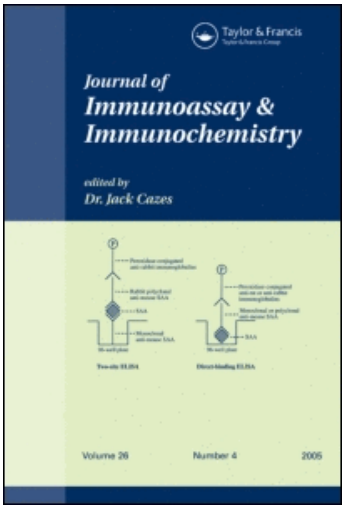

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DIRECT RADIOIMMUNOASSAY OF ACTIVE AND INACTIVE HUMAN GLANDULAR KALLIKREIN : SOME PHYSIOLOGICAL AND PATHOLOGICAL VARIABILITIES.

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ABSTRACT

We have developed a sensitive and specific radioimmunoassay which allows the detection of human glandular kallikrein in biologic fluids at a level of 40 pg / ml. The antisera did not recogniz human plasma kallikrein and glandular kallikrein from other species including marmoset. Furthermore the antibody did not bind pro-kallikrein but was specific for the trypsin activated kallikrein. The antibody inhibited the kininogenase activity of standard kallikrein incubated with human kininogen. However active kallikrein inhibited by inhibitors bound at the active site is still detectable, indicating that the antibody is specific for the structure of the active form but not for the active site. In normotensive subjects, daily urinary kallikrein excretion increased with age until 30, then a decrease was observed. In renal transplanted recipients a progressive increase of the active form was found. A low concentration of immunoreactive active kallikrein was detected in lymphatic fluids of patients suffering from acute pancreatitis treated by lymphatic drainage ; although this

kallikrein is the active immunoreactive form, a very weak kininogenase activity was measured, suggesting a partial inhibition by anti-proteases. These data provide complementary evidence for the physiological and pathological role of glandular kallikrein.
('KEY WORDS:'direct RIA, human urinary kallikrein, aging, renal transplant, lymph).

INTRODUCTION

Glandular kallikrein (E.C. 3,4,21,35) is located in different organs such as pancreas, salivary gland, kidney, prostate and in their exocrine secretions. This wide distribution raises the question of the physiologic significance of kallikrein which has been recently reviewed (1). Kallikrein is a serine protease and one of its major actions is believed to be the generation of potent vasodilatator substances named kinins. Recently, new findings seem to implicate kallikrein in other actions of physiological interest such as peptide and enzyme processing, for example prorenin activation (2), atrial natriuretic peptide processing (3), and direct action on smooth muscle (4). Thus, kininogenase activity may not reflect the only activity of kallikrein. Furthermore the determination of the kininogenase activity in tissue homogenates and in biological fluids, is not devoid of problems because the presence of inhibitors (5). It has also been reported that kallikrein is secreted in both an active and an inactive form with possible interconversion, although the physiological mechanism of such activation remains unknown (1). Usually, inactive kallikrein is measured after *in vitro* trypsin activation. It was therefore of interest to be able to determine the level of kallikrein not only by its kininogenase activity but also its immunoreactivity.

We have purified human urinary kallikrein to apparent homogeneity and produced a specific antiserum. This latter has been used to develop a specific RIA which allows the distinction between active kallikrein and trypsin activatable kallikrein. Using both immunorective detection and enzyme activity measurements, we were able to detect glandular kallikrein in different biological fluids collected either from normal subjects or subjects with several pathologic states.

MATERIALS AND METHODS

Purification of human urinary kallikrein

Human urinary kallikrein was purified from 80 liters of human urine according to a protocol previously described for the purification of rat urinary kallikrein (6). A

four step procedure was used, which included anion exchange chromatography, aprotinin agarose affinity chromatography, followed by a Sephadex G 100 filtration. The final preparation was obtained after an Affigel blue chromatography. The kallikrein preparation was further characterized by SDS Page-electrophoresis, the nature of the kinin formed, the amino acid composition and its kininogenase activity.

