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Antimicrobial Peptides, Innate Immunity, and the Normally Sterile Urinary Tract

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ABSTRACT

Considering the anatomical location of the urethral meatus, it is surprising that urine is normally sterile. The defensive properties of uroepithelia help maintain this sterility as strategically necessary for long-term survival. Epithelia lining the urinary tract prevent adhesion of bacteria by release of Tamm-Horsfall protein, lactoferrin, lipocalin, and constitutive and inducible bactericidal antimicrobial peptides such as α- and β-defensins and cathelicidin. Microbes that overwhelm these early defenses contact uroepithelia and activate an innate immune response through Toll-like receptor 4. With persistence of increasing numbers of microbes, chemokines (IL-8) and cytokines (IL-1 and TNFα) attract and activate large numbers of neutrophils and macrophages that damage tubulointerstitial parenchyma. The risk of serious infection in humans seems quite variable. Cathelicidin, for example, is a vitamin D–dependent gene, and vitamin D stores may influence susceptibility to urinary tract infection in selected individuals. As more knowledge accrues, vitamin D supplementation may someday be useful as adjuvant therapy in this setting.

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Although infections of the urinary tract are among the most common human infections, infections of the urinary system are surprisingly infrequent. In the pediatric population, urinary tract infection can progress to scarring, recurrent infections, and permanent impairment of renal function.[1] However, what is entirely curious is that infection of the urinary tract is not more common. The urethra exits so close to the rectum that it guarantees the urinary tract will forever be exposed to enormous numbers of microorganisms. Despite this peculiar design, most cases of urinary infection that occur in children arise in individuals with anatomical or functional disorders of the bladder and/or the distal collecting system of the kidney.

Perhaps even more odd is that, except for the urethral meatus, the urinary tract appears to be sterile. Because urine can support the growth of microbes, the sterility of the urinary tract must mean that viable bacteria cannot normally ascend to its upper reaches. If we assume a microbe's journey must involve attachment to the epithelia of the lower urinary tract followed by movement upward while in intimate contact with the epithelial surface, one might expect the mechanisms responsible for sterility to be operating at the interface between the microbe and its adjacent epithelium. And because we normally do not find great numbers of neutrophils and other cellular elements in normal urine, sterility cannot be caused by the aggressive "subclinical" recruitment of mononuclear cells onto the luminal surfaces of the urinary tract.

Virulence of Urinary Bacterial Pathogens

Some insight into the mechanisms of defense in the urinary tract can be appreciated by examining the properties that enable microbes to cause infection. Not surprisingly, they include the capacity to form pili and fimbria, structures that permit the microbes to attach to the epithelial surface.[2] Similarly, the presence of flagella enhances virulence by permitting the organisms to move toward the kidney against the flow of the urinary stream.[3] The genetically determined capacity of certain microbes to invade a uroepithelial cell, adapt to the intracellular milieu, and effectively multiply, thereby causing chronic bladder infections, represents a striking microbial adaptation.[2] Lastly, the organisms that utilize the urinary tract as "fertile soil," generally the gram-negative microbes common to the distal bowel, require sufficient concentrations of iron to support growth. To meet their nutritional need for iron they produce organic iron scavengers called siderophores, which are empty when secreted and are avidly recovered when loaded with iron. Microbes that cause urinary tract infections have been found to modify this iron-capture system in ways that inform us of the antimicrobial defenses built around the iron economy.[4]

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Innate Immunity *Versus* Adaptive Immunity

Recent studies have shed light on the antimicrobial defenses that prevent microbes from gaining a foothold on the urinary epithelium. These studies highlight the importance of the innate immune system in the defense of the urinary tract.

Innate immunity represents that branch of host defense that is hard-wired to respond to microorganisms. It consists of receptors that are structured to recognize unique microbial components and effectors that can rapidly respond to the "usual" assaults that have molded our evolution. Its effectors include antimicrobial peptides (AMP) and proteins that rapidly neutralize an invader, chemokines and cytokines that attract phagocytes to a threatened site and enhance their microbicidal capacity, and the phagocytes themselves. Innate immune defenses generally operate at a basal level before infection and are induced to higher levels of expression after microbial invasion.

