Identification of Membrane-bound CR1 (CD35) in Human Urine: Evidence for Its Release by Glomerular Podocytes

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Summary

Complement receptor 1 (CR1) is present on erythrocytes (E-CR1), various leucocytes, and renal glomerular epithelial cells (podocytes). In addition, plasma contains a soluble form of CR1 (sCR1) By using a specific ELISA, CR1 was detected in the urine (uCR1) of normal individuals (excretion rate in 12 subjects, 3.12 ± 1.15 μg/24 h). Contrary to sCR1, uCR1 was pelleted by centrifugation at 200,000 g for 60 min. Analysis by sucrose density gradient ultracentrifugation showed that uCR1 was sedimenting in fractions larger than 19 S, whereas sCR1 was found as expected in fractions smaller than 19 S. The addition of detergents reduced the apparent size of uCR1 to that of sCR1. After gel filtration on Sephacryl-300 of normal urine, the fractions containing uCR1 were found to be enriched in cholesterol and phospholipids. The membrane-association of uCR1 was demonstrated by analyzing immunoaffinity purified uCR1 by electron microscopy which revealed membrane-bound vesicles. The apparent molecular mass of uCR1 was 15 kD larger than E-CR1 and sCR1 when assessed by SDS-PAGE and immunoblotting. This difference in size could not be explained on the basis of glycosylation only, since pretreatment with N-glycosidase F reduced the size of all forms of CR1; however, the difference in regular molecular mass was not abrogated. The structural alleles described for E-CR1 were also found for uCR1. The urine of patients who had undergone renal transplantation contained alleles of uCR1 which were discordant with E-CR1 in 7 of 11 individuals, indicating that uCR1 originated from the kidney. uCR1 was shown to bind C3b-coated immune complexes, suggesting that the function of CR1 was not destroyed in urine. A decrease in uCR1 excretion was observed in 3 of 10 patients with systemic lupus erythematosus, corresponding to the three who had severe proliferative nephritis, and in three of three patients with focal sclerosis, but not in six other patients with proteinuria. Taken together, these data suggest that glomerular podocytes release CR1-coated vesicles into the urine. The function of this release remains to be defined, but it may be used as a marker for podocyte injury.

C R1 (CD35, C3b/C4b receptor) is a transmembrane protein expressed on several circulating cells including E, PMN, monocytes, B-lymphocytes, and some T-lymphocytes (1, 2). Some specific epithelial cells have been reported to express CR1 as well (3, 4). In particular CR1 is present on renal glomerular epithelial cells (podocytes) (4, 5). These receptors were initially identified by the adherence of C3b-coated sheep E and bacteria to glomeruli in frozen sections of human kidney (6, 7). Using immunoelectron microscopy, Kazatchkine et al. (4) have shown that in glomeruli, CR1 antigenic determinants were localized only on podocytes. More recently, Fischer et al. (5) demonstrated that podocyte CR1

shares the functional, antigenic, and biochemical properties of E-CR1, and estimated that each podocyte bears ∼200,000 CR1. The biological function of CR1 on podocytes remains speculative. It has been suggested that the presence of CR1 on podocytes might be necessary for the inactivation of C3b in a compartment (primitive urine) which is devoid of complement inhibitors (5). Except for cells derived from the circulation, no other normal renal cell in the kidney produces or expresses CR1 (8, 9). Thus, CR1 can be considered as a specific marker for podocytes in the kidney. A loss of CR1 antigen on podocytes has been found in different nephropathies such as severe proliferative nephritis of SLE, focal scle-

rosis (hyalinosis), and crescentic nephritis (4, 10–13). Altered patterns of staining were also found in amyloidosis and in diabetic nephropathy (4).

CR1 is also found in plasma as a soluble protein (sCR1)[1] whose function and size are indistinguishable from E-CR1 (14). sCR1 might originate from the various cells that express CR1 as suggested by the recent observation that PMN, lymphocytes, and monocytes release soluble CR1 in vitro (15). The concentration of plasma sCR1 in normal individuals is 34 ng/ml. Elevated levels were found in patients with renal failure and the concentration of sCR1 returned to normal after successful renal transplantation suggesting that the kidney might be, in part, responsible for the catabolism of sCR1 (15).

This study was undertaken to determine whether CR1 is present in urine, and if so, whether it originates from renal podocytes, from circulating cells, or from the glomerular filtration of sCR1.

Materials and Methods

Determination of CR1 in Plasma (sCR1), Urine (uCR1), and on E (E-CR1). The concentrations of uCR1 were determined by using an ELISA previously described for measuring sCR1 (15). In brief, a mAb (3D9) against CR1 was coated on the wells. After incubation of the sample to be tested, the CR1 bound to the first mAb was revealed with a biotinylated second mAb (J3D3) recognizing another epitope of CR1. Recombinant soluble human CR1 (r-CR1, kindly provided by Smith Kline Beecham Pharmaceuticals, King of Prussia, PA) was used as a standard. For uCR1 determinations, fresh urine was centrifuged at 1,500 g for 15 min at room temperature and samples of 10–50 μl were applied to the mAb (3D9)-coated wells in a final vol of 100 μl of PBS-Tween 0.05%. The assay was then performed as for sCR1. The stability of urine samples was tested by keeping them at 4°C or room temperature for various periods of time before performing the ELISA. Urine samples were also ultracentrifuged for 60 min at 50,000 and 200,000 g before being tested. E-CR1 numbers were determined by direct binding of radiolabeled 3D9 mAb to E as previously described (16).

Analysis of Urine by Ultracentrifugation on Sucrose Density Gradients (SDGU) and Gel Filtration. Fresh urine samples were centrifuged at 1,500 g for 15 min at room temperature and concentrated 10-fold using a PM30 (43 mm) membrane with a cut-off of 30 kD (Amicon, Grace SA, Walliselen ZH, Switzerland). Samples of 200 μl of concentrated urine were layered onto 5-ml linear sucrose density gradients (10–50% wt/wt) (17). The gradients were centrifuged for 120 min at 200,000 g at room temperature, and 24 fractions of 200 μl were collected from the bottom. CR1 was measured in the fractions using the ELISA described above. Several controls were included. (a) 100 μl of human plasma which had been diluted 1:1 in PBS was layered onto identical gradients, and the position of sCR1 was determined. (b) Vesicles released from E during ATP depletion are enriched in E-CR1 (18). Such E-vesicles were obtained as reported previously (18) and analyzed by SDGU. (c) The position of IgM in the gradient was determined by using radiolabeled monoclonal IgM. In another series of experiments, 0.5% (final con-

centration) SDS or 0.5% Triton was added to the samples of concentrated urine, E-vesicles, and serum before ultracentrifugation.

