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URINE PLASMIN-LIKE SUBSTANCES AS AN INDEX OF KIDNEY ALLOGRAFT REJECTIONS[1]

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SUMMARY

Using solid state radioimmunoassays developed by the first author, changes in the urine level of plasmin-like substances (PLS) and fibrin degradation products (FDP) before and after human kidney transplantation were determined in 49 transplant patients. Averages of urine PLS and FDP in a normal population of 51 persons were 0.13 ± 0.10 (SD) and 0.14 ± 0.07 μg/ml, respectively. In all transplant patients there was an initial rise of both PLS and FDP in urine immediately after transplantation. This elevation peaked on days 4 and 5 and the PLS and FDP levels returned to normal range within 2 weeks in patients without evidence of rejection. A secondary rise of urine PLS was detected before or with a rise in serum creatinine in all of the patients experiencing rejections. Of 11 patients who showed a rejection episode within 2 weeks of transplantation, the secondary rise of urine PLS was detectable in 55% of the patients slightly before the serum creatinine level changes; of 6 patients with a rejection episode more than 2 weeks after transplantation, 100% showed a secondary PLS rise 6.7 ± 2.3 (SE) days before the serum creatinine increased. The appearance of the secondary rise of urine FDP in the rejecting recipients was slightly later than the rise of PLS. Serial determination of urine PLS levels following human kidney transplantation appears to be an early index of rejections which occurs more than 2 weeks after transplantation, although the clinical usefulness of this measurement is probably limited.

Several reports indicate that following human kidney transplantation fibrin degradation products (FDP) appear in urine and that the amount of urine FDP increases during graft rejection (*1–3, 5, 9, 21, 25*). It has been suggested that the appearance of FDP in urine is the result of the digestive effect of plasmin on fibrin which has been deposited on kidney capillary beds during rejection (*25*). To date, urine plasmin or plasminogen levels after kidney transplantation have not been studied. A very sensitive radioimmunoassay system (solid state radioimmunoassay) has been developed by the first author in order to measure substances which have common antigenicity to heterologous antiplasminogen antibodies. Using this assay, changes in the levels of these plasmin-like substances (PLS) in urine before and after human kidney allotransplantation have been studied and correlated with FDP levels and with rejection episodes.

MATERIALS AND METHODS

Patients. Forty-nine kidney transplants were studied. Sixteen patients received kidneys from living related donors and 33 from cadaveric donors. The recipients were treated with triple immunosuppressive therapy consisting of cyclophosphamide or azathioprine, prednisone, and antilymphocyte globulin. The onset of rejec-

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tion was designated as the day when serum creatinine was considered to have increased significantly. Increase of urine protein and decrease of urine sodium or urine volume were considered supporting evidence for rejection. Serum creatinines and other renal chemical parameters of rejection were measured daily during the first 4 weeks after transplantation and less frequently thereafter. The end of a rejection episode was designated as the day when the elevated serum creatinine level began to fall. The patients were followed for up to 7 months after transplantation. The recipients were divided into five groups: group 1, no rejection episode during the observation period; group 2, doubtful rejections; group 3, rejection episodes within 2 weeks of transplantation; group 4, rejection episode after more than 2 weeks following transplantation; and group 5, serious postoperative complications of any kind regardless of rejections.

Urine collections. The urine was collected for testing in almost all cases at least once before transplantation, three times per week for 2 weeks after transplantation, and twice per week thereafter. The first morning urine was collected in a test tube containing ϵ-aminocaproic acid (EACA) at a concentration of 2.5 mg/ml of urine and kept at 4 C for a few hr. The sample was centrifuged at 4,000 rpm for 10 min at 4 C and the supernatant was stored at −70 C. Urine containing gross blood was excluded.

Radioiodination of rabbit anti-human plasminogen IgG with ^{125}iodine. Rabbit anti-human plasminogen IgG was extracted from commercial antisera (Behring Diagnostic, American Hoechst Corp., Woodbury, New York) using one-third saturated ammonium sulfate precipitation followed by DEAE cellulose (Whatman DE32; H. Reeve Angel Inc., London, England) column chromatography with phosphate buffer, pH 8.0, 0.005 M. Radioiodination with ^{125}iodine (Amersham/Searle Corp., Des Plaines, Illinois) of the rabbit anti-human plasminogen IgG was done according to Greenwood's method (*7*). The specific activity of the labeled IgG was 0.72 to 0.80 mc/mg. The labeled IgG produced only one precipitin band in radioimmunodiffusion in agar (*23*) against normal human plasma as well as against a standard human plasminogen (American National Red Cross, New York, New York).

