

## CIRCULATING FACTOR ASSOCIATED WITH INCREASED GLOMERULAR PERMEABILITY TO ALBUMIN IN RECURRENT FOCAL SEGMENTAL GLOMERULOSCLEROSIS

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**Abstract Background.** Heavy proteinuria and progressive renal injury recur after transplantation in up to 40 percent of patients with renal failure caused by idiopathic focal segmental glomerulosclerosis. A circulating factor may be responsible for this recurrence.

**Methods.** To determine whether patients with focal segmental glomerulosclerosis have a circulating factor capable of causing glomerular injury, we tested serum samples from 100 patients with the disorder in an in vitro assay of glomerular permeability to albumin. Of the 56 patients who had undergone renal transplantation, 33 had recurrences. Sixty-four patients, many of whom had undergone transplantation, were being treated with dialysis. Thirty-one patients with other renal diseases and nine normal subjects were also studied.

**Results.** The 33 patients with recurrent focal segmental glomerulosclerosis after transplantation had a higher

mean ( $\pm$ SE) value for permeability to albumin ( $0.47 \pm 0.06$ ) than the normal subjects ( $0.06 \pm 0.07$ ) or the patients who did not have recurrences ( $0.14 \pm 0.06$ ). After plasmapheresis in six patients with recurrences, the permeability was reduced (from  $0.79 \pm 0.06$  to  $0.10 \pm 0.05$ ,  $P = 0.008$ ), and proteinuria was significantly decreased. Patients with corticosteroid-sensitive nephrotic syndrome or with membranous nephropathy after transplantation had low levels of serum activity. The circulating factor bound to protein A and hydrophobic-interaction columns and had an apparent molecular mass of about 50 kd.

**Conclusions.** A circulating factor found in some patients with focal segmental glomerulosclerosis is associated with recurrent disease after renal transplantation and may be responsible for initiating the renal injury. (N Engl J Med 1996;334:878-83.)

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**F**OCAL segmental glomerulosclerosis develops and progresses to renal failure in some patients who have idiopathic nephrotic syndrome that is resistant to treatment with corticosteroids and cytotoxic drugs.<sup>1-3</sup> After transplantation, up to 40 percent of such patients have recurrences.<sup>4-9</sup> The glomerular abnormalities in patients with established disease include focal and segmental glomerulosclerosis and hyalinosis, although fusion of epithelial-cell foot processes may be the only abnormality early in the course of the disease.<sup>10</sup>

It has been proposed that because some patients with recurrent focal segmental glomerulosclerosis have a response to treatment with plasmapheresis,<sup>11-16</sup> there may be a circulating factor that alters the glomerular barrier to protein filtration.<sup>17</sup> In support of this possibility, several plasma factors have been reported to increase vascular permeability<sup>18-23</sup> or cause proteinuria in animals.<sup>16,24-26</sup> We have developed an in vitro method for assessing the permeability of glomeruli<sup>27</sup> and have documented that incubation of glomeruli with protamine,<sup>27</sup> superoxide,<sup>28</sup> activated leukocytes,<sup>29</sup> or Heymann

antibody and complement<sup>30</sup> increases glomerular permeability to albumin. In the present study, we used this assay to demonstrate that serum from patients with focal segmental glomerulosclerosis causes an increase in glomerular permeability to albumin. In addition, we determined the prevalence of permeability activity in patients with the disorder, the association between increased permeability and the recurrence of disease after renal transplantation, and some characteristics of the circulating factor.

## METHODS

### Study Subjects

We studied 100 patients with focal segmental glomerulosclerosis, 31 patients with other renal diseases, and 9 normal subjects (Table 1). The diagnosis of focal segmental glomerulosclerosis in native kidneys was based on renal biopsies in patients evaluated for proteinuria or renal insufficiency and on the presence of segmental obliteration of capillaries by increased extracellular matrix in some glomeruli. Mesangial proliferation and segmental, coarsely granular deposits of IgM and complement in capillary walls were seen in some biopsy specimens. Among the patients who had received renal transplants who underwent biopsies to evaluate proteinuria, recurrent focal segmental glomerulosclerosis was diagnosed on the basis of the same changes as those observed in native kidneys<sup>14</sup> or on the basis of foot-process fusion on electron-microscopical examination in patients without other abnormalities. Corticosteroid-sensitive nephrotic syndrome, glomerulonephritis, hypertensive nephropathy, and polycystic kidney disease were diagnosed on the basis of clinical findings or renal biopsies. Membranous nephropathy in renal allografts was diagnosed on the basis of characteristic subepithelial deposits on electron microscopy.<sup>31</sup> The study was approved by the Human Subjects Committee at the University of Kansas Medical Center, and all subjects gave informed consent.