150 ml fresh centrifuged urine supplemented with 30 mg of BSA (Sigma Chemical Co., St. Louis, MO) was concentrated 30-fold using the PM30 Amicon membrane. The concentrated urine (5 ml) was applied to a column (Sephacryl-300, 2.5 × 90 cm, vol., 600 ml; Pharmacia, Uppsala, Sweden) equilibrated with PBS at a flow rate of 50 ml/h. Fractions of 3 ml were collected and assessed for the presence of CR1. Similar analyses were performed with undiluted serum (5 ml).

In the Sephacryl-300 elution profiles of concentrated urine, the four fractions containing the highest CR1 concentration were pooled and concentrated with a Centricon 10 (Amicon, Beverly, MA) previously coated with PBS-BSA 1% to a final vol of 150 μl. The phospholipid and total cholesterol content of these pooled concentrates of partially purified uCR1 was analyzed with commercially available enzyme assays (respectively, Phospholipids B, Wako Chemicals GmbH, Neuss, Germany; and CHOD-PAP, Boehringer Mannheim, Germany) (19). The total protein content was determined by the method of Lowry et al. (20).

Electron Microscopy of Immunopurified uCR1. 50 μl of magnetic particles covalently coated with goat anti–mouse Ab (Advanced Magnetics Inc., Cambridge, MA) was incubated with 20 μg of anti-CR1 mAb 3D9, or control anticomplement factor D mAb 72-96-25 (21), in a final vol of 250 μl PBS-BSA 0.1% for 5 h at 4°C. After three washes with PBS, the mAb-coated particles were incubated with samples of 150 μl of pooled concentrate of the partially purified uCR1 (containing 100 ng of CR1 antigen as determined in the ELISA) for 2 h at 4°C. After three washes, the magnetic particles were fixed in 2% glutaraldehyde, dehydrated, and processed for electron microscopy as previously described (22). Thin sections were examined in a Philips EM301 electron microscope.

Analysis of uCR1 by Immunoblotting. Pooled concentrates of uCR1 partially purified by Sephacryl-300 or the pellet of urine which had been ultracentrifuged for 60 min at 200,000 g were electrophoresed under nonreducing conditions on a 4% SDS-PAGE gel. Proteins were blotted onto nitrocellulose membranes, blots were incubated overnight with 2 × 10⁵ cpm/ml of [125I]-3D9 mAb, washed, and autoradiographed. uCR1 was compared to E-CR1, PMN-CR1, and sCR1. Erythrocyte membranes and E-vesicles were prepared as previously described (18). PMN membranes were obtained as follows: fresh PMN were purified from 30 ml of peripheral blood using a Ficoll-Hypaque gradient (Pharmacia) and hypotonic lysis (23). The cells (5 × 10⁶/ml) were resuspended in 0.25 M glucose in the presence of 0.5 mM DFP, 1 mM PMSF, and 10 mM EDTA, and were subsequently homogenized with a device (ball homogenizer) as described by Balch et al. (24). Briefly, the cell suspension was forced repeatedly (via attached syringes) through the device. Approximately 8–12 passes were required to break most of the cells. The crude homogenate was centrifuged at 600 g for 10 min at 4°C to remove residual cells and nuclei. The supernatant was ultracentrifuged at 27,000 g for 7 min at 4°C to remove lysosomes and mitochondria. Finally the supernatant (containing PMN membranes) was ultracentrifuged at 200,000 g for 1 h at 4°C. The pellet was resuspended in a 100–200 μl vol of PBS containing 0.5 mM DFP, 1 mM PMSF, and 10 mM EDTA. sCR1 was purified from serum by immunoaffinity on 3D9 mAb–Sepharose as previously described (15).

Partially purified uCR1 and E-CR1 solubilized from E or E-vesicle membranes were also immunoprecipitated and subsequently analyzed by immunoblotting. In brief, samples of 100 μl of uCR1 and E-CR1 (containing ~100 ng of CR1 antigen as determined in the ELISA) were incubated with 3 μg of anti-CR1 mAb 3D9,

or control anti-factor D mAb, in PBS-BSA 1%–Tween 0.1% (300 μl final vol), for 4 h at room temperature with gentle mixing. The CR1–anti-CR1 complexes were precipitated using 100 μl of protein A–Sepharose CL4B (50%, vol/vol) as immunosorbent (Pharmacia). After centrifugation at 200 g and three washes in cold PBS, the samples were denatured for 20 min at room temperature in the absence of reducing agent, and were electrophoresed and revealed after immunoblotting as described above.

In other experiments, partially purified uCR1, E membranes, and E-vesicles from the same individual were treated with N-glycosidase F (Boehringer Mannheim AG, Rotkreuz, Switzerland) in 0.25 M sodium acetate, 10 mM EDTA, 0.1% SDS, and 0.6% Triton, for 16 h at 37°C, with enzyme concentrations at 10 U/ml. These samples were subsequently analyzed by immunoblotting as described above.

