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Abstract

Monoclonal antibodies were raised against urine proteins from diabetic patients. An antibody, YO-2, stained three protein bands with apparent molecular weights of 66, 49, and 36 kDa. These bands were not reactive with an anti-human albumin antibody. The urine levels of YO-2-reactive antigen in the normal control were 0.97 +/- 0.37 U/g-Cr (units per gram of urine creatinine) (mean +/- SD). Those of the normo-, micro-, and macroalbuminuric diabetic patients, respectively, were 1.38 +/- 1.36, 2.87 +/- 2.07, and 3.92 +/- 3.33 U/g-Cr. They were significantly higher in the micro- and macroalbuminuric patients. The urine levels of YO-2-reactive antigen had no significant correlation with the urine albumin levels and hemoglobin A1c. We concluded that; a) monoclonal antibody YO-2 recognized a non-albumin urine antigen increasingly excreted in diabetic patients with nephropathy, b) recent glycemic control of diabetes would not significantly affect the urinary excretion rate of YO-2-reactive antigen, and c) the excretion rate and probably the mechanism of YO-2-reactive protein differed from those of albumin. The urine levels of YO-2-reactive antigen could be a clinical marker of diabetic nephropathy.

KEYWORDS: diabetes, nephropathy, monoclonal antibody, microalbuminuria

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Increased Urinary Excretion of Non-Albumin Antigen Detected with YO-2, a Novel Monoclonal Antibody, in Diabetic Patients

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Monoclonal antibodies were raised against urine proteins from diabetic patients. An antibody, YO-2, stained three protein bands with apparent molecular weights of 66, 49, and 36 kDa. These bands were not reactive with an anti-human albumin antibody. The urine levels of YO-2-reactive antigen in the normal control were 0.97 ± 0.37 U/g-Cr (units per gram of urine creatinine) (mean $\pm$ SD). Those of the normo-, micro-, and macroalbuminuric diabetic patients, respectively, were 1.38 ± 1.36, 2.87 ± 2.07, and 3.92 ± 3.33 U/g-Cr. They were significantly higher in the micro- and macroalbuminuric patients. The urine levels of YO-2-reactive antigen had no significant correlation with the urine albumin levels and hemoglobin A_{1c}. We concluded that; a) monoclonal antibody YO-2 recognized a non-albumin urine antigen increasingly excreted in diabetic patients with nephropathy, b) recent glycemic control of diabetes would not significantly affect the urinary excretion rate of YO-2-reactive antigen, and c) the excretion rate and probably the mechanism of YO-2-reactive protein differed from those of albumin. The urine levels of YO-2-reactive antigen could be a clinical marker of diabetic nephropathy.

Key words: diabetes, nephropathy, monoclonal antibody, microalbuminuria

Diabetic nephropathy is a major cause of morbidity and mortality of diabetic patients. The Diabetes Control and Complication Trial has recently established that near-normal glycemic control of insulin-dependent diabetes mellitus (IDDM) can substantially reduce the incidence and progress of diabetic complications including nephropathy (1). On the other hand, diabetic nephropathy does not develop in all diabetic patients. About 35 % of IDDM patients and 3 to 25 % of non-insulin-dependent diabetes mellitus (NIDDM) patients develop diabetic nephropathy (2, 3). It is crucial to identify the high-risk patients in whom nephropathy will develop and progress to provide individualized treatment.

The urinary albumin excretion rate (UAE) in normal individuals is less than $20\,\mu g/min$ (4). A subclinical increase in UAE, ($20 < UAE \leq 200\ \mu g/min$) is termed "microalbuminuria", and clinical studies have shown that microalbuninuria often leads to the development and progression of diabetic nephropathy in IDDM (5-7). However, another study indicated that microalbuminuria was not always a strong predictor of diabetic nephropathy (8). Furthermore, although the clinical implications of microalbuminuria in NIDDM are not fully understood, it was shown to be associated with increased macrovascular morbidity and mortality (9, 10). However, in contrast to IDDM in which more than 80 % of patients with microalbuminuria progressed to overt nephropathy within a decade (11, 12), only 20 % of those with NIDDM advanced to overt proteinuria (12). Furthermore, instead of a higher prevalence of microalbuminuria and albuminuria in NIDDM (13-17), the incidence of renal impairment is relatively lower (9, 13).

