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Cellular mechanisms of interaction between uropathogenic *Escherichia coli* and renal epithelial cells

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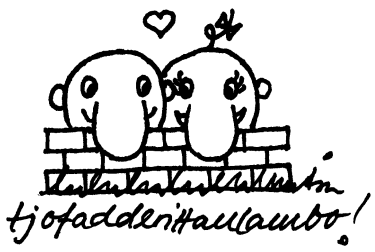

Abstract

Urinary tract infections will often, particularly in children, lead to renal scar formation or hypoplastic small kidneys post-infectiously. The most common causative agent is uropathogenic *Escherichia coli (E. coli)*. The severity of the renal injury is considered to be determined by the host immunological response and the bacterial virulence factors.

Here, we have examined cellular mechanisms by which the host cells respond to *E. coli* exposure.

We found a novel role for the *E. coli* exotoxin, α-hemolysin (Hly) as an inducer of a low frequency intracellular calcium (Ca^{2+}_i) oscillation that acts as a second messenger to induce release of pro-inflammatory cytokines in rat proximal tubule (RPT) cells. The Ca^{2+}_i oscillation exhibited a constant periodicity of 12 minutes and was generated by a combination of calcium influx through voltage-dependent calcium channels and the release from intracellular calcium stores through IP_3 receptor activation. We show indicating evidence that Hly can act through a cell-membrane receptor.

We also found that Hly exerts a dual function on RPT cells. At high concentrations Hly induces sustained elevation of Ca^{2+}_i leading to cell lysis whereas at low concentrations it induces Ca^{2+}_i oscillations that may serve as a host-cell defense response.

Infant rat renal tissue seems to have a fully developed innate immune defense to bacterial toxins. Cytokine release, LPS signaling pathway through Toll-like receptor-4 and Hly induced Ca^{2+}_i oscillations responded similarly in infant and adult renal cells. We could show that *E. coli* pyelonephritis in the infant rat kidney caused a decrease in cell proliferation and increased apoptosis in the renal cortex distant from the site of infection. The higher vulnerability in infants to post-pyelonephritogenic renal growth retardation is likely due to intrarenal cellular effects in the growing kidney, maybe due to bacterial secreted factors.

In conclusion, we show a novel, dual role for the *E. coli* exotoxin, Hly as a virulence factor in childhood pyelonephritis. The higher susceptibility to pyelonephritis in infants is not likely due to an immature innate response to bacterial toxins but rather to anatomical and epithelial cell immaturity that permits bacteria and toxins to accumulate in the renal tissue. Our results shed a new light on the role of pore-forming toxin in disease. Increased knowledge in this field may have therapeutic implications.

Publications

The present study is based on the following papers, which will be referred to by their Roman numerals:

I Uhlén P*, **Laestadius Å***, Jahnukainen T, Söderblom T, Bäckhed F, Celsi G, Brismar H, Normark S, Aperia A and Richter-Dahlfors A
α-haemolysin of uropathogenic E.coli induces Ca^{2+} oscillations in renal epithelial cells.
Nature 405, 694-697 (2000)
*These authors contributed equally to this work.

II **Laestadius Å**, Richter-Dahlfors A and Aperia A
Sublytical effects of Escherichia coli α-hemolysin on rat renal proximal tubule cells.
(Submitted Manuscript)

III **Laestadius Å**, Söderblom T, Aperia A and Richter-Dahlfors A
Developmental aspects of E.coli induced innate responses in renal epithelial cells.
(Submitted Manuscript)

IV Serlachius E, Sundelin B, Eklöf AC, Jahnke M, **Laestadius Å** and Aperia A
Pyelonephritis provokes growth retardation and apoptosis in infant rat renal cortex.
Kidney International 51:1855-1862 (1997)

Contents

Abbreviations

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor
Ca^{2+}_i	Intracellular calcium
cfu	Colony forming units
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
CRAC	Calcium release activated calcium channels
E.coli	*Escherichia coli*
ER	Endoplasmatic reticulum
Hly	α-hemolysin (protein)
HlyA	α-hemolysin (gene)
IL-6	Interleukin-6
IL-8	Interleukin-8
IP_3	Inositol 1,4,5-trisphosphate
LPS	Lipopolysaccharide
PCNA	Proliferating cell nuclear antigen
PLC	Phospholipase C
QBP/LAOBP	glutamine-binding protein /lysine-arginine-ornithine-binding protein
RPT cells	Rat proximal tubule cells
TLR4	Toll-like receptor 4
UTI	Urinary tract infection
VOC	Voltage operated calcium channels

Background

Introduction

Childhood pyelonephritis is a common cause of renal cortical scar-formation or hypoplastic small kidneys. Pyelonephritis in children is more common and often more severe compared to adults. The most common causative agent is uropathogenic *Escherichia coli (E. coli)*. The course of infection is believed to depend on the host cell response to bacterial virulence factors. The first renal cell type exposed to the infecting bacterium is the epithelial cell. Exposure to bacterial virulence factors will induce the epithelial cell to release pro-inflammatory cytokines to trigger an immunological response. Among the virulence factors one can find the bacterial attachment organels, endotoxins such as lipopolyssacharide (LPS) and exotoxins such as α-hemolysin (Hly). Toxins act in a paracrine fashion to interfere with the invading target tissue. The cellular mechanisms of how uropathogenic *E. coli* secreted toxins interact with renal epithelial cells to modulate host immune response and determine age-dependent vulnerability to pyelonephritis is poorly understood.