In general, the innate immune system is poised to respond to a challenge within minutes to hours of invasion. The complete defensive response is designed to unfold on a time scale more rapid than the regeneration time of a microbe. The microbe is killed before it can either recover or propagate. In contrast, the adaptive immune system, which depends on the selection of highly specific antibodies and T cell receptor–bearing lymphocytes, is designed to protect days to weeks after injury, on a time scale far too slow to deal with the immediate consequences of a rapidly unfolding microbial attack. Another feature of the innate immune system is that it is clinically silent. The system operates without signs of inflammation. When the system is working properly a state of health is acknowledged. It is when the innate system fails that the signs of progressive inflammation become clinically evident.

Antimicrobial Peptides

AMPs are widely distributed throughout nature and have been discovered in certain bacteria, protozoa, fungi, plants, and multicellular animals.[5] About 1000 different, naturally occurring peptides have been discovered to date. In general, AMPs are small, between 20 to 60 amino acids, positively charged because of the presence of lysine and/or arginine residues, and amphipathic. This latter property permits them to achieve high concentrations in both an aqueous environment or within a membrane. AMPs act on a microbe's membrane and do so rapidly (within seconds to minutes) and lethally. AMPs have many different structures, and so long as the peptides retain their cationic charge and amphipathicity, they retain activity. As a consequence, the sequences seem to be less important than the structural properties of the peptide.

The AMPs found in the pig or the cow, for example, are different in structure and sequence from one another, and from those found in man. The target of AMPs that underlies their specificity for microbes is the difference in lipid composition between the membranes that surround microbes and cells in our body. For reasons that remain unclear, microbes such as bacteria and fungi have plasma membranes that contain phospholipids with negatively charged headgroups on the outer leaflet, the side exposed to the outside. In contrast, multicellular animals are built of cells that are surrounded by membranes populated with zwitterionic phospholipids on the outer leaflet; anionic phospholipids tend to be segregated in the interior leaflet. This fundamental difference in membrane design is exploited by multicellular organisms in the design of AMPs and clearly has remained effective over millennia. Resistance to these AMPs, in general, is difficult to develop because it requires "redesign" of membrane lipid composition and topology.[5,6] AMPs are produced by "professional" phagocytic cells and by all epithelial cells that come in contact with microbes. Some AMPs are constitutively expressed, and others are only expressed when the organism (or a tissue) has been injured or exposed to microbes.

The current universe of AMPs expressed by animals is the product of a dynamic co-evolution. Microorganisms likely evolve some level of resistance against the AMPs that arm an animal (or plant) and reflexively force the new selection of AMP sequence polymorphisms that recover antimicrobial activity. The AMPs that a modern animal expresses likely reflect the microbial challenges of the niche that the animal inhabits, including both those that are present in the environment and those that exist as commensal inhabitants of their body. Hans Boman, a pioneer in this field and the discoverer of the basis of insect immunity, has argued that AMPs evolved to permit us to live in harmony with commensal flora.[7]

Defensins and Cathelicidin

The most well-studied AMPs in man are the defensins and cathelicidin (also called LL-37). The defensins are highly structured compact peptides, constrained by three sets of disulfide bonds; the α and β classes differ with respect to the manner of folding. Cathelicidin is a linear peptide that assumes an α-helical structure on contact with a membrane. Cathelicidin is represented by one gene[8] and is expressed on all epithelial surfaces[9] and by circulating white cells, including neutrophils, monocytes, natural killer cells, and $\gamma\delta$ T cells.[10] In general, the expression of cathelicidin in most epithelial sites is induced by local injury or infection.

