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Proteinuria induced by anti-dipeptidyl peptidase IV (gp108); role of circulating and glomerular antigen

Y. NATORI, N. SHINDO* & Y. NATORI *Division of Pathophysiology and* **Pathology, Clinical Research Institute, National Medical Centre, Tokyo, Japan*

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SUMMARY

Massive proteinuria is induced in rats by administration of rabbit antibody to dipeptidyl peptidase IV (DPPIV, gp108), a glycoprotein present on glomerular cell membranes and in serum. This study was undertaken to know which antigen, glomerular or serum DPPIV, is responsible for forming immune complex in glomeruli and development of proteinuria. An i.p. injection of the antibody resulted in a rapid decrease of serum DPPIV and a gradual increase of rabbit IgG deposited along glomerular capillary wall for 4–8 h. Abnormal proteinuria appeared within 8 h, peaked on day 2 (> 200 mg/24 h) and then declined. An increase of urinary protein and glomerular deposition of IgG also occurred, when the antibody was injected into serum DPPIV-depleted rats that had received preinjection of anti-DPPIV antibody. These results suggest that proteinuria is induced by direct binding of anti-DPPIV antibody to the membrane antigen of glomerular cells.

Keywords dipeptidyl peptidase IV proteinuria *in situ* immune complex circulating antigen

INTRODUCTION

Studies on experimental models of glomerulonephritis have revealed that glomerular immune deposits can be formed by the *in situ* binding of antibody to intrinsic or planted glomerular antigens as well as by the trapping of circulating immune complexes [1]. Heymann nephritis (HN), a model for membranous nephropathy, can be induced in rats by the injection of antibodies to renal tubular membrane fraction (Fx1A) [2,3]. In this model, immune deposits are found along the glomerular basement membrane in the subepithelial space. Although some studies have shown the involvement of circulating immune complexes in the pathogenesis of this model [4,5], it is believed that glomerular immune deposits in HN are formed mainly by *in situ* binding of antibody [6,7]. gp330, the main pathogenic antigen of HN, has been recently demonstrated on the foot processes of glomerular epithelial cells beneath the basement membranes [8]. Furthermore, anti-gp330 antibody was detected at an identical site after injection into rats [9], thus providing molecular evidence of *in situ* immune complex formation in the glomeruli of HN rats. Nonetheless, it is still unclear whether other antigen(s) are involved in the development of HN, or whether trapping of circulating immune complex has also occurred and is necessary for the development of HN.

Dipeptidyl peptidase IV (DPPIV, or gp108 [10]) is another major antigen of Fx1A, which may be involved in HN [10–12]. An injection of rabbit anti-DPPIV antibody into rats induces acute massive proteinuria and glomerular deposition of rabbit IgG [10]. DPPIV is widely distributed in tissues and cells of mammals. It is predominantly expressed on the brush border membranes of both renal tubules and intestinal microvilli, but is also found along glomerular capillary walls [13,14] and in serum [13,15]. Thus, there are several possible mechanisms of glomerular immune complex formation when anti-DPPIV antibody is injected into rats. The present study was undertaken to elucidate the source of the target antigen responsible for the induction of proteinuria in this model. For that purpose, we followed the contents of both the antigen and the antibody in serum and glomeruli, and used serum DPPIV-depleted rats. The results show that DPPIV located along the glomerular capillary wall is important in the induction of proteinuria.

Correspondence: Yasuhiro Natori PhD, Clinical Research Institute, National Medical Centre, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162, Japan.

MATERIALS AND METHODS

Preparation of anti-DPPIV rabbit antibody

DPPIV was purified from Fx1A of Wistar rat kidneys as described previously [10]. Anti-DPPIV antibody was prepared by immunizing albino male rabbits with 0·65 mg of purified DPPIV in Freund's complete adjuvant (FCA), followed by booster injections of 0·32 mg at 3 weeks and of 0·1 mg at 6, 8 and 14 weeks. All booster injections were in Freund's incomplete adjuvant (FIA). The animals were repeatedly bled after the second booster, and the antiserum titre was determined by enzyme immunoassay as described below. Antisera containing a high activity of anti-DPPIV antibody were pooled and heated at

56°C for 30 min, followed by purification of the gammaglobulin fraction by 33% ammonium sulphate precipitation.