Kidney development and pyelonephritis in children

Kidney development

Normal human kidney development continues postnatally. In humans, the formation of new nephrons is completed before the 32nd gestational week. After the formation of new nephrons, tubules grow by cell proliferation and cell enlargement. Maturation continues until 2 years of age. During the most rapid phase of kidney growth, renal tubule cells undergo terminal differentiation. Kidney growth slows down after puberty (Larsson and Aperia, 1991).

Cell growth is determined by cell proliferation, apoptosis (or programmed cell death) and hypertrophy. A balance between cell death and proliferation regulates the control of cell number and modulation of organ structure (Celsi et al., 1986; Coles et al., 1993; Koseki et al., 1992). Apoptosis is distinct from necrosis as it is a "suicide-program" that is turned on to kill itself without eliciting an inflammatory response (Cohen, 1993). Apoptosis is thus an important mechanism to remove unwanted cells either during embryogenesis, normal cell turnover or in pathophysiological disorders to remove injured cells.

Many proteins are expressed age-dependently during development and organ maturation continues postnatally. This can be exemplified by the observation that many of the transporting proteins in tubular cells are less abundant and might be in another state of activity in newborns and infants than in adults. The urine concentration capacity is subsequently lower in the infant (Aperia, 1992). It has also been shown that transepithelial passage is higher in infant tubular segments than in adult. A less tightly linked epithelial lining may account for this phenomenon (Horster and Larsson, 1976). With a higher intrarenal pressure, such as vesicoureteral reflux, the epithelial lining would allow an unregulated fluid passage and would also be more easily disrupted.

The infant kidney has not only an immature regulatory capacity but also an immature cellular structure compared to adult kidney. It is therefore possible that there is a different interaction between the bacteria and the immature renal cell compared to the interaction between the bacteria and the mature cell.

Childhood pyelonephritis

Acute pyelonephritis is most common in infancy and early childhood and affects approximately 1-2% of all children (Winberg et al., 1974). Pyelonephritis is almost always an ascending lower urinary tract infection; the bacteria finds its way up to the kidney from the bladder through the ureters. The disease is accompanied with

fever, malaise flank pain and in severe cases is a life-threatening disease. The most common causative agent, uropathogenic *E. coli* is accounting for approximately 85% of the pyelonephritogenic strains, other pathogens are Proteus, Klebsiella and Enterobacter (Rubin, 1996).

The outcome of the infection will be decided by the host immunological response and the virulence factors expressed by the bacteria (Johnson et al., 1998; Lomberg et al., 1989b). In addition to the individual genetic repertoire in the immune system (Knight and Kwiatkowski, 1999; Lindstedt et al., 1991), anatomical factors such as reflux, seem to be of great importance (Lomberg et al., 1989a). The incidence of vesicoureteral reflux, often associated with renal parenchymal scarring, is also higher in small children than in adults (Ditchfield et al., 1994; Gordon, 1995; Smellie et al., 1975). Children, especially less than 3 years old, are more vulnerable than adults to develop renal cortical scars or hypoplastic small kidneys post-infectiously (Benador et al., 1997; Berg and Johansson, 1983; Ditchfield et al., 1994; Jakobsson et al., 1994). Post-pyelonephritic renal scarring, with or without reflux, reaches throughout the cortex and is usually seen in the upper or lower renal poles as focal thinning of the renal tissue (Smellie et al., 1975). The cortical scar formation can be visualized as uptake defects in technetium-99m dimercaptosuccinic acid (DMSA) scan (Jakobsson et al., 1992). Renal growth retardation is often seen in children less than one year as a general reduction in renal parenchymal thickness (Hellstrom et al., 1987). Animal studies have shown that post-pyelonephritogenic growth retardation leads to a reduction in DNA content in the kidneys with or without a reduced kidney size. This can be explained by a compensatory hypertrophy of the remaining cells while the number of functional nephrons is reduced (Hannerz et al., 1989). Childhood pyelonephritis should therefore be considered a risk factor for renal functional impairment. Scarring or renal growth retardation in the infants´ developing kidney may result in progressive renal functional disorders, often resulting in hypertension or renal failure later in life (Jacobson et al., 1992; Smellie et al., 1975).