The above observations and controversial correlation between microalbumin and histological glomerular changes (18-21) justify further search for additional clinical markers for diabetic nephropathy, especially in NIDDM. Urinary excretions of non-albumin proteins were studied in diabetic patients (22). Urinary excretion of *N*-acetyl-β-D-glucosaminidase, retinol-binding protein and β_2-microglobulin were reported to be markers of tubular

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damage (23, 24). Excretion of immunoglobulin light chain and α_1-microglobulin were correlated with the level of hemoglobin A_{1c} (HbA_{1c}) (25), which reflects recent glycemic control of diabetes (26). However, their clinical values as predictors of diabetic nephropathy have not been established.

In the present study, we prepared the non-albumin urine protein fraction from patients with poorly controlled NIDDM without macroalbuminuria and raised monoclonal antibodies (MoAb) against it. MoAb YO-2 formed complexes with non-albumin proteins. The urine levels of the protein detected with YO-2 were significantly higher in diabetic patients with micro- and macroalbuminuria, but they were not correlated with either urine levels of albumin or HbA_{1c}.

Materials and Methods

Urine samples for the antigen. Non-albumin urine proteins were obtained from patients with poorly controlled diabetes (HbA_{1c} more than 8.0%) without macroalbuminuria. Urine samples from 20 patients were combined, centrifuged at 30,000 × g for 30 min, filtered through a glass fiber paper (WhatmanGF/B, Whatman International Ltd., Maidstone, England), and freeze-dried. They were reconstructed with a minimum volume of water, applied to a Superose 6-HR 10/30 column in a fast protein liquid chromatography system (Pharmacia LKB Biotechnology, Uppsala, Sweden), and eluted with phosphate-buffered saline (PBS). Fractions eluted after the albumin peak were combined, freeze-dried and used as the partially purified antigen (ppAg). The normal control antigen was prepared in the same way from urine samples from 10 healthy volunteers.

Urine samples for the assay. First morning urine samples were collected from 89 diabetic patients: 85 NIDDM and 4 IDDM, and 19 healthy volunteers (27). Since excluding the patients with IDDM did not affect the results, patients with both IDDM and NIDDM were included in the present study. Diabetic nephropathy was classified into three groups according to the urinary albumin: creatinine ratio (urinary albumin index; UAI): normoalbuminuria (UAI $\leq$ 15 mg/g-Cr), microalbuminuria (15 < UAI $\leq$ 200 mg/g-Cr), and macroalbuminuria (UAI > 200 mg/g-Cr) (28). Table 1 shows the patients' clinical data. Urine samples filtered through a 0.22-μm membrane (Millipore Ltd., Tokyo, Japan) were kept frozen at $-30^{\circ}C$ until assay.

Monoclonal antibody. MoAb was prepared as described by Kennett (29). The ppAg (100 μg as protein) in complete Freund's adjuvant were subcutaneously injected into the back of a female 8 weeks old BALB/c mouse. The same amount of the ppAg in the incomplete Freund's adjuvant were repeatedly given 4 times every 4 weeks. The spleen cells obtained 3 days after the last injection were hybridized with P3-X63-Ag8-U1 myeloma cells using polyethylene glycol 4000 (Sigma Chemical Co., St. Louis, MO, USA). Culture supernatants were assayed for antigen reactivity as described below. The ppAg and normal control antigen were used at 20 μg/ml in 50 mM carbonate buffer (pH 9.6).

The isotype of MoAb was determined using a mouse monoclonal antibody isotyping kit (Amersham International, Little Chalfont, Buckinghamshire, England). The IgM fraction was partially purified using Superose 6-HR 10/30 column chromatography.

Antigen specificity of MoAb YO-2. The ppAg and human albumin (Sigma) were subjected to Superose 6-HR 10/30 column chromatography. Fractions of 0.5 ml were collected and assayed for their reactivities with MoAb YO-2 and rabbit anti-human albumin IgG (Inter-Cell Technologies, Inc., Hopewell, NJ, USA).

