Acta Medica et Biologica
Vol. 40, (Suppl.) 31-47, 1992

Reflux Nephropathy
—Role of Bacterial Antigens and Urinary Proteins—

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Summary. **Monoclonal antibodies (MoAbs) were raised against pyelonephritis-associated P-pili (PAP-pili) and Tamm-Horsfall glycoprotein (THGP). These MoAbs were μ-, κ-chain specific and reacted with polypeptides of 18 kD (PAP-pili) or 90 kD (THGP). The epitope of the MoAb directed against PAP-pili was present at the apical portion of the pilus. MoAbs against THGP recognized 4 distinct epitopes, and one MoAb stained immunofluorescent material inside both Bowmann's capsule and renal tubular cells on frozen sections from a kidney without obstructive or interstitial changes. Analysis of *E. coli* phenotypes with MoAbs against PAP-pili and with polyvalent O and Kl antisera showed that organisms positive for PAP-pili, O1, and Kl antigens were the more common causes of acute pyelonephritis. The chemiluminescence response of polymorphonuclear leukocytes was prominent following stimulation with O or K antigens isolated form pyelonephritic or enteropathogenic *E. coli* strains. Pyelonephritopathogenic *E. coli* may thus be a small number of strains which stimulate an energetic leukocyte response that produces more severe renal lesions. Pyelonephritis patients had high urinary free light chain levels regardless of the presence of vesicoureteral reflux or recurrent infection. THGP bound nonspecifically to these light chains and to IgA. In patients with reflux nephropathy, heavy tubular excretion of such proteins and their binding to THGP may interfere with urine outflow. Recurrent infection by virulent organisms and interactions between THGP and other urinary proteins may thus aggravate reflux nephropathy.**

INTRODUCTION

Reflux nephropathy is the term now used to describe the irregular segmental scarring and contraction of the kidney which may occur in association with persistent vesicoureteral reflux (VUR). There are a number of points regarding the mechanism of renal scarring which remain unclear, but bacterial infection appears to play a major role in its development.

In this report, we present the results of three studies on the pathogenesis of reflux nephropathy:

1) The production of monoclonal antibodies (MoAbs) which identify pyelonephritis-associated P-pili (PAP-pili) and Tamm-Horsfall glycoprotein (THGP).

2) Assessment of the antigenic phenotypes of *Escherichia coli* in clinical isolates and of their virulence factors affecting polymorphonuclear leukocyte function.

3) Determination of the interaction between THGP and various urinary proteins.

1) The Production of Monoclonal Antibodies (MoAbs) Which Identify Pyelonephritis-associated P-pili (PAP-pili) and Tamm-Horsfall Glycoprotein (THGP).

INTRODUCTION

PAP-pili play an important role in bacterial adhesion to receptors on the surface membranes of upper urinary tract cells.[1] However, their detection with human erythrocytes is difficult, since individuals with $\bar{p}$-type erythrocytes are very rare in Japan.[2] THGP is the most abundant protein in normal urine, but its precise physiological function and its role in the pathogenesis of reflux nephropathy are as yet undefined. We raised MoAbs in this study to produce reliable immunological reagents for the detection of PAP-pili or THGP. We report here on the characterization of these MoAbs.

MATERIALS AND METHODS

Purification of PAP-pili

Escherichia coli (E. coli) 06 : H(−) : Kl(−) : F12 : haemolysin (−), a human pyelonephritis isolate which expressed a PAP-pili-positive haemagglutinin, was used for the purification of PAP-pili. PAP-pili were purified by the method of Normark et al.[3] with slight modifications. After the supernatant of homogenate of the organism was subjected to three cycles of salt precipitation with 0.1 M $MgCl_2$, the precipitate was further purified using a Synsorb P_1 affinity column (10×60 mm) (Chembiomed. Co., Ltd., Canada). The affinity-purified PAP-pili thus obtained were used in this study.

Purification of THGP

THGP was purified from the pooled urine obtained from 20 normal adult volunteers according to the method of Dawnay et al.[4] The THGP fraction was precipitated by three cycles of salt precipitation with 0.58 M NaCl, and the crude THGP precipitate was then incubated overnight at room temperature in phosphate buffer containing 8 M urea.[4] Urea-treated THGP was then purified by column chromatography with Superose™ 12 (Pharmacia LKB, USA).

Production of Monoclonal Antibodies (MoAbs)

Monoclonal antibodies (MoAbs) against PAP-pili or THGP were prepared from the culture filtrate of cloned hybridoma cell lines formed by fusion[5] of the PAI myeloma cell line[6] and sensitized BALB/c mouse splenic lymphocytes. Iscove's modified Dulbecco's medium (Gibco Lab., USA) containing 10% fetal bovine serum (FBS) (MA Bioproducts Inc., USA) was used as a complete medium for cell culture. Briefly, female BALB/c mice were immunized intraperitoneally with 0.5 mL of affinity-puried PAP-pili or THGP (100 μg/mL of distilled water) emulsified in the same volume of Freund's complete adjuvant (Difco, USA). Two and 3 weeks later, the mice received a tail vein injection of 0.2 mL of the same solution of antigen minus the adjuvant. Three days after the last immunization, spleen cells were collected by teasing and fused to the PAI myeloma cell line using polyethylene glycol (IBL, Japan). HAT and HT media (IBL) containing 10% FBS were employed for selection culture of the fused cells. The hybridomas were then screened for antibody production by an enzyme-linked immunosorbent assay (ELISA). Antibody-producing cells were cloned by limiting dilution and retested by ELISA. Selected clones were expanded in tissue culture with complete medium for the collection of supernatants. The supernatants were tested for immunoglobulin isotypes using a Mouse Monoclonal Antibody Isotyping Kit (Amersham International, Plc., UK). The supernatants produced were purified with a Monoclonal Antibody Affinity Isolation System Anti-mouse IgM (American Qualex Isolation Inc., USA). The antibody solution thus obtained was dialysed against 5 mM trishydroxychloride buffer (Tris-HCl), pH 8.3, containing 0.3 M NaCl, and then concentrated to approximately 1/20 of the original volume by ultrafiltration using PM 10 (Amicon Corp., USA).

ELISA

Microtiter wells were coated overnight at 4℃ with 50 μL of the purified PAP-pili or THGP solution (200 μg/mL of 50 mM carbonate bicarbonate buffer, pH 9.2), and then rinsed in 200 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 1% bovine serum albumin fraction V (BSA) (Wako Co., Ltd., Japan). Tris-HCl (200 mM, pH 7.5) containing 0.5 M NaCl and 0.05% Tween 20 (Wako) was used as the washing and dilution buffer for the ELISA. A monoclonal culture supernatant or the affinity purified MoAb (diluted 1/10) was used as the first antibody, and a goat $F(ab')_2$ anti-mouse IgM or IgG peroxidase (Tago Inc., USA) (diluted 1/250) was used as the second antibody. The wells were then stained with a Horseradish Peroxidase Color Development Reagent Kit (BioRad Lab., USA).

SDS-PAGE and Western blotting

Electrophoretic separation was carried out on 8% polyacrylamide slab gels (80×80×3 mm), as reported previously.[7] Samples were routinely heated in a solution of sodium dodecylsulfate (SDS) (Sigma Chem. Co., USA) for 1 hr at 60℃, as reported previously.[7] A buffer, pH 8.3, containing 25 mM trishydroxycloride, 192 mM glycine, and 0.1% SDS was used as the electrode buffer. Twenty microliter aliquots of samples were loaded onto the gels and electrophoresis was performed at a constant current (serially 10 mA for 30 min and 20 mA for 150 min). Protein-containing subunits were identified with a silver staining kit (BioRad Lab., USA). After electrophoretic separation, the proteins were electroeluted onto a nitrocellulose sheet (BioRad) overnight at 40 volts in a buffer, pH 8.3, costaing 25 mM trishydroxy-

cloride, 192 mM glicine and 20% methanol. The transferred proteins were identified with the affinity purified MoAb solution (diluted 1/10) and goat $F(ab')_2$ anti-mouse IgM peroxidase (diluted 1/250). The same buffer as that used for the ELISA was employed for the dilution of antibodies and washing of the nitrocellulose sheet.