Immunisation procedure

Three male rabbits (Fauve de Bourgogne 2 months old) were immunized against purified human urinary kallikrein. Immunization was performed according to the dorsal multiple site protocol (7). Briefly, each injection consisted of 30 μg of purified kallikrein in 1 ml isotonic saline emulsified with 1 ml complete Freund's adjuvant. Every 6 weeks, a booster injection was given, containing the same amount of kallikrein emulsified in incomplete Freund's adjuvant. Prior to the booster injection, blood was collected from the ear artery into dry plastic tube without any vasodilatators or anticoagulant. Blood was kept at 4°C for 24 h, and then centrifuged at 3000 x g for 30 min. The antisera were collected, kept 1 h at 56°C in a water bath and then stored frozen at -20°C.

Screening of antibodies

The presence of antibodies was monitored by their specific binding capacity with ^{125}I labeled urinary kallikrein. Purified urinary kallikrein (1μg) was iodinated using the Chloramine T method and purified by a G 100 Sephadex filtration, as previously described (8-9). Binding incubations were performed in Phosphate buffer 0.01 M pH 7.4 containing 0.14 M NaCl and 1% BSA (RIA grade Sigma). Separation of bound and free kallikrein was achieved, using the polyethylene glycol method (10). The radioactivity of the pellet after centrifugation was counted in a gamma spectrophotometer (PACKARD multi RIA system). Antibodies were further characterized by passive immunodiffusion against various closely related proteins. Specificity of these antibodies was also tested with kallikrein from different species (marmoset, rat, mice ,dog and rabbit).

Radioimmunoassay protocol

Incubations were run in triplicate in phosphate buffer 0.01 M pH 7 containing 0.14 M NaCl and 1% BSA in a final volume of 0.4 ml. The composition of the medium was as follows : 0.1 ml of labelled antigen (approximatively 8000 cpm), 0.1 ml of unknown samples or different concentrations of standard (from 20 pg to 10 ng), the final dilution of the antibody was from $1/10^6$ to $1/1.5\ 10^6$ to ensure 35 to 40% of specific binding. Incubation time was 48 h at 4°C. Polyethylene glycol at 15% final concentration, was used to separate the bound and free kallikrein in the presence of

1% of gamma globulins added into the incubation medium. The radioactivity of the unwashed pellet was counted in a fully automated gamma spectrophotometer (PACKARD multi RIA system). Values for unknowns were calculated using a computerised Logit Log or spline function, where B and Bo were the radioactivity respectively bound in the presence or the absence of unlabelled kallikrein. Results were expressed as amounts of urinary kallikrein per ml of sample or per 24 h urinary volume (μg/ml or μg/24 h) .

Kallikrein activity measurements

The kininogenase activity was determined using a kinin radioimmunoassay, as previously reported (11). Results are expressed in μg of bradykinin generated per minute of incubation per ml of sample or per 24h urinary volume (μg BK / ml or μg BK / 24 h). The amidolytic activity was measured using the synthetic substrate D-Val-Leu-Arg-pNa (commercial source from Kabi Vitrum) as already published (12). Activity was expressed in nanomoles of product reversed per minute per ml of sample or per 24 h urinary volume (nM / ml or nM / 24h).

SDS gel electrophoresis

Vertical gel electrophoresis were performed in SDS-Tris-Glycine buffer pH 8.3(4 mA/gel ,2h). Acrylamide concentration was 12% in the separating gel and 4% in the stacking gel as previously described(13). The gels were sliced into 2mm slices and further eluted overnight in 0.5 ml phospate buffered saline pH 7.4. The eluted fractions were tested for the presence of immunoreactive kallikrein.

Normal biological samples :Physiological studies

Normal Volunteers : three separate 24 hour urine collections were obtained from each subject at one week intervals from 100 normal caucasian normotensive subjects (age from 4 to 89 years , 51 males and 49 females). Urine samples were collected throughout two 12 h periods and urine creatinine was measured. Kallikrein was determined either on crude or dialysed samples or in samples kept frozen. Kallikrein was assessed by concentration (μg/24 h), kininogenase (μg BK/24 h) and amidase activity (μM/24 h) before and after trypsin treatment. Timed collections from 8 am to 8 pm at 2 h intervals and from 8 pm to 8 am from 9 normal subjects (age from 25 to 49, 4 males and 5 females) were studied to establish possible diurnal variations.