### Isolation of Glomeruli and Measurement of Permeability to Albumin

Glomerular-capillary permeability to albumin was measured by determining the degree of capillary expansion produced by an oncotic gradient. This assay is based on the principle that the effective pres-

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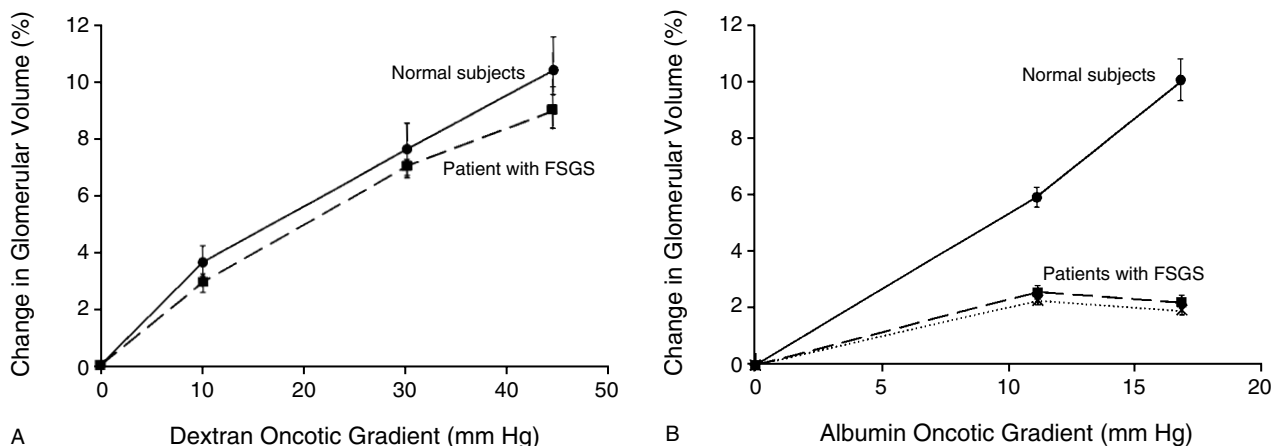


Figure 1. Mean ( $\pm$ SE) Change in Glomerular Volume in Response to Oncotic Gradients of Dextran and Albumin.

After glomeruli were incubated in solutions containing 2 percent pooled serum from normal subjects or 2 percent serum from a patient with focal segmental glomerulosclerosis (FSGS), the increase in glomerular volume in response to dextran was similar (Panel A). The glomerular response to albumin, however, was markedly diminished after incubation with serum from the same patient with FSGS (broken line) or another patient with the disorder (dotted line), as compared with the response after incubation with normal serum (Panel B). For each gradient, the values shown represent the mean values for 15 glomeruli.

sure resulting from an oncotic gradient is equal to the product of the measured gradient and the reflection coefficient of the membrane — in this case, the glomerular capillary.<sup>27</sup>

Glomeruli were isolated from the superficial renal cortex of normal male Sprague-Dawley rats with the use of standard sieving techniques in a previously described isolation medium containing 4 g of bovine serum albumin per deciliter as an oncotic agent.<sup>32</sup> The glomeruli were then incubated for 10 minutes at 37°C in isolation medium containing a 1:50 dilution of serum from a study participant, pooled normal serum, or fractions of plasma. Unless otherwise specified, the fractions were diluted with 25 mM TRIS-hydrochloride (pH 7.8) containing 0.9 percent sodium chloride so that the protein concentrations ranged from 3 to 5 mg per milliliter. After the 10-minute incubation period, the glomeruli were observed by video microscopy before and after the medium was replaced by fresh medium containing 1 g of bovine serum albumin per deciliter. This process resulted in an oncotic gradient across the capillary wall, distention of individual capillaries, and an increase in the glomerular volume. The glomerular volume was calculated as  $V = 4/3\pi(D/2)^3$ , where D represents the average of four diameters measured at 45-degree angles to one another. Glomerular permeability to albumin was calculated as follows<sup>27-30</sup>:

