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Characterization of complement C6 deficiency in a PVG/c rat strain

M. G. A. VAN DIXHOORN, J. J. TIMMERMAN, D. J. VAN GIJLSWIJK-JANSSEN, Y. MUIZERT, C. VERWEIJ*, R. G. DISCIPIO† & M. R. DAHA *Department of Nephrology and *Rheumatology, University Hospital Leiden, Leiden, The Netherlands, and †La Jolla Institute for Experimental Medicine, La Jolla, CA, USA*

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SUMMARY

Complement C6 plays an important role in the effector phase of complement-mediated cell lysis. Recently, a PVG/c rat strain deficient in haemolytic C6 activity was discovered. In the present study we show that these rats lack both antigenic and functional C6, and that repetitive immunization of these rats with PVG/c^+ serum results in generation of specific anti-rat C6 antibodies. The observed absence of rat C6 was further investigated at the genomic and transcriptional level using a 492-bp cDNA of rat C6, cloned from a rat liver cDNA library using full length human C6 as a probe. Northern blot analysis revealed the presence of C6 mRNA in livers of both PVG/c^- and PVG/c^+ rats, corresponding to a size of $\approx 3{\cdot}3$ kb, although the level of C6 mRNA expression was ≈ 100-fold less in PVG/c^- rats. In addition, using rat C6-specific primers, positive signals were obtained in kidneys of both rat strains by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Southern blot analysis of digested genomic DNA did not reveal evidence for large C6 gene deletions. We conclude that the lack of C6 protein in the PVG/c^- rat strain is not due to a (large) C6 gene deletion, but presumably is caused by an unstable mRNA or a point mutation in the C6 gene resulting in an aberrant transcription of the C6 gene. Alternatively, a gene coding for a product involved in C6 biosynthesis that acts *in trans* may carry a mutation.

Keywords complement C6 deficiency rat cDNA glomerulonephritis

INTRODUCTION

The complement system is an important humoral effector mechanism during inflammation and infection. With the discovery of the capacity of cell-free, fresh sera to kill and lyse bacteria [1], the protective role of complement against bacterial infections was recognized.

Host defence mechanisms that are dependent on complement activation include opsonization and phagocytosis of invading organisms, attraction and activation of phagocytes at inflammatory sites, solubilization and clearance of immune complexes and direct lysis of many targets such as enveloped viruses, Gram-negative bacteria and eukaryotic cells recognized as foreign by the host [2].

The crucial role of complement in protection of the host against a wide variety of diseases is evident from genetic defects described for most of the complement components in man (reviewed in [3,4]).

At present there are several approaches to study the role of complement in experimental animal models. Circulating complement levels can be eliminated by the administration of a biological agent such as cobra venom factor [5] or by soluble CR1 (complement receptor for C3b) [6]. Furthermore, the development of homologous recombinant knockout techniques has resulted in mice deficient for factor B, C1q, C3 or C4 [7–9]. In addition, a number of spontaneously occurring complement deficiencies in animals has further aided to elucidate the role of different complement components in various inflammatory reactions [10]. For instance, rabbits lacking C6 [11] have delayed allograft rejection [12], suggesting that the membrane attack complex (MAC) acts as an important mediator during acute graft rejection.

A PVG/c rat strain deficient for rat complement component C6 was discovered by chance [13] and has served as a unique animal model to study the MAC in experimental medicine [14,15]. Although tissue antigens did not seem to differ between PVG/c^- and C6-sufficient PVG/c^+ rats, the question still remained whether these rats completely lack C6.

This study was performed to obtain insight into the C6 defect of the PVG/c^- rat strain, at protein, genomic and transcriptional levels. A complement-mediated experimental model of mesangial proliferative glomerulonephritis (GN) [16] was induced in PVG/c^+ and PVG/c^- rats to assess whether MAC can be generated in glomeruli of kidneys of both types of rats. Next, a specific rat C6

Correspondence: Mieneke van Dixhoorn, Department of Nephrology, Building 1, C3P, PO Box 9600, 2300 RC Leiden, The Netherlands.

cDNA was cloned from a rat liver cDNA bank and served as a probe in RNA and DNA analysis studies.

Protein analysis revealed that in contrast to normal PVG/c^+ rats, no detectable, circulating, haemolytically active C6 is present in sera of PVG/c^- rats. In addition, upon immunization with PVG/c^+ serum, anti-rat C6 antibodies became readily detectable in sera of PVG/c^- rats. Analysis of C6 at the mRNA and genomic DNA level revealed no gross C6 gene abnormalities. Therefore, we suggest that a mutation in the C6 gene that effects either C6 gene transcription or mRNA stabilization may be responsible for the observed disorder.

MATERIALS AND METHODS

Materials

Nickel chloride, imidazole, casein, 3′,3′-diaminobenzidine (DAB) tablets, 2,2′-amino-*bis*-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), bovine serum albumin (BSA), PMSF, benzamidine (Sigma Chemical Co., St Louis, MO), digoxygenin-ester (DIG-NHS), sheep F(ab′) anti-DIG–horseradish peroxidase (HRP), sheep F(ab′) anti-DIG–FITC (Boehringer Mannheim GmbH, Mannheim, Germany) and fetal calf serum (FCS; Hyclone Labs Inc., Logan, UT) were obtained as indicated. M-MLV Reverse Transcriptase, Oligo (dT) 12–18 primer (GIBCO BRL, Gaithersburg, MD), dNTP, Superdex HR200, Sepharose Q, Affigel, nick column and Protein A Sepharose (Pharmacia, Woerden, The Netherlands), Taq polymerase (Perkin Elmer, Norwalk, CT), Hybond N+, α-^{32}P-dCTP, KpnI and the Sequenase version 2.0 DNA sequencing kit (Amersham, Cleveland, OH) were purchased as indicated. The TA cloning kit (Invitrogen, Leek, The Netherlands), the λgt10 cDNA rat liver library (Clontech Labs, Palo Alto, CA), reinforced nitrocellulose (Schleicher and Schuell, Dassel, Germany), Freund's adjuvant (Difco Labs, Detroit, MI), sheep erythrocytes (Bio Trading, Mijdrecht, The Netherlands) were obtained as indicated. Mouse IgG2a anti-Thy-1.1 MoAbs [16] were purified from ascites by Protein A Sepharose 4B column, as described before [17], and mouse MoAbs directed against rat C6 were kindly provided by Dr W. G. Couser (Division of Nephrology, University of Washington, Seattle, WA). Streptavidin–HRP was obtained from the CLB (Amsterdam, The Netherlands) and FITC-conjugated goat anti-mouse IgG2a from Nordic (Tilburg, The Netherlands). FITC-conjugated rabbit anti-rat C3, biotin-conjugated rabbit anti-rat IgA, HRP-conjugated goat anti-mouse IgG and HRP-conjugated rabbit anti-goat antibodies were developed in our own laboratory. Macrophages were detected with FITC-conjugated mouse anti-rat macrophage MoAb, ED-1 (kindly provided by Dr C. D. Dijkstra, Free University of Amsterdam, Amsterdam, The Netherlands) (F/P ratio 3·3; 1·3 mg/ml; dilution 1:50) [18].