Pathological samples

Urine from transplanted patients was collected daily from the first day to the seventh day following transplantation . During that period patients did not received any immnuno suppressive treatement.

Lymphatic fluids were obtained by direct thoracic drainage from patients suffering from acute necro-cortico pancreatitis on the first day of their admission to the intensive care unit. These samples were immediately centrifuged and kept frozen untill analysis.

RESULTS

Antisera production

Three out of three immunized rabbits produced antibodies. They revealed differences in titer and sensitivity . A progressive increase in these parameters was noted with increasing time following immunization. The best antibody choosen for the study was obtained after the fourth administration of antigen (i.e. 6 months after the first immunization). Lyophilisation of the antisera did not affect its binding capacity with ^{125}I labelled kallikrein. The IgG fraction obtained after purification on Protein A showed the same binding capacity and specificity. Purified urinary kallikrein was iodinated and appeared homogeneous after filtration on Sephadex G 100 ; a single peak of immunoreactivity was observed as demonstrated on figure 1 A. Dilution curves are shown in figure 1 B, the antibody K3 was used for the radioimmunoassay, 50% of specific binding was obtained at a final dilution of $1/10^6$.

Characteristics of the radioimmunoassay of active kallikrein

A standard curve resulting from 10 separate experiments is shown on figure 2. In the absence of unlabelled kallikrein, 50% of the added ^{125}I-human kallikrein was specifically bound using a final antiserum dilution of $1/10^6$. Maximum stability of the binding was observed after 48 h incubation at 4°C. The non specific binding was 7 ± 2%. The lower limit of detection was 20 pg of unlabelled kallikrein with an average displacement to 93 ± 4% of the total binding with an intraassay variation of 4 ± 1% and an interassay of 9 ± 2 %. The displacement range was between 50 pg and 10 ng. 50% displacement was obtained with 1.5 ng. Recovery of purified kallikrein added to urine was 94 ± 8%. When a standard curve using aprotinine inactivated kallikrein was established, no difference was shown by comparison with a normal standard curve run with non inactivated kallikrein. The curves obtained with human urine either from normal subjects (0.5 to 10 µl) or from renal transplant (10 to 100 µl) and with human lymph eluted from SDS gel (50 to 100 µl) were parrallel with the human urinary standard curve (figure 2). On the other hand no significant displacement was observed with trypsin (25 to 100ng) or with purified rat urinary kallikrein (25 to 100ng). In addition no cross reactivity was detected with human plasma kallikrein