Immunofluorescent microscopy (IF)

When the MoAbs against PAP-pili were used, bacterial strains were washed with 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (10 mM PBS), and were smeared onto albumin-coated slide glasses. In the case of the MoAbs against THGP, 20-μm frozen kidney sections were obtained from the renal biopsy specimen of a patient with minimal change nephrosis, who had no interstitial disease and no evidence of VUR. Sections were fixed onto albumin-coated slide glasses. After blocking with 10 mM PBS containing 1% BSA for 1 hr at room temperature, the slide glasses were reacted with the MoAb solution (diluted 1/10) or with culture filtrate. After washing in 10 mM PBS, the MoAb-treated slide glasses were stained with goat $F(ab')_2$ anti-mouse IgM FITC (TAgo Inc., USA) (diluted 1/50 with 10 mM PBS). The presence or absence of immunofluorescence was then determined using a Nikon immunofluorescent microscope (Nikon Co. Ltd., Japan).

Electron and immunoelectron microscopy (EM and IEM)

Bacterial surface pili or preparations of pilus fragments were negatively stained[8] with 1.5% sodium phosphotungstic acid, pH 7.0, containing 0.1% BSA for 30 sec on 300 mesh copper grids (Oken Co. Ltd., Japan) coated with 2% collodion membrane (Oken). They were then examined using a Hitachi H-800 electron microscope (Hitachi Co. Ltd., Japan) For immunoelectron microscopy, the affinity purified MoAb (diluted 1/10) and goat anti-mouse IgM conjugated to 20-nm colloidal gold particles (diluted 1/250) (EY Lab. Inc., USA) were applied before the specimens were stained negatively as described above.

Haemagglutination inhibition (HI) assay[9]

The wells of a microplate were fixed with 12.5 ng of THGP and reacted with serial dilutions of a monoclonal culture filtrate. Then tanned sheep erythrocytes coated with the monoclonal culture filtrate were added, and haemagglution inhibition was observed at a final erythrocyte concentration 2.5%.

Determination of protein content

The amount of protein in the samples was determined by dye method with a protein assay kit (Bio-Rad) or by the absorbance at OD_{280nm} using a BSA solution as the standard.

RESUITS

Purification of PAP-pili

Despite three cycles of salt precipitation, the precipitated fraction (120 μg of protein determined by the dye method) showed not only a number of clear protein bands (16, 18, 22.5, 24.5, 31, 34, 42, 44, 52, 55, 61 and 68 kD) but also many faint bands on the silver-stained gel (Fig. 1, lane c). The salt-precipitated fraction was therefore further purified using a Synsorb P_1 column. Affinity purified PAP-pili (20 μg of protein by the dye method) then revealed only a single band at 18 kD on the silver-stained gel (Fig. 1, lane d).

Purification of THGP

THGP precipitated with 0.58 M NaCl showed 2 peaks when subjected to Superose™ 12 column chromatography after 8 M urea treatment (Fig. 2). The 8-10 mL fraction was collected (Fig. 2), and this showed a single band at 90 kD on SDS-PAGE.

Production of monoclonal antibody

Four cell lines producing antibody against PAP-pili and 6 cell lines producing antibody against THGP were identified. All cloned cell lines produced the μ-, κ-chain specific MoAbs. These clones were expanded for the collection of supernatants, which were purified using the Monoclonal Antibody Affinity Isolation System. The MoAbs against PAP-pili or THGP thus obtained showed single reaction bands at 18 kD or 90 kD, respectively, when incubated with nitrocellulose sheets to which crude PAP-pili or THGP had been electrotransferred by Western blotting. IF analysis with these MoAbs showed that they reacted specifically to PAP-pili-positive *E. coli* or to the tubular cells in frozen kidney sections, respectively (Fig. 3-a). When MoAbs No. 4 or No. 6 against THGP were used, the frozen kidney sections showed

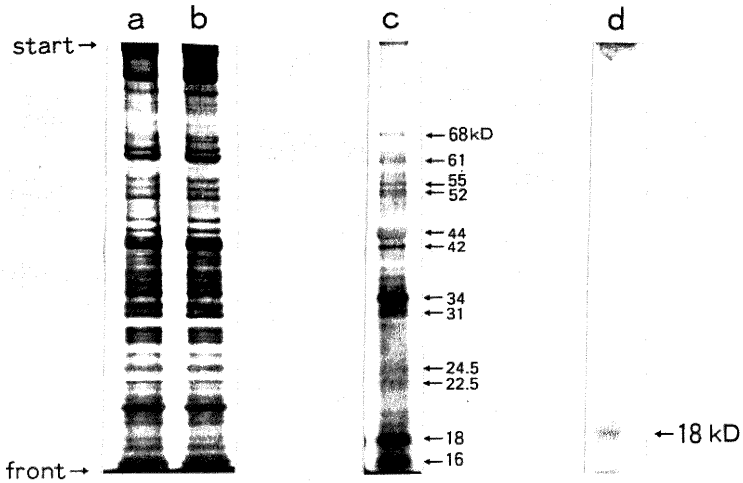


Fig. 1. SDS-PAGE analysis of the homogenate of *E. coli* 06 : H(−) : K1(−) : F12 : hemolysin(−), and of the purified pilus preparation. Lane a) b): homogenate of *E. coli* (5×10^3 cfu). Lane c): the fraction precipitated with 0.1 M/L $MgCl_2$ (120 μg of protein). Lane d): the PAP-pili fraction purified by column chromatography with Synsorb P_1 (20 μg of protein). Lanes a-d) were silver stained.

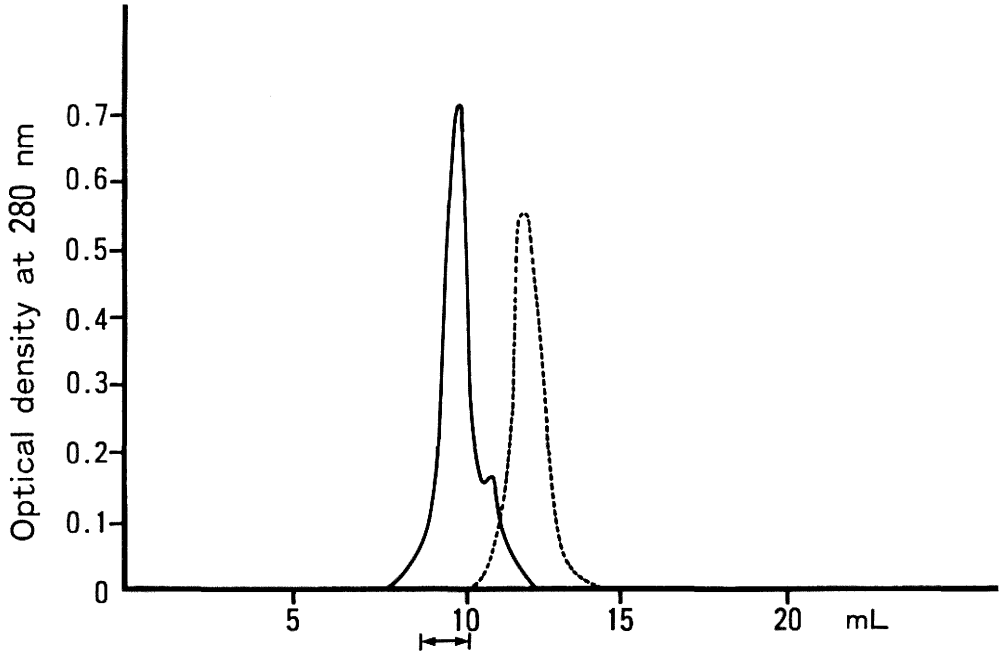


Fig. 2. The elution profile of Tamm-Horsfall glycoprotein on a Superose™ 12 column. The THGP fraction precipitated from normal pooled urine with 0.58 M/L NaCl was solubilized in an elution buffer, and introduced to the column. A fraction of 8-10 mL was collected. Unbroken line: THGP. Borken line: bovine serum albumin. Column: Superose™ 12 (10×300 mm), elution fuffer: 0.04 M/L $NaHPO_4$/0.16 M/L NaH_2PO_4, pH 6.4, containing 2 M/L urea, flow rate: 0.5 mL/min, sample: 0.2 mL (1.5 mg of salt precipitate with 0.58 M/L NaCl)