Assays for DPPIV and anti-DPPIV antibody

The presence of anti-DPPIV antibody in the sera of rats injected with anti-DPPIV rabbit antibody made it impossible to determine the DPPIV content in sera by sandwich enzyme immunoassay [15]. Therefore, the DPPIV content was measured on the basis of enzyme activity using photometry [11], despite the lower sensitivity of this method. The reaction mixture (0·5 ml) containing 50 mM Tris-HCl pH 8·0, 1% Triton X-100, 1·5 mM Gly-Pro-*p*-nitroanilide tosylate (Sigma Chemical Co., St Louis, MO) and 10 μl of test serum was incubated at 37°C for 2 h and the increase in absorbance at 410 nm was measured. The presence of anti-DPPIV antibody (100 μg/ml) in the reaction mixture did not interfere with the enzyme activity (absorbance at 410 nm: normal rabbit gammaglobulin *versus* anti-DPPIV antibody, 0·834 ± 0·018 *versus* 0·901 ± 0·003).

Anti-DPPIV antibody was measured by enzyme immunoassay [15]. Wells of microtitration plates (Nunc Immunoplate I, Roskilde, Denmark) were coated with 100 μl of 2 μg/ml DPPIV, and incubated with the antibody solution followed by incubations with peroxidase-conjugated goat anti-rabbit IgG antibody (Cappel/Organon Teknica Corp., West Chester, PA), and substrate solutions. The absorbance at 486 nm was measured with an ELISA Analyzer SLT-210 (Salzburger Elektronic Industry, Austria).

Proteinuria induced by anti-DPPIV antibody

Male, six-week-old Lewis rats (weighing 130–140 g, Charles River Japan Inc., Atsugi, Japan) were used. A group of seven rats was injected intraperitoneally with 50 mg of gammaglobulin fraction of anti-DPPIV rabbit antisera and fed in metabolic cages. Blood samples were collected at 1, 2, 4, 6, 8, 12 and 24 h and at 2, 3, 4, 5, 6, 8 and 10 days after the antibody injection. Urine was collected every 24 h. Levels of DPPIV and anti-DPPIV rabbit antibody in sera were measured as described above, and urinary proteins were determined by the biuret method after trichloroacetic acid precipitation. Three control rats were injected with 50 mg of gammaglobulin fraction of normal rabbit serum.

To determine urinary protein excretion over a short period, five rats injected with 50 mg of anti-DPPIV antibody and four control rats injected with 50 mg of normal rabbit gammaglobulin were kept in small mouse-cages without feed and their urine was collected for 8 h. Urinary proteins, in these short-period experiments, were determined by the method of Lowry *et al.* [16] because it is more sensitive than the biuret method.

Immunohistochemistry for anti-DPPIV-injected rats

Thirty-one rats injected with 50 mg of anti-DPPIV antibody were killed at 1, 4, 8 and 24 h and at 2, 3, 5 and 7 days after injection using the following numbers of animals: 2, 6, 6, 4, 7, 2, 2 and 2, respectively. Four control rats injected with 50 mg of gammaglobulin fraction of normal rabbit serum were killed at 4, 8 and 24 h and at 2 days after injection, one at each time. A portion of each kidney from all rats was snap-frozen in hexane cooled with acetone/dry ice. Direct immunofluorescence was performed on cryostat sections of kidneys stained with FITC-conjugated goat anti-rabbit IgG, anti-rat IgG or anti-rat C3 (Cappel/Organon Teknica Corp.). For light microscopy, the above mentioned specimens were fixed in 20% buffered formalin, sectioned and stained with haematoxylin–eosin.