IC Adherence Assays. Radioiodinated hepatitis B surface antigen (HBsAg)/Ab complexes were prepared as previously reported (25). C3b-coated HBsAg/Ab complexes were formed by a 15-min incubation of HBsAg/Ab with normal human serum at 37°C, followed by SDGU to partially purify the complexes. The binding of C3b-coated HBsAg/Ab complexes (15 μl = 5 ng Ag) to partially purified uCR1 or to CR1 of E-vesicles obtained from the same individual (bearing the A allele of CR1) was measured as follows: 15 μl of complexes was added to 185 μl of a uCR1 or E-vesicle suspension (in PBS-BSA 0.1% buffer) containing fixed number of CR1 molecules (1.6 × 10^9 CR1). The suspensions were incubated for 30 min at 37°C. Bound C3b-coated HBsAg/Ab complexes were separated from unbound material through oil by centrifugation at 40,000 g. Control experiments showed that C3b-coated HBsAg/Ab complexes were retained on the water/oil interface, and were pelleted only when bound to uCR1 or to CR1 of E-vesicles. Similar assays were performed to assess the specificity of the binding the C3b-coated HBsAg/Ab complexes to uCR1 or to E-vesicles: uCR1 and E-vesicles were preincubated for 30 min at 37°C with an excess of anti-CR1 mAb 3D9 (10,000-fold excess of 3D9 over CR1) before incubation with C3b-coated HBsAg/Ab complexes. As a nonspecific control, the anticomplement factor D mAb was used (21). All measurements were done in duplicate.

Urine and Blood Samples from Normal Individuals and Patients. In 12 normal individuals, 24-h urine collections were used to define the mean uCR1 daily excretion rate, and the ratio uCR1/u-creatinine was calculated. In two individuals, the uCR1 and u-creatine were determined separately in the urine of each micturition over 24 h. Blood samples were collected to measure sCR1 and E-CR1. Similar measurements were performed in different groups of patients: (a) 9 patients with SLE satisfying ⩾ 4 of the 1982 revised criteria of the American Rheumatism Association; (b) 10 patients with various renal diseases on renal biopsy including minimal change disease (2), membranous nephropathy (2), focal sclerosis (3), and diabetic nephropathy (3). In addition, urine and blood samples were collected in 11 patients who had undergone renal transplantation (at least 6 mo before the study) to compare their respective uCR1 and E-CR1 phenotypes. Similar analysis were performed in four patients who had undergone bone marrow transplantation (at least 6 mo before the study) as a treatment for hematological malignancies.

Statistical Analysis. Data were compared by using the Student's t test or the nonparametric Mann-Whitney test. Correlations were studied by regression analysis.

Results

Detection of CR1 Antigen in Urine. In the human kidney, visceral epithelial cells of glomeruli (podocytes) express CR1

(4). The possibility that such cells release CR1 into the urine was investigated. Fresh urinary samples were centrifuged at 1,500 g to remove cell debris and assayed for CR1 antigen by a sandwich ELISA using two mAb. The specificity of this ELISA has been established previously for soluble CR1 in plasma (sCR1) (15). CR1 was detected in urine (uCR1), and there was a dose–response relationship between the volume of urine (between 10 and 50 μl) and the amount of CR1 antigen detected. To confirm the specificity of the ELISA signal in urinary samples, fresh urine was depleted of uCR1 by immunoadsorption chromatography using Sepharose-bound mAb. Such uCR1-depleted urine was negative in the ELISA (data not shown). In the first normal urines (n = 12) tested, the concentration of uCR1 varied from 0.96 to 4.2 ng/ml (mean = 2.38 ± 1.29 ng/ml) corresponding to an approximately 10-fold lesser concentration than sCR1 in plasma. To examine the effects of different storage conditions on the concentration of uCR1, urine samples from three normal individuals (previously centrifuged at 1,500 g for 15 min) were stored for 2 h at various temperatures or frozen at −80°C. No significant differences in uCR1 concentrations were observed between 18 and 37°C, however, the storage of urine at 4°C was responsible for a significant drop of uCR1 in one urine sample, from 2.1 to 0.6 ng/ml, which may have been due to the formation of urate crystals at 4°C in this sample. Freezing urine at −80°C did not modify uCR1 concentrations. Thus, all urine samples were left at room temperature before being frozen at −80°C.

In 12 normal individuals, 24-h urine collections were performed (Table 1). The mean daily excretion rate of uCR1 was 3.12 ± 1.15 (1 SD) μg/24 h (range, 1.39 – 5.22 μg/24 h). In these samples, the ratio of a uCR1/u-creatinine was 0.23 ± 0.08 (1 SD) μg/mmol. In two individuals in whom uCR1

Table 1. *Daily Excretion Rate of uCR1 in Normal Individuals*

Subject	uCR1	U-creatinine	uCR1/creatinine ratio
	(μg/24 h)	*(mmol/24 h)*	*(μg/mmol)*
1	4.22	12.5	0.34
2	3.08	9.2	0.33
3	1.39	8.4	0.17
4	2.66	12.3	0.22
5	3.65	13.2	0.28
6	1.85	11.9	0.16
7	2.68	15.5	0.17
8	5.22	14.8	0.35
9	4.26	18.6	0.23
10	1.78	14.5	0.12
11	2.83	18.1	0.16
12	3.87	21.3	0.18
Mean (± 1 SD)	3.12 ± 1.15	14.2 ± 3.8	0.23 ± 0.08

Table 2. *Effect of Ultracentrifugation on CR1 Concentration in Urine (uCR1), E-vesicles (E-CR1), and Serum (sCR1)*

| | CR1 (ng/ml) | | | | |
| | Urine samples* | | | | |
	1	2	3	E-vesicles‡	Serum§
Before centrifugation	3.71	3.05	2.10	20.1	35.0
After 60 min centrifugation					
at 50,000 *g*	1.47	1.07	0.39	0	ND
at 200,000 *g*	0	0	0	ND	34.8

* Fresh urine samples from three different normal donors.
‡ E-vesicle suspensions in PBS.
§ Fresh serum from a normal donor.

concentrations were determined separately in the urine obtained from each micturition during 24 h, there was a direct correlation between the uCR1 and the creatinine concentrations (r = 0.89 and r = 0.82, in both: $p < 0.05$), corresponding to mean uCR1/u-creatinine ratios of 0.25 and 0.21. It is noteworthy that no correlation was found between uCR1 elimination and sCR1 or E-CR1 numbers (data not shown).

