range 1.1–2.3 mg/liter), compared to 51 mg/liter in their healthy littermates ($n = 52$, range 26–98 mg/liter).

Discussion

We have previously suggested that hereditary porcine MPGN type II may be caused by deficiency of a C regulatory protein (17). C regulatory proteins may be divided into those present in plasma and those attached to cellular membranes, and several such regulators are known in humans. Plasma regulators are C1 inhibitor, C4b binding protein, factor D, FH, factor I, factor J, properdin, vitronectin, and clusterin. Membrane-bound regulators are decay-accelerating factor, membrane cofactor protein, complement receptor type 1, homologous restriction factor, glycoporphin A and CD59. The density of membrane bound regulators varies considerably between different types of cells, but all cells probably express at least some of them.

Initial transfusion experiments clearly demonstrated that the deficient regulatory protein of MPGN piglets was present in plasma from normal pigs and not on erythrocytes. By fractionating normal porcine plasma and monitoring the C inhibitory activity of the fractions in vivo, we finally found that the activity was confined to one single protein. Intravenous administration of 5 mg/kg of this protein showed a similar C inhibitory effect as the transfusion of 30 ml/kg of citrated plasma, indicating a normal plasma concentration of the protein of ~ 200 mg/liter. The single chain protein had a mol wt of 150 kD, showed factor I cofactor activity in vitro, showed immunologic cross-reactivity with HFH, and the NH₂-terminal amino acid sequence was homologous with human, murine, and rabbit FH. Taken together these data strongly indicate that the purified protein was PFH.

Specific antibodies against PFH were produced. In Western

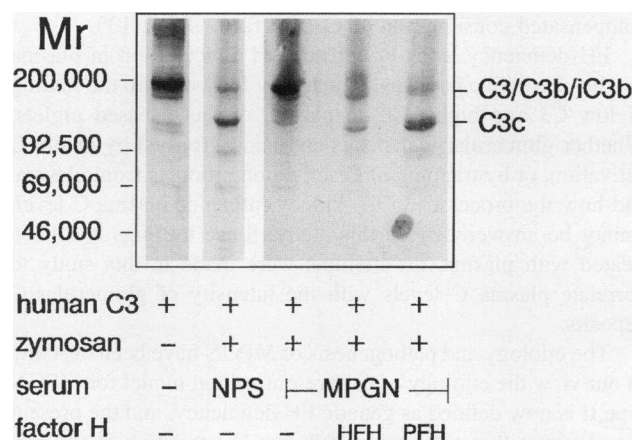


Figure 6. Factor I cofactor activity in porcine serum. NPS or MPGN-serum (MPGN) was mixed with purified human C3 (0.1 g/liter) and zymosan (10 g/liter), with or without the addition of purified HFH or PFH (0.1 g/liter). The mixtures (not C3 only) were incubated for 24 h at 37°C before SDS-PAGE was performed under nonreducing conditions using 0.1 μ l sample per lane. The gel was blotted onto nitrocellulose and stained with goat anti-human C3 (diluted 10⁻³) and horseradish peroxidase-linked mouse anti-goat Ig (diluted 10⁻³). The antiserum against human C3 showed insignificant cross-reactivity against porcine C3 in Western blotting.

	1	5	10	15
Porcine:	X	X	X	X
Human:	E	D	C	N
Murine:	E	D	C	K
Rabbit:	E	D	C	K

Figure 7. NH₂-terminal sequences (from the left) of porcine, human, murine, and rabbit FH. The sequences for porcine and rabbit proteins have been determined by automated Edman degradation, whereas the sequences of the human and murine proteins have been deduced from cDNA clones (25–27). X, residue which we could not determine; (), uncertain residue; –, residue identical to the porcine residue.

blotting all MPGN-affected piglets were shown to be subtotally deficient in the 150-kD PFH. The rabbit antiserum, but not mAb 7C3, in addition reacted with a 31-kD protein present in similar amounts in healthy and MPGN-affected piglets. This protein probably represents an FH related protein. Three similar small FH-related proteins are known in humans. A 49-kD protein occurs by alternative splicing of FH mRNA. Two separate genes encode for one polypeptide each, which because of different glycosylation, occur as 42/37- and 29/24-kD doublets in SDS-PAGE (23, 28, 29). An enzyme immunoassay for PFH revealed levels of FH in MPGN-affected piglets of ~ 3% compared to their healthy littermates, and half-normal levels in their parents compared to adult pigs not associated with the disease.

Substitution therapy in the form of weekly plasma transfusions significantly improved survival of the MPGN-affected piglets. The used dose (mainly 30 ml/kg) was, however, insufficient to normalize the level of C activation, revealed as persistent supranormal TCC (Fig. 1). Furthermore, hardly any remaining C inhibitory effect was seen 3 d after a transfusion. This shows that relatively modest amounts of FH was needed to convert the MPGN into a nonlethal course.