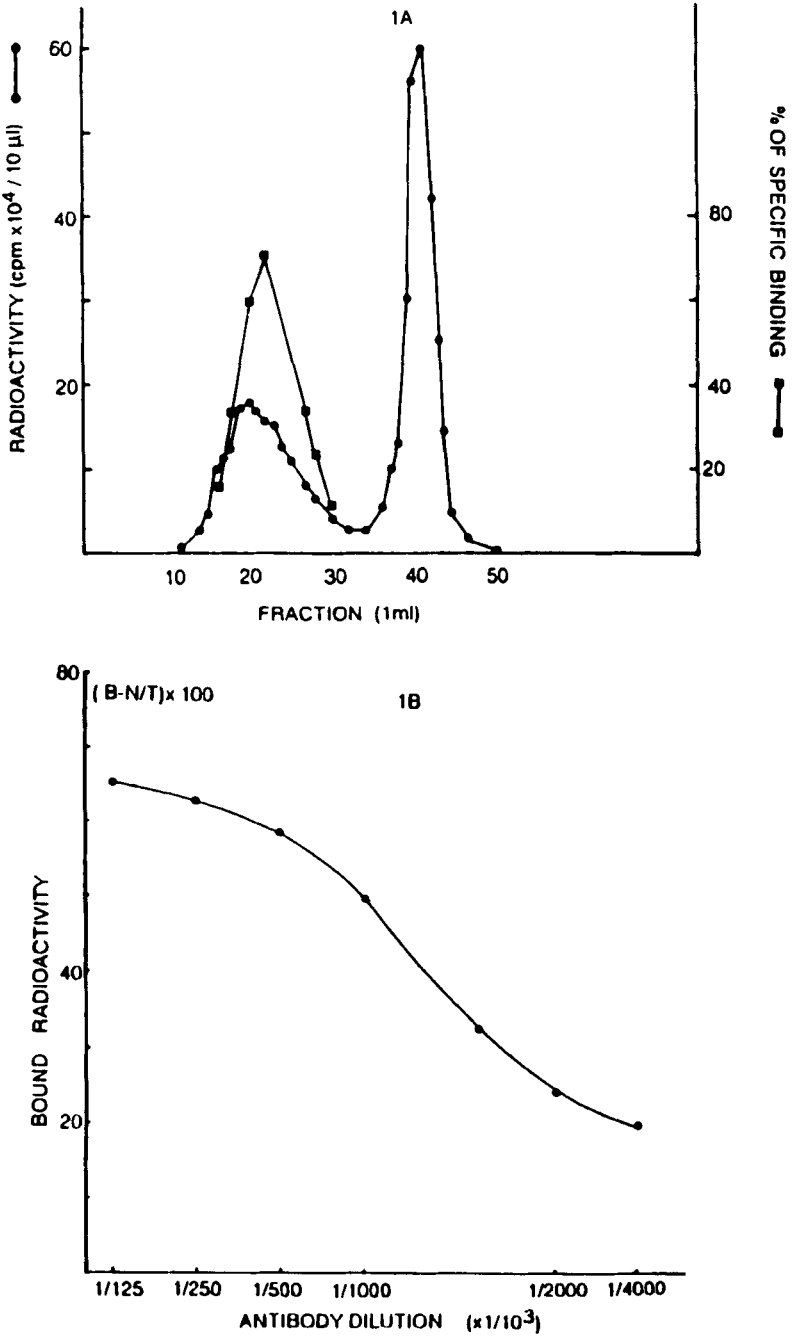

FIGURE1 : Fig 1A - Chromatography of iodinated human urinary kallikrein on Sephadex G100 : (●—●) radioactivity , (■—■) Immunoreactivity. Fig 1B- Antibody titration curve of serum K3 used for radioimmunoassay .