uCR1 Is Membrane Bound. The next step was to determine whether uCR1 was free, similarly to sCR1 in plasma, or attached to membrane fragments. It has been shown that E which have been attacked by complement or depleted of ATP release small fragments of cell membranes in the form of vesicles (18, 26). These vesicles contain several membrane proteins including CR1 (E-CR1). After ultracentrifugation for 1 h at 50,000 *g*, the CR1 bound to E-vesicles is pelleted, whereas sCR1 from plasma remains in solution even after centrifugation at 200,000 *g* (15, 18, and Table 2). The concentration of a uCR1 was reduced in the supernatant after centrifugation at 50,000 *g* for 1 h, and no uCR1 remained
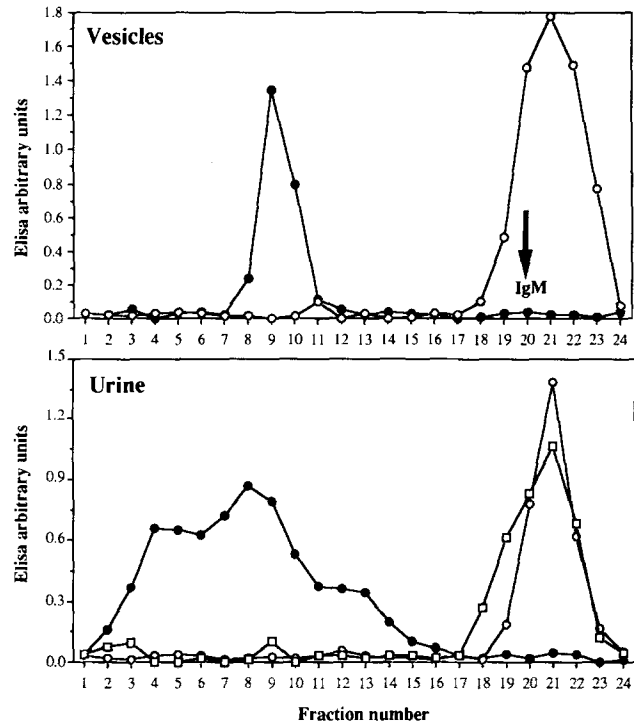

Figure 1. SDGU of E-vesicles derived from ATP-depleted human E (*top*) and urine from a normal individual (*bottom*). Fractions were assayed for CR1 antigen. Samples of E-vesicles or urine (●) were ultracentrifuged as described in Materials and Methods. Similar samples were treated with a 0.5% solution of SDS (○) or Triton (□, *bottom*) before application onto the gradients. The bottom of the gradient is on the left. (*Arrow*) Position of radiolabeled IgM.

in solution after 200,000 *g* (Table 2). The u-CR1 was recovered in the pellet that had been resuspended in buffer (data not shown).

Urine samples were then subjected to ultracentrifugation on sucrose density gradients and compared with sCR1 from plasma and to E-vesicle–bound CR1 obtained after ATP depletion of human E (Fig. 1). sCR1 was found in late fractions

Table 3. *Proteins and Lipids Associated with Partially Purified uCR1 from Four Normal Subjects*

Preparation (CR1 phenotype)	uCR1	Protein	Cholesterol	Phospholipids	Cholesterol/ phospholipids ratio
	μg/ml				
1 AA	0.737	1,170	191.1	144.0	1.33
2 AA	0.199	820	223.0	138.5	1.61
3 BB	0.053	800	116.8	77.7	1.50
4 AA*					
(1)	0.540	1,100	172.6	119.6	1.44
(2)	0.170	312	54.8	38.6	1.42

* Done twice at 1-mo interval.

having a smaller sedimentation rate than IgM (data not shown), whereas uCR1 and E-vesicle–bound CR1 were found in the initial fractions, with uCR1 being recovered over a wider range of fractions. However, when uCR1 or E-vesicle were incubated with two types of detergents (0.5% SDS or 0.5% Triton) in order to dissolve membranes before ultracentrifugation, the CR1 antigen was displaced and recovered in the same fractions as sCR1. The difference in size between uCR1 and sCR1 was also demonstrated after gel filtration chromatography on Sephacryl-300, i.e., uCR1 antigen was

present in the initial fractions ($\geqslant 10^6$ M_r, whereas sCR1 from plasma was recovered as expected between IgM and IgG (data not shown).