Pyelonephritogenic *E. coli* and virulence factors

Pyelonephritogenic *E. coli* strains

Uropathogenic *E. coli* is a gram-negative bacterium and subset of the normal gut flora that possesses virulence characteristics enabling it to colonize the urinary tract. The uropathogenic strains can be classified according to cellular markers (serotype). It is known that certain lipopolysaccharides, capsules and flagellar antigens (O:K:H) are associated with *E. coli* strains related cystitis or pyelonephritis (Blanco et al., 1996; Orskov et al., 1982). Other virulence factors important for pathogenicity are adhesion molecules, Hly, aerobactin and serum resistance (Siegfried et al., 1994).

The virulence factors are usually co-expressed and organized in pathogenicity islands, which are large chromosomal regions in the bacterial genome (Hacker et al., 1997). This feature often makes it difficult to distinguish the contribution of each individual virulence factor for pathogenicity.

Adhesion molecules in uropathogenic *E. coli*

To cause disease, *E. coli* has to have the ability to adhere to the host target cell. Uropathogenic *E. coli* expresses different adhesion molecules, pili or fimbriae. Depending on the type of adhesion molecule different strains have different location preferences. For example type-1 piliated *E. coli* strains are often found in cystitis while P-fimbriae are often expressed by pyelonephritogenic strains. The cellular target receptor intracellular, the signaling pathways and the importance for innate immune responses have been extensively characterized for adhesion molecules (Frendeus et al., 2001; Hedlund et al., 2001; Lindstedt et al., 1991; Mulvey et al., 1998; Sauer et al., 2000).

The endotoxin, lipopolysaccharide and its signaling pathway

Lipopolysaccharide (LPS), the major constituent of the outer membrane of gram-negative bacteria, is generally considered to be the strongest immuno-stimulatory agent in bacterial infections. It consists of a highly variable O-specific chain, less variable core oligosaccharide and a lipid component, lipid A. The O-antigen could be considered as a shield to make the bacterium less immunogenic and is important in determining bacterial virulence. The lipid A anchors the molecule in the bacterial cell wall and is believed to be the most immunogenic part (Darveau, 1998). LPS can be shed from the bacteria and is believed to signal via Toll-like receptor 4 (TLR4). The released LPS initially has to bind soluble or membrane bound CD14, which can be considered a co-receptor for TLR4. The LPS-CD14 complex binds and activates the transmembrane bound TLR4. Activation of the intracellular signaling pathway eventually leads to nuclear translocation of NF-κB, and the concomitant activation of inflammatory response genes such as cytokine genes (Beutler, 2000; Poltorak et al., 1998; Wiese et al., 1999).

The exotoxin, α-hemolysin and its role in pathogenesis

Hly, member of the RTX (repeats-in-toxin) family, has long been considered to be a pore-forming toxin (Bhakdi et al., 1996; Bhakdi et al., 1986) capable of lysing a wide variety of target cells such as erythrocytes, leukocytes, endothelial cells and epithelial cells. Hly is an exotoxin produced, modified and secreted from the bacteria in an active state. It is genetically organized in an operon and is co-expressed with its own modification and secretion machinery: *hlyCABD/tolC*. HlyC is an acyltransferase that couples two acyl chains to HlyA that is essential for target cell membrane interaction. HlyB, HlyD and TolC are part of the secretion machinery, and HlyA is secreted through a secretion I system (Blight et al., 1994; Koronakis and Hughes, 1988; Stanley et al., 1998). Hly, a 107 kDa protein, has three functional domains required for target cell recognition and hemolytic activity:

the fatty acyl groups, the hydrophobic domain and the calcium binding repeat domain, which will bind 3-5 calcium ions (Welch, 2001). Although HlyA is considered a pore-forming toxin all attempts to visualize pore formation have been unsuccessful. A few investigators have suggested that Hly, in model membranes, forms a pore permeable to potassium and calcium and molecules <2 nm (Bhakdi and Tranum-Jensen, 1988). How the toxin is interacting with its target cell is also unclear. Some investigators believe that Hly unspecifically interacts with any cell membrane (Bhakdi et al., 1996; Hyland et al., 2001). Others believe that Hly interacts with a specific receptor; it has been shown that Hly binds to leukocyte function associate antigen, LFA-1, on leucocytes (Lally et al., 1997) and to glycophorin on erythrocytes (Cortajarena et al., 2001).