Hereditary FH deficiency has been reported in 12 human beings. Two of these had clearly defined MPGN type II, five presented other types or undefined MPGN. Levy et al. (7) described two brothers presenting with hematuria at 14 and 4 mo old, respectively. Electron microscopic evaluation of renal biopsies revealed MPGN type II in both. Their FH levels were ~ 10% of normal. Thompson and Winterborn described an 8-mo-old boy who presented hemolytic uremic syndrome and his 3-yr-old healthy brother (13). Both had low amounts of FH in serum, 6 and 7% of normal value, respectively. Electron microscopic evaluation of a renal biopsy from the proband revealed capillary wall thickening due to subendothelial deposits, agreeing with MPGN type I. Lopez-Larrea et al. described three siblings with undetectable FH (< 5%) who all presented MPGN, not further specified (14). Brai et al. described three siblings with undetectable FH (< 1%). One of these also had a partial C2 deficiency and presented SLE with nephritis (15). Nielsen et al. described a 15-yr-old girl with no detectable FH in plasma (< 1%). She presented two episodes of meningococcal disease, but was otherwise completely healthy (11). Fijen et al. described an FH-deficient SLE patient who presented with meningococcal disease (30). A functional FH deficiency associated with MPGN type II (atypical) was described by Meri et al. (31). This patient had a monoclonal λ light chain that bound FH and interfered with its C inhibitory function.

MPGN type II is a rare condition, and FH deficiency is extremely rare in humans. The association of the two conditions as observed in a few individuals may suggest a causal relationship. This is supported by the observation that plasma factors which block FH function, either by binding to the alternative C3 convertase, or by binding to FH itself, also are associated with MPGN type II (5, 31). It is notable, however, that human beings with complete FH deficiency have a good chance to be spared from kidney disease. Furthermore, many patients with C3 nephritic factor present with other types of glomerulonephritis than MPGN type II (1), and some of them have no signs of glomerulonephritis at all (32).

In the described FH deficiency in pigs, all affected animals (homozygous deficient) developed renal failure within a few weeks of birth. The disease invariably followed a lethal course, and the renal morphology was consistently compatible with

MPGN type II (16). We will elsewhere present an extended family study which clearly shows a simple autosomal recessive inheritance of the disease and an absolute concordance between FH deficiency and MPGN type II (Jansen, J. H., K. Høgåsen, and A. M. Grøndahl, unpublished results). Renal tissue and blood samples were available in 239 piglets from 28 litters. From these, 64 piglets had renal changes diagnostic for MPGN. The same 64 piglets also had FH deficiency and hypocomplementemia, in contrast to none of the healthy littermates.

The tolerance for FH deficiency seems to be much lower in pigs compared with humans. This species difference may have several explanations. First, there may be differences in the repertoire of plasma and cellular C inhibitory proteins, as well as differences in the ability of these inhibitors to substitute for FH deficiency. Second, there may be differences in the amount of C components and their tendency for spontaneous activation. Third, there may be differences in the degree of C compatibility of host surfaces; to what degree host surfaces fail to amplify alternative pathway C activation. Fourth, the MPGN-affected piglets might have a nephritogenic genetic deficiency in addition to the FH deficiency. The last explanation is improbable, since our extensive family study showed no examples of discrepancy between FH deficiency and MPGN.

Our data provide some information about the kinetics of C activation products. They confirm that TCC has a short $t_{1/2}$ in vivo. This $t_{1/2}$ has been estimated to 30–50 min in rabbits (33) and 50 min in humans (34). A relatively high concentration of FH was necessary to suppress C activation in vivo since 30 ml/kg corresponds to about half the normal plasma volume in piglets. Even this amount of plasma was insufficient to normalize TCC, and the effect lasted < 3 d, indicating that most of the infused FH was consumed during this period. Since the $t_{1/2}$ of FH in man is ~ 76 h (35), our data may indicate either shorter $t_{1/2}$ of FH in piglets, or alternatively, an increased metabolism of FH in MPGN-affected piglets. However, our data agree with the finding that FH levels ~ 10% are associated with considerably increased activity in the alternative pathway with uncompensated consumption of C3 and factor B (7, 13).

FH deficiency leads to unrestricted C activation in plasma via the alternative pathway, which may fully explain the finding of low C3 and high TCC in plasma of the diseased piglets. Whether glomerular C deposits are mainly formed by in situ C activation, or by trapping of C activation products from plasma, and how the processes in the kidney influence plasma C level, cannot be answered from this study. Since the pigs had been treated with plasma, no attempts were made in this study to correlate plasma C levels with the intensity of glomerular C deposits.

The etiology and pathogenesis of MPGN have been obscure. In our view the etiology of the present animal model for MPGN type II is now defined as genetic FH deficiency, and the present data suggest that alternative pathway C activation is the key event in the pathogenesis of MPGN type II.

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