To analyze whether uCR1 was found in fractions containing cell membranes, the four fractions of the Sephacryl-300 chromatography containing the highest concentration of uCR1 antigen were pooled and concentrated, and lipid contents were determined. Four different urine samples were analyzed and in all there was an almost fixed ratio of cholesterol to phospholipids (mean = 1.45) (Table 3). uCR1 from one individual
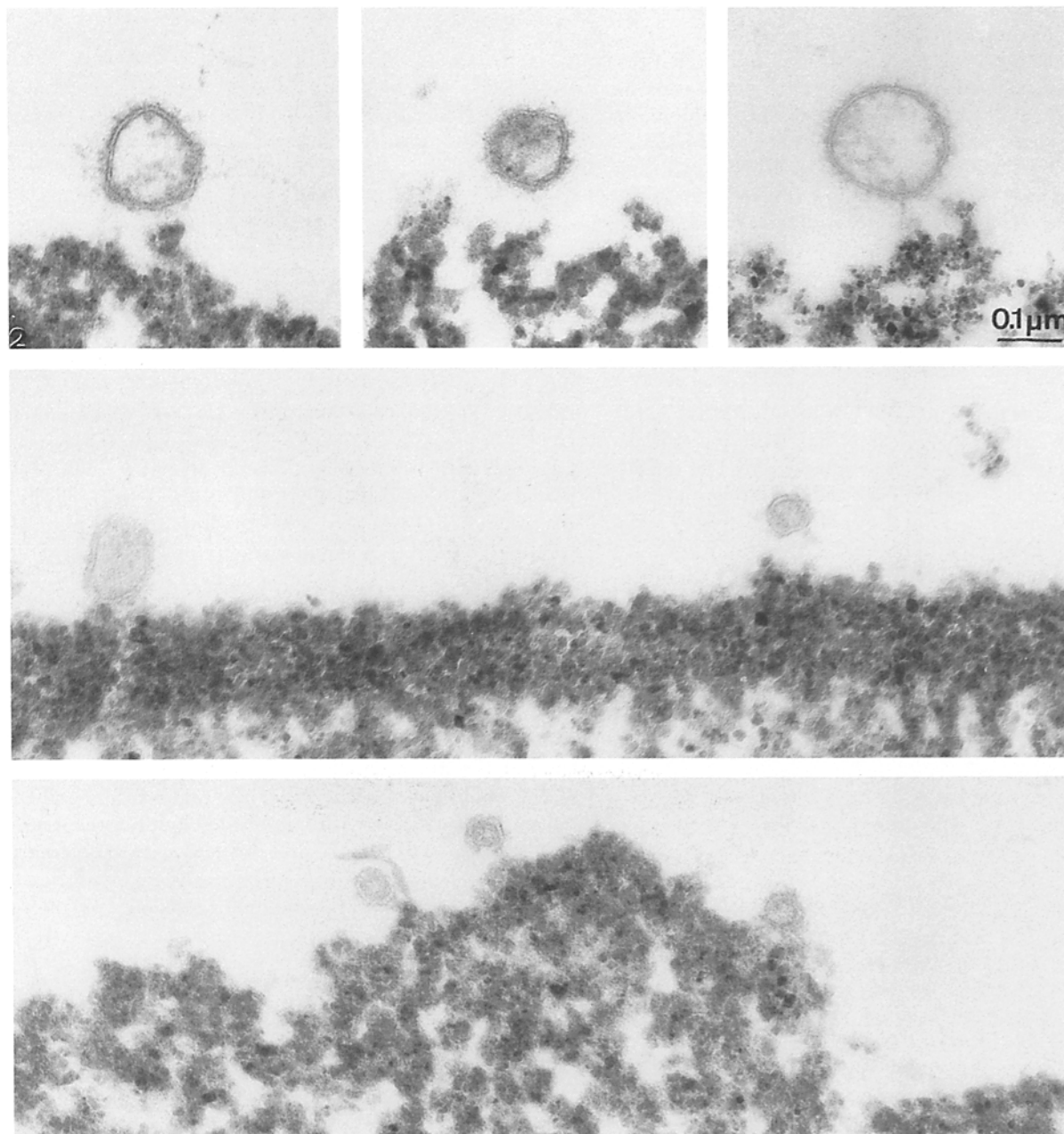

Figure 2. Electron microscopy of urinary vesicles purified by immunoaffinity with anti-CR1 coated magnetic particles. Representative examples of vesicles fixed onto the magnetic particles are shown.

was purified twice at 1-mo intervals. In both samples, the relative concentrations of uCR1, total proteins, and lipids were very similar.

However, this analysis only indicated the copurification of uCR1 with membranes. A direct evidence for the association between uCR1 and membranes was obtained by ultrastructural studies. uCR1 partially purified by Sephacryl-300 was immunopurified with magnetic particles coated with mAb against CR1 (3D9), and fixed. By electron microscopy, double membrane structures typical of cell membranes were seen attached to the magnetic particles, most in the form of vesicles of various sizes without any visible cytoplasmic structures inside (Fig. 2). No such vesicles were seen attached to the beads in the control experiment performed with a nonrelevant mAb (anticomplement factor D mAb).

Characterization of uCR1. The structural characteristics of uCR1 were analyzed next. After partial purification by Sephacryl-300, uCR1 was applied to 4% SDS-PAGE under nonreducing conditions. The gels were blotted onto nitrocellulose membranes that were probed with ^{125}I-3D9 mAb and autoradiographed. Two observations were made. First, the apparent molecular mass of uCR1 was slightly larger (15 kD) than that of E-CR1 (Fig. 3). Second, the structural polymorphism of E-CR1 was also found for uCR1 (27–30), and for the 10 normal individuals tested, the polymorphism of uCR1 was identical to that of E-CR1. These individuals included the AA, AB, BB, and AD phenotypes. Differences in N-glycosylation have shown to account for cell-specific molecular weight differences of CR1 between PMN and E (31). To assess the possible role of N-linked oligosaccharides in the difference in the molecular weights observed for E-CR1 and uCR1, partially purified uCR1 and E-CR1 derived from solubilized E membranes or E-vesicles were treated with N-glycosidase F. The size of both uCR1 and E-CR1 was reduced by treatment with N-glycosidase F, however the difference in molecular weight was not totally abrogated (Fig. 4). This experiment was performed four times, using two different donors, and the results repeatedly showed the persistence of
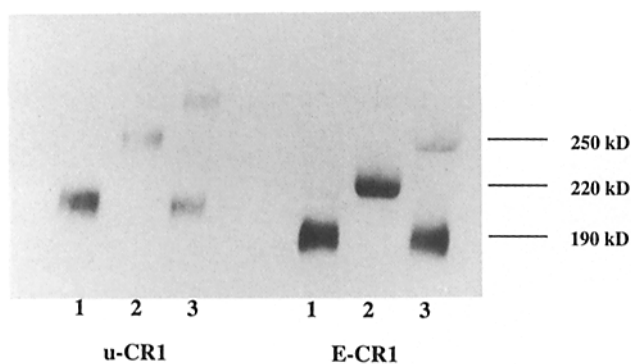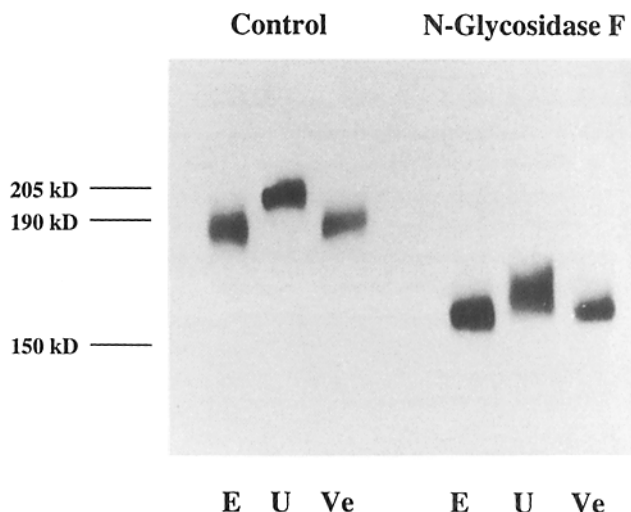

