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The Role of Tamm-Horsfall Protein in the Pathogenesis of Reflux Nephropathy and Chronic Pyelonephritis

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Recurrent bacterial infection of the kidney was previously thought to be responsible for the renal scarring typical of chronic pyelonephritis until recent studies suggested that recurrent bacteriuria rarely produces chronic pyelonephritis in the absence of obstructive uropathy. In contrast, the association between vesicoureteral reflux (VUR) and chronic pyelonephritis has been observed frequently in the absence of urinary infection. Although the mechanism by which VUR injures the kidney has not been defined, recent observations have suggested that some component of urine might serve as an antigenic determinant involved in the immunopathogenesis of renal scarring in VUR. Therefore, the present studies investigated the immunopathogenic role of Tamm-Horsfall protein (THP) in *(1)* a rabbit model of tubulointerstitial nephritis; *(2)* a swine model of reflux nephropathy; and *(3)* patients with recurrent nephrolithiasis. The antigenic similarities between THP and uropathic bacteria were also studied.

Our observations indicate that autoimmune responses to THP may occur after exposure to THP by intravenous challenge in rabbits, by urinary reflux in pigs, and in recurrent nephrolithiasis in man. Also, extracts of uropathic coliforms competitively inhibit the binding of human THP to its antibody. These studies suggest that autoimmune responses to THP may be the pathogenetic mechanism by which these factors, including bacteriuria, contribute to "chronic pyelonephritis."

Historically, the renal entity of "chronic atrophic pyelonephritis" emerged from a series of clinical and pathological observations beginning with E.L. Wagner's in 1882 [1] and extending to Weiss and Parker's in 1939 [2]. Most of the cases were in children and young adults who had a history of recurrent urinary tract infection dating back to early childhood. In some of these cases, however, there was no such history of urinary infection, and the same observations are also true today.

Although the role of bacterial infection as the major determinant for the development of *acute* pyelonephritis has been well documented for years, the nature of the renal scarring typical of *chronic "atrophic" pyelonephritis* has been a matter of controversy for at least three decades. In fact, for many years most of us were convinced (and some may still be), that recurrent bacterial infection of the kidney is primarily responsible for the pathologic entity of "chronic pyelonephritis." Pathologically, the term "chronic pyelonephritis" describes shrunken, irregular, coarsely scarred kidneys with focal thinning of the renal parenchyma, often from an original dimension of three centimeters in thickness to a final depth of only two millimeters [3]. However, after almost three decades of following patients, primarily women, with recurrent

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urinary tract infections, we have learned that recurrent bacteriuria alone, that is, in the absence of some form of obstructive uropathy, rarely, if ever, leads to chronic renal failure and to the pathologic entity of *"chronic atrophic pyelonephritis"* [4,5]. During this time, we also learned that, whereas many etiologic agents have been shown to produce chronic tubulointerstitial damage, only a limited number of conditions can lead to the pathologic picture of chronic pyelonephritis [3,6]. These are: *(a)* vesicoureteral reflux [7]; *(b)* urinary obstruction [6]; *(c)* analgesic nephropathy; *(d)* unusual forms of papillary necrosis which occur in association with sickle cell disease, focal tuberculosis, or severe dehydration in infants [8]; and *(e)* segmental hypoplasia (the Ask-Upmark kidney) [9]. Even though this latter entity (Ask-Upmark kidney) was considered, initially, to be a development anomaly, more recent studies suggest that the majority of patients with segmental hypoplasia have vesicoureteral reflux, and that this condition is almost certainly part of the spectrum of reflux nephropathy [3,6,10]. Furthermore, we now know that typical pyelonephritic scarring, in the vast majority of patients, is due to bacterial infection superimposed on an anatomic urinary tract anomaly, urinary obstruction, or, most commonly, *vesicoureteral reflux* [3,6,11].