$$P_{\text{albumin}} = 1 - (\Delta \text{ volume}_{\text{experimental glomerulus}} / \Delta \text{ volume}_{\text{control}}),$$

where  $P_{\text{albumin}}$  denotes glomerular permeability to albumin and

$$\Delta \text{ volume} = \frac{\text{final volume} - \text{initial volume}}{\text{initial volume}}$$

Four to five glomeruli were used to test each sample, and the mean value for permeability to albumin was calculated. When multiple assays were performed with serum from the same patient, the average value for permeability to albumin was used. The measurements were made without knowledge of the diagnosis or the manipulations the sample had undergone.

Glomerular permeability to albumin can be calculated from changes in the glomerular volume in response to an albumin gradient only if the response to an impermeable solute is not altered by the experimental manipulations.<sup>27</sup> Glomeruli incubated with isolation medium (4 g of bovine serum albumin per deciliter) containing serum from patients with focal segmental glomerulosclerosis were transferred to medium containing neutral dextran with a molecular weight of 252,000. The glomerular responses to the oncotic gradients produced by this impermeable solute were assessed with the use of a series of lower concentrations of dextran. As shown in Figure 1A,

glomeruli that had been incubated with serum from patients with focal segmental glomerulosclerosis had a response to dextran gradients that was similar to the response of glomeruli incubated with serum from normal subjects. In contrast, the glomeruli incubated with serum from the patients with focal segmental glomerulosclerosis had a diminished response to concentrations of bovine serum albumin (Fig. 1B).

#### Reliability of the Assay

The correlation among multiple determinations of glomerular permeability to albumin with the use of serum from 35 patients was 0.72 ( $P < 0.001$ ). There was 83 percent agreement between the first and second measurements of permeability to albumin, with agreement defined as a difference of less than 0.3 between the two values. Storage of serum at  $-20^\circ\text{C}$  for up to two years had no apparent effect on serum activity.

Incubation of five human glomeruli (obtained from a nephrectomy specimen) with serum from a patient with recurrent focal segmental glomerulosclerosis resulted in a mean ( $\pm$ SE) increase in the permeability value to  $0.79 \pm 0.11$ , which was similar to the value after five rat glomeruli had been incubated with the same serum ( $0.82 \pm 0.05$ ).

#### Fractionation of Serum

Each of the fractionation steps was carried out with discarded plasma from plasmapheresis in at least four different patients. After the lipoproteins had been removed,<sup>33</sup> protein was precipitated at ammonium sulfate saturations of 50 to 80 percent. The precipitates were dissolved in assay dilution buffer and tested for activity. Because activity was detected only in the 70 to 80 percent precipitate, only this precipitate was used for further fractionation. The precipitate was fractionated by affinity-column chromatography with Affigel-Blue (Bio-Rad, Hercules, Calif.), concanavalin A Sepharose (Sigma, St. Louis), heparin agarose (Bio-Rad), or protein A agarose (Bio-Rad); cation-exchange chromatography with Mono-S (Bio-Rad); anion-exchange chromatography with Mono-Q (Bio-Rad) or DEAE-Sepharose (Sigma); hydrophobic-interaction chromatography (HIC-methyl, Bio-Rad); and Sephacryl S-300 size-exclusion chromatography (Pharmacia-LKB, Piscataway, N.J.).