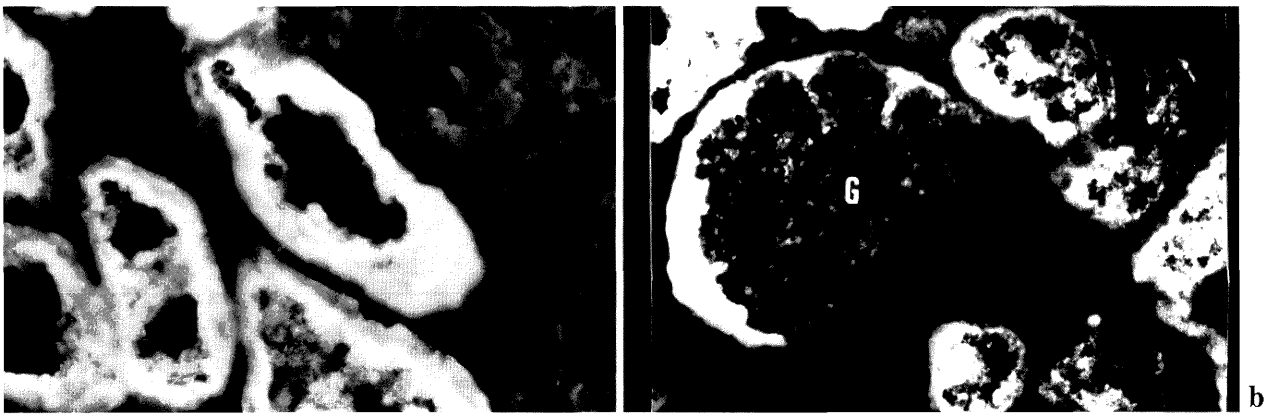


Fig. 3. Immunofluorescent microscopy using monoclonal antibodies raised against Tamm-Horsfall glycoprotein. a): Tubular cells of frozen kidney sections were stained positively by culture filtrate from any of the hybridomas. b): Immunofluorescent material was observed inside Bowmann's capsule as well as in the tubular cells when the culture filtrate from hybridomas No. 4 or No. 6 was used.

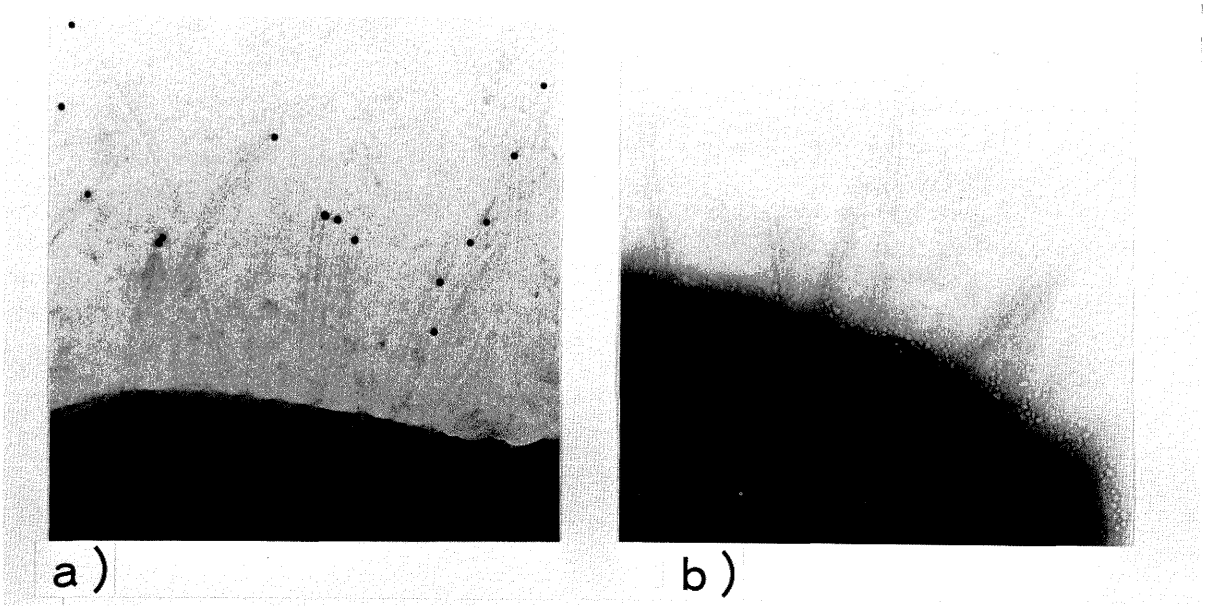


Fig. 4. Immunoelectron micrographs of PAP-pili of *Esherichia coli*. *E. coli* were incubated with an MoAb against PAP-pili and 20 nm gold particles (a) or with normal BALB/c mouse serum, and gold particles (b). These specimens were shadowed with 1.5% phosphotungstic acid, pH 7.0.

positive immunofluorescence inside Bowmann's capsule as well as in the tubular cells (Fig. 3-b). IEM using the MoAbs against PAP-pili obtained from 4 cloned cell lines demonstrated gold particles at the apical portions of the pilus filaments (Fig. 4-a). The 6 cloned cell lines producing antibodies against THGP secreted 4 different MoAbs according to the results of the haemagglutination inhibition test (Table 1). MoAbs No. 2 and No. 5 recognized the same epitope of THGP, while MoAbs No. 4 and No. 6 both recognized another epitope.

DISCUSSION

The MoAbs directed against PAP-pili reacted specifically with an 18 kD polypeptide, as indicated by Western blotting. IEM using colloidal gold parti-

Table 1. Hemagglutination ingibition titers of the monoclonal antibodies raised against Tamm-Horsfall glycoprotein.

Inhibiting MoAb	Erythrocytes coated with MoAb					
	No. 1	No. 2	No. 3	No. 4	No. 4	No. 6
No. 1	2,140	<10	<10	<10	<10	<10
No. 2	<10	730	<10	<10	730	<10
No. 3	<10	<10	3,660	<10	<10	<10
No. 4	<10	<10	<10	<730	<10	1,250
No. 5	<10	1,250	<10	<10	1,250	<10
No. 6	<10	<10	<10	1,250	<10	1,250

Each well was coated with 12.5 ng of THGP, and then reacted with serially diluted inhibiting MoAb before erythrocytes coated with MoAbs were added. Numbers indicate the inhibition titers.

cle labeling revealed that the MoAbs against PAP-pili reacted with an epitope near the apical portions of the pilus filaments. According to Normark et al., [10-13] PAP-pili are heteropolymers with a major subunit (PapA) which accounts for the bulk of the pilus, and some other minor subunits. Among these subunits, PapG is responsible for the binding specifity of the pili and must be identical to the protein that primarily interacts with the αGalβ(1-4)Gal recepor.[11] The epitope of the present MoAbs might be the PapG adhesin, but the molecular weight showed no agreement to their results. This may be because there are a large number of PapG variants among globoside-binding *E. coli* as they suggested.[14,15] THGP is a large filamentous molecule with a very high molecular weight, and the reported molecular weight of its smallest homogenous subunit is about 90-100 kD.[16] The culture filtrates from 6 cloned cell lines reacted with a 90 kD polypeptide and specifically stained renal tubular cells by IF, indicating that these 6 clones produced MoAbs against THGP. The HI assay indicated that these MoAbs detected 4 distinct epitopes. Blunisholz et al.[17] have previously reported 7 distinct epitopes of THGP, so it may be possible that the antigenic determinants of this molecule are not uniform. IF microscopy performed with the culture filtrate from cell line No. 6 showed immunofluorescent material inside Bowmann's capsule as well as in the renal tubular cells of kidney sections obtained form patient without obstructive or interstitial changes. The abnormal interstitial localization of THGP seen in certain pathological states has suggested a role for it in the pathogenesis of various renal diseases.[18] Interstitial THGP deposits and circulating antibodies to THGP have been found in a porcine model of reflux nephropathy.[19] Tubulointerstitial nephritis localized to the ascending limb of the loop of Henle occurs in the rabbit[20] and the rat[21] following the administration of homologous THGP. However, all these previous atudies were undertaken using polyclonal antibodies to THGP. Our present findings with MoAbs raise some questions about the pathogenesis of reflux nephropathy. Abnormal localization of THGP may not actually be evidence of intratubular urinary backflow and it may thus not play a major role in the pathogenesis of reflux nephropathy. In this context, Chambers et al.[22] have reported that there was no correlation between the distribution of THGP and the degree of tubulointerstitial damage, urinary THGP excretion, urinary β_2-microglobulin excretion, glomerular filtration rate, urinary concentrating ability, or the incidence of pyuria.

2) Assessment of the Antigenic Phenotypes of *Escherichia Coli* in Clinical Isolates and of Their Virulence Factors Affecting Polymorphonuclear Leukocyte Function

INTRODUCTION

E. coli is the most common etilolgic agent of urinary tract infection (UTI).[23] The bacterial factors[24,25] that affect virulence include the polysaccharide O and K antigens and haemolysin production, in addition to the pili or fimbriae.