The close association between chronic pyelonephritis and vesicoureteral reflux was first established in 1960 by Hodson and Edwards [7], and has since been confirmed by many other observers, so that this relationship is now widely recognized. Vesicoureteral reflux is frequently present (about 50 percent) in adults with classic pyelonephritic scarring and is almost always present (more than 90 percent) in children with this renal pathology [12,13,14]. Furthermore, in a significant proportion of cases, vesicoureteral reflux-associated scarring will ultimately progress to end-stage renal disease requiring dialysis or transplantation [3,6,14]. In some centers, from 15 to 20 percent of adults under 50 years of age and more than 30 percent of children requiring renal substitution have this diagnosis [3,6]. Two additional points should be emphasized. First, the term *"reflux nephropathy,"* introduced by Bailey in 1973 [11], has now replaced the term chronic pyelonephritis, for reflux-associated renal scarring. Second, reflux nephropathy, in the absence of urinary tract infection, has been observed to progress to severe (end-stage) renal scarring in children [11,15,16,17] and in experimental animal models of reflux [18,19]. In fact, of those patients with renal failure resulting from reflux nephropathy, only 60 percent have a history of urinary tract infection [3]. It is the latter observation, namely, that the renal scarring associated with reflux nephropathy can occur in the absence of infection, that has led to much of the controversy about the pathogenesis of these lesions. Thus, the role of infection in reflux nephropathy has been debated frequently. Although there is a history of recurrent urinary tract infection in most children with this disease, others present early in adult life with gross reflux, severe renal damage, and no history of urinary tract infection [3]. What is the relationship, then, of reflux, infection, and renal scarring? The following observations by various investigators are pertinent.

1. Vesicoureteral reflux is present in 30 to 50 percent of children with recurrent urinary tract infection and is the most common abnormality associated with infection regardless of whether the children are symptomatic or asymptomatic [20,21,22].

2. Vesicoureteral reflux is present in more than 90 percent of children with pyelonephritic scarring, but not all children with vesicoureteral reflux (only 30 to 60 percent) go on to develop renal scars [13,22].

3. Pyelonephritic scarring is also seen in 25 percent of children with urinary tract infection, particularly those with recurrent symptomatic bacteriuria [23–32].

These findings, however do not explain the mechanism by which vesicoureteral reflux injures the kidney. Subsequently, a series of observations led to the suggestion that some normal component of urine might serve as an antigenic determinant involved in the immunopathogenesis of renal scarring in vesicoureteral reflux. Specifically, *(1)* intrarenal reflux (IRR) was observed in some patients, mostly in children less than five years of age, who had the more severe grades of vesicoureteral reflux [33–38]; *(2)* intrarenal reflux occurred primarily in the polar regions of the kidney and was associated with focal renal scarring, i.e., typical reflux nephropathy [3,6,39]; *(3)* the polar distribution of intrarenal reflux was shown, both in children and in pigs, to be related to differences in the orifices of the ducts of Bellini, e.g., slit-like in simple single papillae, which are relatively resistant to intrarenal reflux, but wide openings in central depressions of compound papillae which occur commonly in the polar regions (about 75 percent of human kidneys have at least one compound papilla) [40–42]; *(4)* a direct relationship was observed between age and the pressure required to produce intrarenal reflux in human kidneys, i.e., lower pressures are required to produce intrarenal reflux in kidneys from younger children [6,43]; and *(5)* the observation that normal urinary proteins, particularly Tamm-Horsfall protein, could be detected in the renal interstitium [44,45], renal veins and lymphatics [46,47], and peri-renal lymph nodes [48,49] in association with intrarenal reflux. Thus, these observations suggested that some normal component of urine might serve as an antigenic determinant involved in the immunopathogenesis of renal scarring in vesicoureteral reflux or in other acquired states which produce retrograde urine flow. An immunopathologic response to such an antigen might help explain why the histologic lesions of reflux nephropathy, chronic pyelonephritis, or chronic tubulointerstitial nephritis are characterized by mononuclear cellular infiltrates and fibrosis rather than polymorphonuclear leukocytes. Furthermore, such antigenic substances, or immune responses to them, might serve as non-invasive markers of vesicoureteral reflux and have the potential to detect those patients at risk for reflux nephropathy and renal scarring which may ultimately lead to end-stage renal disease. For these reasons, the normal urinary protein, Tamm-Horsfall protein (THP), was investigated in an attempt to determine what role, if any, it played in the pathogenesis of the renal scarring, i.e., chronic pyelonephritis, observed in those patients with sterile vesicoureteral reflux. Tamm-Horsfall protein is synthesized in the tubular epithelial cells of the thick ascending limb of the loop of Henle and the distal convoluted tubules, where, under normal conditions, it adheres to the luminal surface [50–54]. Tamm-Horsfall protein is secreted into the urine in quantities of approximately 25 μg/ml and has not been conclusively demonstrated elsewhere in the body. Its exact physiologic function is unknown, although it may influence the water permeability characteristics of the loop of Henle [55] and, conceivably, might protect urinary tract mucosa from the effects of bacteria [55,56] or viruses [50,51].