The ppAg and human albumin were subjected to

Table 1 Clinical data of the patients

	Number of patients	Sex (F/M)	Age (Years)	Duration of DM (Years)	HbA_{1c} (%)	Retinopathy (%)		
						(−)	Simple	Proliferative[b]
Normal control	19	8/11	61±4	—	nd[a]	—	—	—
Normoalbuminuria	50	22/28	61±10	14±12	7.6±1.8	56	21	23
Microalbuminuria	25	7/18	65±8	16±9	7.6±1.3	41	36	23
Macroalbuminuria	14	2/12	67±9	16±7	7.7±1.4	14	29	57

[a]Not determined; normal range is within 6.2%. [b]Including preproliferative retinopathy. DM: Diabetes mellitus.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. They were separated in a 10 % polyacrylamide gel in the reducing condition and transferred to a nitrocellulose membrane. After blocking nonspecific binding with 1 % gelatin in PBS and washing with PBS containing 0.05 % of Tween 20 (T-PBS), the membrane was incubated with MoAb YO-2 (16 μg/ml) or rabbit anti-human albumin IgG (9.3 μg/ml) at 4°C overnight, washed again, and then reacted with peroxidase-conjugated goat anti-mouse IgG + IgM (H + L) (Jackson ImmunoResearch Lab., Inc., West Grove, PA, USA) or peroxidase-conjugated goat anti-rabbit IgG. The membranes were exposed to X-ray film after treating them with Amersham Western blotting detection reagents (Amersham International).

Enzyme-linked immunosorbent assay (ELISA). Urine samples were diluted 50 times with 50 mM carbonate buffer (pH 9.6). Thirty microliters of each sample was applied to the wells of a 96-well immunoplate (Dynatech Lab. Inc., Chantilly, VA, USA) and incubated at 4°C overnight to immobilize the antigen. The wells were washed with T-PBS, blocked with 1 % gelatin in PBS, and then added 30 μl of MoAb YO-2 (20.5 μg/ml). After one hour incubation and through washing with T-PBS, 50 μl/well of peroxidase-conjugated goat anti-mouse IgG + IgM (H + L) (90 μg/l) were added. The plate was then incubated at room temperature for one h, washed extensively with T-PBS, added 100 μl of the substrate solution containing 1.23 mM *O*-phenylenediamine (Zymed Lab., San Francisco, CA, USA) and 0.03 % of H_2O_2 in 0.05 M citrate phosphate buffer (pH 5.0). The peroxidase reaction was stopped after 5 min by adding 50 μl of 2 N HCl. The absorbance at 492 nm was read in a MTP-120 ELISA reader (Corona Electric Co., Ibaragi, Japan). The standard curve was constructed in each assay using the serially diluted ppAg. The amount of antigen contained in 9.6 μg/well of the ppAg was arbitrarily defined to be one unit.

Other analytical methods. Urinary albumin was determined by the immunodiffusion method (Denkaseiken Co., Ltd., Tokyo, Japan) and creatinine by a urease-indophenol method (Iatron Lab., Inc,, Tokyo, Japan). The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. HbA_{1c} was measured by high-performance liquid chromatography (HPLC, HLC-723GHbIII, Tosoh, Tokyo, Japan).

Statistical analysis. Results are expressed as an arithmetic mean with standard deviation (mean ± SD). Statistical differences among more than three groups were calculated by the Kruskal-Wallis rank test and that between two groups by the Mann-Whitney U-test. Categorical data were analyzed with a contingency table method (chi-square test and Fisher's exact test). Correlations were determined using Pearson's correlation coefficient.