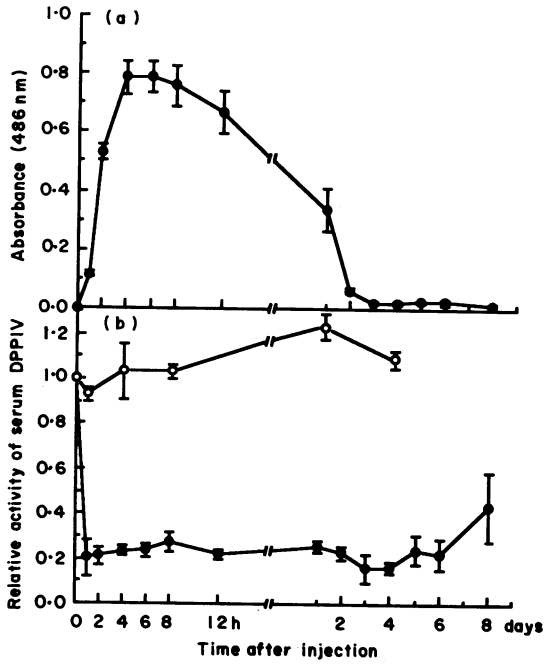

Fig. 1. Anti-dipeptidyl peptidase IV (DPPIV) antibody (a) and DPPIV activity (b) in sera of rats after the injection of anti-DPPIV antibody (●) or normal rabbit gammaglobulin (○). DPPIV content was expressed as relative activity to normal level (day 0).

Localization of glomerular DPPIV in three rats 4 days after anti-DPPIV antibody injection was compared with that of a normal rabbit gammaglobulin-injected rat by indirect immunofluorescence using anti-DPPIV antibody and FITC-conjugated goat anti-rabbit IgG.

Repeated injection of anti-DPPIV antibody

Nine rats were injected intraperitoneally with anti-DPPIV antibody twice, on days 0 and 4. Urine samples from six rats were collected every 24 h up to day 9 and samples of their blood were collected on days 0, 1, 2, 4, 5, 6 and 9. Three rats were killed 4 h after the second injection and their kidneys were used for direct immunofluorescence. Seven control rats were injected intraperitoneally with anti-DPPIV antibody on day 0 and with normal rabbit gammaglobulin on day 4: five control rats were for urine collection and two for direct immunofluorescence.

Statistical analysis

Student's *t*-test for paired or unpaired data was used to analyse the results of urinary protein excretion. All values are expressed as means ± s.d.

RESULTS

An i.p. injection of anti-DPPIV antibody into rats resulted in a gradual increase in the level of anti-DPPIV antibody in the circulation, reaching a maximum between 4 h and 8 h and then declining (Fig. 1a). Serum DPPIV enzyme activity rapidly decreased within 1 h and remained low for 6 days (Fig. 1b). Abnormal proteinuria appeared on day 1 as previously reported

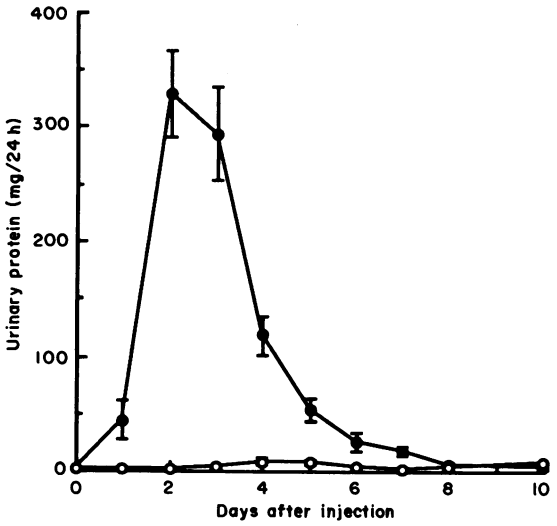

Fig. 2. Urinary protein excretion of rats injected with anti-dipeptidyl peptidase IV (DPPIV) antibody (●) or normal rabbit gammaglobulin (○).

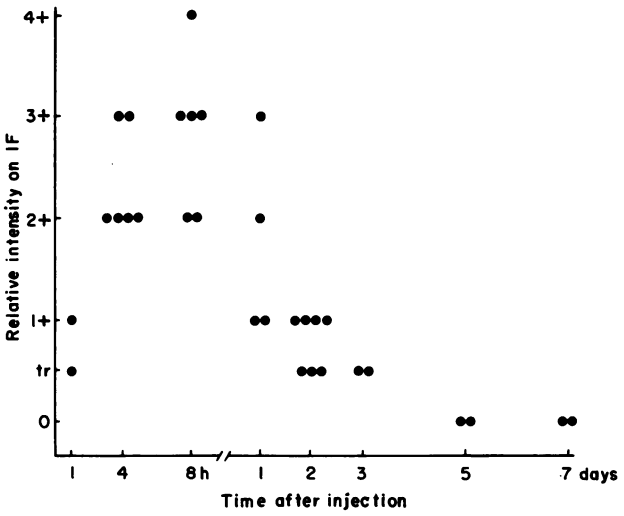

Fig. 3. Direct immunofluorescence (IF) for rabbit IgG in glomeruli of rats after the injection of anti-dipeptidyl peptidase IV (DPPIV) antibody. The figure demonstrates the relative intensity (0–4+) of glomerular deposits of rabbit IgG for each rat, which was determined under blind examination.