Solid state radioimmunoassay for PLS. PLS in urine samples was radioimmunoassayed using Mori's method (*19*) with modifications, which was originally developed for measuring antimicrosomal and antithyroglobulin antibodies. Each well of a disposable microtiter plate (Cooke Engineering Co., Alexandria, Virginia) was filled with normal human serum which had been diluted 1:50 with carbonate-bicarbonate buffer (pH 9.0, 0.02 M) containing 0.25% EACA. Three wells of the plate were used as a control filled with only the buffer. The plate was placed in a humid box at 25 C for 2 hr and then washed three times with Tris-glycine-phosphate saline (TGPS) (pH 7.6; 0.025 M Tris-0.025 M glycine-0.02 M KH_2PO_4-0.9% NaCl). The wells were filled with TGPS containing 1% bovine serum albumin (BSA) and 0.01% horse γ-globulins (HGG), then the plate was incubated for 20 min in the same way as above and washed three times with TGPS. The plates coated with human serum could be kept in a humid box at 4 C for several hr without damaging the antigenic activity of the coated proteins.

Standard plasminogen solution (original concentration, 1.8 mg/ml) was serially diluted by TGPS containing 2% BSA-0.02% HGG and 0.25% EACA. Standard mixtures and sample mixtures were made as follows: 5 volumes of each of the diluted standard plasminogen, plus 1 volume of ^{125}iodine-labeled antiplasminogen IgG in TGPS at a concentration of 3.6 to 4.0 μc/ml, plus 3 volumes of TGPS. The final concentrations of the standard plasminogen ranged between 8.0 and 0.002 μg/ml. One of the standard mixtures was made containing no plasminogen. Sample mixtures were prepared so that urine was diluted 1:9, using 1 volume of urine, 5 volumes of TGPS containing BSA, HGG, and EACA, 1 volume of the labeled IgG, and 2 volumes of TGPS. When the PLS level of the sample examined exceeded 8.0 μg/ml, the sample was appropriately diluted with TGPS. The mixtures were incubated at 25 C for 1 hr, then 250 μl of each mixture were transferred in triplicate to the plate already coated by normal human serum. The standard mixture containing no plasminogen was put in both the serum-coated wells (positive control) and the uncoated wells (negative control). The plate was incubated at 25 C for 20 hr in a humid box. The mixtures were removed by suction and the plate was washed with tap water. After drying, each well was separated and the radioactivities of the wells were counted with a crystal well-type gamma counter (Beta/Gamma Liquimat;

Picker Nuclear, White Plains, New York). ϵ-aminocaproic acid inhibits the activity of plasminogen activator and plasmin, which could affect the assay. Bovine serum albumin is generally used as a stabilizer in radioimmunoassays. Horse γ-globulin in sample mixtures inhibits cross-reaction of antihorse γ-globulin antibody against ^{125}I-labeled rabbit anti-human plasminogen IgG. Because many of the patients were treated with horse anti-human antilymphocyte globulin, some may have had antibodies against horse globulin.

Calculation of the urine PLS concentration was performed in the following way: positive control count minus negative control count was defined as 100% net bound count in each plate. Per cent bound of standard mixtures or sample mixtures was determined by the following formula:

$$\frac{\text{standard or sample count} - \text{negative control count}}{\text{100\% net bound count}} \times 100$$

A standard curve was made from the percentage bound for each standard mixture. The PLS concentration of each sample was determined from the standard curve. Although each plate gave a slightly variable count even for the same sample, this variability in counts was eliminated by using the above defined calculation for the percentage bound. The sensitivity of the assay was determined and 0.02 μg/ml of plasminogen in the standard mixture was found to be the minimum amount detectable. Four kinds of dilutions of a sample were assayed six times to study reproducibility of this method. Mean values and standard deviations of each dilution were 0.38 ± 0.05, 0.97 ± 0.01, 4.0 ± 0.74, and 10.3 ± 1.1 (μg/ml).