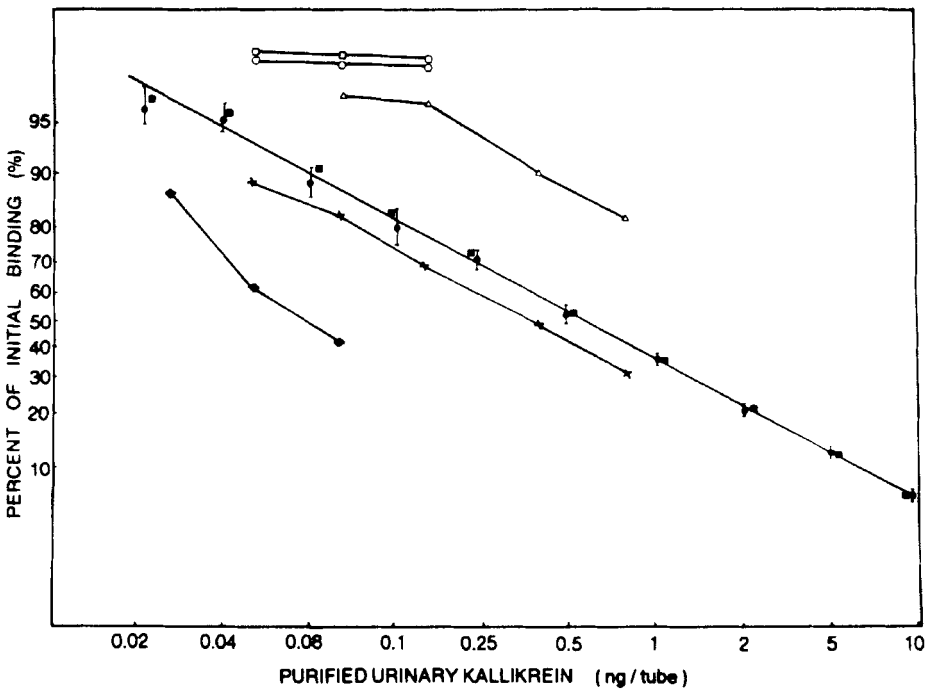

FIGURE 2 : Standard curve . B/Bo X 100 (100X ratio of bound cpm to cpm bound at the zero dose) is plotted versus the log of standard kallikrein concentration : (●—●) purified urinary kallikrein . (■—■) purified urinary kallikrein inhibited with aprotinine .(✱—✱) normal human urine .(△—△)urine from renal transplant recipient . (◆—◆) human lymph . (□—□) rat urinary kallikrein . (○—○) trypsin .

(commercial source from Kabi Vitrum), urokinase (From Choay), and glandular kallikrein from other species such as dog, rabbit and marmoset. Dialysis of samples did not improve the immunoreactivity, but extensive dialysis resulted in a decrease of the immunoreactivity after 24 h.

Direct radioimmunological determination of inactive kallikrein

For this purpose urine samples were incubated in the presence or the absence of trypsin and then analyzed to determine whether trypsin activation induced changes in immunoreactive kallikrein concentration.

It can be seen that immunoreactive kallikrein measurements were highly correlated with kininogenase activity assay (r = 0.952 figure 3 A) and amidolytic assay

($r = 0.954$ figure 3 B). Furthermore these correlations remained significant after trypsinisation of the samples ($r = 0.984$,figure 3A and $r = 0.98$ figure 3B). Therefore *in vitro* trypsin activation increased respectively immunoreactive kallikrein by 45.4 ± 5.5%, kininogenase activity by 47 ± 6.7 % and amidolytic activity by 41.8 ±12%. As a consequence, these percentage increases between the respective assays were significantly correlated : $r = 0.937$ for the increases in immunoreactive kallikrein and kininogenase activity and $r = 0.88$ for the increases in immunoreactive kallikrein and amidolytic activity (figure 3C).

Physiological variations

The mean average 24h urinary kallikrein excretion (UKE) in randomized normotensive subjects on uncontrolled salt diets without any medication was 136± 56 µg/24 h. The average urinary concentration was 146±62 ng/ml or 98±22 ng/mg creat. In the same age group the inter individual coefficient of variation was 25±14%. The intra individual coefficient of variation measured in three consecutive 24 h urine collection was 12±5%.

A larger number of volunteers may be needed to study the influence of age on UKE. Our study points out significant age related differences illustrated in figure 4. UKE increased with age reaching maximal values between 30 to 40 years and then a significant decrease was observed in oldest subjects over 50 years. The values in children were lower than those of adults when expressed in µg/24h. However the increase observed between 10 and 30 years was not significant when the results were expressed per mg of creatinine. The age related decrease observed in adults persisted independent of the method of expressing the results. No statistical differences between male and female could be demonstrated. Nycthemeral variations are represented in figure 5, UKE remained at an unchanged level although the daily UKE showed an intra-individual variation of 14.5±6.7%. Individual urinary creatinine excretion remained also steady, ranging from 48 to 131 mg/h, with an average intra-individual coefficient of variation of 13.5±4.5 %.

Urinary kallikrein excretion following transplantation

As shown on figure 6 urinary immunoreactive active kallikrein excretion in renal transplant patients immediately after transplantation is significantly lower than control values (12.4±3.1 versus 136±56 µg/24 h, $p<0.01$). However during the week, following renal transplantation, UKE rose significantly from day 1to day 7 (12.4±3.1 versus 58±26 µg/24 h ,$n=17$, $p<001$).