[10] and continued for about 7 days (Fig. 2). The protein excretion rate peaked on day 2 (> 200 mg/24 h), followed by a gradual decline. An injection of normal rabbit gammaglobulin induced neither a decrease of serum DPPIV nor abnormal proteinuria (Figs 1b and 2). Rats injected with anti-DPPIV antibody demonstrated a statistically significant increase in urinary protein excretion in the first 8 h after injection compared with rats given normal rabbit gammaglobulin fraction ($1{\cdot}5 \pm 0{\cdot}4$ *versus* $0{\cdot}4 \pm 0{\cdot}2$ mg/8 h, $P < 0{\cdot}01$).

Light microscopic examination revealed normal cellularity of glomeruli at all time periods. Glomerular deposition of rabbit IgG by direct immunofluorescence coincided with the appearance of the antibody in the circulation (Figs 3 and 4). Rabbit IgG was faintly seen along the glomerular capillary walls 1 h after injection, and increased for 4–8 h. On day 1, rabbit IgG was found in the tubules as well as the glomeruli of kidneys from three of four rats. Glomerular deposits of rabbit IgG decreased on days 2 and 3, whereas continued strong fluorescence was seen in the lumina of proximal tubules. On days 5 and 7 no rabbit IgG was found in glomeruli despite the presence of slight but persistent abnormal proteinuria. No rat IgG or C3 was found in the glomeruli of any rats, and no rabbit IgG was found in glomeruli of control rats.

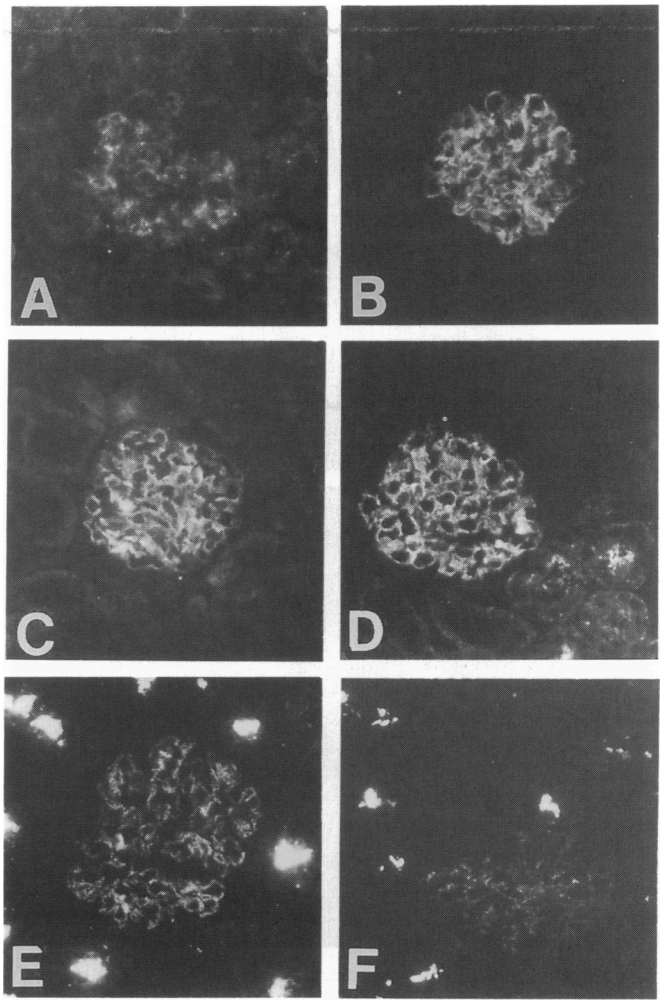

Fig. 4. Direct immunofluorescence for rabbit IgG of kidney sections of rats injected with anti-dipeptidyl peptidase IV (DPPIV) antibody after (a) 1 h, (b) 4 h, (c) 8 h, (d) 24 h, (e) 2 days or (f) 3 days. Immune deposits are observed along the glomerular capillary walls, and from 24 h after the injection they are also seen in the proximal tubules. (× 135.)