#### Statistical Analysis

The results are expressed as means  $\pm$ SE. Comparisons among groups were made with one-way analyses of variance, Student's t-test, or chi-square tests. The reliability of repeated testing was assessed on

Table 1. Demographic and Clinical Characteristics of the Study Subjects and Values for Permeability Activity.\*

GROUP	AGE	SEX	DIALYSIS†	SERUM CREATININE	SERUM ALBUMIN	SERUM CHOLESTEROL	PERMEABILITY ACTIVITY
	yr	% female	no. of patients	mg/dl	g/dl	mg/dl	
Normal subjects (n=9)	—	—	—	—	—	—	0.06±0.07
Patients with focal segmental glomerulosclerosis							
Without transplants (n=44)	28±3	26	27	8.2±0.6	3.4±0.1	187±10	0.18±0.04
With transplants							
No recurrence (n=23)	37±3	38	20	9.9±1.0	3.5±0.1	176±14	0.14±0.06
Recurrence (n=33)	29±3	23	17	5.5±0.8	3.3±0.2	282±31	0.47±0.06‡
Patients with other renal diseases							
Corticosteroid-sensitive nephrotic syndrome (n=9)	5±1	56	0	0.4±0.1	2.2±0.5	NA	-0.50±0.05
Membranous nephropathy after transplantation (n=5)	43±5	0	0	3.1±0.8	3.0±0.5	401±48	0.19±0.04
End-stage renal disease (n=17)	47±4	75	17	13.0±1.0	3.9±1.0	178±9	0.22±0.10

\*Plus-minus values are means ±SE. The values for permeability activity are the average values in assays of each serum sample. To convert the values for serum creatinine to micromoles per liter, multiply by 88.4; to convert the values for serum cholesterol to millimoles per liter, multiply by 0.02586. NA denotes not available.

†Numbers indicate the numbers of patients undergoing dialysis at the time of sample collection.

‡P=0.003 for the comparison with the normal subjects; P<0.001 for the comparison with the patients who had focal segmental glomerulosclerosis without a recurrence after transplantation.

the basis of Pearson's correlation coefficient and the percentage of agreement. In studies to determine the relation of serum activity to the recurrence of disease after transplantation, patients were grouped according to the average value for permeability to albumin. A test for linearity of trends was used to assess the relation between the recurrence rate and the value for permeability.

## RESULTS

Incubation with pooled normal serum did not alter glomerular permeability to albumin. Medium containing pooled normal serum was therefore used as the control in subsequent experiments. In assays with serum samples from the nine normal subjects, the mean

(±SE) value for glomerular permeability to albumin was  $0.06 \pm 0.07$  (Table 1).

### Patients with Focal Segmental Glomerulosclerosis

Among the 44 patients with focal segmental glomerulosclerosis who had not received renal transplants, 27 of whom were being treated with dialysis when serum samples were obtained, the mean value for glomerular permeability to albumin was  $0.18 \pm 0.04$ . Twenty percent of these patients had values of 0.50 or higher. Among the 56 patients who had undergone renal transplantation and were followed for at least six months, the permeability values were  $0.14 \pm 0.06$  for those without recurrent focal segmental glomerulosclerosis and  $0.47 \pm 0.06$  for those with recurrent disease. The relation between glomerular permeability to albumin and the prevalence of recurrent focal segmental glomerulosclerosis was examined by stratifying patients according to the permeability value, with arbitrary breaks in the distribution of the values. The frequency of recurrent focal segmental glomerulosclerosis increased from 31 percent in the group with values below 0.10 to 100 percent in those with values above 0.69 ( $P < 0.001$ ) (Fig. 2).

Among the 30 patients with focal segmental glomerulosclerosis from whom serum samples were obtained in the year preceding transplantation, the cumulative incidence of recurrent disease after transplantation was 17 percent in the patients with permeability values under 0.50 and 86 percent in those with values equal to or higher than 0.50 (Table 2). Thus, the relative risk of recurrent focal segmental glomerulosclerosis was about five times higher in the patients with pretransplantation values of at least 0.50.