The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was obtained from the American Type Culture Collection (ATCC, Rockville, MD), the full length human C6 probe [19] was kindly provided by Dr R. G. DiScipio (La Jolla Institute for Experimental Medicine, La Jolla, CA) and tyramide–FITC was purchased from NEN-Dupont Research Products (Boston, MA).

Three-month-old PVG/c rats weighing 200 g were obtained from two different companies. PVG/c rats with normal complement activity (PVG/c^+) were obtained from Harlan Sprague Dawley (Cambridge, UK). Complement-deficient PVG/c rats (PVG/c^-) were originally obtained from Bantin and Kingman (Edmonds, WA) and age- and sex-matched rats were bred in our own animal facility. Wistar rats were obtained from our own breeding facility. All rats were housed in an accredited animal facility with free access to normal rat chow and water.

Methods

Gel filtration of PVG/c^- and PVG/c^+ serum. Sera of PVG/c^- and PVG/c^+ rats were separated according to size using a Superdex HR200 column (10/30) equilibrated with 50 mM sodium phosphate buffer containing 150 mM NaCl pH 7·0. Two hundred microlitres of each sample were applied to the column at a flow rate of 0·5 ml/min and fractions of 1·0 ml were collected. In every other fraction the protein and rat IgA concentration were assessed by BCA protein assay (Pierce, Rockford, IL) and a rat IgA ELISA [17], respectively. The presence of rat C2 and C6 in the fractions was determined by haemolytic assays.

Rat C6 and C2 haemolytic assay. For the quantification of haemolytically active rat C2, sheep erythrocytes coated with purified human C4 and bovine C1 were used (EAC14) as described previously [15], with slight modifications. The fractions of the gel filtration column were diluted 1:50 in half-isotonic Veronal buffered saline containing 0·05% gelatin, 0·15 mM $CaCl_2$, 0·5 mM $MgCl_2$ and 3% (w/v) dextrose (DGVB++), and 100 μl of each fraction were incubated with 1×10^7 EAC14 for 10 min at 30°C. After the addition of guinea pig complement (300 μl of a 1:30 dilution in half-isotonic Veronal-buffered saline containing 0·05% gelatin and 0·04 M EDTA) for 60 min at 37°C, unlysed EAC14 were sedimented by centrifugation for 7 min at 600 ***g*** and subsequently the number of effective haemolytic sites generated (Z) was calculated [20] and haemolytic activities in the fractions of the PVG/c^- rat were calculated relative to the maximal haemolytic activity of C2 in PVG/c^+ serum.

Haemolytically active rat C6 was determined using human C6-deficient serum by haemolytic assay, as described previously [13]. In this assay a dilution of 1:6400 of PVG/c^+ serum still induced at least 80% lysis of sheep erythrocytes. For the detection of rat C6 in PVG/c^- serum a dilution of 1:20 was used.

Immunization of PVG/c^- rats with PVG/c^+ serum. Three PVG/c^- rats were immunized intraperitoneally with 0·5 ml fresh PVG/c^+ serum. In addition, a simultaneous subcutaneous (s.c.) injection of 750 μl PVG/c^+ serum and Freund's complete adjuvant (FCA) in a 1:1 ratio was administered at different sites. On days 14, 28 and 42 the rats were boosted subcutaneously with 0·5 ml PVG/c^+ serum and Freund's incomplete adjuvant (FIA) in a 1:1 ratio. Blood samples were taken on days 0, 14, 28 and 42 by tail bleeding and on day 70 blood was collected from the three rats by intra-arterial puncture, after which the rats were killed. Sera were stored at −80°C until use.

Purification of affinity-purified polyclonal (PVG/c^-) rat anti-rat C6 antibodies. Rat C6 was isolated from 100 ml fresh Wistar serum by step-wise $(NH_4)_2SO_4$ precipitation [21], followed by fast performance liquid chromatography (FPLC) using a Sepharose Q and gel filtration on a HR200 column. The fractions were tested for protein in a BCA protein assay and rat C6 haemolytic activity. Subsequently, semi-purified rat C6 was coupled to 10 ml Affigel by overnight head-over-head rotation at 4°C (72·4% binding), as described earlier [22]. The pooled sera from day 70 of the immunized rats were precipitated with $(NH_4)_2SO_4$, dissolved in distilled H_2O and dialysed against PBS and subsequently incubated overnight at 4°C by head-over-head rotation with 10 ml of rat C6 Affigel. Bound specific antibodies were eluted with 0·1 M glycine, 0·3 M NaCl, 2 mM EDTA pH 2·8, neutralized with 1·0 M Tris–HCl

pH 8·0, concentrated and dialysed overnight against PBS. The protein concentration was determined by BCA protein assay and was found to be 100 μg/ml. The polyclonal rat anti-rat C6 antibodies were then conjugated with DIG-NHS according to the manufacturer's instructions.

Detection of rat IgA and rat C6 by ELISA. Rat IgA was detected by ELISA as described previously [17]. For the assessment of rat anti-rat C6 antibodies, microtitre plates were coated for 2 h at 37°C with 1 μg/well of the MoAb directed against rat C6. The plates were washed three times with PBS–Tween and incubated for 1 h with PVG/c$^-$, PVG/c$^+$ and Wistar serum diluted 1:50 in PBS–Tween containing 1% casein (PBS–Tween–Cas). After washing, plates were incubated for 1 h at 37°C with a dose–response of the immune sera from PVG/c$^-$ rats (dilutions starting at 1:50 in PBS–Tween containing 1% FCS (PBS–Tween–FCS)). Bound antibodies were detected with subsequent incubation for 1 h at 37°C with biotin-conjugated monoclonal anti-rat κ antibody (1:4000), followed by incubation with streptavidin–HRP (1:500 in PBS–Tween–FCS) for 1 h at 37°C. Plates were subsequently incubated with the HRP conjugate, ABTS, and the amount of substrate generated was determined using a Titertek Multiskan plate reader at optical density (OD) 415 nm.