On day 4, when the serum DPPIV level was still low, immunoreactivity of glomerular DPPIV was found to be similar to that of control rats (Fig. 5), indicating that these rats were serum DPPIV-depleted but contained normal levels of glomerular DPPIV. Anti-DPPIV antibody was again injected intraperitoneally into these rats, 4 days after the first antibody injection. Glomerular deposition of rabbit IgG was observed 4 h after the second injection (Fig. 6a) in the same pattern and intensity as that in rats 4 h after the first injection, and resulted in an increase of urinary protein excretion (Fig. 7). An injection of normal

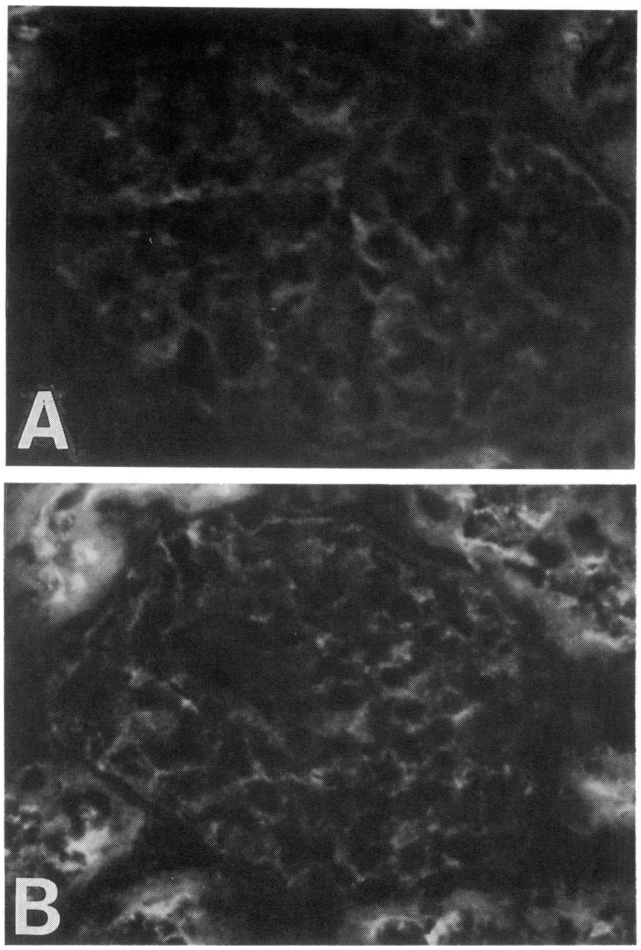

Fig. 5. Indirect immunofluorescence for dipeptidyl peptidase IV (DPPIV) of kidney sections of rats injected with anti-DPPIV antibody (a) or with normal rabbit gammaglobulin (b) and killed 4 days after the injection (× 300).