### Effect of Plasmapheresis

Ten serum samples were obtained during a 46-month period from one patient who had previously undergone

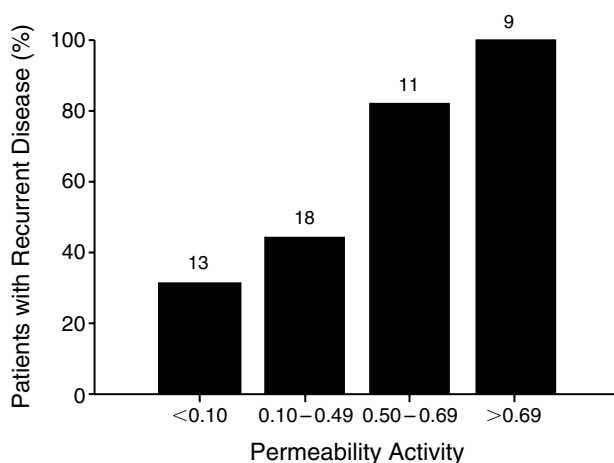


Figure 2. Relation between Permeability Activity and Recurrent Focal Segmental Glomerulosclerosis after Renal Transplantation. Patients followed for six months or more after renal transplantation were divided arbitrarily into four groups according to the serum value for permeability activity. The frequency of recurrent focal segmental glomerulosclerosis increased with increasing values ( $P < 0.001$ ). The number of patients in each group is shown above the bars.

Table 2. Frequency of Recurrent Focal Segmental Glomerulosclerosis in 30 Patients after Transplantation, According to the Value for Permeability Activity in Serum Samples Obtained before Transplantation.

PERMEABILITY ACTIVITY*	NO RECURRENCE		RECURRENCE	
	no. of patients (%)			
<0.50	19		4	(17)
≥0.50	1		6	(86)
Total	20		10	(33)

\*P<0.001 for the comparison between the patients with values under 0.50 and those with values greater than or equal to 0.50.

renal transplantation and had subsequently had a fulminant recurrence of focal segmental glomerulosclerosis requiring a return to hemodialysis. The permeability values were all high, ranging from 0.66 to 0.98 and averaging  $0.84 \pm 0.03$ . The coefficient of variation of 12 replicate assays of activity in a single sample from this patient was 11 percent. A series of three plasmapheresis treatments resulted in a marked reduction in the level of activity (Fig. 3), which then increased gradually after the last treatment.

Plasmapheresis was also carried out in six other patients during the course of treatment for recurrent focal segmental glomerulosclerosis. As shown in Figure 4A, each patient had a high level of permeability activity before plasmapheresis ( $0.79 \pm 0.06$ ), with a decreasing level of activity after plasmapheresis ( $0.10 \pm 0.05$ ,  $P=0.008$ ), and activity was detected in the plasmapheresis fluid from each patient. Urinary protein excretion decreased from  $8.9 \pm 2.5$  to  $0.9 \pm 0.2$  g per gram of creatinine after plasmapheresis ( $P=0.02$ ) (Fig. 4B). The details of the plasmapheresis protocols and the clinical course of three of these patients have been reported elsewhere.<sup>14</sup>

#### Patients with Other Renal Diseases

The mean value for permeability activity in serum samples from the nine children with corticosteroid-sensitive nephrotic syndrome was  $0.50 \pm 0.05$  (Table 1). This value was not significantly different from the value in serum from normal subjects. Among the five patients with membranous nephropathy in renal allografts, each of whom had proteinuria and hypercholesterolemia and was receiving prednisone, azathioprine, and cyclosporine, the mean permeability activity was  $0.19 \pm 0.04$ .<sup>31</sup> We also studied 17 patients with end-stage renal disease due to glomerulonephritis, hypertension, or polycystic kidney disease. The mean value in serum from these patients was  $0.22 \pm 0.10$ , which was not significantly different from the value in serum from the normal subjects.

#### Initial Characterization of the Factor

The permeability activity in serum samples was not altered by heating the serum to 60°C for 20 minutes, in-

dicating that complement was not required for the production of glomerular injury. In contrast, the activity was abolished by boiling the serum for 10 minutes.

Plasmapheresis fluid from six patients with recurrent focal segmental glomerulosclerosis and high levels of permeability activity was fractionated with ammonium sulfate. Only the precipitate obtained at 70 to 80 percent saturation was active.

Preliminary chromatographic studies were carried out with the 80 percent precipitate. The activity was eluted with the bound fraction from Affigel-Blue, heparin agarose, protein A agarose, DEAE-Sephacel, Mono-Q, or HIC-methyl columns. In each case the active fraction accounted for less than 5 percent of the protein applied to the column. The activity was eluted with the unbound fraction from concanavalin A Sepharose and the Mono-S column and was eluted from the Sephacryl S-300 column with a large peak corresponding to a molecular mass of about 50 kd. The activity of the active fractions depended on the protein concentration and was maximal at a concentration as low as 0.1 µg per milliliter of incubation medium.