A *pathophysiologic* role for Tamm-Horsfall protein in the development of pyelonephritic scarring associated with reflux has been suggested by several clinical and experimental observations. Interstitial deposits of Tamm-Horsfall protein have been detected in approximately 50 percent of patients with medullary cystic disease, approximately 30 percent of 100 human kidneys from patients with "chronic pyelonephritis," reflux nephropathy, or obstructive uropathy, as compared with less than 2 percent of patients with glomerulonephritis [44–46]. Although these Tamm-Horsfall protein deposits appear to have come from adjacent ruptured tubules, the amorphous Tamm-Horsfall protein is frequently surrounded by focal mononuclear cell infiltrates.

Also, acute ureteral obstruction in mice [57], rats [58], and swine [59] produces extravasated interstitial deposits of Tamm-Horsfall protein within one to two hours. Serologic responses (IgG) to human Tamm-Horsfall protein have been observed in patients with recent urinary obstruction, vesicoureteral reflux, and acute pyelonephritis, but not in patients with cystitis, asymptomatic bacteriuria, reflux nephropathy with scarring, or end-stage renal disease [60–68].

The following studies from my laboratory, initiated by Dr. Robert Marier and colleagues [60], and thereafter by Drs. Andrew Mayrer, Eric Berke, and Peggy Miniter [68–70], were designed to investigate further the immunopathogenic role of Tamm-Horsfall protein in a rabbit model of tubulointerstitial nephritis, and in a swine model of reflux nephropathy. Mayrer and Miniter also examined the immune responses to Tamm-Horsfall protein in patients with recurrent nephrolithiasis, who are at risk of developing renal damage and chronic pyelonephritis [68], and also explored the antigenic similarities between Tamm-Horsfall protein and protein-containing components of uropathic bacteria [68,71].

Rabbit Model of Tubulointerstitial Nephritis One hundred twenty-one rabbits were challenged by the intravenous route every twenty-eight days for up to 48 weeks with one of the following sterile solutions: *(1)* homologous rabbit urine containing rabbit Tamm-Horsfall protein; *(2)* rabbit urine selectively depleted of Tamm-Horsfall protein; *(3)* autologous rabbit urine containing Tamm-Horsfall protein and obtained by bladder catheterization; or *(4)* purified rabbit Tamm-Horsfall protein. Unchallenged, age-matched rabbits were used as normal controls.