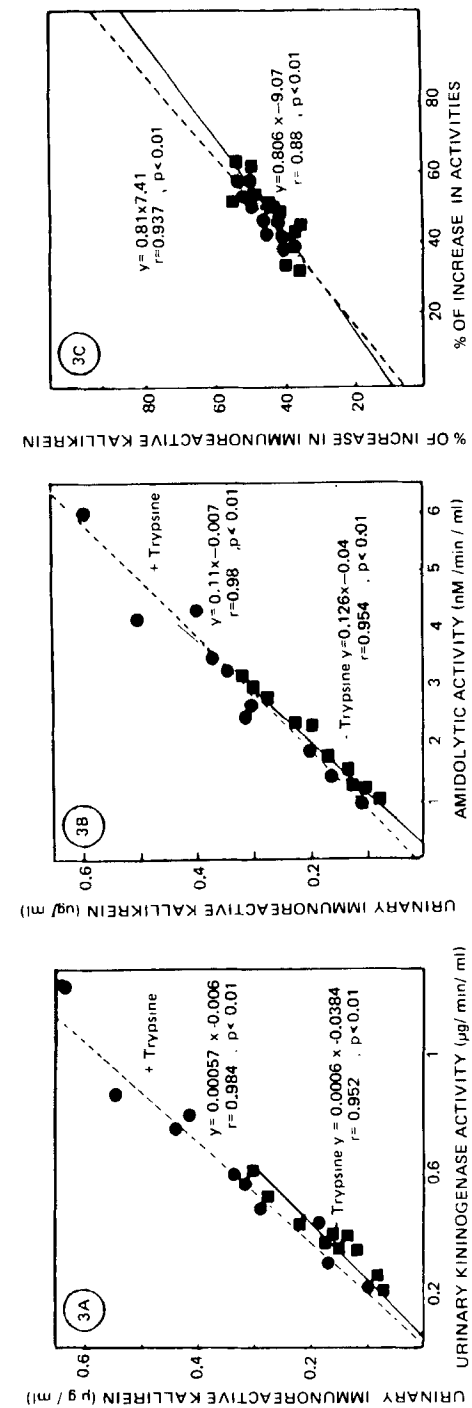

FIGURE 3 : Comparison of the direct detection of immunoreactive kallikrein with others measurements of kallikrein . Fig 3A : with kininogenase activity (■—■) and trypsin activatable kininogenase (●—●). Fig 3B : With amidolytic activity (■—■) and trypsin activatable amidolytic activity (●—●) . Fig 3C : With the percentage of increase following trypsin activation of kininogenase activity (■—■) and amidolytic activity (●—●).

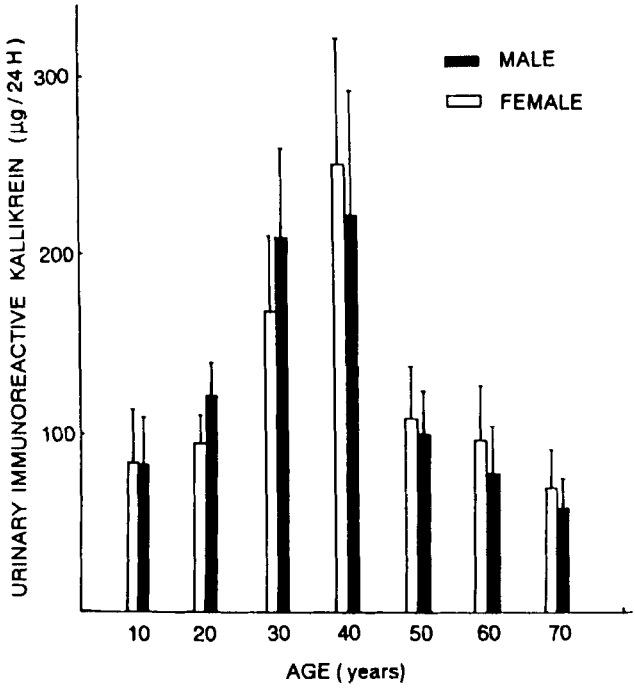

FIGURE 4 : Relationship between age and daily urinary kallikrein excretion without adjustment for creatinine excretion .