In this study, the incidence of various virulence factors (PAP-pili and pyelonephritopathogenic O and K antigens) was determined in *E. coli* cultured from the urine of UTI patients. The interactions between these bacterial antigens and polymorphonuclear leukocyte (PMN) function were also assessed.

MATERIALS AND METHODS

Strains, culture conditions, and patients

The *E. coli* strains used in the present study were isolated from urine cultures obtained from 57 UTI patients and 20 patients with asymptomatic bacteriuria who were treated at the Department of Paediatrics of kinki University School of Medicine from May 1, 1984 to March 31, 1988. UTI was defined as the detection of one (or more) cultures with at least 10^5 colony forming units (cfu)/mL together with pyuria (i.e., more than 5 leukocytes per high power field in the urinary sediment) in a patient with

symptoms.[26] A diagnosis of acute pyelonephritis was based on the presence of bacteriuria, a temperature of at least 38.5℃, and no previous history of UTI, together with at least one of the following: an increased erythrocyte sedimentation rate (more than 25 mm/h), a C-reactive protein level over 20 mg/L, a transient decrease in renal urine concentrating capacity (<800 mOsm/L), transient changes of renal tubular function, or pain in the renal angle.[26,27] Recurrent pyelonephritis (RPN) was defined by the presence of a past history of UTI together with the symptoms and signs of acute pyelonephritis. Acute cystitis was defined by the presence of burning and frequency of urination in patients with a temperature under 38.5℃, and with normal laboratory findings other than those related to the urinalysis.[26] A diagnosis of chronic pyelonephritis was based on histological examination[28] of a renal biopsy specimen together with the detection of bacteriuria and pyuria. In patients who had UTI symptoms but did not fulfill the criteria for any of the groups mentioned above, a diagnosis of nonspecific UTI was made.[29] Asymptomatic bacteriuria was defined as the homogeneous growth of an organism at more than 10^5 cfu/mL from at least two consecutive urine specimens obtained from a patient with no corresponding symptoms and signs at a regular check-up.[26] Control urinary*E. coli* were isolated as contaminants[24] from urine containing less than 10^2 cfu/mL, and were obtained from 31 patients without any present or past history of UTI (table 2). Clean-voided midstream specimens were introduced to a VITEK AMS (VITEK System Inc., USA) within 3 h of collection, and the cultured urinary *E. coli* were stored.

Table 2. Characteristics of the groups with various types of urinary tract infection and the control patients.

Diagnosis	Number of cases	Sex		Age of years old, median (range)
		Boys	Girls	
Acute pyelonephritis (APN)	16	9	7	$1\frac{6}{12}$ ($\frac{1}{12}$ ~ 8)
Recurrent pyelonephritis (RPN)	9	0	9	9 ($\frac{2}{12}$ ~13)
Acute cystitis (AC)	8	3	5	$5\frac{6}{12}$ (3 ~ 11)
Chronic pyelonephritis (CPN)	8	6	2	10 (2 ~ 12)
Non-specific urinary tract infection (N-UTI)	16	4	12	$1\frac{6}{12}$ ($1\frac{1}{12}$ ~ 13)
Asymptomatic bacteriuria (ABU)	20	9	11	2 ($\frac{1}{12}$ ~ 15)
Control	31	17	14	2 ($\frac{1}{12}$ ~ 13)

Serotyping

Antigen O grouping and possessing of the K1 antigen were tested by bacterial agglutination according to the method of Kaijser[30] using O1, O2, O4, O6, O75 (Difco, USA), and K1 antisera (Denka-Seiken Co. Ltd., Japan).

PAP-pili analysis of *E. coli*

E. coli PAP-pili were analysed by immunofluorescent microscopy (IF) using a monoclonal antibody (MoAb) against PAP-pili, as mentioned in chapter 1.

Extraction and purification of O and K antigens

Strains O1: H7: K1(−): F11, 06: H(−): K1(−): F12, and O75: H(−): K1: F7 (human pyelonephritis isolates), a strain of enteropathogenic *E. coli* 044: H18: K74, and a standard strain O14: H(−): K7, provided by the Research Institute for Microbial Deseases, Osaka University, were used for the purificaton of O antigens. All strains were PAP-pili positive and produced no haemolysin. The O2: H4: K1: haemolysin(+): PAP-pili(−) strain, provided by the National Institute of Health, and the enteropathogenic and standard strains mentioned above were used for the purification of K-antigen. The O1, O6, O75, O44 and O14 antigens were extracted by a method based on that of Bolanos et al.,[31] and the purified K1, K74 and K7 antigens were extracted by a method based on that of Kasper et al.[32]

Chemiluminescence response of polymorphonuclear leukocytes (PMNs) stimulated by O or K antigens

The chemiluminescence response of polymorphonuclear leukocytes (PMNs) obtained from normal volunteers was measured by a previously reported method after stimulation with O or K antigens.[33] In brief, 1×10^6 PMNs, suspended in Hanks' balanced salt solution (Nisshin Kagaku Co., Ltd., Japan) (300

μL) and 50 μL of luminole (100 μg of luminole) were introduced into a tube and allowed to dark adapt for 10 min. The PMNs were then stimulated with 50 μL of O or K antigen solution. Isolation of peripheral PMNs was carried out by Farrante's one-step method.[34]

Statistical methods

The chi-squared test and student's t test were used to evaluate the significance of results.

RESULTS

Relationship between clinical findings and antigenic phenotypes of *E. coli*

The antigenic phenotypes of *E. coli* were initially compared with the clinical diagnosis. The rate of isolation of PAP-pili-positive *E. coli* by IF was 63% in patients with acute pyelonephritis and 67% in those with recurrent pyelonephritis, and it was more frequent in both groups compared to the control group ($p<0.01$) (Table 3). *E. coli* isolated from patients with acute or chronic pyelonephritis were apt to be of the O1 and O2 serotypes ($p<0.05$ and $p<0.01$, respectively). In contrast, strains from patients with acute cystitis and asymptomatic bacteriuria were frequently of the O6 and O4 serotypes (both $p<0.05$) (Table 3). *E. coli* of the O6, O4, and O1 serotypes were the 3 most frequent isolates out of the 5 different pyelonephritopathogenic O serotypes which were found. The K1 antigen was present in only a few strains, but there was a difference in the rate of isolation of K1-positive *E. coli* between the patients with acute pyelonephritis and the controls ($p<0.05$) (Table 3). Two strains which showed a simultanous positive reaction to PAP-pili, O1 and K1 serotypes were isolated from 2 patients with acute pyelonephritis. There were another 2 strains found which showed a positive reaction to both PAP-pili and the K1 serotype, one from a case of acute pyelonephritis and the other from a case of recurrent pyelonephritis (Table 3). PAP-pili positivity was significantly associated with the O1 serotype ($p<0.05$) (Table 4).

Relationship between bacterial virulence factors and polymorphonuclear leukocyte function

The PMNs from normal volunteers showed a dose-dependent increase in chemiluminescence with the addition of 50–750 μg of O6 or 25–250 μg of K1 antigen (Figs. 5 and 6). The chemiluminescence produced by various other O or K antigens was compared with that produced by 250 μg of O or 100 μg of K antigen, respectively. The stimulatory effect of the O1 or O44 antigens was greater than that of the O75, O6 or O14 antigen ($p<0.01$ and $p<0.05$, respectively) (Table 5). The K1 or K74 antigens stimulated PMNs more

Table 3. The clinical diagnosis and the number of phenotypes of *Escherichia coli*.

Diagnosis	PAP-pili		K1			O-serotype						
	pos	neg	pos	neg	n.e.	O 1	O 2	O 4	O 6	O 75	neg	n.e.
Acute pyelonephritis	10**[2](4)<1>	6	5*[2]<1>	11	0	4*[2](2)	1(1)	2	0	1(1)	7	1
Recurrent pyelonephritis	6**(1)<1>	3	1<1>	7	1	1(1)	0	0	1	1	4	2
Acute cystitis	1	7	2	6	0	0	0	1	2*	1	2	2
Chronic pyelonephritis	1(1)	7	0	8	0	0	3**(1)	0	3	0	2	0
Non-specific urinary tract infection	2	14	3	11	2	1	0	1	2	0	6	6
Asymptomatic bacteriuria	3(2)	17	1	19	0	1	2(1)	5*(1)	2	0	8	2
Control patients	3	28	1	29	1	0	0	1	1	2(1)	23	4

Numbers indicate those of the strains. pos: positive, neg: negative, n.e.: not examined. **$P<0.01$, *$p<0.05$ compared with controls. [ ], ( ) and < > indicate the number of strains which showed simultaneous positive reactions to PAP-pili and the O and K1 serotypes, PaP-pili and the O serotype, and PAP-pili and the K1 serotype, respectively.