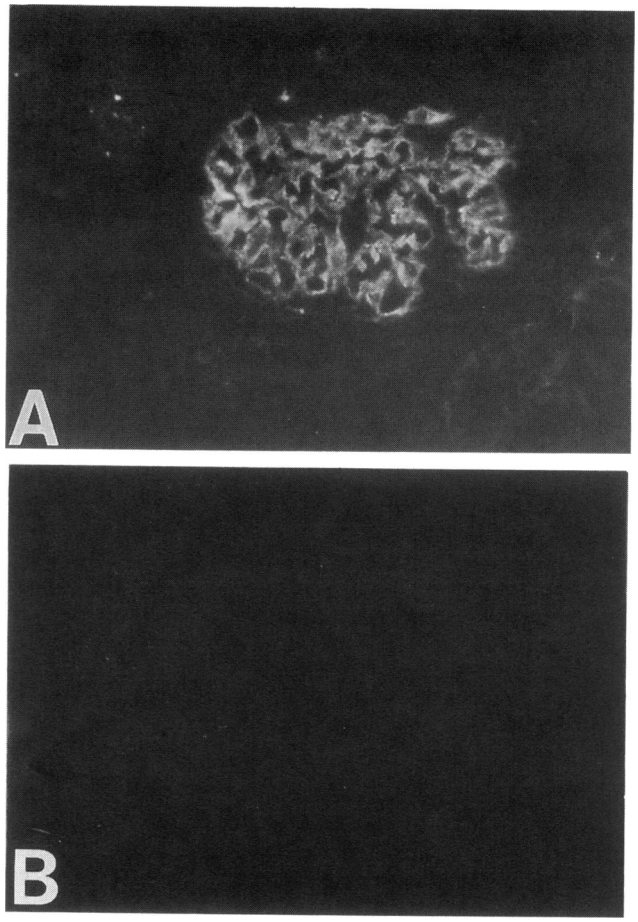

Fig. 6. (a) Direct immunofluorescence for rabbit IgG in glomerulus of a rat injected twice (days 0 and 4) with anti-dipeptidyl peptidase IV (DPPIV) antibody and killed 4 h after the second injection. The pattern and the intensity of fluorescence is nearly the same as that of Fig. 4 (b). (b) Direct immunofluorescence for rabbit IgG in glomerulus of a control rat injected with anti-DPPIV antibody on day 0 and with normal rabbit gammaglobulin on day 4. (× 165).

rabbit gammaglobulin into serum DPPIV-depleted rats did not cause an increase of urinary protein excretion (Fig. 7) or additional glomerular deposition of rabbit IgG (Fig. 6b).

DISCUSSION

Over 20 years ago, it was first determined that certain of the antigens in Fx1A are also present in serum. This observation has been used to support the hypothesis of glomerular trapping of circulating immune complexes [4,17]. Since DPPIV, one of the major antigens in Fx1A, is present in serum, we asked whether serum DPPIV could form circulating immune complexes that are trapped in glomeruli and lead to development of proteinuria.

Most of the enzyme activity of DPPIV was removed within 1 h from the circulation after the injection of anti-DPPIV antibody, while anti-DPPIV antibody in the circulation peaked during 4 and 8 h. Glomerular deposition of rabbit IgG peaked more than 4 h after antibody injection, coincident with the peak of the antibody level in serum. These results support the idea that anti-DPPIV antibody that had slowly appeared from peritoneal space into the circulation formed *in situ* immune complex by direct binding to glomerular DPPIV, resulting in the induction of proteinuria. However, it still seemed possible that circulating immune complexes formed with serum DPPIV were sequestered from the circulation rapidly, and then gradually redistributed to the kidney.

To assess the role of serum DPPIV in this model, we performed the experiment using serum DPPIV-depleted rats, which can be made by an injection of anti-DPPIV antibody. Four days after the injection of anti-DPPIV antibody, serum DPPIV of those rats was 10–20% of normal level, whereas their glomerular DPPIV was nearly normal. An injection of anti-DPPIV antibody into serum DPPIV-depleted rats clearly induced glomerular deposition of rabbit IgG and proteinuria, suggesting that the role of serum DPPIV in this model is small, if any, and that the binding of antibody to the intrinsic antigen of glomeruli is responsible for development of proteinuria. Direct binding of anti-DPPIV (gp90) antibody to glomerular antigen was shown by Bagchus *et al.* [18] in a perfusion study.

Because serum DPPIV-depleted rats still contained the low level of enzyme activity of DPPIV in serum, the possibility that serum DPPIV plays a role in this model can not be completely excluded. Although the enzyme assay system used in this study is specific for DPPIV [19], the remaining enzyme activity in