#### DISCUSSION

The diagnosis of focal segmental glomerulosclerosis is based on histologic criteria, including scarring that involves glomeruli in a heterogeneous pattern both within the population of glomeruli and within segments of individual glomeruli. Focal sclerosis can occur in systemic diseases, including human immunodeficiency virus infection, as well as in the absence of known systemic illness. The clinical course of the disorder is characterized by moderate to marked proteinuria and progressive renal insufficiency. The nephrotic syndrome and progressive glomerular sclerosis recur in 30 to 40 percent of patients after renal transplantation. Treatment of patients with recurrences has included immunosuppression with

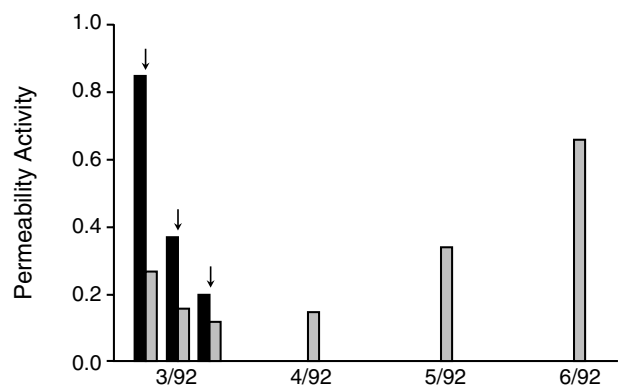


Figure 3. Permeability Activity before Plasmapheresis (Black Bars) and after Plasmapheresis (Gray Bars) in a Patient with Recurrent Focal Segmental Glomerulosclerosis after Renal Transplantation.

The three plasmapheresis treatments (arrows) were performed on alternate days, with serum samples obtained at the end of each treatment.

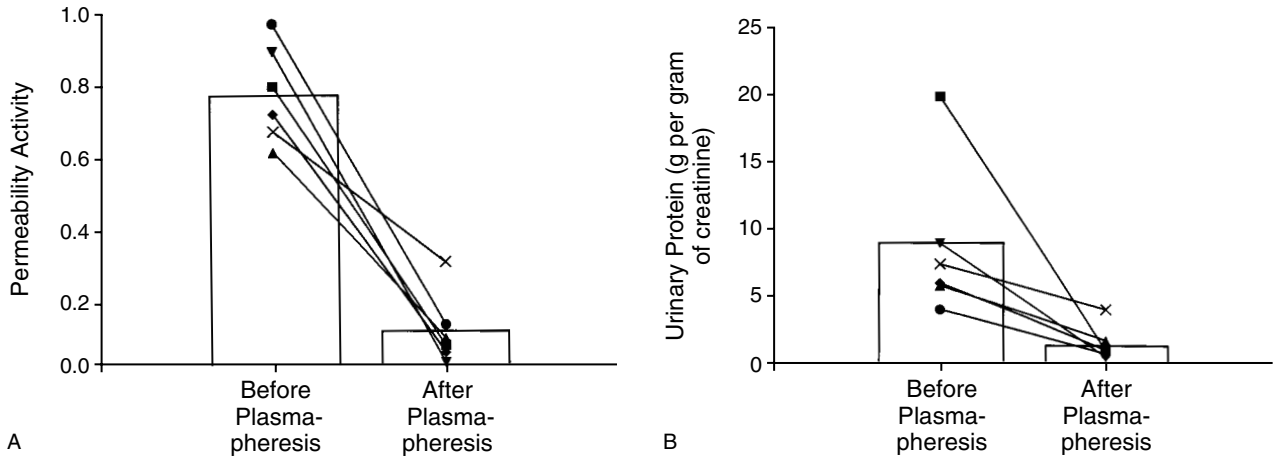


Figure 4. Permeability Activity (Panel A) and the Ratio of Urinary Protein to Creatinine (Panel B) before and after 4 to 14 Plasma-pheresis Treatments in Six Transplant Recipients with Recurrent Focal Segmental Glomerulosclerosis.