Results

Establishment of monoclonal antibodies reactive with the ppAg. We finally established 6 hybridoma clones, YO-1 to -6, producing monoclonal antibodies to the ppAg from diabetic patients. Determination of the immunoglobulin isotype showed that all of the antibodies were IgM. The reactivities of the 6 antibodies, YO-1 to -6, were determined by ELISA using the ppAg and normal control antigen. Comparison of the antibody reactivities against the ppAg and against the normal control antigen showed that the reactivities of two clones (YO-1 and YO-2) to ppAg were more than two times higher than the control antigen (data not shown). MoAg YO-1 reacted not only with the ppAg but also with human albumin. On the other hand, MoAb YO-2 reacted only with the ppAg used as an immungen (data not shown). Therefore, only MoAb YO-2 was selected for further study.

Antigen specificity of MoAb YO-2. To identify the antigen molecule(s) which react with MoAb YO-2, ELISA analysis was carried out. As shown in Fig. 1B, the MoAb YO-2-reactive antigen (YO-2-Ag) was eluted from a Superose 6-HR 10/30 column just after albumin. It should be noted here that albumin was not completely removed from the ppAg (Fig. 1).

Immunoblotting of the electrophoretically separated urine sample showed three MoAb-YO-2-reactive bands with molecular weights of 66, 49, and 36 kDa (Fig. 2A). MoAb-YO-2 did not react with human albumin (Fig. 2B). The urine sample exhibited a major 67-kDa band and several smaller bands with apparent molecular weight of 54, 45, 31, and 16 kDa (Fig. 2C). Immunoblotting of the purified albumin with a polyclonal anti-albumin antibody showed a single band at 67 kDa (Fig. 2D).

Urine levels of the YO-2-Ag in diabetic patients. The urine levels of YO-2-Ag were determined by ELISA (Fig. 3). Normal control values (healthy volunteers) were 0.97 ± 0.37 U/g-Cr (mean ±

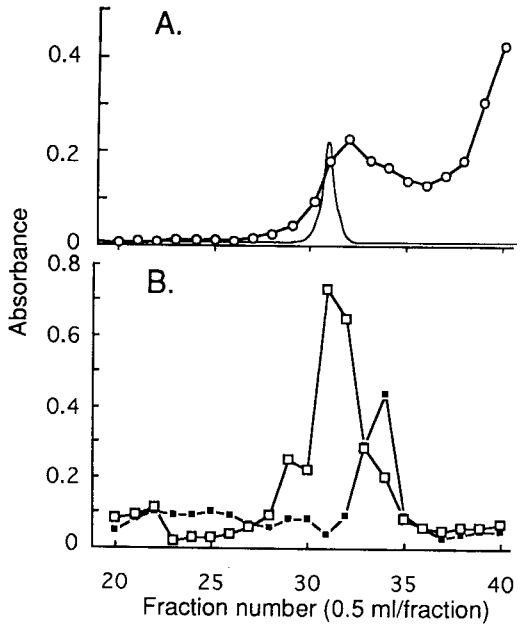

Fig. 1 Molecular sieve column chromatography of the MoAb YO-2-reactive urine antigen. The partially purified urine antigen was separated in a Superose 6-HR 10/30 column. **A**: protein concentration of the urine sample was determined with a BCA protein assay kit (OD 570nm: ○—○) and that of the purified albumin with the absorbance at 280nm (——). **B**: Each fraction was assayed for MoAb YO-2 reactivity (OD 472nm: ■—■) and anti-albumin reactivity (OD 472nm: □—□).

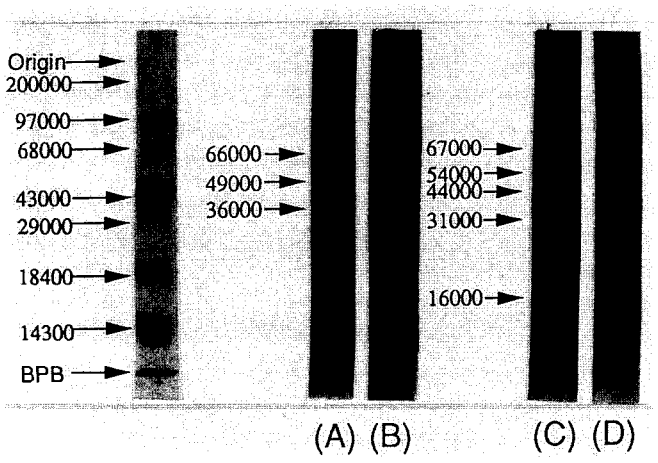

Fig. 2 Polyacrylamide gel electrophoresis and immunoblotting of the MoAb YO-2-reactive antigen. The partially purified urine antigen (**A** and **C**) and human albumin (**B** and **D**) were separated by SDS-PAGE and immunostained with MoAb YO-2 (**A** and **B**) or anti-albumin antibody (**C** and **D**).