Table 4. Relationship between the phenotypes of *Escherichia coli* isolated from urine.

	O-serotype						
	O1	O2	O4	O6	O75	neg	n.e.
PAP-pili							
pos	5*	2	2	0	2	11	4
neg	2	4	8	11	3	41	13

Numbers indicate those of the strains.
pos: positive.
neg: negative.
n.e.: not examined.
*$p<0.05$ compared with O-negative strains.

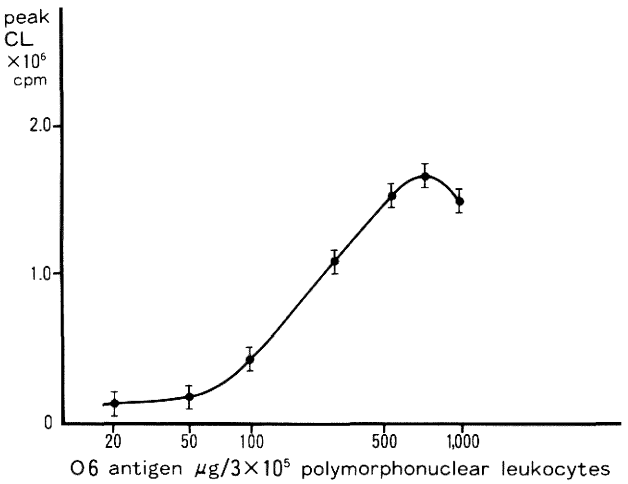


Fig. 5. The chemiluminescence (CL) response of polymorphonuclear leukocytes stimulated by the *Escherichia coli* O6 antigen.

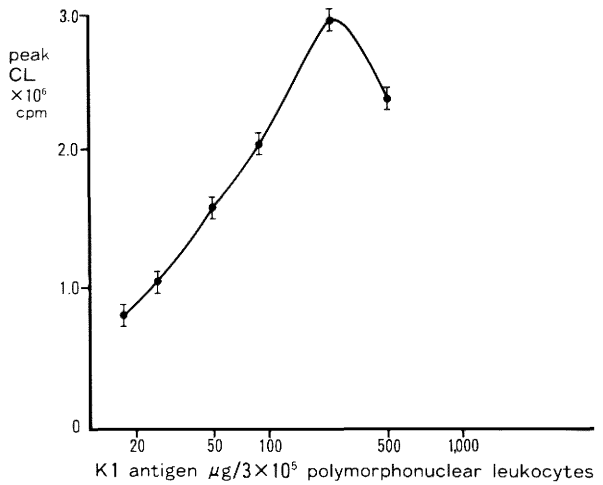


Fig. 6. The chemiluminescence (CL) response of polymorphonuclear leukocytes stimulated by the *Escherichia coli* K1 antigen.

prominently than did K7 ($p<0.01$) (Table 6).

DISCUSSION

PAP-pili-positive *E. coli* were frequently isolated by IF in patients with acute and recurrent pyelonephritis. Since similar findings have previously been reported in studies using HA,[1] IF,[35,36] and gene analysis,[37] PAP-pili appear to play an important role in bacterial adhesion to receptors on the surface membranes of the upper urinary tract epithelial cells.[1] Also, strains from acute pyelonephritis patients frequently expressed the K1 antigen, which is known to be one of the virulence factors for severe infection.[38] Our present results suggest that the *E. coli* causing pyelonephritis belong to a small number of strains, because they showed a simultaneous positive reaction to PAP-pili and the K1 antigen or one of the 5 different pyelonephritopathogenic O serotypes. We also found that strains with certain O serotypes were frequently found in urine. In the present study, the chemiluminescence response of PMNs showed a direct correlation with the amount or kinds of O or K antigens possessed by the *E. coli* strains tested. The O antigens obtained from the strains isolated from pyelonephritis patients or those from the enteropathogenic strains of *E. coli* caused more stimulation of normal PMNs than the antigens from the standard strain. The chemiluminescence response of PMNs stimulated by the K1 antigens obtained from pyelonephritic strains or the K74 antigens obtained from enteropathogenic strains were also greater than those stimulated by antigens from the standard strain. PMNs produce superoxide during the phagocytosis and killing of bacteria, and this process is reflected by the chemiluminescence response.[39] Superoxide is harmful to nomal human tissues as well as to bacteria. These results imply that the pyelonephritopathogenic strains of *E. coli* may be a small number of clones, as was proposed by O'Hanley et al.[37] on the basis of DNA analysis, and that these small number of clones may produce more severe renal lesions because of their stimulation of excessive superoxide production by host PMNs.

3) Determination of the Interaction between THGP and Various Urinary Proteins

INTRODUCTION

THGP is a urinary glycoprotein that has been im-

Table 5. The chemiluminescence (CL) response of polymorphonuclear leukocytes from normal volunteers stimulated by various *Escherichia coli* O antigens.

Age (years old)	Sex (M: male, F: female)	*Escherichia coli* O-antigens				
		O 1	O 6	O 75	O 44	O 14
22	F	0.994	0.824	0.393	0.943	0.823
29	M	0.858	0.703	0.234	1.092	0.703
28	M	1.177	0.668	0.354	1.331	1.059
33	M	1.062	0.777	0.199	1.079	0.639
20	F	0.811	0.695	0.231	0.695	0.501
27	M	1.122	0.913	0.411	0.953	0.785
34	M	1.179	0.923	0.295	1.216	0.884
31	M	1.091	0.931	0.243	0.970	0.524
28	M	1.152	1.175	0.635	1.429	1.191
27	M	1.120	0.598	0.441	0.959	0.497
mean±s.d.		1.057±0.130	0.821±0.170	0.344±0.133	1.067±0.214	0.759±0.239

s.d.: standard deviation.
Numbers indicate the peak CL response ($\times 10^6$ cpm) stimulated by 250 μg of O antigen/3×10^5 polymorphonuclear leukocytes.
The O1 and O44 antigens showed a stronger stimulating effect than the O75 ($p<0.01$) and the O1 or the O14 antigens ($p<0.05$).

Table 6. The chemiluminescence (CL) response of polymorphonuclear leukocytes from normal volunteers stimulated by various *Escherichia coli* K antigens.

Age (years old)	Sex (M: male, F: female)	*Escherichia coli* K-antigens		
		K1	K74	K7
26	M	1.780	2.294	0.554
21	F	1.867	2.655	0.456
29	M	1.846	2.447	0.343
26	M	1.435	2.044	n.e.
28	M	3.422	3.172	0.501
27	M	2.409	3.423	0.549
33	M	1.436	1.775	0.253
mean ±s.d.		2.028 ±0.697	2.544 ±0.591	0.443 ±0.121

n.e.: not examined.
s.d.: standard deviation.
Numbers indicate the peak CL response ($\times 10^6$ cpm) stimulated by 100 μg of K antigen/3×10^5 polymorphonuclear leukocytes. The K1 and K 75 antigens produced more stimulation than K7 antigen ($p<0.01$).

plicated in the pathogenesis of tubulointerstitial nephritis associated with vesicoureteric reflux (VUR) or reflux nephropathy. The results presented in chapter 1, however, raised some questions about its actual role in the pathogenesis of reflux nephropathy. The conflict with the findings of previous studies may have arisen due to the ability of THGP to bind to unrelated proteins, as reported by Schachner et al.[40] Accordingly, this study investigated the interaction of THGP with various urinary proteins.

MATERIALS AND METHODS

Patients

Twenty-four hour samples were collected (using a method reported previously[7]) from 26 patients with pyelonephritis-associated VUR, 16 with acute poststreptococcal glomerulonephritis, 6 with minimal change nephrosis, and 9 with acute lymphoblastic leukemia. All patients were treated by the Departments of Pediarics or Urology at Kinki Uinversity School of Medicine from Aprill 1, 1984 to March 30, 1990. Twenty-six age-matched normal children were used as the controls. Patients with pyelonephritis were classified into three groups. Those with active pyelonephritis and VUR formed group A. Group B had suffered from recurrence of infection during a postoperative observation period ranging from 6 months to 2 years. Patients who had no postoperative recurrence of pyelonephritis formed group C.