The α-defensins, HNP1 to HNP4, are present in high concentrations in neutrophils and provide the neutrophil with its nonoxidative microbicidal activity. Other members of the α family (HD5 and HD6) are expressed in the Paneth cells of the small intestine, where they are continuously secreted from the intestinal crypt into the lumen. It is believed that these abundant AMPs both protect the stem cells of the intestine and influence the species composition of the intestinal commensal.[11,12]

β-Defensins are widely expressed throughout our epithelia. More than 30 loci exist,[13] but only 4 (HBD1 to HBD4) have been studied in any detail. The α-defensin family and most of the β-defensins are clustered on chromosome 8p23.1; the locus exhibits copy number polymorphism ranging from 2 to 12 copies per diploid genome.[14] mRNA concentration correlates with copy number,

suggesting that variations in this cluster could affect clinical outcome. Indeed, clinical significance of inherited defensin gene copy number has been linked to a colonic form of Crohn's disease; mucosal expression of HBD2 is lower than normal in tissue taken from individuals with the colonic form of Crohn's disease and individuals who appear to be more likely than unaffected people to have <4 copies/diploid genome of the HBD2 repeat.[15] No comparable study has been conducted evaluating the association between defensin gene copy number and the incidence of urinary tract infections.

Both defensins and cathelicidin appear to be chemoattractants for certain circulating white cells. Both the α- and β-defensins attract immature dendritic cells; β-defensins interact *via* a specific receptor, CCR6.[16] Cathelicidin attracts neutrophils as well as circulating and tissue-derived monocytes; this AMP specifically interacts with fMLP-receptors on these cells.[17] I imagine this chemoattractant property provides AMP-based defenses with "secondary" back-up that is coordinated with signals stimulating AMP expression. The optimal chemoattractant concentrations of defensins and cathelicidin are in the micromolar range, considerably higher than observed for traditional chemokines. As a consequence, only those white cells in the local environment where these AMPs appear will be attracted, limiting the intensity of white cell recruitment to local resident cells.

Both defensins and cathelicidin are expressed along the human urinary tract. The challenge in understanding their role has been to distinguish whether their presence is truly anti-infective, as suggested by *in vitro* studies, or whether they serve another as yet unknown function. HBD1 is constitutively produced by the collecting ducts, distal tubules, and loops of Henle.[18] As such, this AMP is positioned to defend the kidney from ascending infectious microbes. Concentrations of HBD1 in urine are below effective antimicrobial concentrations. It is assumed that the fluid layer in direct contact with the luminal surface of the tubular epithelia, into which AMPs are initially secreted, contain sufficiently higher anti-infective concentrations of AMPs; an AMP-rich biofilm is presumed present, continuously replenished as it is swept into the bulk stream of urine. The presence of these quick-acting, anti-infective agents would be expected to damage or kill microbes that gain a foothold on epithelia, and so provides a chemical shield.

In the setting of chronic kidney infections, expression of HBD2 (absent in the healthy kidney) is induced in the same anatomical regions as HBD1. The fundamental hypothesis is that an additional level of antimicrobial defense must be called into action after infection to complement-constitutive AMP defenses.[19] The strongest evidence that defensins actually play a role in the defense of the kidney comes from studies of a geneticall-engineered mouse in which a defensin gene analogous to the constitutively expressed HBD1 was deleted. In contrast to wild-type mice, these null animals do not maintain sterility of their urine; about 30% of the healthy *Defb1*$^{-/-}$ mice had *Staphylococcus* species in bladder urine.[20]

Other Antimicrobial Proteins Implicated in Bacterial Defense of the Urinary Tract

In man, several antimicrobial proteins have also been implicated in innate defense of the epithelium. Of particular interest in the setting of the kidney are Tamm-Horsfall protein (THP), lactoferrin, and lipocalin. THP, an abundant urinary glycoprotein, is not itself antimicrobial; it serves an anti-infective role by impeding the ability of certain uropathogenic microbes from adhering to epithelia.[21] THP interferes with epithelial adhesion of *Escherichia coli* through interaction with fimbrial structures. Recent studies of mice expressing null alleles for THP demonstrate that *E. coli* introduced into the bladders are cleared less rapidly than in wild-type mice.[21] Furthermore, these THP-deficient mice develop chronic bladder wall inflammation, suggesting that the abnormal microbial persistence provokes a secondary pathologic response.