Table 2 Number of cases with the abnormally high urine YO-2-reactive antigen levels in the diabetic patients with nephropathy

	Number of cases	Urine YO-2-Ag levels[a] Number of cases	
		Normal	High
Normal control	19	18(95)	1(5)
Normalalbuminuria[b]	50	42(84)	8(16)
Microalbuminuria[c]	25	7(28)	18(72)
Macroalbuminuria[d]	14	4(29)	10(71)

[a] $P < 0.0001$ among four groups.
[b] $P = 0.2242$ between normal control and normoalbuminuria.
[c] $P < 0.0001$ between normal control and microalbuminuria. $P < 0.0001$ between normo- and microalbuminuria.
[d] $P = 0.0001$ between normal control and macroalbuminuria. $P = 0.0002$ between normo- and macroalbuminuria. $P = 0.6629$ between micro-and macroalbuminuria.

SD). Those of normoalbuminuric patients (1.38 ± 1.36 U/g-Cr) were comparable to the normal values. The micro- and macroalbuminuric patients had levels of 2.87 ± 2.07 and 3.92 ± 3.33 U/g-Cr, respectively. The P values between the normal control and microalbuminuria groups, between the normo- and microalbuminuria groups, between the normo- and macroalbuminuria groups, and between the normal control and macroalbuminuria groups were less than 0.0001. Differences between the normal control and normoalbuminuria groups and between the micro- and macroalbuminuria groups were insignificant.

When the mean + 2 × SD of the normal control (1.71 U/g-Cr) was taken as the upper limit of normal, 5, 16, 72, and 71 % of the normal control, normo-, micro-, and macroalbuminuric patients, respectively, had abnormally high levels (Table 2). Differences between the normal control and microalbuminuria groups, between the normal control and macroalbuminuria groups, between the normo- and microalbuminuria groups, and between the normo- and macroglobulinuria groups were significant ($P < 0.001$).

There was no significant correlation between the urine levels of YO-2-Ag and those of albumin ($r = 0.184$, $P = 0.11$, Fig. 4). The correlation between the urine levels of YO-2-Ag and HbA_{1c} was not significant either ($r = 0.063$, $P = 0.55$, Fig. 5).

The urine levels of YO-2-Ag of the patients without retinopathy were 1.83 ± 1.60 U/g-Cr(mean ± SD), and those of patients with simple retinopathy and with prolifer-

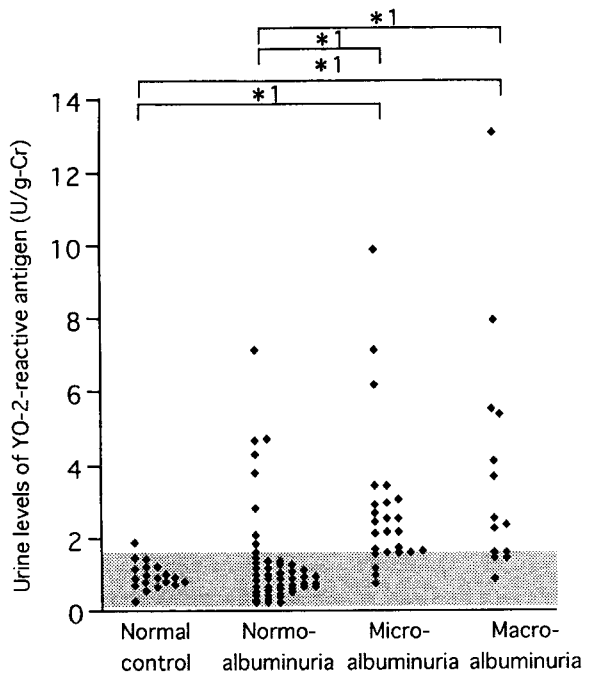

Fig. 3 Urine levels of the MoAb YO-2-reactive antigen in diabetic patients. The shaded area represents the normal range. *1, $P < 0.0001$.