The following serial studies were obtained at two- to four-week intervals: *(1)* renal histopathology as determined by hematoxylin and eosin, Masson's to detect fibrosis, periodic acid Schiff (PAS) to define distal tubular segments as well as to detect Tamm-Horsfall protein, peroxidase-antiperoxidase immunohistochemical staining for Tamm-Horsfall protein to identify the thick ascending limb of the loop of Henle, light immunofluorescence microscopy (IFM) for determinations of immunoglobulin deposition using fluorescein-conjugated anti-rabbit immunoglobulins (IgG, IgM, and IgA) raised in sheep and goats (Cappel Laboratories, Cochranville, PA), and electron microscopy for ultrastructural examination for more precise characterization of infiltrates, fibrosis, and localization; *(2)* serum IgG anti-Tamm-Horsfall protein antibody levels by ^{125}I-staphylococcal protein A radioimmunoassay [60]; *(3)* cell-mediated immunity, using rabbit peripheral blood lymphocytes obtained by gelatin sedimentation, Ficoll-Hypaque gradient centrifugation and nylon wool columns to enrich for T cells, evaluated by a cytotoxicity assay and blast transformation [69]. The cytotoxicity assay quantitates the cytotoxic effects of lymphocytes from challenged rabbits against "innocent bystander" mouse A9 fibroblast target cells in the presence of a soluble protein antigen and is a specific test of delayed-type hypersensitivity [69]. Lymphocyte transformation was measured by tritiated thymidine uptake.

Light microscopy of kidney sections demonstrated a characteristic focal tubulointerstitial nephritis at the corticomedullary junction in 9 percent of rabbits challenged with homologous urine after two to 12 weeks, and in 83 percent after 16 to 48 weeks of challenge. The same lesions were observed in 78 percent of rabbits challenged with autologous urine and 79 percent challenged with purified rabbit Tamm-Horsfall protein. In comparison, none of the unchallenged controls and 7 percent (one rabbit) of those challenged with Tamm-Horsfall protein-depleted homologous urine developed tubulointerstitial nephritis. Tubulointerstitial nephritis developed more rapidly in

rabbits challenged with purified rabbit Tamm-Horsfall protein than in rabbits challenged with homologous urine. Early lesions consisted of focal mononuclear cell infiltrates that encircled isolated tubular segments identified as thick ascending limbs of the loop of Henle. Later lesions were more severe and contained more fibroblasts and collagen, and more tubular atrophy, and often extended in a radial manner within medullary rays. Immunofluorescence studies failed to demonstrate significant immunoglobulin deposition in any of the challenged animals [68]. Electron microscopy studies demonstrated mononuclear cells along the thick ascending limb of the loop of Henle and fibrosis.

Serum anti-Tamm-Horsfall protein antibody elevations (IgG) were common in rabbits challenged with either urine or Tamm-Horsfall protein and correlated with the finding of tubulointerstitial nephritis.

Cell-mediated immune responses to Tamm-Horsfall protein were observed in approximately one-half the rabbits challenged with either urine contaning Tamm-Horsfall protein or purified Tamm-Horsfall protein. In contrast, only three of 35 rabbits in the two control groups (unchallenged and Tamm-Horsfall protein-depleted urine) had cytotoxic lymphocytes. The presence of cytotoxic lymphocytes correlated with the development of tubulointerstitial nephritis. These studies suggested that rabbit Tamm-Horsfall protein is the major antigen in urine to which lymphocytes are sensitized, that the effector cells are functional T lymphocytes since they are non-adherent to nylon wool columns, and that the soluble supernatant (lymphokine) of the interaction between antigen and effector lymphocyte is responsible for cytotoxicity.