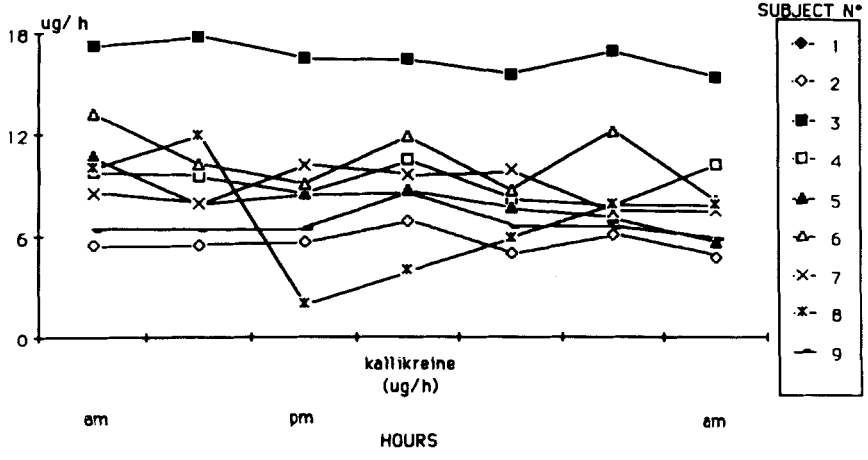

FIGURE 5 : Diurnal variations in urinary immunoreactive kallikrein in 9 normotensive subjects.

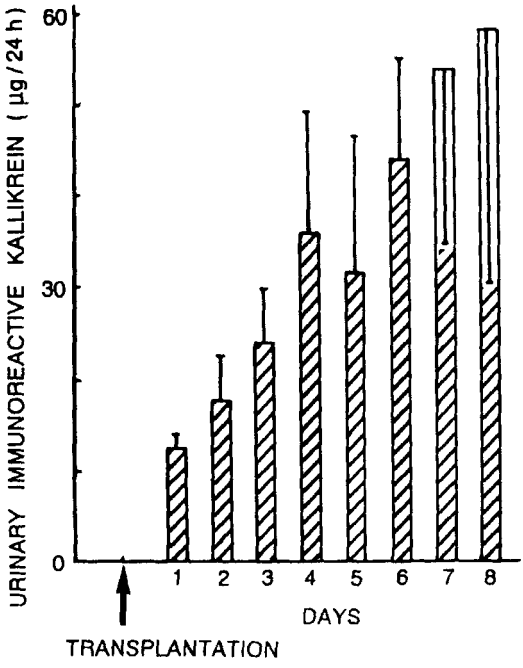

FIGURE 6 : Pattern of urinary immunoreactive kallikrein excretion in 17 renal transplant recipients during 8 consecutive days following transplantation .

Presence of kallikrein in lymph

Direct detection of glandular immunoreactive kallikrein in lymph was not possible. Addition of lymph increased the non specific binding from 6±2 to 25±12 %. However once lymph samples were subjected to SDS gel electrophoresis, the fractions eluted from the gel gave a parallel curve with kallikrein standard (figure 2). The immunoreactive profile of the fraction of a SDS gel electrophoresis is shown in figure 7A. Trypsinisation of the eluate did not induce any change in the measurement and the fractions exhibited a very weak kininogenase activity. In addition SDS gel electrophoresis did not alter the kininogenase activity of standard kallikrein. The immunoreactive fractions detected after lymph electrophoresis, exhibited the same Rf as purified glandular kallikrein (figure 7B). Recovery of standard kallikrein was 61± 7 % (n = 4). When standard kallikrein was added to lymph sample a simple immunoreactive peak was still observed (figure 7C).