Radioimmunoassay for FDP. The principle of the radioimmunoassay for FDP was the same as that for PLS, but there were several differences in the assays. Antifibrinogen IgG was extracted from commercial rabbit anti-human fibrinogen antisera (Behring Diagnostics). The specific activity of the antifibrinogen IgG labeled with ^{125}iodine was 0.9 mc/mg, and for the radioimmunoassay the labeled IgG was diluted to 2.5 μc/ml with phosphate-buffered saline (PBS), pH 7.6, 0.02 M. Human fibrinogen (Nutritional Biochemicals Corp., Cleveland, Ohio) was diluted to the concentration of 0.5 mg/ml with carbonate-bicarbonate buffer (pH 9.6; 0.002 M), filtered through Whatman No. 1 paper, and used for coating microtiter plates. PBS was used for diluting media instead of TGPS. For making standard fibrinogen solution, human fibrinogen in PBS was gel-filtered through Sephadex G-200 (Pharmacia, Piscataway, New Jersey) and the filtrate of the first main peak was collected. After arrangement of the protein concentration at 3.4 mg/ml, the fibrinogen solution was serially diluted by PBS containing 2% BSA, 0.02% HGG, and 0.25% EACA. The final concentrations of the fibrinogen in standard mixtures were 15.1 to 0.038 μg/ml. The minimum amount of fibrinogen detected by this method was found to be 0.08 μg/ml. The reproducibility of this assay was slightly better than for the plasmin assay.

RESULTS

Normal level and preoperative range. Averages of urine PLS and FDP in a normal human population of 51 persons were 0.13 ± 0.12 (SD) and 0.14 ± 0.07 μg/ml, respectively. Preoperative levels of urine PLS and FDP in 14 primary transplant patients were very variable, ranging from 0.14 to 11.7 μg/ml for PLS and 0.38 to 46.0 μg/ml for FDP.

Group 1. Of the 49 transplants, 21 had no rejections during the observation period. In all 21 patients, an elevation of urine PLS was observed starting on 1 day after transplantation and lasting for 4 to 5 days, then falling gradually (Fig. 1). The average value of urine PLS was 3.16 ± 4.80 (SD) μg/ml for the first 5 days, 0.86 ± 1.25 from the 6th to 10th days, 0.40 ± 0.26 for the 11th to the 15th day, 0.26 ± 0.12 from the 16th to 20th day, and 0.26 ± 0.11 thereafter. Except for two patients, the urine PLS values were stabilized at a lower level than 0.5 μg/ml within 14 days following transplantation. The general pattern of urine FDP in this group was similar to that of PLS (Fig. 2).

Group 2. Of the 49 transplants, 3 had rejection episodes which were only suspected by renal function tests. However, urine PLS remained higher than 0.5 μg/ml for all of the patients for more than 14 days following transplantation. The urine PLS pattern was very similar to that of group 3, which had a rejection episode within 2 weeks after transplantation.

Group 3. There were 13 cases with definite rejection episodes within 2 weeks of transplantation. The urine PLS pattern (Fig. 3) was quite different from that seen in group 1. The

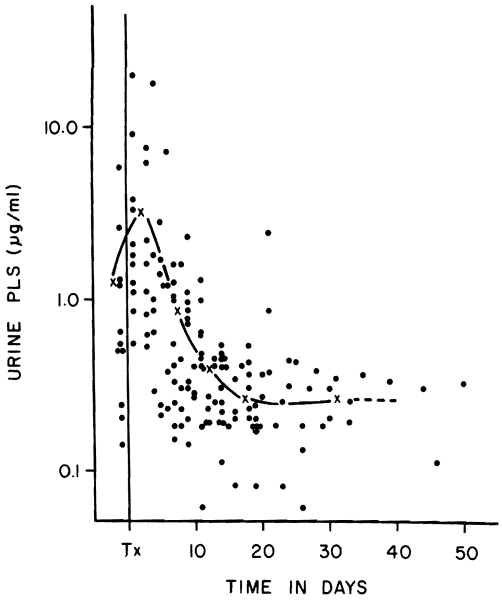

FIGURE 1. Urine PLS levels (μg/ml) in 21 patients with no observed rejection episodes. Mean values before transplantation and at intervals after transplantation are indicated (×——×).