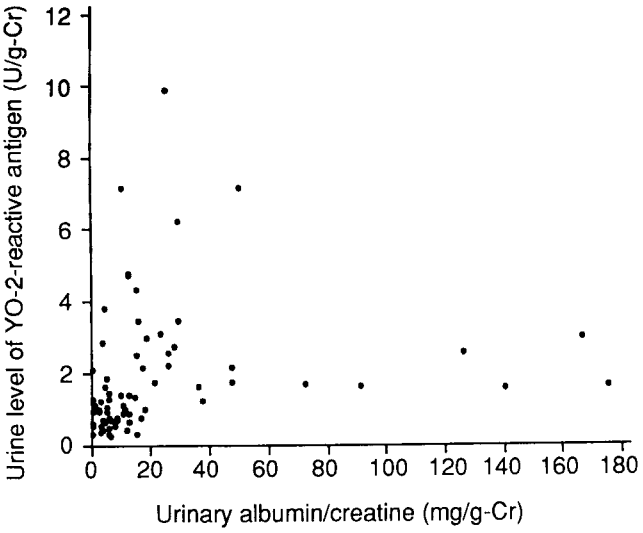

Fig. 4 Correlation between the urine levels of the MoAb YO-2-reactive antigen and the urinary albumin: creatinine ratio (urinary albumin index) between the normo- and microalbuminuria groups. Correlation coefficient was $r = 0.184$ ($P = 0.11$).

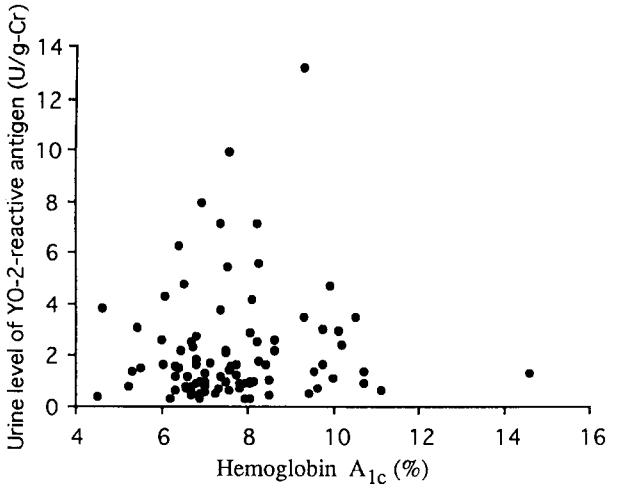

Fig. 5 Correlation between the urine levels of the MoAb YO-2-reactive antigen and hemoglobin A_{1c} among three diabetic groups. Correlation coefficient was $r = 0.063$ ($P = 0.13$).

ative retinopathy were 2.48 ± 2.25 and 2.58 ± 2.63 U/g-Cr, respectively. There was no significant difference in the urine levels of YO-2-Ag between patients with and without retinopathy ($P = 0.1325$).

There was no significant difference in the urine levels of YO-2-Ag between men and women patients, and there was no significant correlation between the urine levels of YO-2-Ag and the age of the patients.

Discussion

Diabetic nephropathy is a major prognostic factor of diabetic patients. It develops if metabolic control remains inadequate for more than 5–10 years and is associated with irreversible glomerular changes. However, a genetic factor is also suggested to be involved in the pathogenesis of diabetic nephropathy (30). Since strict glycemic control prevents the development and progress of nephropathy (1), it will be invaluable to be able to predict nephropathy. At present, microalbuminuria is the most reliable predictor for future development of nephropathy in IDDM (5–7). However, it was pointed out that microalbuminuria was not always a strong predictor of diabetic nephropathy (8), and the implications of microalbuminuria in NIDDM have not been identified. Only 20 % and more than 80 % of microalbuminuric patients with NIDDM and IDDM, respectively, progressed to overt nephropathy over a

decade (11, 12). Patients with NIDDM have a higher incidence of microalbuminuria but a lower incidence of nephropathy (9, 13-17).