Approximately 50% of the uropathogenic *E. coli* strains express Hly, and Hly is one of the factors showing a strong correlation with pyelonphritogenic strains (Brauner et al., 1990; Siegfried et al., 1994). Although this exotoxin is often expressed among uropathogenic *E. coli* strains, the role in pathogenesis is poorly understood. *In vitro* studies show that Hly secreted by uropathogenic *E. coli,* by its cytolytic action, can kill renal epithelial cells (Mobley et al., 1990; Trifillis et al., 1994). These studies suggest that the major role for Hly in pathogenesis is to disrupt the epithelial lining in the urinary tract by killing the target epithelial cell. It has also been show that Hly expressing *E. coli* may cause a more aggressive disease in both humans and mice than non-Hly expressing *E. coli* (Jacobson et al., 1994; O'Hanley et al., 1991). How Hly is interacting with its target cell, the role of Hly in urinary tract infections (UTI) and its contribution to renal parenchymal tissue damage is still unclear.

Innate immunity and cytokine response

Innate immunity

The innate immune system is the first-line host defense that serves to limit the infection in the early hours after exposure to invading microorganisms. The mucosal inflammatory response will be activated upon evolutionary conserved receptor binding to components of the invading pathogen (Hoffmann et al., 1999). The host cell response will determine the outcome of the infection. In the urinary tract, the close presence of the bacteria will interact with the epithelial cell, which will produce and secrete pro-inflammatory cytokines and chemokines, e.g. Interleukin (IL)-6 and IL-8 (Agace et al., 1993; Brauner et al., 2001; Rugo et al., 1992). The cytokine and chemokine release will attract polymorphonuclear cells, such as neutrophils, to clear the infection. The epithelial cell could thus be considered as the conductor of the innate immune response and IL-6 and IL-8 as early markers of an innate immune response to bacterial infection.

Cytokine response

Different bacterial virulence factors can stimulate the epithelial cell to release the cytokine IL-6. This is a multi-functional cytokine that is believed to have both local and system immunomodulatory effects. IL-6 has been shown to be important for bacterial clearance (Khalil et al., 2000). IL-6 can also be measured in the urine of children with pyelonephritis and maybe used as a prognostic marker for post-pyelonephritic renal damage (Tullus et al., 1994a; Tullus et al., 1994b). The chemokine IL-8 will also be secreted in the initial phase of infection.

IL-8, also essential for bacterial clearance, will attract neutrophils to the site of infection and cause transepithelial migration to kill the invading pathogen (Godaly et al., 1997). IL-8 can also be measured in urine from patients with pyelonephritis (Jacobson et al., 1994; Tullus et al., 1994a). Several studies have shown that monocytes in preterm infants have lower or equal cytokine basal secretion

compared to adults and that cytokine release is increased upon bacterial stimulation, either proportional to adult or a relative increase (Berner et al., 2002; Elsasser-Beile et al., 1995). The importance of the different levels of secreted cytokine release in newborn versus adult for bacterial susceptibility remains to be elucidated.

Intracellular calcium signaling

Ionized calcium is a key carrier of information in the cell. To prevent the toxic effects of a sustained increase in intracellular calcium (Ca^{2+}_i), both excitable and non-excitable cells use trains of calcium spikes for activation of a number of both rapid and sustained events (Berridge, 1997; Berridge et al., 1998). This signaling system has a high level of specificity, since cells can decode the frequency and amplitude of Ca^{2+}_i oscillations. It has been shown that artificially induced low frequency and low amplitude Ca^{2+}_i oscillations give rise to a more pronounced activation of NF-κB than high frequency oscillations or sustained elevated Ca^{2+}_i of higher amplitude (Dolmetsch et al., 1997). According to general concepts, Ca^{2+}_i oscillations are caused by a synchronized opening and closing of inositol 1,4,5-trisphosphate (IP_3) receptors in the endoplasmatic reticulum (ER) and calcium release activated calcium channels (CRAC) in the plasma membrane (Ma et al., 2000; Mikoshiba, 1997). There is evidence suggesting that there is a direct contact between IP_3 receptor and CRAC via cytoskeleton proteins. Phospholipase C (PLC)–coupled receptors generate the chemical messenger IP_3 that binds to IP_3 receptors to release Ca^{2+} from ER. Activation of voltage-gated calcium channels (VOC) also seems to be required (Barritt, 1999). Previous studies on the effect on slow oscillations were performed in artificial systems and despite the importance of Ca^{2+}_i oscillations for cellular signaling few physiological inducers have so far been identified.