Figure 4. N-glycosidase F treatment of uCR1 and E-CR1 from a normal individual homozygous for the A allele. Partially purified uCR1 (*U*) and E-CR1 from solubilized E membranes (*E*) and E-vesicles (*Ve*) (see Materials and Methods) were treated with N-glycosidase F or buffer alone (*Control*). After enzymatic treatment, the samples were analyzed by 4% SDS-PAGE and immunoblotting.

a small difference in molecular weight after N-glycosidase F treatment. Thus the variation in apparent molecular weight was not entirely due to the presence of N-linked oligosaccharides. The difference in size was also seen when uCR1 and E-CR1 from E were immunoprecipitated with the anti-CR1 3D9 mAb before being electrophoresed, i.e., uCR1 appeared slightly larger than E-CR1 (data not shown). Finally, the size of uCR1 was also 15 kD larger than sCR1 purified from serum (sCR1) by immunoaffinity on anti-CR1 (3D9) mAb-Sepharose (15) (Fig. 5), and ~5–10 kD larger than CR1 obtained from solubilized PMN membranes (Fig. 6).

uCR1 Originates from the Kidney. Vesicle-bound uCR1 may originate from the kidney podocytes, circulating cells, or the soluble form in plasma. We took advantage of the structural polymorphism of CR1 to investigate this question. In patients who had undergone renal transplantation, a discrepancy between the structural alleles of CR1 between E-CR1 and uCR1 would indicate that uCR1 derived from the grafted kidney. In 11 patients with transplanted kidneys, the structural alleles of uCR1 and E-CR1 differed on seven occasions (Fig. 7 and Table 4).

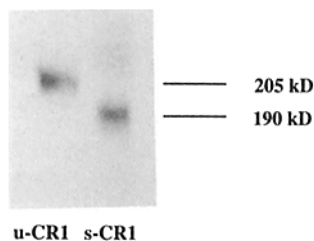We also analyzed E-CR1 and uCR1 structural polymor-

Figure 3. uCR1 and E-CR1 phenotypes in three normal individuals bearing different CR1 alleles. Respectively, lanes: (*1*) the AA (190 kD), (*2*) the BB (220 kD), and (*3*) the AD (190 and 250 kD) alleles. Samples of partially purified uCR1 and E-CR1 from solubilized E membranes were analyzed by 4% SDS-PAGE and immunoblotting with ^{125}I-3D9 mAb.

Figure 5. Comparison of uCR1 and sCR1 from a normal individual homozygous for the A allele. sCR1 was purified from serum by immunoadsorption. The samples were analyzed by 4% SDS-PAGE and immunoblotting.
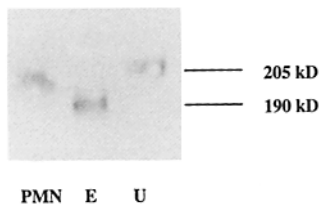
Figure 6. Comparison of PMN-CR1 (*PMN*), E-CR1 (*E*) and uCR1 (*U*) from a single donor homozygous for the A allele. Solubilized PMN and E membranes were prepared as described in Materials and Methods. The samples were analyzed by 4% SDS-PAGE and immunoblotting.

phism in the reverse situation, i.e., after bone marrow transplantation. Four patients were studied; the donor was always a family relative. In one patient a discordance between E-CR1 and uCR1 was found (AA and AB respectively), and as expected the donor (brother) was AA (data not shown). Thus the E-CR1 allele (AA) had been acquired after bone marrow transplantation.

uCR1 Mediates Immune Adherence. The capacity of uCR1 to bind C3b was analyzed using radioiodinated C3b-coated HBs Ag/Ab immune complexes as described in Materials and Methods. uCR1-containing vesicles were able to bind immune complexes (Fig. 8). The binding was specific, since immune complexes were not pelleted in the control experiments performed either in the absence of uCR1 or in the presence of uCR1 and a 10,000-fold excess of anti-CR1 3D9 mAb (which blocks the C3b-binding sites of CR1). Although the immune adherence increased directly with the amount of uCR1 used, the efficiency of the binding was lower than that of E-CR1 since, at an equal CR1 concentration, E-CR1 of E-vesicles bound more immune complexes than uCR1 (Fig. 8).

Levels of uCR1 in Patients with SLE and Various Renal Diseases. Acquired loss of renal podocyte CR1 has been described in several diseases in which glomeruli are severely damaged, such as in proliferative lupus nephritis, focal hyalinosis, and crescentic nephritis (4, 7, 10–13). In a preliminary study of nine patients with SLE, we found a low uCR1 in the three patients with histological evidence of severe proliferative lupus nephritis (Table 5). There was no correlation between the activity of the disease or E-CR1 numbers and uCR1. Out of 10 patients with various renal diseases, 3 had low uCR1, all of whom had focal sclerosis (Table 6). There was no correlation between uCR1 and proteinuria in these patients.

Discussion

The major conclusion of this study is that a distinct form of CR1 (uCR1) is present in human urine. Its unique char-
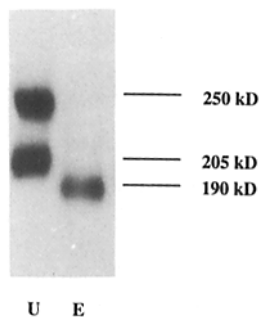

Figure 7. Discordance between uCR1 (*U*) and E-CR1 (*E*) phenotypes in a patient who underwent renal transplantation. The samples were analyzed by 4% SDS-PAGE and immunoblotting. This patient had E-CR1 of the A phenotype (corresponding genotype AA), and uCR1 of the AB phenotype (corresponding to the genotype of the kidney donor).