initial rise of urine PLS immediately after transplantation was followed by a secondary rise, which appeared shortly before or almost at the same time as when the serum creatinine level started to increase. The mean difference in time between the secondary rise in urine PLS and the elevation of serum creatinine in 11 of the 13 cases was 0.3 ± 0.6 (SE) days (Table 1). The secondary rise of PLS exceeded 0.5 μg/ml of urine in all of the 13 cases and exceeded 10 μg/ml in 4 cases. Almost simultaneously with the end of rejection this secondary elevation disappeared, except for one patient in whom it remained elevated even after renal function had recovered. The urine FDP pattern in this group was the same as the PLS, except that the secondary rise of FDP level usually did not occur earlier than the onset of rejection. The FDP level was over 2.0 μg/ml during the rejection episodes except for one case.

Group 4. There were 12 cases with a rejection episode more than 2 weeks after transplantation. In all 12 patients, urine PLS level became normal after the initial rise following transplantation and then elevated to a level of more than 0.5 μg/ml when the rejection was evident by renal function tests. With six patients whose samples were available for examination within 3 days before the onset of rejection, the time relationship of the secondary rise in urine PLS to the onset of rejection was examined (Fig. 4). All six patients showed a secondary elevation, which appeared definitely before serum creatinine criteria gave evidence of rejection. The mean difference in time between the rise in urine PLS and serum creatinine was 6.7 ± 2.3 (SE) days (Table 1). The pattern of FDP of this group was again similar to that of PLS, but the elevation of FDP started a few days later than PLS; the FDP value during rejection exceeded 20 μg/ml for 10 of the 12 patients.

Group 5. There were eight patients who had major complications after transplantation: one lung infection attributable to cytomegalovirus, two acute tubular necroses, and five complications requiring operative intervention including one gastrectomy, one amputation of a lower leg, and three operations for urological complications. Urine levels of PLS and FDP did not change in the patient with cytomegalovirus infection, but they were markedly elevated with

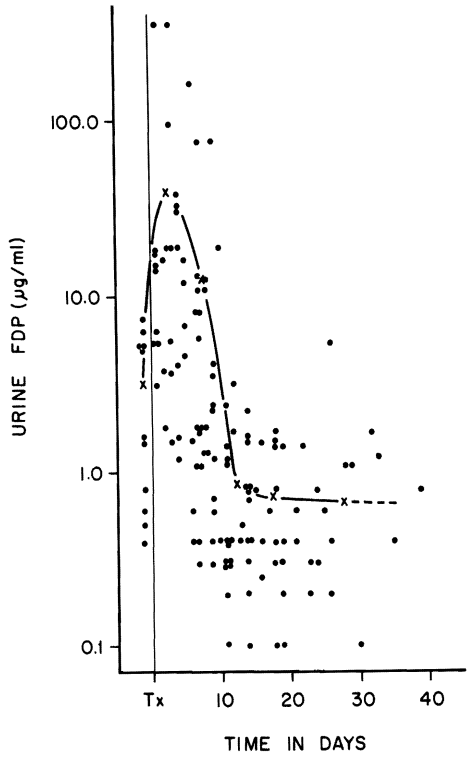

FIGURE 2. Urine FDP levels (μg/ml) in 21 patients with no observed rejection episodes. Mean values before transplantation and at intervals after transplantation are indicated (×——×).

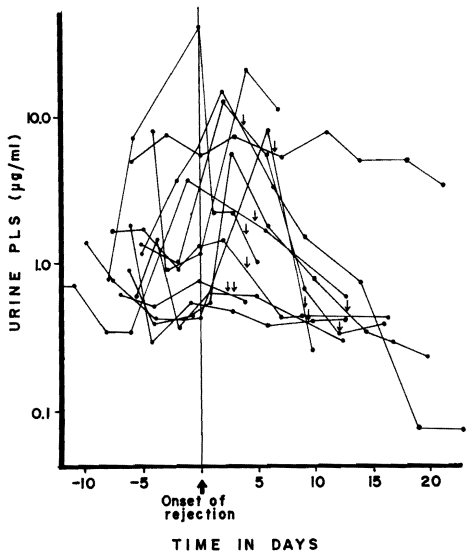

FIGURE 3. Urine PLS levels (μg/ml) in 13 patients with initial rejection episodes during the first 2 weeks after transplantation. The time of onset of rejection was determined by initial rise of serum creatinine. The end of each rejection episode as measured by fall of serum creatinine is indicated (↓).

acute tubular necrosis and slightly elevated after secondary surgical procedures. Three patients had a rejection episode concomitant with these complications, and elevation of urine PLS and FDP was observed with these rejections.