Western blotting. Sera of a PVG/c$^-$ and a PVG/c$^+$ rat were precipitated with PEG 6000 and double lanes each containing 10 μg of total protein per sample were separated on a 7% acrylamide SDS–PAGE gel according to the method of Laemmli [23]. The gel was blotted [24] and one half was stained for rat C6 using the MoAb against rat C6, and the other half with DIG-conjugated polyclonal rat anti-rat C6 antibodies. Bound antibodies were detected by subsequent incubation with HRP-conjugated goat anti-mouse IgG or HRP-conjugated sheep (Fab′) anti-DIG antibodies, respectively. Between incubation steps the blots were washed with PBS–Tween, and finally the bands were visualized by DAB/nickel/imidazole staining [25].

Induction of experimental mesangial proliferative GN. Thy-1.1 nephritis was induced in rats by injecting male PVG/c$^-$ and PVG/c$^+$ rats intravenously with 1 mg/kg body weight of purified mouse IgG2a anti-Thy-1.1 MoAbs (ER4G) as described before [17]. One single injection of ER4G in rats results in complement-mediated mesangiolysis after 1 day. Subsequently, mesangial cell (MC) proliferation and extracellular matrix (ECM) expansion are observed in the glomeruli of the rats. The disease is further characterized by proteinuria reaching levels of 300 mg/24 h in the first week of the development of nephritis [16]. Control rats were injected with the same volume of PBS. Renal biopsies were taken under halothane anaesthesia 1 day after disease induction, processed and examined by immunofluorescence (IF) microscopy for the presence of mouse IgG, rat C3, and influx of monocytes and macrophages as described previously [17]. For the detection of rat C6 slides were preincubated with PBS containing a 1:3000 dilution of a 30% H_2O_2 solution to block endogenous peroxidase. Thereafter the slides were washed in PBS and incubated with DIG-conjugated anti-rat C6 antibodies (diluted 1:100 in 0·1 M Tris–HCl containing 0·15 M NaCl and 0·5% Boehringer Blocking reagent (TNB)), followed by incubation with HRP-conjugated sheep F(ab′) anti-DIG fragments (diluted 1:500 in TNB). Slides were subsequently incubated with tyramide-FITC (NEN-Dupont) (diluted 1:2000 in 0·2 M Tris–HCl pH 8·8, 10 mM imidazole containing 0·003% H_2O_2) for 30 min at room temperature. Between the 1-h incubation steps the slides were washed twice with PBS–Tween and twice with PBS.

Cloning and characterization of a rat C6 cDNA. A rat C6 cDNA clone was isolated from a rat liver cDNA library in λgt10 ($1{\cdot}4\times10^6$ independent clones; average insert size 1·2 kb) by standard hybridization screening at low stringency using a 3·3-kb full length human C6 cDNA as a probe, according to the manufacturer's instructions. Double-positive clones were subjected to a second and third screening round on 94-cm^2 plates until single phage colonies could be picked. A 492-bp C6 rat cDNA insert was subcloned in the plasmid pCRII using the TA cloning kit and confirmed as C6 cDNA based upon homology of the sequence with the human C6 cDNA sequence.

Radiolabelled probes. A KpnI 3·3-kb full length human C6 cDNA insert and the 490-bp rat C6 cDNA fragment were radiolabelled to a specific radioactivity of $\approx 10^8$ ct/min/μg DNA using random primer annealing [26]. Non-incorporated radioactivity was separated from labelled cDNA using a nick column (Pharmacia). Before hybridization the probes (5 ng/ml) were denatured by heating for 10 min at 100°C.

Northern blot analysis. A total of 20 μg of RNA was separated on formaldehyde containing 1% agarose gel and blotted to nitrocellulose filters. Gel electrophoresis and RNA transfer were performed as described [27]. The filters were prehybridized for 1 h and hybridized overnight in hybridization mixture (50% (v/v) formamide containing 5×SSPE, 5×Denhardt, 0·1% (w/v) SDS and 10 μg/ml of herring sperm DNA) at 37°C (low stringency) or 42°C (high stringency) with the human or rat C6 probe, respectively. Washing of the filters was performed twice for 30 min at low stringency with 2×SSC containing 0·1% (w/v) SDS at 50°C for the human C6 probe or twice for 30 min at high stringency with 0·2×SSC containing 0·1% (w/v) SDS at 65°C for the rat C6 probe. To quantify the amount of RNA loaded per lane the filters were rehybridized at low stringency with a GAPDH probe. Between each hybridization step the filters were stripped with 0·1% (w/v) SDS for 5 min at 90°C and exposed overnight in order to verify that no remaining signal was left on the filter.

Southern blot analysis. Southern blotting of genomic DNA was carried out as described by Southern & Wahl [28]. Chromosomal DNA (10 μg) was digested with EcoRI and BamHI, loaded on a 0·8% agarose gel and subsequently blotted to Hybond N+ using 0·4 M NaOH. The filter was hybridized at high stringency with the rat C6 cDNA probe as described above.

Reverse transcriptase-polymerase chain reaction. Total cellular RNA isolated from kidney or liver tissue was reverse transcribed into cDNA by oligo dT priming, as described previously from our laboratory [29]. For rat C6, oligonucleotide primers were constructed from the cDNA rat C6 sequence (corresponding to nucleotide positions 30–48 (P1, 5′-TGCCTCAAACCAGTCGTT-3′; sense cDNA primer) and 364–344 (P2, 5′-GCAGATGAAGT-GAAGTACTGA-3′; antisense polymerase chain reaction (PCR) primer)). Amplified DNA (10 μl) was electrophoresed on a 1·0% agarose gel and transferred overnight to Hybond N+ with 0·4 M NaOH. After neutralization in 2×SSC the filters were dried and stored at room temperature until use.

RESULTS

Size separation of PVG serum using gel filtration

It has been demonstrated previously that serum of PVG/c$^-$ rats contains no haemolytically active circulating C6 levels and 10% C2 compared with serum of PVG/c$^+$ rats [15]. Size fractionation of PVG/c$^+$ serum resulted in peaks of haemolytically active C6 and