Radiological signs

Intravenous pyelography and cystography were used

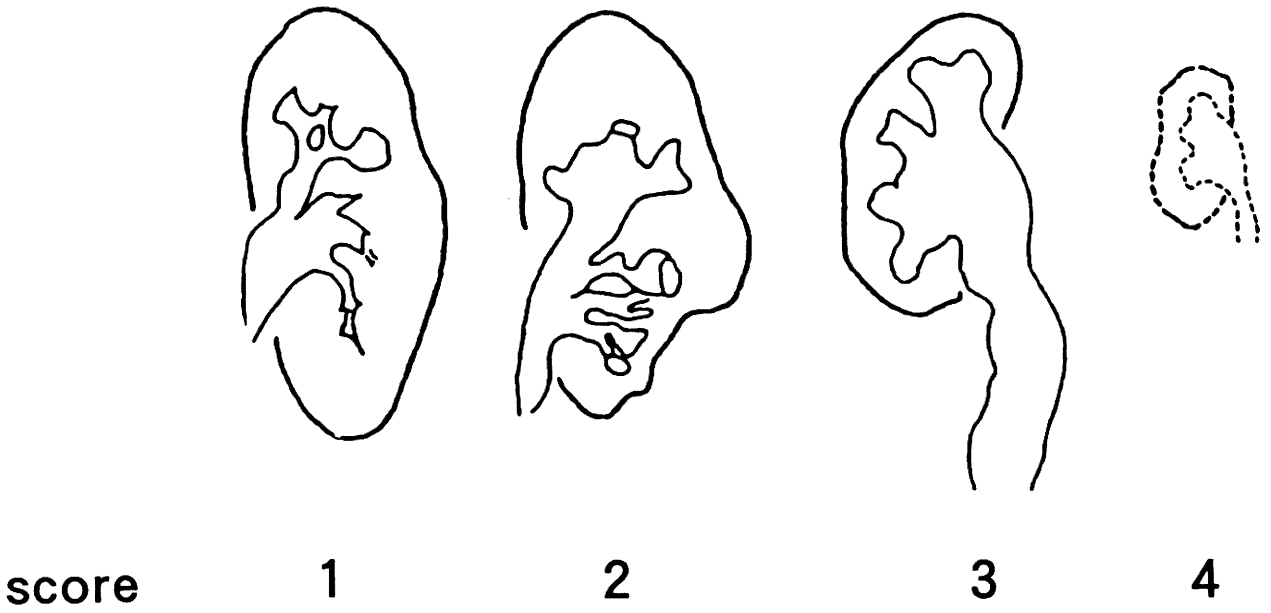


Fig. 7. Renal scarring scoring system. 0: No scarring. 1: Mild scarring with not more than two scarred areas. 2: Severe scarring; more generalized, but some areas of normal parenchyma are still present. 3: Back-pressure type kidney; infective changes (irregular thinning of the renal parenchyma) superimposed on the generalized calyceal deformity usually found in obstructive atrophy. 4: End-stage shrunken kidney, with little or no function.

to investigate the morphology of the urinary tract according to the criteria of Smellie et al.[41)] VUR was classified into 4 grades of severity,[41)] and scarred kidneys were arbitrarily categorized as one of the following 4 types (Fig. 7): (a) Mild scarring (not more then two scarred areas) was given a score of 1. (b) Severe scarring (more generalized scars, with some areas of normal tissue) was given a score of 2. (c) Backpressure type (generalized calyceal deformity, with variable but generalized thinning of the renal parenchyma) was given a score of 3. (d) An end-stage kidney (little or no function and indistinguishable from a dysplastic or hypoplastic kidney) was given a score of 4. (e) A score of 0 indicated no scarring.

Proteins and antibodies

The following human proteins and antibodies were used. Commercially available products included human IgG and human IgA (Protagen AG, Switzerland), human transferrin, human fibronectin, anti-human IgG goat serum, anti-human IgA goat serum, anti-human kappa-chain-specific (free) goat serum, anti-human albumin goat serum, peroxidase-conjugated anti-goat IgG rabbit serum, and peroxidase conjugated anti-mouse IgM (all from Cappel, USA), and β_2-microglobulin (Chemicon, USA). Urinary kappa free light chain was purified from pooled urine as reported previously.[7)] Urinary albumin was purified from nephrotic urine by affinity chromatography with Affi-gel blue (BioRad) according to the method of Ghiggeri et al.[42)]

Quantitative determination of κ-free light chain

The level of κ-free lignt chains in urine was determined by a dot immunobinding assay, as previously reported.[7)]

Analytical isoelectric focusing (IEF) of proteins

Analytical IEF was carried out on polyacrylamide slab gels or Immobiline Dry Plates pH 4-7 (Pharmacia LKB, USA) according to the manufacturer's instructions, using an LKB Multiphor System. The polymerization solution included 10 mL of 29.1% acrylamide, 10 mL of 0.9% N-methylene-bis-acryla-

mide, 7 mL of 87% glycerol, and 3 mL of 30% Amphiline pH 2.5-4.5, and was adjusted with distilled water to a final volume of 60 mL.

Purification of THGP

The purification technique for THGP and the production of MoAbs against THGP were described in chapter 1.

Interactions between THGP and other proteins

Interactions between THGP and other proteins were studied by ELISA according to the method of Schachner et al.[40] To the wells of a micro-ELISA plate, 50 μL of the following reagents were added sequentially: THGP or water alone (control) was incubated for 18 h at 4℃; 10 mM PBS containing 1% BSA was incubated for 2 h (no wash); human protein solution was incubated for 5 hr; anti-human protein goat serum (1:200 in 10 mM PBS containing 1% BSA) was incubated for 18 h at 4℃; and horseradish peroxidase-conjugated anti-goat rabbit serum (1:500 in 10 mM PBS containing 1% BSA) was incubated for 2 h at 37℃: Coloration was obtained with a Horseradish Peroxidase Color Developing Kit (BioRad, USA). THGP was added after coating the wells with human protein and 10 mM PBS containing 1% BSA in reverse, and MoAbs against THGP (chapter 1) and horseradish peroxidase-conjugated anti-mouse IgM serum were used in some experiments.

RESULTS

Patients with pyelonephritis plus VUR or with leukemia excreted larger amounts of κ-free light chains (FLC) than the normal controls ($p<0.05$). High urinary levels of FLC were observed in the patients regardless of their association with VUR or infection. All patients in groups A, B, and C excreted greater amounts of FLC than the normal controls ($p<0.001$, $p<0.01$, and $p<0.05$, respectively) (Feg. 8). Urinary FLC excretion was greatest in patients with scarred

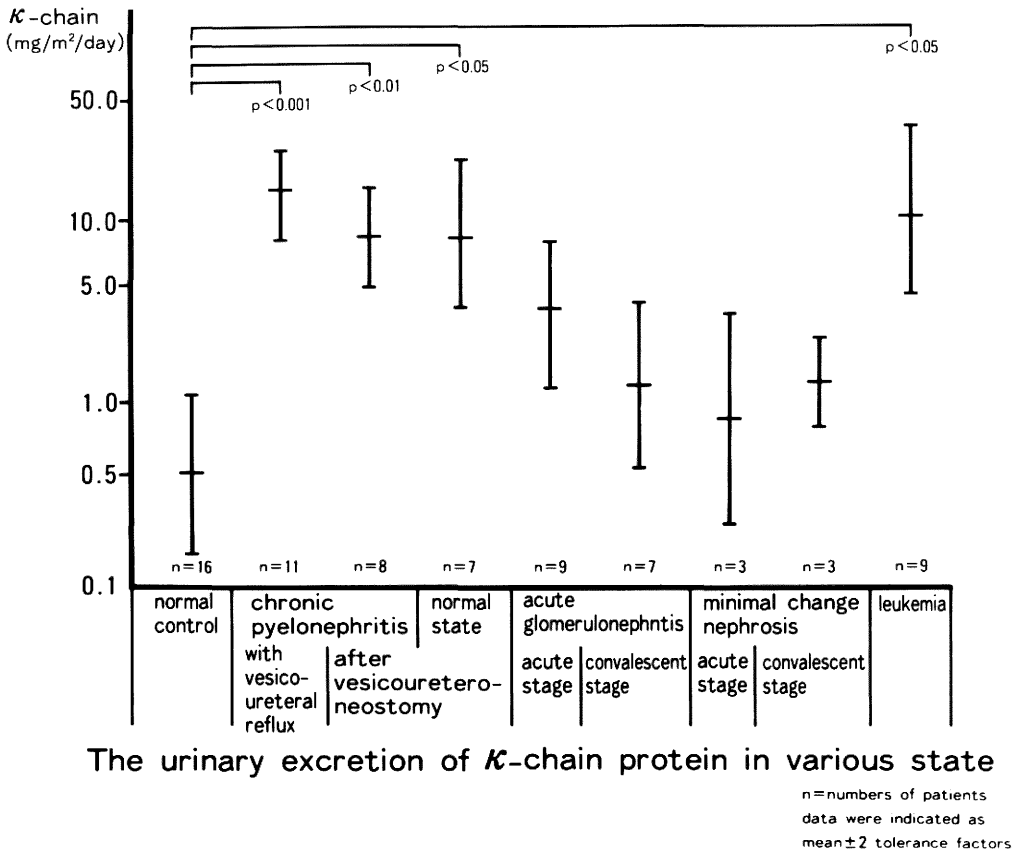


Fig. 8. The urinary excretion of κ-free light chains in various disease states. n: number of patients. Data are the mean ± 2 tolerance factors.