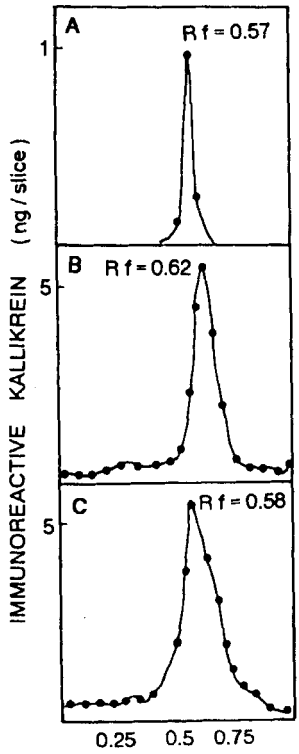

FIGURE 7 : Polyacrylamide gel electrophoresis . Fig 7A : Lymph (100 µl) is applied to the gel . Fig 7B : purified human urinary kallikrein (1µg) is applied to the gel . Fig 7C : Lymph and purified urinary kallikrein are applied to the gel .

DISCUSSION

Our results demonstrate that we have produced a specific antibody against human glandular kallikrein. Radioimmunoassay of human tissue kallikrein has been quite widely reported (5-8-14-15) however as no commercial kits are available it remains necessary for each laboratory to validate its own assay. Our antibody recognizes only the active form as trypsin treatment of the sample induced the same percentage of increase in the kininogenase activity. It is possible the heptapeptide (Ala-Pro-Pro-Ile-Gln-Ser-Arg) identified recently as the profragment liberated during

the activation of prokallikrein (16) inhibits the immunological binding with the antibody. Interestingly the inhibited active form is still immunologicaly reactive because the presence of inhibitor (aprotinin in our study) covalently bound at the active site has no influence on the direct immunoreactive detection, indicating that the kallikrein molecule remains immunologically unchanged. The data reported here indicate that our antibody recognizes the active form but is not oriented toward the active site. This is of particular interest because many inhibitors are present in biological fluids and kallikrein may exist as an active form bound to small inhibitors.

This radioimmunoassay was first used to investigate UKE under normal conditions. UKE showed considerable inter-individual and intra-individual differences, however the mean value was in the same range as previously reported (5-8). The large variation observed in normotensive subjects has been previously observed (17-18-19) and may suggest the importance of environmental and genetic factors. The data indicate also an age dependent effect on UKE. 24 h UKE increased in young children but this increase was blunted when normalized per mg of creatinine. Using an esterase method such variations have been previously reported (17-18). In contrast with these previous studies, we did not observe any variation in the creatinine adjusted UKE. Secondly, an age related decrease was also observed independent of the method of expressing the results. Since a decline in UKE has been suggested as a preclinical indicator of some hypertensive states, the age related decrease in UKE may be, in some way , involved in hypertension in older people.

Glandular kallikrein has already been measured in urine of renal transplant patients (20-21-22-23). It is generally agreed that UKE is decreased in renal transplant recipients. It has been suggested that urinary kallikrein may be a useful indicator of tubular function in this group (20) particularly in association with graft rejection. Although kallikrein excretion has been reported as being either increased (21) or decreased (20-22-23). The differences may be explained by the methodologies used and by the interference by enzymes other than kallikrein (23) ; it is difficult to evaluate the utility of kallikrein as a marker of renal tubular function. Indeed a graft rejection episode is a rather complex situation and many causative agents may be involved : whether variations of UKE result from renal tubular damage or occur before a tissue lesion remains to be demonstrated.

Thus in our study we did not try to use UKE as predictive index of graft rejection, but assessed its longitudinal UKE during recovery of satisfactory renal function. The enhancement of UKE resulted essentially from a significant rise in the excretion of the active form.

The development of this radioimmunoassay also allowed the detection of immunoreactive kallikrein in lymph of patients suffering from pancreatitis. The thoracic drainage being justified by the severity of the disease. Although other workers have measured esterase activity in dog renal lymph (24) or rat renal lymph (25-26), to our knowledge no previous study has identified tissue kallikrein in human lymph . This immunoreactive kallikrein is essentially in the active form, however a very weak kininogenase activity was observed suggesting that this lymphatic kallikrein exist as an inhibitor complex. It seems that in lymph prokallikrein is processed into active kallikrein but the active form is present in a complex inhibitor form. However whether this lymphatic kallikrein exists in normal subjects or is linked to the pancreatitis remains a topic for future studies.

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