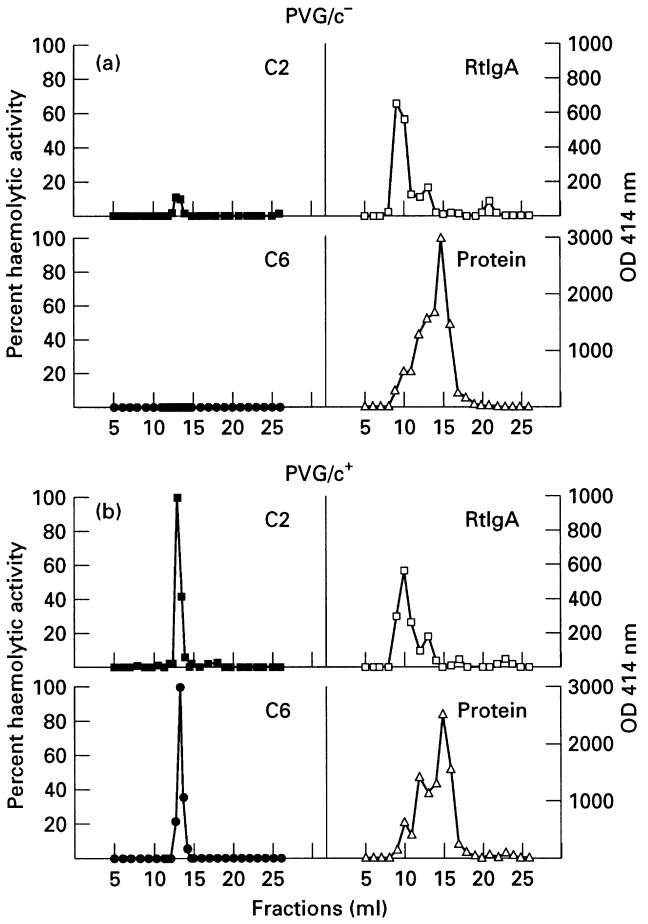

Fig. 1. Gel filtration of PVG/c$^-$ (a) and PVG/c$^+$ (b) serum. The fractions were tested for rat C2 and C6 haemolytic activity. As an internal control fractions were tested for rat IgA and total protein concentration. The peaks for C2 and C6 correspond to a size of ≈120 kD.

C2 (Fig. 1). When PVG/c$^-$ serum was fractionated the relative percentage of haemolytic activity in the fractions was only maximally 0% and 10% compared with the PVG/c$^+$ serum samples for C6 and C2, respectively. To justify the above comparison, the amount of serum applied to the column was assessed by measuring rat IgA and total protein content in the fractions of both elution profiles. The outcome for both sera was comparable.

Immunization of PVG/c$^-$ rats with rat serum containing C6

To determine whether PVG/c$^-$ rats were able to develop an immune response against rat C6, three PVG/c$^-$ rats were immunized and boosted with PVG/c$^+$ serum emulsified in FCA. On day 70 after the first immunization, serum of PVG/c$^-$ rats showed positive binding in an ELISA, in which rat C6 served as the capturing antigen (Fig. 2). Pre-immune sera of all three rats did not react with rat C6 in this ELISA. Antiserum of these rats after immunization showed comparable reactivity in the rat C6 ELISA when PVG/c$^+$ or Wistar serum was used as the antigen source (not shown). From each of three rats, 3 ml serum were obtained on day 70, pooled and used for affinity purification of anti-rat C6 antibodies. After acid elution a total of 100 μg of affinity-purified polyclonal rat anti-rat C6 antibodies was obtained. After DIG conjugation these polyclonal rat anti-rat C6 antibodies were used

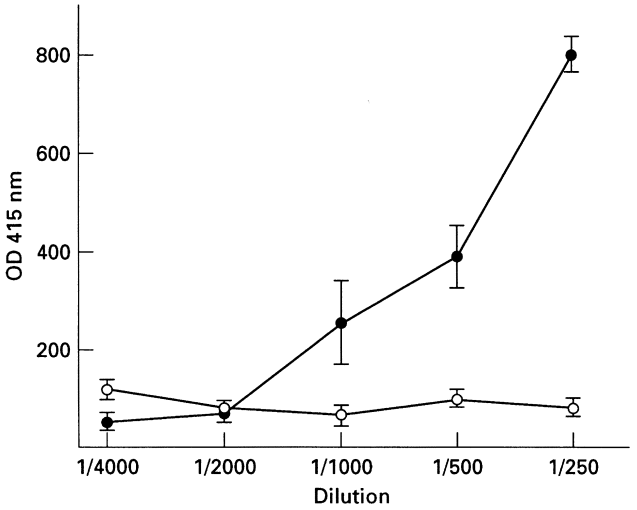

Fig. 2. Detection of anti-rat C6 antibodies in serum of PVG/c$^-$ rats as assessed by ELISA. PVG/c$^-$ rats were repetitively immunized with PVG/c$^+$ serum and anti-rat C6 antibodies were assessed using ELISA plates coated with the anti-rat C6 MoAb incubated subsequently with PVG/c$^+$ serum, to serve as a coating-antigen-source. Depicted are averages of three PVG/c$^-$ rats ± s.d. ●, 70 days after immunization; ○, pre-immunization.

for Western blotting and IF (Figs 3 and 4e). In serum of PVG/c$^+$ rats one strong positive band of 90 kD was identified by Western blot, while no detectable band was found in serum of PVG/c$^-$ rats. When a parallel blot was stained with monoclonal mouse anti-rat C6, again a positive band of similar size was seen in PVG/c$^+$ serum and not in PVG/c$^-$ serum (Fig. 3).

Complement activation in vivo *after induction of GN*

Induction of anti-Thy-1.1 nephritis using mouse IgG2a anti-Thy-1.1 antibodies (ER4G) results in glomerular deposition of IgG and subsequent activation of complement up to the C9 stage [16]. To assess complement activation in the two rat strains *in vivo*, rats were injected intravenously with a single dose of complement-fixing ER4G. One day after injection, renal cortex cryostat sections were examined for the presence of mouse IgG2a, rat C3 and C6 and influx of monocytes/macrophages. In both ER4G-infused PVG/c$^-$ and PVG/c$^+$ rats an equally intense mesangial staining was

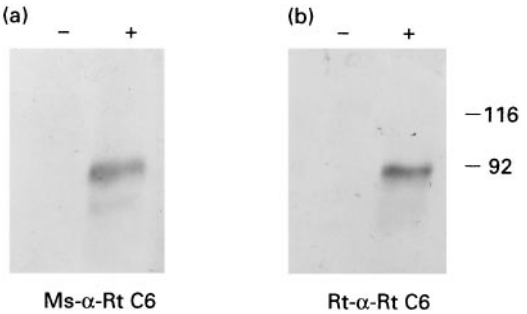

Fig. 3. Western blot analysis of serum obtained from PVG/c$^+$ (lanes indicated by +) or PVG/c$^-$ rats (lanes indicated by −). One blot was stained with mouse MoAb directed against rat C6 (a). A parallel blot was stained with the polyclonal rat anti-rat C6 antibodies obtained from PVG/c$^-$ rats, immunized with PVG/c$^+$ serum (b). Positive bands of 90 kD appear only in lanes of PVG/c$^+$ rat serum.