Predictability of rejection according to PLS and FDP determinations. In order to see the efficiency of urine PLS and FDP determinations in predicting rejection episodes, the day on which the secondary rise in PLS and FDP was detected for the first time was compared with the day of the onset of rejection judged by the change in serum creatinine level in groups 3 and 4 (Table 1). Two of the 13 patients in group 3 were excluded from this analysis because of nonavailability of urine samples within 3 days prior to rejection. Of the remaining 11 patients, 6 (55%) demonstrated a detectable rise of PLS at a slightly earlier time than the rejection was diagnosed by serum creatinine criteria; in these 11 cases the mean difference in time between the rise in urine PLS and serum creatinine was 0.3 ± 0.6 (SE) days. The elevation of urine FDP in this group was detected later than the serum creatinine increase in 8 of the 11 patients, which made the time difference −0.7 ± 0.7 days. Six patients from group 4 were available for this comparison. In all six cases, the urine PLS elevation preceded a rise in serum creatinine by a mean of 6.7 ± 2.3 days. Three of the six cases showed an elevation in urine FDP which preceded a rise in serum creatinine. The mean difference in time between the elevation of urine FDP and rise in serum creatinine in these six patients was 2.5 ± 1.2 days. The relative ability of urine PLS versus FDP determinations to predict rejection in these six patients was not statistically different ($P < 0.20$).

DISCUSSION

Because there is no effective therapy for hyperacute or chronic rejection, most of the tests that have been developed to detect rejection are directed at identifying acute rejection episodes, which may be reversible by treatment with corticosteroid and possibly by graft radiotherapy. Numerous immunological tests have been devised for this purpose, including leukocyte aggregation tests (*10*), spontaneous blastogenic transformation (*16*), peripheral mononuclear cell RNA synthesis (*22*), leukocyte migration inhibition (*24*), mixed leukocyte culture inhibition (*26*), and changes in serum immunoglobulin and complement levels (*15, 30*). A number of other tests for rejection have been devised which generally depend on looking at the products of secondary damage to the graft, including lysozymuria (*8*), elevation of blood and urinary histamine (*18*), and increased urinary FDP (*1-3, 5, 9, 21, 25*). Renal biopsies have been studied for this purpose (*17*), but there is no morphological finding that is completely specific for rejection.

The clinician still relies heavily on physiological tests of graft function for making a diagnosis of rejection. Oliguria, increased serum creatinine, and decreased creatinine clearance are the most useful clues to rejection, but obstructive uropathy and major vascular occlusion must be eliminated from the differential diagnosis before the exclusion diagnosis of rejection becomes tenable. It is generally agreed that acute rejection should be treated as promptly as possible. It is therefore important to continue to search for tests for rejection which can detect this process more specifically and earlier than current methods.

Changes in urine level of PLS and FDP before and after kidney transplantation were analyzed for this purpose. In all patients with transplants, an initial rise of both PLS and

TABLE 1. Time of elevation of serum creatinine, urine PLS, and urine FDP in 17 patients with definite rejection episodes

Patients	Donor source	Days after transplantation		
		Serum creatinine elevation	Urine PLS elevation	Urine FDP elevation
Group 3 (Rejection within 2 weeks after transplantation)				
C. D. (2nd transplantation)	Cadaveric	8	5	5
J. H.	Cadaveric	6	3	6
M. G. (3rd transplantation)	Cadaveric	14	13	20
M. L.	Cadaveric	7	6	6
E. P.	Cadaveric	8	7	7
C. M.	Cadaveric	10	9	13
C. D.	Cadaveric	5	5	5
D. I.	Living Related	7	8	8
D. J.	Cadaveric	9	10	10
G. S.	Cadaveric	7	9	9
J. H.	Living Related	14	17	14
			0.3 ± 0.6[a]	-0.7 ± 0.7[a]
Group 4 (Rejection after 2 weeks following transplantation)				
M. L. (2nd rejection)	Cadaveric	39	22	35
M. L. (3rd rejection)	Cadaveric	60	53	53
M. G.	Cadaveric	20	13	20
A. F.	Cadaveric	19	15	19
B. S.	Cadaveric	15	11	15
F. D.	Cadaveric	28	27	24
			6.7 ± 2.3[a]	2.5 ± 1.2[a]

[a] The mean difference in time (days) between the onset of rejection measured by initial rise of serum creatinine and the onset of rejection measured by rise of urine PLS or urine FDP (mean ± SE).