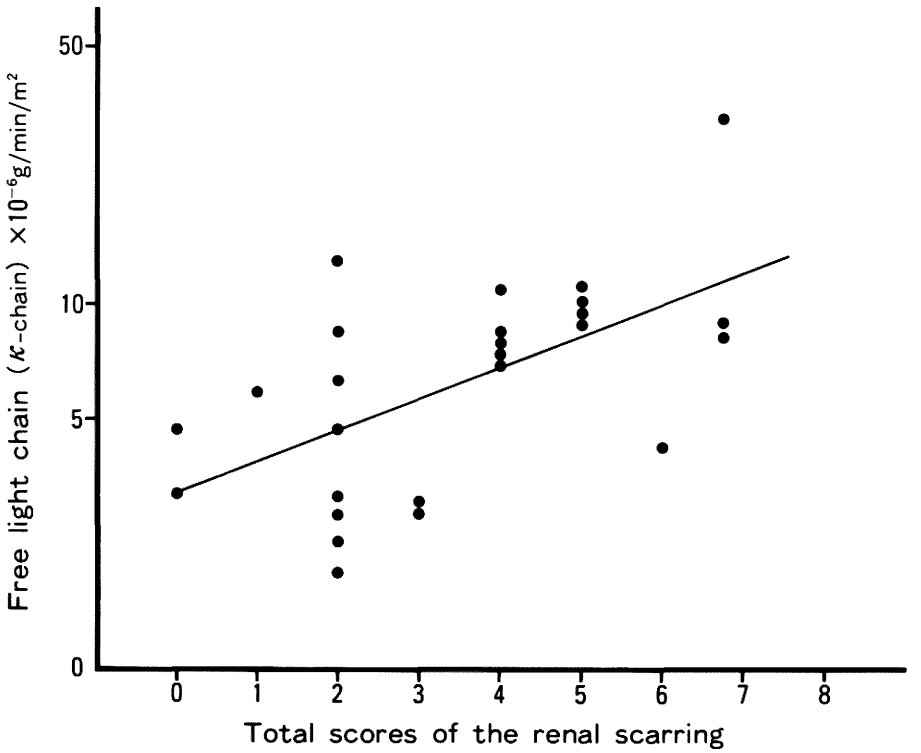


Fig. 9. Relationship between the urinary excretion of κ-free light chains and the grade of renal scarring. Total scarring scores were the sum of the scores for both kidneys.

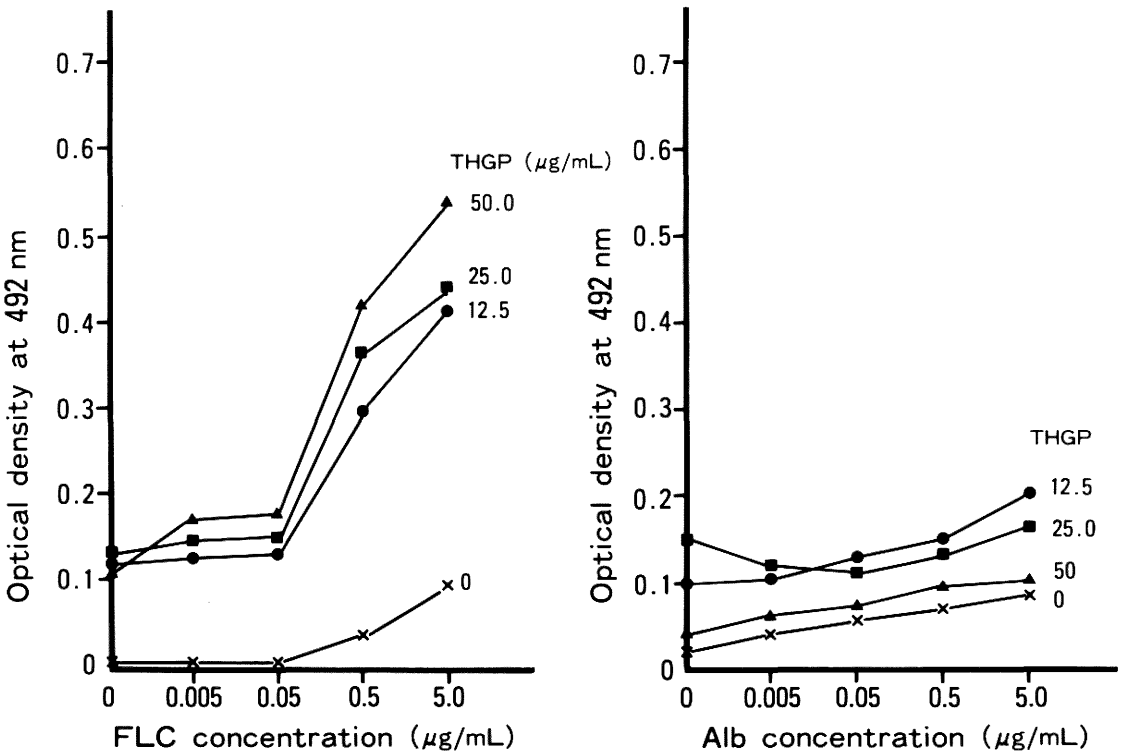


Fig. 10. Tamm-Horsfall glycoprotein binding to free light chains (FLC) or albumin (Alb).
Each well of a microplate was coated with solutions of either 50 μg/mL (▲), 25 μg/mL (■), 12.5 μg/mL (●) or no (×) THGP, before ELISA with FLC or Alb.

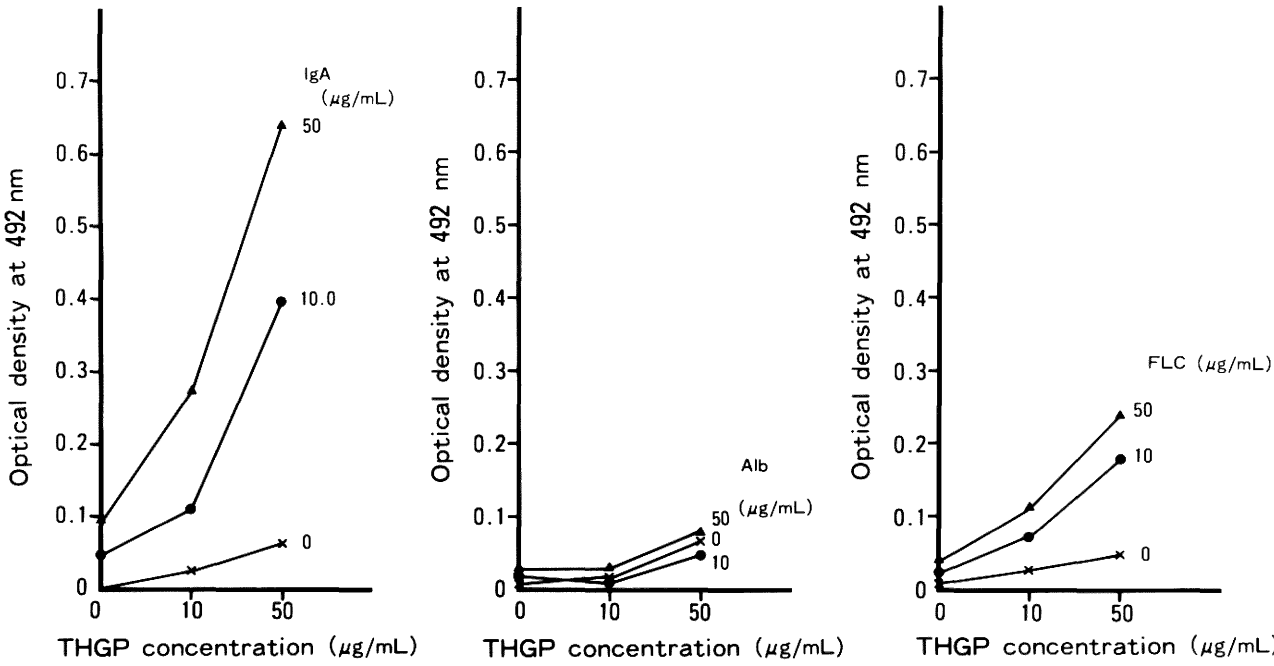


Fig. 11. Tamm-Horsfall glycoprotein binding to a variety of different solid-phase proteins. Before ELISA with THGP, microplate wells were coated with solutions of either 50 μg/mL (▲), 10 μg/mL (●) or no (×) immunoglobulin A (IgA), albumin (Alb) or free light chains (FLC), respectively.