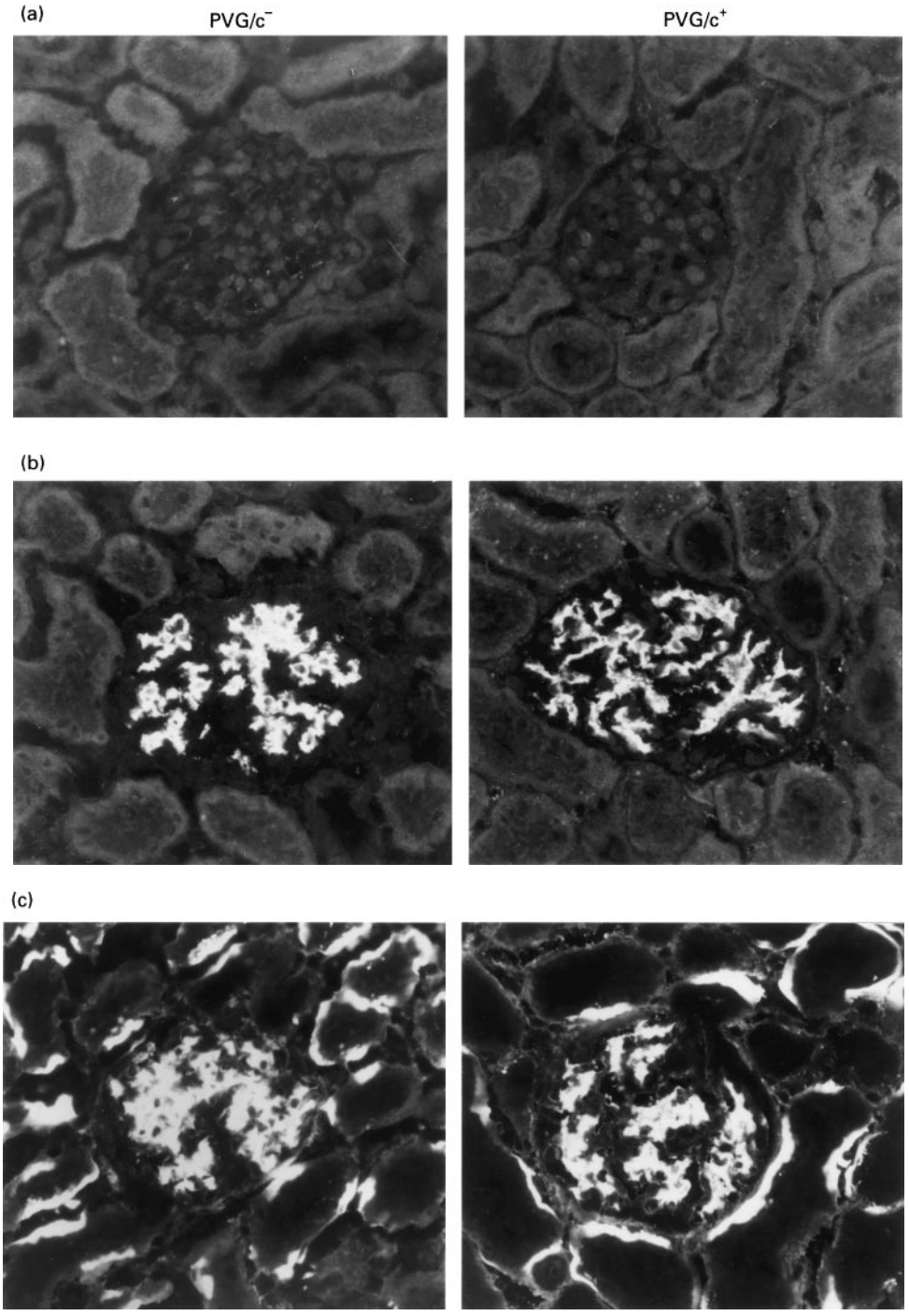

Fig. 4. (cont. on next page) Immunofluorescence staining for mouse IgG2a (a,b), rat complement component C3 (c), and C6 (d,e,f) in kidneys of PVG/c^+ and PVG/c^- rats. Rats were infused with PBS (a and f) or ER4G (b, c, d and e) and after 1 day kidney biopsies were taken. Sections were stained with FITC-conjugated goat anti-mouse IgG2a (a,b), FITC-conjugated rabbit anti-rat C3 (c), DIG-conjugated mouse anti-rat C6 (d,f) or DIG-conjugated rat anti-rat C6 antibodies (e). Binding of the DIG conjugates was assessed using anti-DIG–horseradish peroxidase (HRP) antibodies followed by incubation with tyramide-FITC. Despite comparable C3 deposition in glomeruli of PVG/c^+ and PVG/c^- rats, only in PVG/c^+ rats was glomerular C6 deposition clearly observed (c, d and e). In contrast to PVG/c^+ rats, no tubular rat C6 staining was seen in biopsies of PVG/c^- rats.

observed for mouse IgG2a (Fig. 4b), whereas PBS-treated rats were negative for mouse IgG2a staining (Fig. 4a). Furthermore, glomerular rat C3 staining was similar in PVG/c^+ and PVG/c^- rats (Fig. 4c). Tubular staining for C3 was observed in both types of rats, a feature which is also seen in normal PVG/c^- and PVG/c^+ control rats (not shown). When the kidney sections were analysed for the presence of rat C6 using MoAbs directed against C6, only in PVG/c^+ rats and not in PVG/c^- rats was a strong positive