Porcine Model of Reflux Nephropathy Twenty-one female miniature pigs underwent cystotomy and right ureteral orifice meatotomy with placement of a nonconstricting 3- or 4-mm omega-shaped ring around the proximal urethra. Intravenous pyelography, suprapubic aspirates of bladder urines for cultures, serum creatinine, and serum IgG anti-pig Tamm-Horsfall protein antibody titers as compared to baseline control serum in the same pig were obtained at two-week intervals. In addition, cell-mediated immunity was quantitated by lymphocyte-mediated cytotoxicity against target fibroblasts in the presence of pig Tamm-Horsfall protein. The kinetics of the pyelographic changes in porcine reflux nephropathy indicate that bladder dilatation occurred after approximately one month, ureteral dilatation after 39 days, renal insufficiency after 57 days, and renal scarring after 71 days [68]. Serum anti-Tamm-Horsfall protein antibody titer elevations developed in association with ureteral dilatation. Serum titers decreased in association with removal of the urethral omega ring, which was required to reverse severe uremia. Pyelographic evidence of coarse focal scarring occurred in 15 of 21 animals at this time. Lymphocyte-mediated cytotoxicity was studied in pigs pre-operatively and when intravenous pyelography demonstrated bladder dilatation only, ureteral dilatation, and hydronephrosis with uremia. Abnormal lymphocyte cytotoxicity was seen most frequently when pigs developed ureteral dilatation. Histopathologic examination of the kidneys demonstrated chronic tubulointerstitial nephritis characterized by mononuclear cellular infiltrates, often associated with interstitial Tamm-Horsfall protein deposits, and large focal scars.

The observations that lymphocyte-mediated cytotoxicity developed (in challenged rabbits and refluxing pigs) against susceptible target fibroblasts in the presence of Tamm-Horsfall protein suggest that cell-mediated immunity to Tamm-Horsfall protein plays an important role in the pathogenesis of tubulointerstitial nephritis.

Earlier animal models of tubulointerstitial nephritis by Neilson and Phillips [72–75] also suggest that cell-mediated immunity to renal tubular antigens plays an important role in the pathogenesis of renal damage and fibrogenesis and that the elaboration of soluble factors by sensitized lymphocytes is a primary mechanism of renal damage.

Immune Responses to Tamm-Horsfall Protein in Patients with Nephrolithiasis Serum IgG anti-human Tamm-Horsfall protein antibody and peripheral lymphocyte-mediated cytotoxicity and lymphocyte transformation in the presence of human Tamm-Horsfall protein were determined in 49 patients attending the Yale–New Haven Hospital Renal Stone Clinic who provided informed consent for a venipuncture. All patients (32 men and 17 women) had recurrent nephrolithiasis. Twelve of the 49 showed elevated antibody titers to human Tamm-Horsfall protein as compared with none of 12 normal control subjects. Those patients with elevated antibody titers, as compared with those with normal antibody titers, had the first stone develop more recently, and also had another more recent stone event (50 percent vs. 11 percent, $p < 0.02$). Cell-mediated immunity to Tamm-Horsfall protein was studied in 24 patients, and 13 had either abnormal lymphocyte transformation or lymphocyte-mediated cytotoxicity, whereas 11 patients had neither abnormality [68].

Relationship of Uropathic Coliforms to Human Tamm-Horsfall Protein The potential for cross-reactivity between proteins obtained from uropathic coliforms and human Tamm-Horsfall protein was investigated by competition inhibition radioimmunoassay [60,69]. In this assay, 50 μl of rabbit anti-human Tamm-Horsfall protein IgG was adsorbed to the wells of microtiter plates and 40 μl of test antigens plus 10 μl of ^{125}I-human Tamm-Horsfall protein (50,000 cpm) were added, incubated, washed, and counted. Test antigens included veronal buffer extracts of *Escherichia coli* 014:K7(L):NM, an untypable *E. coli* (ECY9), *Klebsiella oxytoca,* and *Streptococcus faecali.* Extracts of *Aspergilla fumigatus* and *Legionella pneumophilia* were also tested. Veronal extracts of *E coli* 014 were further fractionated on a Sephadex G-150 column, a column of DEAE-Sephacel anion exchange resin, and on a Sepharose-4B anti-human Tamm-Horsfall protein IgG affinity column [68]. Veronal extracts and specific fractions were characterized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and stained with Coomassie blue. Furthermore, unstained gels were electroblotted on to nitrocellulose paper, incubated with rabbit antiserum to Tamm-Horsfall protein, overlaid with ^{125}I staphylococcal protein A, and autoradiographed [68]. Antisera raised in rabbits to *Aspergillus fumigatus* and *Candida albicans,* as previously described [76,77], were used as negative controls. The results of these studies indicated that non-radioactive human Tamm-Horsfall protein was the most effective inhibitor of the binding of anti-human Tamm-Horsfall protein antibody (IgG) to its antigen, human Tamm-Horsfall protein. Pig and rabbit Tamm-Horsfall protein inhibited binding but at greater concentrations [68]. Crude veronal extract of *E. coli* 014, ECY9, and *K. oxytoca* also inhibited binding but at concentrations reflecting lesser potencies. Inhibition was not due to mechanical or precipitogenic influences by veronal extract on ^{125}I-human Tamm-Horsfall protein. Comparable concentrations of *A. fumigatus* extracts, albumin, and ribonuclease A did not inhibit binding of ^{125}I-human Tamm-Horsfall protein to its solid phase antibody [68]. Furthermore, the second of three peak fractions of *E. coli* 014 veronal extract, eluted from a column of Sephadex G-150, was found to have tenfold more competitive inhibitory capacity than did the parent veronal extract and contained protein-containing subunits of less than 70,000 molecular weight. A fraction of veronal extract