Plasmapheresis was carried out daily for two to four days and then on alternate days. Permeability activity was measured in serum samples obtained immediately before the first treatment and within 24 hours after the last treatment. Urinary protein and creatinine were measured before treatment and 4 to 10 days after the last treatment. The bars indicate the mean values.

high-dose cyclosporine and trials of plasma exchange or immunoadsorption.

In our study, serum samples from patients with focal segmental glomerulosclerosis increased glomerular permeability to albumin, and the level of activity was highest in serum from patients with recurrent disease after transplantation. No activity was detected in serum from normal subjects and patients with the corticosteroid-sensitive nephrotic syndrome, and only low levels of activity were detected in serum from patients with membranous nephropathy after transplantation and those with chronic renal failure. These findings do not support the view that the serum activity associated with increased glomerular permeability to albumin is a non-specific phenomenon in the nephrotic syndrome, after transplantation, or in renal failure.

Plasmapheresis in patients with recurrent focal segmental glomerulosclerosis lowered the level of serum activity and decreased proteinuria, and activity was detected in the plasmapheresis fluid. The decrease in serum activity after plasmapheresis was consistent with the removal of a substance that is primarily confined to the plasma space and is not rapidly synthesized after its removal. These findings provide support for the hypothesis that a serum factor is responsible for the injury to the glomerular-filtration barrier in patients with recurrent focal segmental glomerulosclerosis and that this factor may contribute to persistent proteinuria in such patients.

Focal segmental glomerulosclerosis and minimal-change disease are often considered manifestations of a single process.<sup>10</sup> Immune mechanisms have been postulated, because patients with minimal-change disease and some patients with focal segmental glomerulosclerosis have a response to corticosteroids and immunosuppressive drugs. The detection of a number of im-

munologic abnormalities in patients with minimal-change disease also supports this hypothesis.<sup>34-39</sup> The presence of a circulating substance has been proposed, because serum or plasma from patients with focal segmental glomerulosclerosis may cause proteinuria in animals.<sup>24-26</sup> In a study of patients with the idiopathic nephrotic syndrome and proteinuria after transplantation, repeated injection of a fraction of plasma protein removed by protein A immunoadsorption caused albuminuria in rats.<sup>16</sup> This fraction may contain a substance that corresponds to the factor we are studying, because both bind to protein A and their molecular mass is similar. A factor produced by hybridomas of T cells from patients with minimal-change disease causes proteinuria in rats,<sup>23</sup> but this factor does not appear to share the biochemical properties of the substance we are studying. Our finding that serum samples from patients with corticosteroid-sensitive nephrotic syndrome have no effect on glomerular permeability *in vitro* does not support the hypothesis that this syndrome and focal segmental glomerulosclerosis have a common cause.

The active factor in our study appears to be larger than any known lymphokines and relatively hydrophobic. Its solubility in 70 percent ammonium sulfate distinguishes it from the majority of serum proteins. On the basis of the chromatographic findings, the factor carries a weak anionic charge at a pH of 6.0. Since it binds to protein A but does not precipitate with immunoglobulins, we postulate that it may be a nonimmunoglobulin protein or a fragment of an immunoglobulin.

The mechanism for glomerular injury in focal segmental glomerulosclerosis remains to be identified. Immunoglobulin and complement deposition, cellular infiltration, and mesangial-cell proliferation are not prominent

in most patients.<sup>10</sup> It has been proposed that in both minimal-change disease and focal segmental glomerulosclerosis, the negative charges of the glomerular capillary are neutralized by a cationic substance.<sup>40</sup> Neutralization of negative charges cannot account for the effects of the active substance in our studies, since it is itself anionic and as little as 0.1  $\mu\text{g}$  per milliliter of partially purified protein caused a maximal increase in glomerular permeability to albumin. We postulate that cellular effects may be responsible for the observed increase in permeability.

We conclude that a circulating factor in serum from patients with focal segmental glomerulosclerosis can cause immediate and marked changes in glomerular permeability to albumin. The serum factor is strongly associated with the recurrence of focal segmental glomerulosclerosis after renal transplantation and may be responsible for proteinuria in patients with this disorder.

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