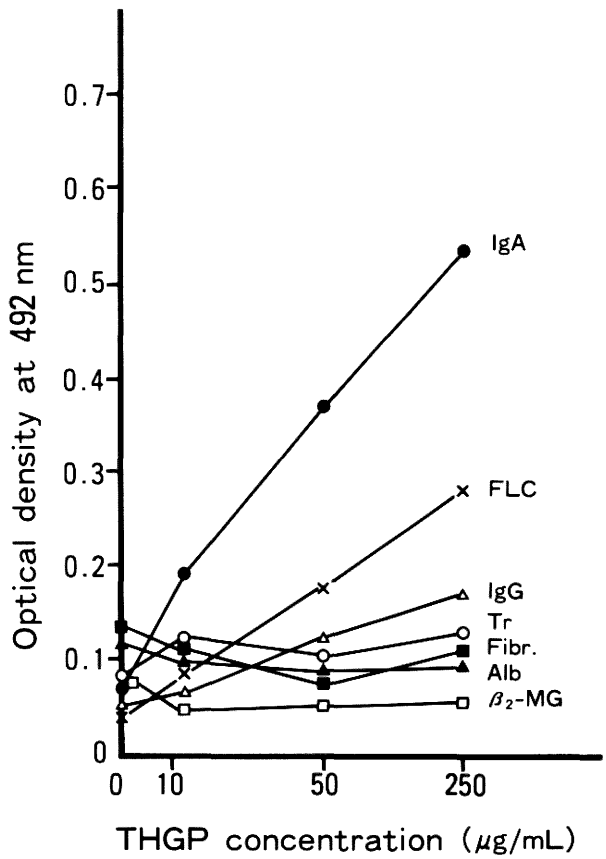
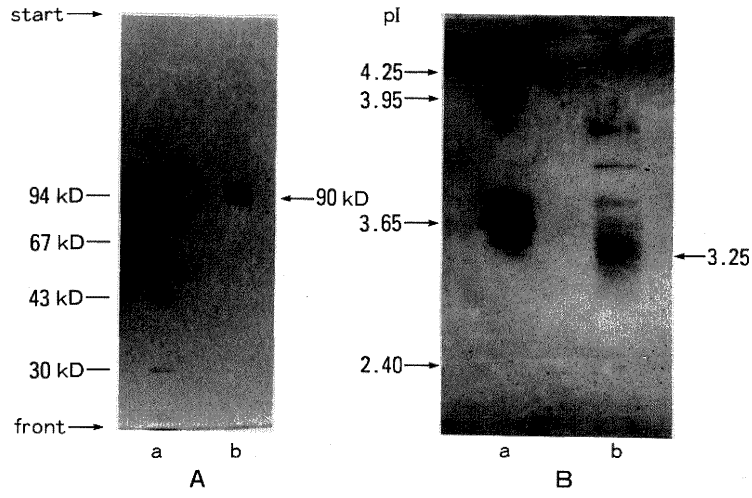
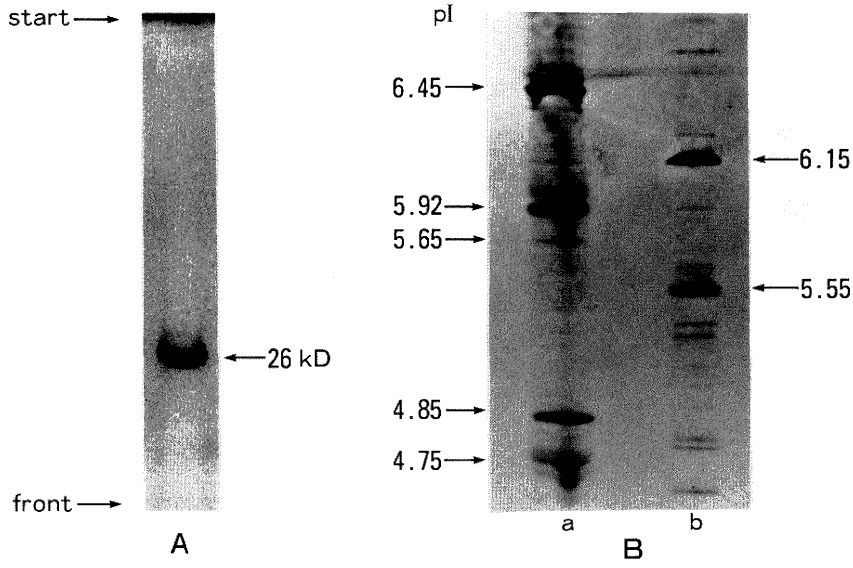


Fig. 12. Tamm-Horsfall glycoprotein binding to a variety of different solid-phase proteins. Before ELISA with THGP, microplate wells were coated with solutions of 50 μg/mL of immunoglobulin A (● IgA), immunoglobulin G (△IgG), transferrin (○Tr), albumin (▲Alb), or fibronectin (■ Fibr), or with 5 μg/mL free light chains (×FLC) ro β-microglobulin (β_2-MG).



The electrophoretic figures of the Tamm-Horsfall glycoprotein (THGP)

Fig. 13. Electrophoresis of Tamm-Horsfall glycoprotein. Ten micrograms of THGP showed a single band at 90 kD on sodium dodecylsulfate-polyacrylamide gel electrophoresis (A, lane b), and a clear band at pI 3.25 with several faint bands by isoelectrofocusing (B). Both gels were stained with Coomassie blue. The bands in lane a) are marker proteins.



The electrophoretic figures of the κ-free light chain (FLC)

Fig. 14. Electrophoresis of free light chains. Thirty micrograms of κ-free light chain showed a single band at 26 kD on SDS-PAGE (A), and 2 clear bands at pI 5.55 and 6.15 (plus several other faint bands) on IEF (B, lane b). Both gels were stained with Coomassie blue. The bands in lane a) are marker proteins.

kidneys. There was a correlation between the urinary FLC excretion and the degree of bilateral renal scarring; i.e., urinary FLC excretion was proportional to the total renal scarring score ($r=0.57$, $p<0.01$) (Fig. 9). FLC was found to bind directly to solid-phase THGP in a dose related manner (Fig. 10), and THGP was also found to bind to solid-phase IgA or FLC in a dose related manner (Fig. 11). However, THGP did not bind other solid-phase proteins such as albumin, transferrin, IgG, fibronectin, and β_2-microglobulin (Fig. 12). Electrophoresis of THGP showed a band at 90 kD and a pI of 3.25 (Fig. 13), while FLC showed a 26 kD band and a pI of 5.55-6.15 (Fig. 14).

DISCUSSION

Patients with pyelonephritis had a high urinary FLC excretion regardless of the presence of VUR or recurrent infection, and urinry FLC excretion was proportional to the severity of their renal scarring. There are several reports that patients with pyelonephritis excrete large amounts of tubular proteins such as FLC, β_2-microglobulin, or lysozyme,[7] and a recent study showed that IgA and secretory IgA were abundant in the urine of pyelonephritis patients.[43,44] In the present study, THGP was shown to bind with FLC or IgA, which are not antibodies to THGP. Non-specific binding between THGP and a variety of other substances occurs because THGP is negatively charged under physiological conditions.[45] FLC can readily obstruct the renal tubules, and positively charged FLC (as studied in the present experiment) is harmful to the kidneys of multiple myeloma patients.[46] In patients with obstructive uropathy or reflux nephropathy, large amounts of FLC or IgA might be excreted into the tubular lumen with recurrent infections and bind to THGP to interfere with urine flow. The interaction between THGP and FLC or IgA might thus be one of the factors aggravating reflux nephropathy, because THGP is the most prominent urinary protein under normal conditions.

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