Lactoferrin and lipocalin are antimicrobial proteins that act by virtue of their capacity to restrict the availability of iron, and essential microbial nutrient. Lactoferrin is expressed in the distal collecting tubules and can be found associated with the luminal surface; lactoferrin can damage microbes both by chelation of iron an by effecting membrane damage, the latter arising as a consequence of interactions mediated by amphipathic cationic sequences.[22] Lactoferrin is not induced after injury or infection. Lipocalin is designed to capture the iron-laden organic siderophores that bacteria utilize to scavenge iron from the environment;[23] lipocalin exhibits bacteristatic activity against organisms that secrete siderophores in iron-limiting media.[23–25] Lipocalin-null mice are more susceptible to systemic infection from organisms that synthesize siderophores.[24,25] Lipocalin is dramatically upregulated throughout the kidney within 3 hours of injury after ischemia-induced acute tubular necrosis (ATN).[24] As yet, the specific stimuli that are directly responsible for the induction of lipocalin are unknown. The relationship between ATN and the induction of lipocalin suggests that it plays some role in repair of the epithelial damage that accompanies ATN.[26,27] However, in mice lacking a functional lipocalin gene, recovery from ischemia-induced ATN does not differ from wild-type mice.[24] The importance of the antimicrobial function of lipocalin in the setting of ATN has not been studied. Although an acute ischemic insult to the kidney is characterized by extensive damage to the epithelial cells of the kidney tubules, involving distortion of tubule structure along with decreased urine flow, secondary infections of these kidneys are uncommon. It is fair to speculate that, during ATN, lipocalin provides a rapidly responsive, protective, antimicrobial function, constraining the invasion by uropathogens during the subsequent phases of recovery.

The Role of Cathelicidin in the Antimicrobial Defense of the Urinary Tract

The sterility of the healthy urinary tract suggests that antimicrobial defenses must be exceedingly rapid, adjusting to microbial presence over a time frame briefer than the replication rate of poten-

tial pathogens. A very recent report on the expression of cathelicidin in the human kidney suggests they are poised to appear explosively should urinary epithelia sense microbial presence.[28]

Low levels of cathelicidin are present in human urine and, as might be expected, at higher concentrations in children with urinary tract infections; the cathelicidin, curiously, appears to be of epithelial origin, because levels do not correlate with urinary neutrophils (as noted above, both epithelial cells and neutrophils produce cathelicidin). Examination of human renal biopsies demonstrate that cathelicidin is continuously synthesized by tubular epithelium in uninfected tissue and released into the tubular lumen without being stored to any significant extent. On exposure of renal explants to *E. coli*, mRNA encoding cathelicidin rose within minutes, followed directly by peptide secretion into the surrounding medium, highlighting the inducibility of this AMP by microbial presence. Synthesis and secretion of cathelicidin continued for hours.

These data suggest that the cathelicidin gene in the human urinary tract is designed to defend epithelia by a remarkable mechanism that coordinates secretion quickly. The time scale is such that microbes setting off the trigger would likely die before they could reproduce. Furthermore, the activated patch of epithelia would continue to defend itself through AMP secretion for hours, perhaps in anticipation of an additional microbial assault. Consistent with a reason for prolonged synthesis is the observation that uropathogenic strains of *E. coli* are more resistant to the bactericidal action of human cathelicidin than *E. coli* strains not associated with urinary tract infections.