Table 4. *Analysis of E-CR1 and uCR1 Phenotypes in Patients Who Underwent Renal Transplantation*

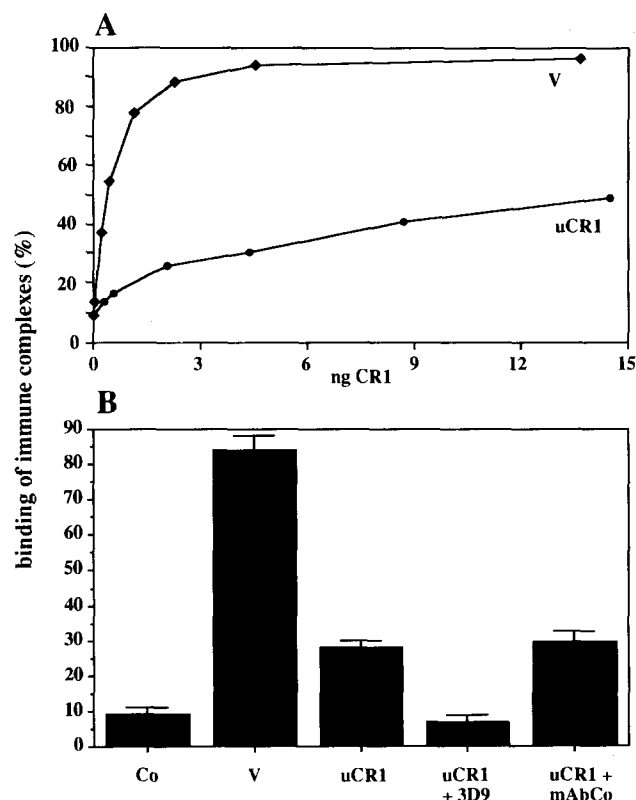| Subject	E-CR1	uCR1
1	AB	A
2	A	AB
3	AB	AD
4	A	A
5	A	A
6	AB	A
7	A	AB
8	A	A
9	A	A
10	A	AB
11	AB	A

Figure 8. Adherence of C3b-coated HBsAg/Ab immune complexes to CR1 on urinary vesicles (*uCR1*) or E-vesicles (*V*). (*A*) There was a dose-dependent increase in the adherence of immune complexes to both types of vesicles. (*B*) The specificity of the binding was shown by incubating immune complexes alone (control, *Co*), with partially purified *uCR1* in the presence of mAb blocking the binding sites of C3b on CR1 (*3D9*) or a control mAb (*mAbCo*), or with E-vesicles. The amount of E-vesicles used was adjusted to offer the same amount of CR1 than uCR1 (4 ng). The percentage of binding was determined after centrifugation through oil.

Table 5. *uCR1 in Patients with SLE*

Patient Age/sex	Main clinical manifestations	E-CR1 (number/E)	sCR1	uCR1	u-Creatine	p-Creatine	Ratio uCR1/u-creatine
			(ng/ml)	*(ng/ml)*	*(mmol/liter)*	*(μmol/liter)*	
1 26/F	Arthritis, serositis	87	36.9	4.80	7.8	74	0.61
2 38/F	Arthralgia, serositis	156	43.6	2.30	3.3	60	0.69
3 18/F	Arthritis, normal renal biopsy	539	44.5	3.65	25	ND	0.15
4 43/F	Nephritis (II)*‡, arthralgia	375	38.7	1.10	3.7	90	0.29
5 44/F	Nephritis (II)	461	57.2	1.40	6.6	ND	0.21
6 33/F	Nephritis (II), hemolytic anemia	219	28.6	5.35	11.1	80	0.48
7 47/F	Nephritis (IV)‡, CNS damage	356	25.8	0.31	5.8	80	0.05
8 21/F	Nephritis (IV), CNS damage	181	25.1	<0.2	2.7	192	<0.07
9 20/F	Nephritis (IV)‡	295	22.0	0.32	7.5	259	0.04

* Renal biopsy performed in patients 3–9. World Health Organization classification of lupus nephritis: II, mesangial hypercellularity and/or deposits; IV, diffuse proliferative glomerulonephritis.
‡ Patients in whom the nephritis was in clinical remission at the time of the urine collection.
CNS, central nervous system.

acteristics suggest that uCR1 is not derived from either CR1 of E or PMN, or from sCR1 found in plasma, but originates from the renal podocytes.

The uCR1 was initially detected in urine using the sandwich ELISA previously shown to be sensitive and specific for sCR1 in plasma (15). We demonstrated that the assay was reliable for detecting CR1 in urine as well. The concentration of uCR1 was ∼2–3 ng/ml, i.e., 10-fold lower than sCR1 in plasma. Because of its high molecular weight, sCR1 is unlikely to be filtered through the glomerulus to any significant amount, suggesting another origin for uCR1.

By carefully examining its properties, we first showed that uCR1 was not, strictly speaking, soluble since it was pelleted after ultracentrifugation whereas under similar conditions sCR1 remains soluble (15). This strongly suggested that uCR1 was bound to cell membrane fragments. Indeed, when analyzed by SDGU, uCR1 was found to sediment in the initial fractions of the gradient (>19 S), well above the fractions containing sCR1 from plasma, and comigrated with CR1 present on E-vesicles derived from ATP-depleted E. E-vesicles have been shown to constitute a population of spheres with a diameter of ∼200 nm (26), and contain several membrane proteins including CR1 (18, 32). After preincubation with detergents (Triton or SDS), both uCR1 and CR1 of E-vesicles sedimented similarly to sCR1, as would be expected from proteins associated with lipid membranes. More direct evidence for the membrane association of uCR1 was provided by electron microscopy of affinity-purified uCR1. The beads

Table 6. *uCR1 in Patients with Various Renal Diseases*

Patient Age/sex	Disease	E-CR1 (number/E)	sCR1	uCR1	u-Creatine	p-Creatine	Ratio uCR1/u-creatine
			(ng/ml)	*(ng/ml)*	*(mmol/liter)*	*(μmol/liter)*	
1 80/M	MCD with NS	612	33.7	14.5	19.3	98	0.75
2 49/M	MCD with NS	480	39.8	2.32	14.7	95	0.15
3 75/F	MN with proteinuria	615	56.5	3.50	9.7	100	0.36
4 21/F	MN with NS	635	17.4	1.16	2.2	65	0.52
5 67/F	DN with NS	395	33.1	1.53	4.7	283	0.32
6 49/F	DN with proteinuria	272	107	1.30	9.6	188	0.14
7 50/F	DN with proteinuria	309	64.5	1.10	3.7	184	0.30
8 34/F	FS with NS	313	59.7	0.13	4.7	179	0.03
9 76/F	FS with NS	480	60.1	0.20	2.8	527	0.07
10 28/F	FS with NS	ND	43.6	0.23	4.7	175	0.05