was prepared by passage through an affinity column containing Sepharose-4B covalently linked to anti-human Tamm-Horsfall protein (IgG). This fraction was comparable to pig Tamm-Horsfall protein in its ability to inhibit binding between ^{125}I-human Tamm-Horsfall protein and its specific antibody [68]. Immunoautoradiographs of polyacrylamide gels, electroblotted on to nitrocellulose paper, and incubated sequentially with rabbit antiserum to human Tamm-Horsfall protein and ^{125}I-labeled staphylococcal protein A indicated that both veronal extract and its ion exchange fractions exhibited three protein-containing subunits of less than 70,000 molecular weight that are bound by anti-human Tamm-Horsfall protein antibody. Extracts of *S. faecalis, A. fumigatus,* and *L. pneumophilia* did not autoradiograph. Veronal extract and its fractions did not autoradiograph after incubations with rabbit anti-Aspergillus or anti-Candida antisera [68,71]. Our results support the previous observations of Fasth and colleagues [78] and indicate that veronal extracts of three uropathic coliforms competitively inhibited binding of human Tamm-Horsfall protein to its antibody, that purified fractions of *E. coli* 014 veronal extract obtained by column chromatography were ten times less potent than pig and rabbit Tamm-Horsfall protein in competition inhibition of human Tamm-Horsfall protein, that some eight protein-containing subunits (<50,000 daltons) were found by polyacrylamide gel electrophoresis of veronal extracts to correlate with Tamm-Horsfall protein inhibition, and that a maximum of three electrophoretic subunits were detected by immunoautoradiography in veronal extracts of each of the three uropathic coliforms examined in these studies [68,71]. Further studies employed purified anti-Tamm-Horsfall protein antibody conjugated to sepharose beads, thereby forming an immunoadsorption column capable of isolating Tamm-Horsfall protein and cross-reactive antigens from solution. The bacterial extracts, however, did not react with the affinity column. This finding suggests that the cross-reactivity seen in the immunoassays is caused by the interaction between the protein extracts of uropathic coliforms and Tamm-Horsfall protein and is not representative of true immunologic cross-reactions for a common antibody. Obviously, more studies are required before these apparent differences can be resolved definitively.

In summary, evidence has been presented that vesicoureteral reflux can lead to renal injury and scarring in the absence of infection and that autoimmune responses to Tamm-Horsfall protein may occur after exposure to Tamm-Horsfall protein by intravenous challenge, urinary reflux, or recurrent nephrolithiasis. This autoimmune response to Tamm-Horsfall protein may be the pathogenetic mechanism by which these factors, including bacteriuria, contribute to chronic pyelonephritis.

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