To further explore the importance of cathelicidin in the defense against urinary tract infection, Chromek *et al.* compared the course of an ascending urinary tract infection in groups of mice in which the cathelicidin gene was deleted or normally expressed.[28] In this murine model, a large inoculum of uropathogenic *E. coli* is introduced into the bladder, the numbers of bacteria adjusted to overwhelm the defenses of the lower urinary tract to produce infection in the kidneys. When examined within 1 hour of bladder inoculation, animals lacking cathelicidin have greater numbers of bacteria adherent to bladder epithelium compared with wild-type mice. As the infection ascends, expression of cathelicidin throughout the renal parenchyma increases, and neutrophils are seen around local sites of infection. A profusion of neutrophils was noted in areas of intense microbial invasion, damaging the renal architecture as a result of the inflammatory response. Cathelicidin-deficient animals experienced a higher rate of ascending infection compared with wild-type animals. Depletion of neutrophils did not significantly increase the susceptibility of either wild-type or cathelicidin-deficient mice for kidney infection, suggesting the functional role of cathelicidin in defense of the kidney is primarily preventative.

Toll Receptor Activation, Chemokines, and Neutrophils

What happens should AMPs and antimicrobial proteins not contain a microbial assault? One imagines the microbes come in contact with epithelial cells, possibly internalize, and/or enter the subepithelial space of the lower and upper portions of the urinary tract. The Toll-like receptor TLR4, the major lipopolysaccharide (LPS) sensor in mammals, is likely recruited at this stage as part of the innate immune defense. TLR4 is present on the luminal surface of bladder epithelium. Activation of this receptor stimulates production of IL-8, a potent neutrophil chemoattractant.[29] The current paradigm thus proposes that, should gram-bacteria (or certain microbial constituents) contact uroepithelial cells, neutrophils will be recruited as a consequence of IL8 stimulation.[30,31] TLR4-deficient mice do not effectively mobilize neutrophils after inoculation of large numbers of *E. coli* into the lower urinary tract, and these mice fail to express the murine version of IL-8. Although this response must be protective, it is curious that TLR4-negative mice clear *E. coli* from the kidney more effectively than wild-type mice, perhaps because of diminished neutrophil-associated damage of the kidney.

A curious complexity surrounds the functioning of TLR4 in the kidney. It appears that a critical LPS coreceptor, CD14, is generally required for TLR4 activation. CD14 is present in low levels in healthy kidney and uroepithelia are only weakly responsive to LPS.[32] However, an endogenous lipid from uroepithelial cells, ceramide, also acts as a TLR4 stimulus, and microbial attachment to the epithelia activates sphingomyelinase, liberating endogenous ceramides that trigger TLR4-dependent responses, such as chemokine production.[32] This scenario has not been described in other human organ systems as yet.

A Simplified Picture of the Innate Host Defenses in the Urinary Tract

The picture that emerges regarding innate antimicrobial defenses and their progressive deployment in the setting of an ascending bacterial infection is depicted in Figure 1. Microbes that enter the urinary tract are prevented generally from initial adherence by proteins such as the constitutively secreted Tamm-Horsfall glycoprotein. Organisms that approach the uroepithelium encounter lactoferrin, which both depletes local concentrations of iron and, upon binding to the microbial membrane, inflicts membrane damage. Both HBD1 defensin and cathelicidin, each constitutively secreted, contribute to the bactericidal properties of the fluid layer immediately adjacent to uroepithelia. Should organisms overwhelm this constitutive defense, they provoke the inducible AMPs, HBD2 defensin, and more cathelicidin. In the case of the latter (and perhaps also for HBD2), uroepithelia have the capacity to explosively synthesize and secrete these microbicidal AMPs onto the uroepithelial surface. In coordination with the induction of cathelicidin and HBD2, as a consequence of the chemoattractant properties of these AMPs, local white blood cells congregate. Monocytes recruited to the site of microbial activity may secrete cytokines (such as IL-1 or TNF), which in turn further stimulate expression of inducible AMPs by epithelial cells. It is likely that most initial microbial assaults are effectively defended in this way.