DN, diabetic nephropathy; *FS*, focal sclerosis; *MCD*, minimal change disease; *MN*, membranous nephropathy; *NS*, nephrotic syndrome.

coated with anti-CR1 mAb bore on their surface small vesicles of varying sizes presenting double membranes typical of cell membranes. Most of these vesicles looked empty and so it was not possible to distinguish between vesicles released by cells and fragments of cell membranes taking a vesicular conformation upon preparation for the electron microscopy. An additional evidence for the membrane association of uCR1 was provided by the analysis of cholesterol to phospholipid ratio of the fractions containing uCR1 after Sephacryl-300 gel filtration. This ratio (1.45) was higher than expected for cell membranes (most often ≤1 [33, 34]) and it might correspond to the specific composition of the vesicles' membrane. Several reports (35–37) have indicated that the release of vesicles by cells is due to the properties of certain phospholipids or sphingolipids of membranes that are specifically incorporated (or not) into the forming bud on the cell membrane. For instance, protasomes which are small vesicles (mean diameter, 150 nm) released by epithelial cells of the prostate into the seminal fluid, have a cholesterol/phospholipid ratio of ∼2 (38, 39).

When assessed by SDS-PAGE under nonreducing conditions, the apparent molecular mass of uCR1 was slightly larger than that of sCR1, E-CR1 (both 15 kD), and PMN-CR1 (5–10 kD). Differences in N-glycosylations have been shown to account for cell-specific molecular weight differences of CR1 between PMN and E (31). After incubation with N-glycosidase-F, which is known to remove all N-linked oligosaccharides, uCR1 lost ∼30 kD, similarly to E-CR1, so that the small difference in size persisted. Thus, the higher molecular weight of uCR1 could not be attributed only to the presence of N-linked oligosaccharides. Whether other types of glycosylations may explain this difference in size remains possible. However it is worth emphasizing that Lublin et al. (31) could not find any evidence for O-glycosylations in lymphoblastoid cell lines. The larger size of uCR1 may be due to a difference in the amino acid sequence. However this seems unlikely since no alternate splicing has been reported for CR1 (2). Another possibility is that urine is responsible for some subtle modifications in the properties and/or structure of uCR1.

The structural polymorphism of CR1 was conserved for uCR1, thus it was possible to define after SDS-PAGE and immunoblotting, the A, B, and D alleles for uCR1, similarly to E-CR1. As expected in all normal individuals tested, the structural polymorphism was identical for uCR1 and E-CR1. This observation allowed us to define whether the membranes bearing uCR1 stemmed from the renal podocytes or from blood-derived cells which are found in normal urine. In patients who had undergone renal transplantation, there was a frequent discrepancy in the structural polymorphism between uCR1 and E-CR1 which suggested very strongly that uCR1 was of donor origin. That the kidney is the origin of uCR1 was further indicated by analyzing the reverse situation, i.e., a patient who had undergone bone marrow transplantation and in whom the structural polymorphism of E-CR1 was similar to that of the donor of the hematopoietic cells, but different from its uCR1. Since only podocytes are known

to express CR1 in the kidney, the logical conclusion was that membranes (vesicles) bearing uCR1 originated from podocytes.

Many circulating and other cells have been shown to release vesicles by budding at the time of complement attack or under other conditions (40–46). Thus, the release of vesicles by podocytes would by no means be unique. Whether CR1 on the surface of these vesicles plays a biological role remains undefined. The presence of "immune adherence" receptors on podocytes, i.e., in a bodily compartment exposed exclusively to primitive urine, has intrigued many investigators (4–13). The most likely functions of CR1 on podocytes are the transport of C3b-coated immune complexes into urine and the inactivation of C3b in pathological circumstances in which plasma proteins come into contact with podocytes. To support this hypothesis was the observation that uCR1 was capable of mediating immune adherence of C3b-coated immune complexes. However, at an equal amount of CR1, immune adherence was much lower than for E-CR1. This difference may be explained by the unique clustered distribution of CR1 on E which favors immune adherence (47, 48), or by a loss of function of uCR1 by its exposure to urine. However, it is worth emphasizing that the uCR1, being membrane bound, is more efficient at reacting with C3b-coated immune complexes than recombinant sCR1 (Pascual, M., S. Sadallah, and J.-A. Schifferli, unpublished observations). Similar observations have been made for another complement inhibitor. Decay accelerating factor on protasomes of seminal fluid is a more potent inhibitor than its soluble urinary counterpart (49).

The continuous vesiculation phenomenon might contribute to the elimination not only of C3b-coated immune complexes, but also of the membrane attack complex of complement. In an experimental rat model of Heymann nephritis, it has been shown that the C5b–C9 complex is transported across podocytes by multivesicular bodies that release small vesicles coated with C5b–C9 into the urinary space (50). Similar vesicles were recovered in rat urine. C5b–C9 has been found in the urine of patients suffering from membranous nephritis (51, 52). Whether such C5b–C9 is bound to vesicles similar or identical to CR1-bearing vesicles has not yet been determined.

The release into the urine of vesicle-bound CR1 from the podocytes may provide us, for the first time, with a specific marker of podocyte injury in various diseases. In the normal individuals tested, the release of uCR1 appeared to be constant over time, correlating with the elimination of creatinine. In some glomerular diseases, CR1 are known to be lost on podocytes. In particular, CR1 expression is markedly reduced in severe lupus nephritis and focal sclerosis (4). In the few patients with these diseases studied here, several had very low excretion of uCR1. No immunohistochemistry analysis has been performed in these patients to demonstrate a concomitant lack of CR1 on glomerular podocytes in the renal biopsies. Studies are now required to assess whether the suggested correlation between uCR1 and CR1 expression on podocytes is clinically useful, and whether uCR1 is an indicator of the severity of glomerular lesions in specific nephropathies.

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