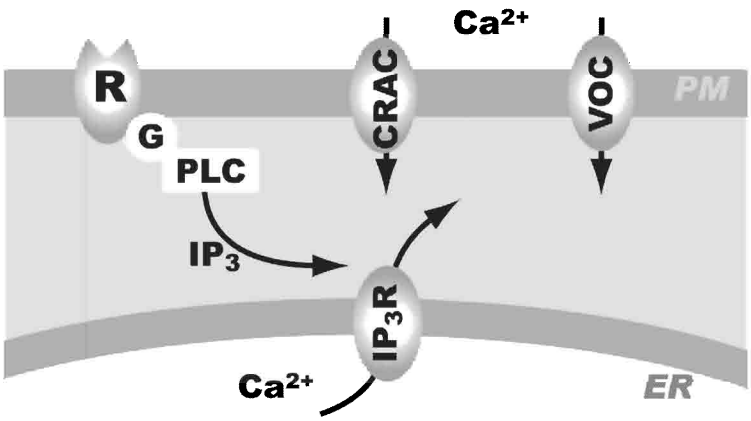

Fig 1. Calcium transporters. R = receptor, G = G-protein coupled receptor, PLC = phospholipase C, IP_3 = inositol 1,4,5-trisphosphate, IP_3R = IP_3 receptor, Ca^{2+} = calcium, CRAC = calcium release activated calcium channels, VOC = voltage-gated calcium channels, ER = endoplasmatic reticulum, PM = plasma membrane.

Aims

1. To study the chain of events in the host cell response to *E. coli* exposure. To study whether Ca^{2+}_i signaling is involved in this chain of events. To study the effects of these events on the cytokine response.

2. To study if the effect of bacterial toxin on renal epithelial cells is dose-dependent and if the common uropathogenic *E. coli* exotoxin α-hemolysin has a dual effect on renal epithelial cells. To initiate characterization of the binding site of this toxin on renal cells.

3. To study if there is an age-dependent difference in the innate immune response in renal epithelial cells exposed to *E. coli* expressed toxins.

4. To study the relative role of growth arrest and of apoptosis for post-pyelonephritic renal growth retardation. To study the age-dependency of this effect.

Methodological aspects

Animals

Sprague-Dawley rats of different ages were used in this study. Infant rat (10 d) kidney development corresponds to 0-2 years human, weaning rat (20 d) to 3-7 years human, and adult rat (40 d) to teenager/adult (Larsson and Aperia, 1991). When anaesthesia was given we used ether (Paper II and IV) or Phenobarbiturate (Paper I-IV). All animal studies were approved by the Swedish Animal Ethical Committee, Karolinska Institutet.

Preparation of primary proximal tubule cells

Primary cultures of rat proximal tubule (RPT) cells were prepared from kidneys taken from 10-, 20- and 40-days old rats as previously described (Larsson et al., 1986), but with a few modifications. Our protocol renders a homogeneous cell preparation of proximal tubule cells that grows in cell clusters, 10-100 cells/cluster, with a cobblestone-like appearance. These cell preparations have been well-characterized and physiological studies made in our lab have shown that if the cells are cultured less than 72 h, the different age-groups keep their phenotypic characteristics, which makes it possible to compare different developmental stages in cell culture (Larsson et al., 1986). (Paper I, II, III)

Preparation of *E. coli*, bacterial supernatant and α-hemolysin (Paper I-IV)

ARD6 is a strain of *E. coli* isolated from a child with pyelonephritis. It is pyelonephritogenic in both human and rat. Its serotype is O6:K13:H1. It expresses type 1C pili and Hly. Hemolytic activity was confirmed in hemolytic assays.

ARD6/pGFPmut2 is similar to ARD6, except for harboring a plasmid with green flourescent protein (GFP), which makes it possible to visualize the bacteria when excited at 481 nm UV light. The strain is cultured in the presence of 100 µg/ml ampicillin. Hemolytic activity was confirmed in hemolytic assays.

DS17 is a strain of *E. coli* isolated during an epidemic outbreak of pyelonephritis in a neonatal ward. It is serotyped O6:K5:H-. It expresses P-fimbriae and Hly. Hemolytic activity was confirmed in hemolytic assays.

W3110 is an *E. coli* K-12 strain. It is an apathogenic lab strain, and does not express an O-antigen. It expresses type-1 fimbriae but does not express Hly. Non-hemolytic activity was confirmed in hemolytic assays.

W3110/pANN202-812 is similar to W3110, except for harboring a plasmid expressing HlyA, including the acyltransferase HlyC, and the HlyA-excretion proteins HlyB and HlyD, necessary to export an active Hly. The strain is cultured in the presence of 100 µg/ml ampicillin. Hemolytic activity was confirmed in hemolytic assays.

W3110/pANN202-812B is similar to W3110/pANN202-812, except for a defect in hlyC, which renders Hly non-acylated. The strain is cultured in the presence of 30 µg/ml chloramphenicol. Non-hemolytic activity was confirmed in hemolytic assays.

The bacteria were cultured overnight in Luria Bertani (LB) medium at 37 ^{o}C unless otherwise stated. Washed bacteria were inoculated in the rat bladder (see Experimental pyelonephritis) or added to the media of primary RPT cell cultures at 10^5 cfu/ml.

When bacterial growth supernatant was added, 10-100 µl/ml of sterile filtered supernatant from an over-night culture of bacteria was used. Heat-inactivation of the supernatant was performed to denature proteins. To neutralize LPS, Polymyxin B (100 U/ml) was added to the supernatant.