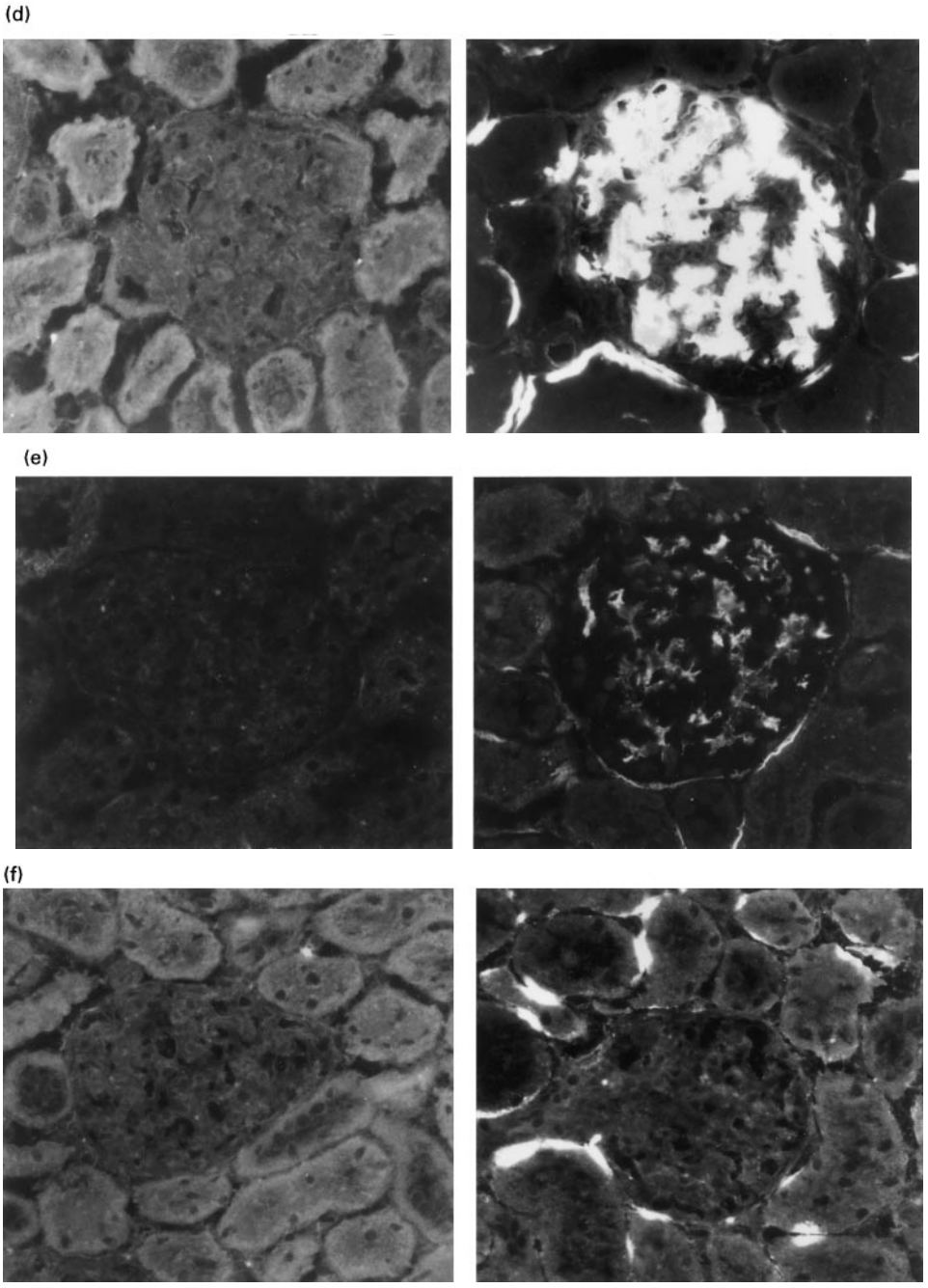

Fig. 4. continued

mesangial staining using the tyramide-FITC method found (Fig. 4d). An identical staining pattern was observed using DIG-conjugated polyclonal rat anti-rat C6 antibodies. PVG/c$^+$ rats exhibited clear mesangial C6 deposits, whereas PVG/c$^-$ rats were completely negative (compare Figs 4d and 4e). Furthermore, tubules of both control PVG/c$^+$ rats and ER4G-treated PVG/c$^+$ rats stained positive for rat C6, whereas PVG/c$^-$ rat biopsies were completely negative (Fig. 4f).

The average number of glomerular monocytes/macrophages 1 day after anti-Thy-1.1 injection was quantified. In PVG/c$^+$ rats an increase from 1·0 ± 1·4 monocytes/macrophages per glomerular cross-section in controls to 8·2 ± 3·1 in ER4G-treated PVG/c$^+$ rats ($P < 0{\cdot}05$) 1 day after the injection was seen. In ER4G-treated PVG/c$^-$ rats no significant increase in the number of glomerular ED1$^+$ cells was observed compared with control PVG/c$^-$ rats (1·7 ± 1·4 *versus* 0·7 ± 0·8, respectively).

Further analysis of the C6 deficiency

The functional and immunohistochemical studies suggest a complete absence of C6 in the PVG/c$^-$ rats. The C6 deficiency was therefore also investigated at the genomic and transcriptional level. A partial rat C6 cDNA was cloned from a rat liver cDNA library. Sequence analysis of the 492-bp rat C6 cDNA fragment was 82% homologous to the region enclosed by positions 2239 and 2727 of

ALIGNMENT OF RAT C6 CDNA SEQUENCE (TOP) TO HUMAN C6 CDNA SEQUENCE
(BP POSITIONS 2239 - 2727)

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CGGGGGATGT CGAATGCCAA CGGACCGAGT GCCTCAAACC AGTCGTTCAG GATGTCCTGA CCATCTCCCC ATTTC   75
--GGGGATGT GGAATGCCAA CGGACGGAGT GCATCAAGCC AGTTGTGCAG GAAGTCCTGA CAATTACACC ATTTC   73

AGAGTGTGTA CAAGATTGGG GAATCCATTG AGCTGACCTG TCCCAGAGGC TTTGTTGTTG CTGGCCCATC GAGGT  150
AGAGATTGTA TAGAATTGGT GAATCCATTG AGCTAACTTG CCCCAAAGGC TTTGTTGTTG CTGGGCCATC AAGGT  148

ATACATGCAA GGGAGACTCC TGGACACCTC CCATTCCAAA TTCACTGAGC TGTGAAAAAG ATACTCTGAC AAAGT  225
ACACATGCCA GGGGAATTCC TGGACACCAC CCATTTCAAA CTCTCTCACC TGTGAAAAAG ATACTCTAAC AAAAT  223

CAAAGGGCCT TTGTCAACCA GGACAAAAGC AATCAGGATC CGAGTGTGTT TGTATGTCCC CAGAAGAAGA CTGTA  300
TAAAAGGCCA TTGTCAGCTG GGACAGAAAC AATCAGGATC TGAATGCATT TGTATGTCTC CAGAAGAAGA CTGTA  298

GCAGTTATTC GGAAGATCTC TGTATATTTG ATGAGGGATC CAGTCAGTAC TTCACTTCAT CTGCTTGCAA ATTTT  375
GCCATCATTC AGAAGATCTC TGTGTGTTTG ACACAGACTC CAACGATTAC TTTACTTCAC CCGCTTGTAA GTTTT  373

TGGCTGAAAA ATGTTTAAAC AGCAACCAGT TCCACTTTGT CCATGCTGGT TCCTGCCAAG AAGGCCCACA GTTAG  450
TGGCTGAGAA ATGTTTAAAT AATCAGCAAC TCCATTTTCT ACATATTGGT TCCTGCCAAG ACGGCCGCCA GTTAG  448

AATGGGGTCT TGAGAGGCTA AAACTCGCAA TGAAGAGCAC AC                                     492
AATGGGGTCT TGAAAGGACA AGACTTTCAT CCAACAGCAC AA                                     490
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Fig. 5. Sequence of the rat C6 cDNA (top) projected against the corresponding region of the full length human C6 cDNA sequence flanked by basepair positions 2239 and 2727. The vertical lines indicate the differences between the cDNA sequences. The rat C6 cDNA sequence was 82% homologous to the human C6 sequence.

the human C6 cDNA sequence (Fig. 5) (Geneworks, IntelliGenetics Inc., Mountain View, CA). No stop codon appeared in the rat C6 sequence (Fig. 5). Furthermore, the deduced amino acid sequence was 79% homologous to the human C6 protein sequence, and no significant sequence homology was found with human C7, C8 or C9.