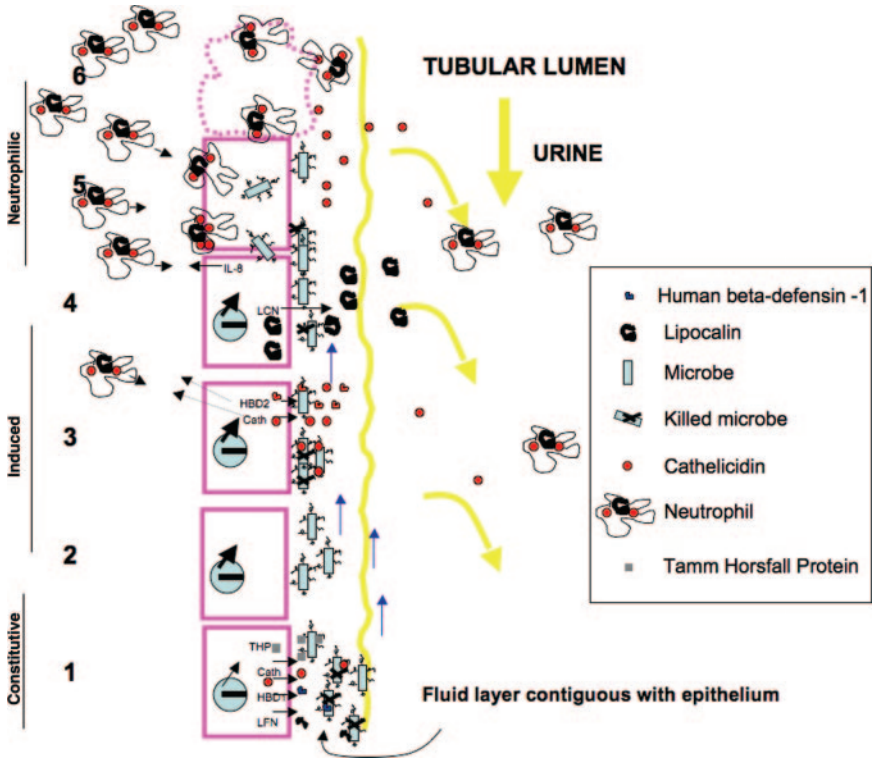

Figure 1. Progressive engagement of innate immune defenses in an ascending infection. Bacteria enter the urinary tract and ascend against the flow of urine. (1) The microbes initially face an array of constitutively expressed antimicrobial proteins and peptides. (2) As surviving microbes ascend, they stimulate the expression of inducible AMPs such as HBD2 and cathelicidin. (3) Secretion of active AMPs occurs within minutes of microbial exposure, both rapidly killing the threatening bacteria that have adhered as well as attracting nearby phagocytes. (4) Advancing microbes attach to the epithelium and stimulate TLR4-dependent and -independent circuits, leading to the secretion of IL-8 and lipocalin. (5) Microbes now face a barrage of neutrophils, which control the advancing infection through phagocytosis and by their secretion of intracellular AMPs and proteins. (6) When the prior defenses have been overwhelmed, continued neutrophil invasion results in extensive tissue destruction.

If microbial organisms succeed in breaching these early defenses, uroepithelia may be susceptible to attachment and damage. Through TLR-mediated pathways (and receptors still uncharacterized), bacterial attachment, propagation, and invasion, as well as cellular injury, stimulates the production and secretion of cytokines such as IL-8 and antimicrobial proteins such as lipocalin. The presence of IL-8 will lead to the rapid recruitment of more neutrophils, providing both anti-infective relief as well as local epithelial damage. Lipocalin, induced by cellular injury, provides antimicrobial defense through sequestration of microbial siderophores. Should these layers of defense fail, the neutrophil influx grows increasingly more aggressive and results in destruction of the normal microanatomy. If this cellular response is overwhelmed systemic infection ensues.

Enhancing Expression of Endogenous AMPs to Protect against Urinary Tract Infections: Might It Be Possible?