HlyA was purified and assayed for hemolytic activity as described (Bhakdi et al., 1986). The purification procedure does not yield a completely pure preparation of the toxin since it will contain trace amounts of LPS. The purification procedure will result in an active, hemolytic Hly as confirmed by hemolytic assays. These assays were performed mixing a serial dilution of bacteria, supernatant or purified Hly with a suspension containing 2.5% sheep erythrocytes. 1,000 hemolytic units (HU) were defined as the dilution giving 60% hemolysis.

Table 1. *E. coli* strains used in this study.

Bacterial strain	**Relevant phenotype**	**Reference**
ARD6	Hly+	(Mattsby-Baltzer et al., 1982)
ARD6/pGFPmut2	Hly+	(Cormack et al., 1996)
DS17	Hly+	(Tullus et al., 1984)
W3110	Hly-	(Bachmann, 1972)
W3110/pANN202-812	Hly+	(Vogel et al., 1988)
W3110/pANN202-812B	Hly-	(Ludwig et al., 1987)

Intracellular calcium imaging

We used a cell image system allowing ratiometric measurement of Ca^{2+}_i on a single cell level. All measurements can be performed and visualized simultaneously with live cells and bacteria, mimicking physiological conditions. RPT cells were incubated with a calcium sensitive dye, Fura-2 acetylmethyl ester (Fura-2/AM), and installed in a temperature-regulated chamber mounted on a

microscope. The fluid in the chamber can be exchanged according to the experimental protocol. The cells were excited every 30 or 60 s, and emission fluorescence was collected and processed. (Paper I, II, III)

Drugs used in calcium signaling experiments

In experiments studying Ca^{2+}_i signaling, we sometimes used pharmacological treatment. RPT cells were pre-incubated for 10-15 minutes with the respective drug before exposure to *E. coli* supernatant. The drugs used in our studies are listed in Table 2. (Paper I, II)

Table 2. Pharmacological treatments in calcium experiments

Drug (concentration used)	**Specificity**
Nifedipine (100 μM)	Antagonist for the L-type voltage-gated calcium channel.
2APB (50 μM)	Cell membrane permeable inhibitor of IP_3 receptor mediated calcium release and inhibitor of CRAC calcium influx.
Bay K 8644 (10 μM)	Agonist for L-type voltage-gated calcium channels.
U73122 (2 μM)	Non-specific inhibitor of phosphoinositide-specific phospholipase C.
CNQX (10-200 μM)	Specific antagonist of the AMPA and kainate receptor ion channel.
MK801 (100 μM)	Ion channel blocker of NMDA receptor ion channel.
Mecamylamine (10 μM)	Receptor selective antagonist of nicotinic acetylcholine receptor ion channel.

Reverse transcriptase polymerase chain reaction (RT-PCR)

When extracting RNA for expression studies on TLR4 and CD14 in renal epithelial cells, the tissue sections should preferably be emptied of contaminating macrophages and leukocytes. These cell types are known to have high expression of TLR4 and CD14, and could give false positive expression. Primary cell culture, kidney perfusion and tubule/glomeruli microdissection was performed as described (Larsson et al., 1986; Nishi et al., 1993). Microdissection was done to study cell specific mRNA expression (Paper III). RT-PCR was used to either detect the presence of mRNA (Paper II and III) or as a semiquantative comparison of detected mRNA from different age groups compared to a house-keeping gene (Paper III).

Cytokine measurements by ELISA

ELISA is an easy, sensitive and reliable method to measure soluble protein if the detecting antibody is of good quality and the samples comparable. Using a human origin cell line, IL-6 and IL-8 could be measured and compared by ELISA as a response to the different treatments. The cell line provides a homogenous cell population, it grows to a given cell density in each plate and there are good antibodies to detect human IL-6 and IL-8 (Paper I). In contrast, rat renal cortical slices consist of a mixed cell population, although the majority of the cells are proximal tubule cells (Aperia et al., 1981). The use of slices enabled us to measure rat IL-6 by ELISA as the number of cells in the compared samples became high enough in the different age groups. The commercial rat IL-6 ELISA kit was not sensitive enough to measure small differences in IL-6 or amounts <20 pmol/ml. Therefore, primary cultures of RPT cells, where the cell number varies between different ages, preparations and samples, could not be used (Paper III).

Experimental pyelonephritis

To mimick a reflux ascending pyelonephritis in human infant and adult, we used an *in vivo* rat model (Hannerz et al., 1989). 20- and 40-day old female rats were catheterized with a double-lumen silver cannula that allowed continuous recording of bladder pressure during infusion of a pyelonephritogenic *E. coli* suspension (10^7 cfu/ml). The pressure control made it possible to record vesicoureteral reflux. The method can be difficult to perform as infusion volumes can vary and it may also result in pressure-induced lesions in the kidney. (Paper IV). In Paper I we were only interested to investigate bacterial-renal cell interaction *in vivo*. The method was modified and the bladders of the anaesthesized 20-day-old rats were catheterized and a predetermined volume of *E. coli* suspension (10^5 cfu/ml) was slowly inoculated into the bladder. The infectious rate was equally good as in the pressure-model.