Messenger RNA isolated from liver and kidney tissue of both types of rats was separated according to size and hybridized at low stringency with the 3·3-kb full length human C6 cDNA probe. After stripping, the same blot was re-hybridized at high stringency with the 492-bp rat C6 cDNA probe (Fig. 6). While a 3·3-kb band was found in the liver of the PVG/c^+ rat using the human C6 probe, no positive signal was detected in the liver of the PVG/c^- rat. Using the rat C6 probe in both the PVG/c^+ as well as in the PVG/c^- rat, a band of 3·3 kb was observed in the liver. Despite the identical size of C6 mRNA in both PVG/c^- and PVG/c^+ rats, the density of the C6 band in the PVG/c^- rat was $\approx$ 100 times less intense than the density of the band of the PVG/c^+ rat.

Rat C6 primers were designed on basis of the rat C6 cDNA sequence and used in reverse transcriptase (RT)-PCR analysis on liver and kidney cDNA of both types of rats. After electrophoresis and Southern hybridization using the rat C6 probe (Fig. 7), one band of 334 bp in the liver and the kidney appeared in lanes of both types of rats. However, the intensity of the PVG/c^- bands was 10-fold less strong compared with those of the PVG/c^+ rats. Hybridization of the same PCR blot at low stringency with the human full length C6 probe revealed positive bands of similar size only in the kidney and the liver of the PVG/c^+ rat, and not in tissue of

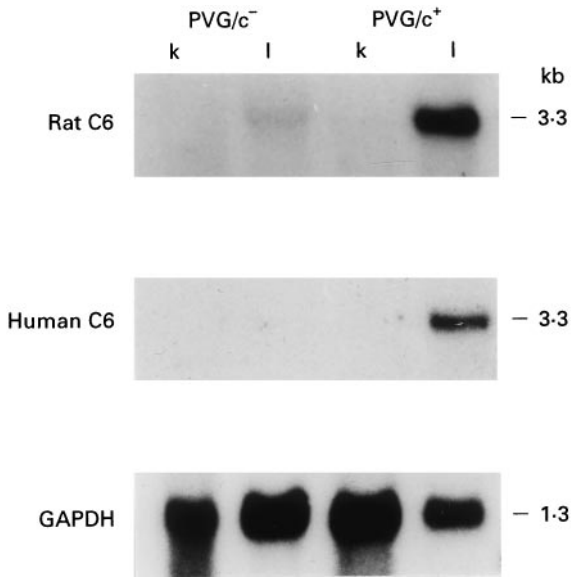

Fig. 6. Northern blot analysis of C6 mRNA expression in livers and kidneys of PVG/c^+ and PVG/c^- rats. Each lane represents 20 μg of liver (l) or kidney (k) mRNA. The blot was hybridized with a specific rat C6 cDNA, a full length human C6 cDNA and as a control a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Using the rat C6 cDNA as a probe, similar bands of 3·3 kb appear in the kidney and the liver of both types of rats.

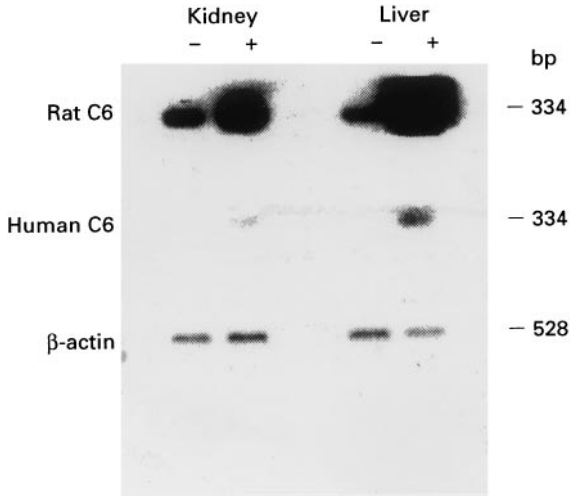

Fig. 7. Reverse transcriptase-polymerase chain reaction **(**RT-PCR) analysis for the presence of rat C6 mRNA in livers and kidneys of PVG/c$^+$ and PVG/c$^-$ rats. mRNA was reverse transcribed into cDNA and PCR analysis was performed using specific primers for rat C6 and β-actin. The rat C6 PCR products were hybridized with the ^{32}P-labelled rat C6 and human C6 probes. Rat C6 cDNA was detected in kidney and liver of PVG/c$^+$ and PVG/c$^-$ rats.

PVG/c$^-$ rats. As a control for equal amounts of cDNA used in the PCR analysis, a β-actin PCR was performed (Fig. 7).

To investigate if there were no large chromosomal C6 deletions in the PVG/c rats, Southern blot analysis was performed. Genomic DNA was isolated and subsequently digested using restriction enzymes, separated according to size and hybridized with the rat C6 cDNA probe at high stringency. In Fig. 8 identical restriction bands appear in BamHI lanes of both types of rats. The additional, larger bands that appear in the EcoRI lane of the PVG/c$^-$ rat can be ascribed to incomplete digestion of chromosomal DNA (not shown), rather than differences in gene composition.