How might we use this information to protect the health of our patients? Perhaps one of the most intriguing possibilities is that we could prevent or treat infections by pharmacologically inducing AMPs.[33–36] Recent studies demonstrate that expression of AMP genes in various epithelial cells can be induced by a variety of nutrients and vitamins. For example, cathelicidin can be induced by vitamin D[37–40] and short-chain fatty acids.[41,42] In the distal colon, fecal bacteria convert complex carbohydrates into short chain fatty acids; these fatty acids are used as a source of energy by the enterocytes. Perhaps it is not surprising that these microbially engendered substances also stimulate expression of cathelicidin by colonocytes. In human *Shigella* dysentery, cathelicidin expression in the rectosigmoid colon is dramatically depressed during the active stage of the disease, but it recovers as the infection clears.[43] Treatment with oral sodium butyrate during early phases of an experimental *Shigella* infection results in the induction of colonic expression of cathelicidin, reduction in the numbers of fecal *Shigella*, and accelerated recovery.[41]

The expression of the gene encoding human cathelicidin is vitamin D–dependent and has a vitamin D receptor site.[37] This is not the case for mice and other fur-covered mammals, where expression of cathelicidin is independent of vitamin D regulation. Through a series of steps, cholecalciferol is hydroxylated at positions 25 and 1 to become fully active. While the liver converts cholecalciferol generated in the skin to calcidiol, the final hydroxylation takes place within specific cells through the enzymatic action of 1-α-hydroxylase (CYP27B1). In both keratinocytes and macrophages, microbes cause the induction of CYP27B1 and the vitamin D receptor by stimulating TLR2 receptors.[37,44] The local synthesis of calcitriol, in turn, leads to induction of vitamin D–dependent genes, including cathelicidin.

In the case of the human macrophage, the vitamin D–dependent synthesis of cathelicidin is required for optimal killing of ingested *Mycobacterium tuberculosis*.[44] The plasma concentrations of calcidiol from dark-skinned people who live in the Northern Hemisphere appear to be below that required to support maximal microbe-stimulated calcitriol/cathelicidin synthesis; the possibility that the high prevalence of tuberculosis in some human populations might be attributable to inadequate levels of vitamin

D has been suggested.[34,44] Clinical studies suggest that vitamin D supplementation can have significant benefit in the treatment of tuberculosis in individuals with suboptimal plasma concentrations of calcidiol.[45–47]

Vitamin D is known to exert suppressive effects on the inflammatory arm of the adaptive immune response.[48] Indeed, vitamin D attenuates the production of the proinflammatory cytokines IFN-γ and TNFα in macrophages exposed to *M. tuberculosis*; at the same time it stimulates their production of cathelicidin. In a sense, calcitriol can be thought of as activating the AMP arm of innate immunity while suppressing the proinflammatory arm of adaptive immunity. In an organ like the kidney, where inflammation can result in irreversible damage, a noninflammatory antimicrobial defense seems prudent.

The 1-α-hydroxylation of calcidiol in the proximal tubule is required for calcium metabolism and bone health. However, 1-α-hydroxylase is present in other sites within the kidney, such as the distal tubules and collecting ducts.[49,50] Perhaps most curious is the observation that 1-α-hydroxylase is induced by LPS in the distal nephron, as studied in cell culture.[51] This leads to the possibility that, as in other settings, calcitriol might be utilized to regulate innate immune defenses in the kidney. Because it appears that vitamin D–deficient humans have lower innate immune function,[34,44] might an association exist between the prevalence of urinary tract infections or asymptomatic bacteriuria and vitamin D stores? Asymptomatic bacteriuria is said to occur in 2% to 10% of pregnant women and in up to 20% of women 80 years of age or older;[52] might this condition be linked to vitamin D deficiency, which has recently been reported to be widespread in both pregnant women and the elderly?[53] Might it be possible to treat certain forms of urinary tract infection with vitamin D supplementation rather than conventional antibiotics? Should we be concerned about compromising defenses of the urinary tract when treating patients with the antifungal itraconazole,[38] ketoconazole, or the HIV-protease inhibitor ritonavir, drugs that are known to pharmacologically inhibit 1-α-hydroxylase?[54] I suspect that as our understanding of the innate antimicrobial mechanisms operating in the kidney becomes more complete, new insights into the treatment and management of urinary tract infections will enter clinical practice.

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DISCLOSURES

None.

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