Detection of DNA synthesis and apoptotis

To determine DNA synthesis in tissue sections, we used immunohistochemical labeling of proliferating cell nulear antigen (PCNA) and autoradiography of ^{3}H-thymidine incorporation. PCNA is a cofactor for DNA polymerase σ and is crucial for DNA replication during cell proliferation, which occurs in mitotic cells during G_1 and S-phase (Connolly and Bogdanffy, 1993; Dietrich, 1993). PCNA is endogenously expressed, no manipulation of the animal needs to be done, labeling for PCNA allows retrospective studies and results can be analyzed in two days. Quality of the antibody, tissue fixation and handling should be considered when interpreting immunohistochemical results. ^{3}H-thymidine is incorporated in proliferating cells during S-phase and is a widely accepted reliable method. The animals have to be injected with the substance approximately 1 h prior to preparing the tissue and autoradiography takes months to develop. The two methods provided compatible results (Paper IV).

To identify apoptotic cells in tissue sections, we used *in situ* immunohistochemical staining of end-labeled endonuclease cleaved DNA (Gavrieli et al., 1992). The method is widely accepted and should be considered semiquantative. (Paper IV)

Data analysis

A power spectrum analysis was performed as previously described (Uhlen et al., 2000). Briefly, single cell calcium oscillations recordings were analyzed to produce a spectrum where the peaks correspond to the different frequencies present in the original data. The dominant peak was determined by comparing the relative power of the peaks in the spectrum. This was calculated by determining the area between the two extremes closest to the peak, divided by the total area of the power spectrum.

Statistical analysis was performed using Student's t-test. All values are presented as mean ± standard errors of mean. (Paper I-IV)

Results

E. coli induced intracellular calcium signaling in renal tubule cells

To study uropathogenic *E. coli* cell interaction and innate immune response in pyelonephritis we chose a combination of *in vivo* and *in vitro* experimental approaches. In a rat model mimicking ascending pyelonephritis, histological staining for ARD6 showed presence of bacteria in the lumen of proximal tubules (PT) in the initial phase of infection (Paper I, fig 1a), indicating that PT cells are the primary target cells for *E. coli* in pyelonphritis. GFP-labeled ARD6 was added to primary cultures of rat proximal tubule (RPT) cells (Paper I, fig 1b). Visual inspection revealed that bacterial attachment was not needed in the initial phase of infection. We had previously shown that renal growth retardation can occur in the absence of inflammatory cells (Paper IV), indicating that bacteria or excreted factors from the bacteria may have a direct signaling effect on renal cells. We were therefore interested to study the cellular signaling mechanisms induced by *E. coli*. To study if *E. coli* could induce a Ca^{2+}_i response in target cells, RPT cells loaded with the calcium-sensitive dye Fura-2, were exposed to a live suspension of ARD6/pGFP (10^5 cfu/ml). The calcium response was recorded with a cell imaging system. Within 30 min, the cells responded with an oscillatory Ca^{2+}_i response (Paper I, fig 1c). Bacterial attachment was not needed and the sterile filtered supernatant of an over night culture of ARD6, induced a similar low frequency oscillatory Ca^{2+}_i response within 5 minutes (Paper I, fig 1d and e). Spectral analysis indicated that the calcium oscillations had a constant, low frequency of 1.4 ± 0.1 mHz, that corresponds to a periodicity of 12 ± 0.7 min (Paper I, fig 2a and b).

Deducing the source of the calcium oscillations

Inhibition of L-type VOC by nifedipine completely abolished the Ca^{2+}_i oscillations (Paper I, fig 3a). RPT cells pre-incubated with 2APB, a cell-permeable IP_3 receptor

antagonist, also inhibited the Ca^{2+}_i oscillations (Paper I, fig 3b). Activation of VOC with Bay K 8644, or inhibition of PLC with U73122 resulted in a deranged Ca^{2+}_i response (Paper I, fig 3c), indicating that *E. coli* induced Ca^{2+}_i oscillations are generated by an interaction between VOC-dependent calcium influx through the cell membrane and IP_3 receptor dependent calcium release from Ca^{2+}_i stores.