FDP immediately after transplantation was observed. This elevation peaked on days 4 and 5 and the level returned to normal range within 2 weeks in patients without evidence of rejection. When patients had a rejection, urine PLS level exceeded 0.5 μg/ml of urine in all cases and the FDP level exceeded 2.0 μg/ml in 88% of the rejecting patients. The group of patients in which the urine PLS level provided the most useful information for an early diagnosis of rejection was those patients who experienced a rejection episode after more than 2 weeks following transplantation (group 4). In all six patients in this group which were available for the early diagnostic studies, the urine PLS elevation preceded the rise in serum creatinine by an average of 6.7 ± 2.3 (SE) days. In contrast with this, only 50% of the patients from this group showed the FDP elevation which antedated the rise in serum creatinine. With patients whose rejection occurred within the first 2 weeks following transplantation (group 3), the urine PLS and FDP assays were less frequently able to predict rejection, i.e., 55% for PLS and 27% for FDP. Both the PLS and FDP assays were not capable of distinguishing rejection from acute tubular necrosis (group 5). The precise mechanism of PLS excretion of urine during acute rejection remains uncertain. An initial process of the acute rejection is presumed to be destruction of vascular endothelial cells in graft tissue by both antibodies and lymphocytes against histocompatibility antigens. In this process, plasminogen would be activated into plasmin by plasminogen activator released from the injured endothelial cells (*20, 28*), which contain much of the plasminogen activator (*12, 29*). The injury of the endothelial cells causes platelet aggregation and fibrin clotting (*4, 11*). The Hegeman factor (*14*) and fibrin produced in this process can also stimulate the PLS system. In addition, blood flow retardation by the platelet aggregation and fibrin clotting results in tissue ischemia. The plasminogen activator is released from the ischemic tissues (*13, 14*). As a result, the amount of PLS would increase in the graft as well as in the circulating blood (*6*), and the PLS would act on fibrin to give rise to FDP.

The PLS level in urine depends on possibly

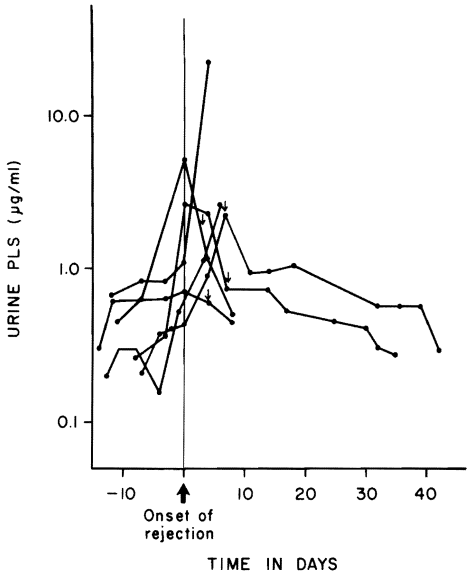

FIGURE 4. Urine PLS levels (μg/ml) in six patients who had a rejection episode which began more than 2 weeks after transplantation. The time of onset of rejection was determined by initial rise of serum creatinine. The end of each rejection episode as measured by fall of serum creatinine is indicated (↓).

both abnormal production of the PLS in the transplanted kidney and abnormal filtration of these proteins through damaged glomeruli. The molecular weight of plasmin monomer is approximately 32,000 (*27*) and is much smaller than those of FDP and albumin. Only the monomer type of plasmin is detected in vivo (Y. Takeda, personal communication). It would not be unreasonable to assume that a large amount of the small molecules produced in the grafted tissue could easily appear in urine at an early stage of rejection. In fact, urine PLS tended to be detected earlier than urine FDP in this study and there was no correlation between the amount of PLS and total proteins in urine after kidney transplantation.

The time needed for the determination of urinary PLS levels, approximately 25 hr, partially limits the clinical value of this test. If the plate incubation time can be shortened, the clinical usefulness of this assay may be increased.

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