In the present study, we tried to find a novel and clinically useful non-albumin urine protein excreted by NIDDM patients which might be used as a prognostic marker. A monoclonal antibody, YO-2, was raised against a non-albumin urine protein from NIDDM patients. The apparent molecular weight of the YO-2-Ag was 66 kDa. Two other bands of 49 and 36 kDa were also positively immunostained, but were supposed to be degradation products of the 66-kDa protein. Albumin was previously shown to be degraded in urine (31) as confirmed in the present study. Although the molecular weight of the antigen was similar to that of human albumin, albumin and YO-2-Ag are clearly different proteins. MoAb YO-2 did not react with the purified human albumin, and a polyclonal antibody against human albumin did not stain the YO-2-reactive bands. Furthermore, we could not find a significant correlation between the urine levels of YO-2-Ag and albumin.

Urinary excretion of non-albumin proteins such as *N*-acetyl-β-D-glucosaminidase, retinol-binding protein, β_2-microglobulin, immunoglobulin light chain and α_1-microglobulin in early diabetic nephropathy was studied (22-25). Their molecular weights, however, differ from that of YO-2-Ag. Human plasma contains several proteins having molecular weights similar to that of the YO-2-Ag (65 to 70 kDa); they are antithrombin III, α_1-fetoglobulin, sex steroid binding protein, prothrombin, and α_1-antichymotrypsin. The clinical implications of urinary excretion of these proteins in diabetic patients have not been reported to our knowledge. Further study is needed to determine whether one of these is the YO-2 reactive antigen or not.

The mean urine levels of YO-2-Ag were significantly higher in the patients with microalbuminuria and macroalbuminuria. However, YO-2-Ag was similar in both the early and late stages of nephropathy. Urine YO-2-Ag levels were normal in 29 % of macroalbuminuric patients and 28 % of microalbuminuric patients. About 16 % of normoalbuminuric patients had abnormally high urine YO-2-Ag levels. These results suggest that YO-2-Ag and microalbumin have different clinical significance. Further prospective study is needed to determine whether the patients with the abnormally high values of both the urine YO-2-Ag and albumin are at increased risk for the development of nephropathy than those with either abnormality alone.

A correlation between HbA_{1c} and the urine excretion rates of several proteins, including α_1-microglobulin and retinol-binding protein, was reported (25, 32). Microalbumin was also reported to be elevated in cases of insufficient metabolic control (33, 34), however the urine YO-2-Ag levels had no correlation with HbA_{1c} levels. It was thus suggested that recent glycemic control of diabetes did not significantly affect the urinary excretion of YO-2-Ag.

In this study, the urine levels of YO-2-Ag were different from those of albumin (Fig. 4). The mechanism of the different excretion rates of the YO-2-Ag and albumin into urine is not clear. Though the exact mechanism for increased albumin excretion into urine in diabetic patients has not yet been fully elucidated, it is postulated that both the size and ionic charge of proteins determine their passage across the barrier between the glomerular capillary and the urinary space of Bowman's capsule (35-38). However, those interpretations did not explain why the excretion rates of YO-2-Ag and albumin are different even though their molecular weights are similar. Other factors such as differences in their tubular reabsorption rate, molecular configuration, or affinity for extracellular matrices may explain the different excretion rates of the two proteins.

In conclusion, a novel monoclonal antibody, YO-2, was raised against urine proteins from diabetic patients. YO-2 recognized a non-albumin urine antigen which was excreted at increased rates in diabetic patients with nephropathy. Recent glycemic control of diabetes did not significantly affect the urinary excretion rate of YO-2-Ag. Also, the excretion rate and probably the mechanism of YO-2-Ag differed from those of albumin. The urine levels of the YO-2-reactive antigen could be a clinical marker of diabetic nephropathy.

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