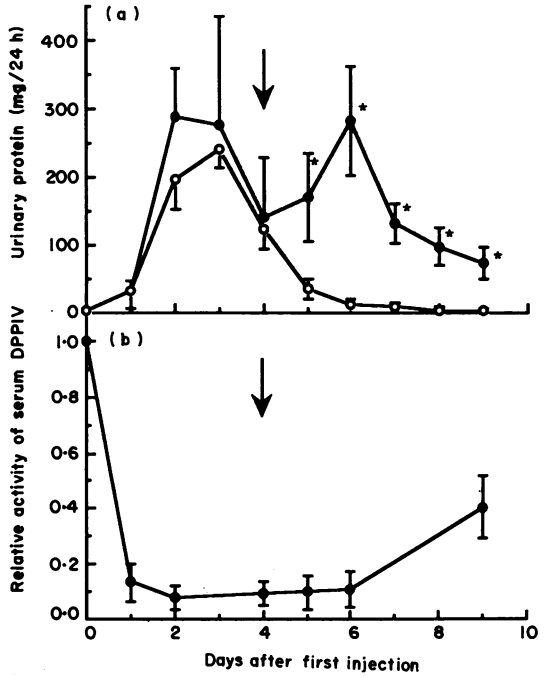

Fig. 7. (a) Urinary protein excretion of rats injected twice with anti-dipeptidyl peptidase IV (DPPIV) antibody on days 0 and 4 (arrow) (●) or control rats injected with anti-DPPIV antibody on day 0 and with normal rabbit gammaglobulin on day 4 (○). $*P<0{\cdot}01$ compared with control rats. (b) Serum DPPIV activity of rats injected twice with anti-DPPIV antibody.

serum DPPIV-depleted rats might be attributed to a different protein from common DPPIV, since it could not be lowered by an additional injection of anti-DPPIV antibody (Fig. 7). The molecular size of most serum DPPIV is identical to that of kidney DPPIV [15], which has the common structure of DPPIV, a homodimer of a 110-kD subunit. On the other hand, Bauvois reported 55-kD DPPIV in mouse fibroblasts [20], which might be an isozyme of DPPIV. The 55-kD DPPIV, therefore, is a possible origin of the remaining enzyme activity of DPPIV in serum DPPIV-depleted rats.

The mechanism of abnormal urinary protein excretion after antibody binding to the membrane antigen of glomerular cells is still unknown. Neither glomerular deposition of C3 nor an increase in glomerular cell number were observed at any time after the antibody injection. Although DPPIV is a membrane enzyme that releases dipeptides from the N-terminus of susceptible peptides or proteins, recent studies have shown that it also has the potential to function as a binding protein to extracellular matrix materials or collagens [20–22]. Anti-DPPIV antibody can interfere with the interaction between cells and collagen *in vitro* [23,24]. Interference of the interaction between glomerular cells and basement membranes might be involved in the mechanism of the induction of proteinuria. Rapid induction of abnormal urinary protein excretion after the antibody injection (within 8 h) might suggest the direct effect of antibody binding. A recent study by van Leer *et al.* [25] has suggested the similar effect of anti-DPPIV antibody in mouse model for lupus nephritis.

Mendrick & Rennke [26] demonstrated that administration of a MoAb to a sialoglycoprotein (SGP-115/107), later identified as DPPIV [27], induced proteinuria and detachment of the epithelium from the underlying basement membrane in rats, without the involvement of complement or infiltrating inflammatory cells. This phenomenon was induced only when FCA was co-injected, suggesting that a non-selective activation of some cells such as macrophages or lymphocytes is essential for the induction of proteinuria. Our model using polyclonal anti-DPPIV antibody does not require the injection of Freund's adjuvant. The reason for this discrepancy is unknown.

Anti-DPPIV antibody bound along the basement membrane disappears from glomeruli much faster than does anti-gp330 [8]. In HN induced by anti-Fx1A antibody, immune deposits along the glomerular basement membranes persist for at least 3 months, along with deposits of rat IgG [3]. Kerjaschki *et al.* showed that anti-gp330 antibody binds to the target antigen on the coated pits of the foot processes of epithelial cells, and forms large immune deposits in the basement membranes [9]. The immune deposits remain in contact with coated pits and become firmly attached to the basement membranes. The known interaction of gp330 with cytoskeletal components [9,28] and the direct binding of gp330 to basement membrane components [9,29] have been considered important for the formation of large immune deposits. Although *in vitro* studies have shown the ability of DPPIV to bind to extracellular matrix component (collagen), anti-DPPIV does not remain in glomeruli for more than 5 days. To elucidate which factor(s) determine the destiny of immune deposits in basement membranes, it will be necessary to study another single antigen–antibody system and to compare the molecular properties or physiological functions of the individual target antigens. Because the pathogenesis of membranous nephropathy in man may be similar to that of HN, such studies may help to identify the target antigen(s) of membranous nephropathy.

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