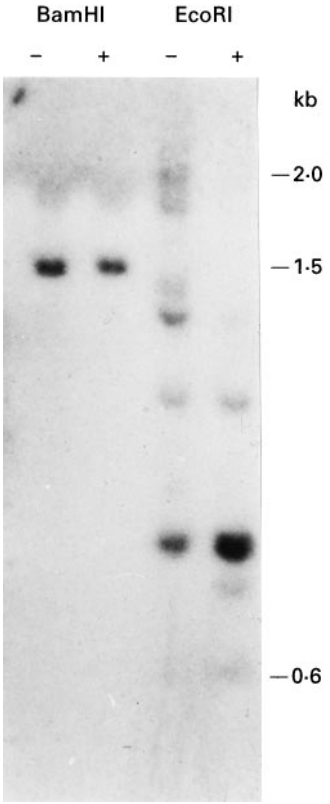

Fig. 8. Southern blot analysis of chromosomal DNA obtained from PVG/c$^+$ and PVG/c$^-$ rats. After restrictive digestion with EcoRI or BamHI, the chromosomal DNA was electrophoresed and blotted. The filter was hybridized with radiolabelled rat C6-specific cDNA. Similar restriction bands appear in BamHI lanes of both types of rats. The additional (larger) bands in the EcoRI lane of the PVG/c$^-$ rat can be ascribed to incomplete digestion of chromosomal DNA.

DISCUSSION

Complement-deficient animals have proved to be of importance for studies on the role of complement in a wide variety of biological systems *in vivo*. In this study we characterized rat C6 deficiency in an inbred PVG/c$^-$ rat strain. While it was established previously that no alloantibodies could be generated in PVG/c$^-$ rats after immunization with syngeneic (PVG/c) spleen cells [13], we found that immunization of these PVG/c$^-$ rats with PVG/c$^+$ or Wistar serum resulted in generation of specific anti-rat C6 antibodies. The polyclonal rat anti-rat C6 antibodies were reactive with a protein of 90 kD in size. MoAbs against rat C6 recognize a protein of similar size in PVG/c$^+$ serum and not in PVG/c$^-$ serum. Furthermore, C6 captured by monoclonal anti-rat C6 is recognized by these polyclonal anti-rat C6 antibodies, indicating that the antibody response in PVG/c$^-$ rats upon immunization with PVG/c$^+$ serum is directed against rat C6. This suggests that the rat C6 is seen as a novel antigen by the PVG/c$^-$ rats. The absence of detectable C6 levels in PVG/c$^-$ rats was also confirmed by high performance liquid chromatography (HPLC) analysis. These findings indeed collectively suggest that PVG/c$^-$ rats completely lack circulating rat C6.

Apart from a complete defect for C6, PVG/c$^-$ rats also appeared to have reduced levels of haemolytically active C2 compared with PVG/c$^+$ rats. It was discovered earlier that addition of highly purified human C6 to PVG/c$^-$ serum fully reconstituted its haemolytic activity [13]. This would suggest that the remaining 10% C2 present in the circulation of PVG/c$^-$ rats is sufficient to generate a functional MAC. Our assessment of rat C2 uses EAC14 together with a 1:30 dilution of guinea pig complement in the presence of EDTA. This assay is not influenced by rat C6, because of excess of C3-C9.

As was demonstrated earlier [15], only PVG/c$^+$ rats exhibit glomerular C6 deposits and macrophage influx upon active renal disease induction. Brandt *et al.* analysed the generation of functional C5a in serum of PVG/c$^-$ and PVG/c$^+$ rats and found comparable amounts of C5a in both types of rats. This would imply that the absence of influx of monocytes/macrophages in PVG/c$^-$ rats after renal disease induction cannot be due to impaired C5 cleavage, but the exact reasons are not clear at this stage. However, since other chemotactic agents, such as IL-1, tumour necrosis factor-alpha (TNF-α) or monocyte chemoattractant protein 1 (MCP-1) (reviewed in [30]) are also known to play a role in the mobilization of monocytes, we suggest that apart from a C6 deficiency, PVG/c$^-$ rats may also aberrantly generate chemoattractants like MCP-1, following infusion of anti-Thy-1.1 antibodies. This, however, needs further investigation. In addition, it was suggested that the formation of sublytic amounts of C5b-9 on the surface of renal cells may result in an increased production of IL-1, TNF-α or MCP-1, leading to worse disease development [31].

The size of the C6 mRNA in both types of rats is identical, i.e. 3·3 kb, indicating that at least no major alterations in the coding sequence of the C6 gene are present. However, the quantity of C6 mRNA present in the liver of PVG/c$^-$ rats appeared to be 100-fold less compared with PVG/c$^+$ rats. This suggests either instability of PVG/c$^-$ mRNA, or the presence of a defect at the C6 transcriptional level, resulting in low C6 mRNA levels. This may be the result of a point mutation within the C6 gene or in the coding region of a gene, the product of which operates *in trans* in C6 biosynthesis. When the more sensitive RT-PCR was performed, C6 mRNA was found in liver and kidney of PVG/c$^-$ rats. Furthermore, preliminary genomic C6 gene analysis suggests that no gross C6 gene deletions are present in the PVG/c$^-$ rats. These findings together argue against a complete absence of the C6 gene.

Naturally occurring animals deficient in C3 [32], C4 [33] or C6 [34] have been described. The C3 deficiency in a strain of guinea pigs (C3 levels 5% of normal) was identified to be due to a co- or post-synthetic processing modification that renders C3 proteins highly susceptible to proteolysis [32]. The authors suggest that the mutation causing the defective phenotype lies in the non-coding region of the C3 gene. None of the actual genetic backgrounds leading to complement deficiencies in these animals has been further characterized so far.

In humans, absence of C6 is the second most common complement deficiency in Caucasoids (incidence 1:60 000 [35]). The 80-kb C6 gene is described to be located on chromosome 5 [36]. Although most C6-deficient patients are described to lack total protein, using a more sensitive ELISA Würzner *et al.* described that small amounts of functionally active C6 and C7 were found in more than 50% of sera of C6- and C7-deficient individuals [37].

C7 markers show a linkage disequilibrium with C6 deficiency in the Cape Coloured population [38]. In humans, the molecular bases of C6, C7, combined subtotal deficiencies of C6 and C7, C8β deficiency, and C9 deficiency have been described [39–43]. These studies in general imply that simple (point) mutations leading to (stop) codon substitutions can result in truncated or low expressed proteins. The simultaneous occurrence of hereditary C6 and C2 deficiency in man has arisen by chance [44,45]. The single C2 deficiency appeared not to be due to a major gene deletion or rearrangement, but as the result of a specific and selective pre-translational regulatory defect in C2 gene expression [46]. This leads to a lack of detectable C2 mRNA and a subsequent lack of C2 protein synthesis.

We hypothesize that the observed C6 defect in this strain of PVG/c rats may be due to a small deletion or point mutation at the genomic level leading to an aberrant rat C6 transcription, resulting in low mRNA levels and subsequent hardly detectable C6 protein. Now that we have developed rat C6 primers to study the regulation of local rat C6 synthesis, these PVG/c$^-$ rats provide a unique model for the analysis of complement and its role in a variety of experimental disease models such as glomerulonephritis.

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