Identification of the inducer

To test if Ca^{2+}_i oscillations are a general phenomenon in response to uropathogenic *E. coli*, we tested other *E. coli* strains. The supernatant from a clinical isolate, DS17, induced Ca^{2+}_i oscillations (Paper I, fig 3e) while the apathogenic strain W3110 did not (Paper I, fig 3f). Virulence factors of uropathogenic *E. coli* are usually expressed in pathogenicity islands. One such factor is Hly. When an *HlyA*-expressing plasmid was introduced into W3110 (W3110/pANN202-812) the supernatant induced Ca^{2+}_i oscillations (Paper I, fig 3g). Purified Hly induced Ca^{2+}_i oscillations in low doses (Paper I, fig 3h) but had no lytic effect in hemolytic assay, whereas high doses of Hly resulted in a sustained elevated Ca^{2+}_i response in RPT cells (Paper I, fig 3i), and showed hemolysis in hemolytic assay. Supernatant from W3110/pANN202-812B, expressing a non-acylated form of Hly that makes it defective in its interaction with the cell membrane, did not induce a Ca^{2+}_i oscillation, indicating that Hly has to properly interact with the cell membrane to induce Ca^{2+}_i oscillations. Polymyxin B treatment did not have any affect on Ca^{2+}_i oscillations induced by ARD6 supernatant or purified Hly (Paper I, fig 3j). These data show that Hly is the inducer of the oscillatory Ca^{2+}_i response in RPT cells.

Pathophysiological effect

The initial activation of the innate immune response includes release of pro-inflammatory cytokines and chemokines, e.g. IL-6 and IL-8. We found that Ca^{2+}_i oscillations, induced by Hly in a renal epithelial cell line, stimulated production of

the cytokine IL-6 and the chemokine IL-8. By blocking the Ca^{2+}_i oscillations with nifedipine, the cytokine release was completely inhibited (Paper I, fig 4b and c). These data implicate that Hly induced Ca^{2+}_i oscillations act as a second messenger to induce release of pro-inflammatory cytokines.

Dose-response of HlyA and epithelial cells

To study whether Hly had a dose-dependent effect on RPT cells we performed a dose-response study with regard to Ca^{2+}_i signaling and cell morphology. RPT cells incubated with low doses (10-50 µl/ml) of supernatant from ARD6 responded with Ca^{2+}_i oscillations (Paper II, fig 1a and c) that were highly regular and slow in the 10-12 minute range (Paper II, Table 1). Within a very narrow range, increase of Hly (100 µl/ml), caused a sustained increase in Ca^{2+}_i in the majority of cells. These cells often exhibited a rapid decrease in Ca^{2+}_i below the basal level after 45-90 minutes, interpreted as a sign of cell lysis (Paper II, fig 1b and d). The cell morphology seemed to be unaffected after 4-24 h incubation with low doses of ARD6 supernatant (Paper II, fig 3a and data not shown) while high concentrations of ARD6 supernatant resulted in lysis in the majority of cells (Paper II, fig 3b). This indicates that Hly secreted from uropathogenic *E. coli* exerts a dual action on RPT cells.

Toxin-cell interaction

We have indirect evidence that Hly acts through a cellular receptor (Paper I and II). To study the site of interaction between Hly and RPT cells we used the glutamate α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), which shares a common binding motif in bacterial proteins (the glutamine-binding protein (QBP)/lysine-arginine-ornithine-binding protein (LAOBP)-like module (O'Hara et al., 1993; Paas et al., 1996). Ca^{2+}_i oscillations were dose-dependently abolished by CNQX, in pretreated

RPT cells exposed to ARD6 supernatant (Paper II, fig 4a-e). As the AMPA receptor is not expressed in the rat kidney (Paper II, fig 5), this indicates that Hly is interacting with a protein with a QPB/LAOBP-motif to induce a Ca^{2+}_i second messenger response.

Age-dependent differences in innate immune response mechanisms to *E. coli*

Toxins excreted from uropathogenic *E. coli* have several alternative pathways to interact with the target renal epithelial cell and to elicit an innate immune response. An age-dependent difference in the innate immune response is a possible mechanism for the higher susceptibility to pyelonephritis observed in infants compared to adults. To study this, we compared renal epithelial cells from 10 d and 40 d old rats exposed to supernatant from ARD6. Basal production of IL-6 from 10 d renal cortical tissue was approximately 20% of that from 40 d tissue. Six h incubation with ARD6 supernatant caused approximately 15-fold increase in 10 d and 5-fold increase in 40 d IL-6 release. Absolute level of IL-6 release in supernatant exposed tissue was however significantly lower at 10 d than at 40 d (Paper III, fig 1). LPS, the main stimulator of cytokine release in UTI, signals via the TLR4. RT-PCR performed on outer renal cortex indicated that expression of TLR4 mRNA was similar in both ages (Paper III, fig 3a and b). Microdissection studies revealed that in both age groups receptor mRNA was expressed in proximal tubules but not in glomeruli (Paper III, fig 3c). Hly-induced Ca^{2+}_i oscillations were found to be well developed in 10 d tubular cells (Paper III, fig 2, Table 1). These data indicate that the innate immune system is equally well developed in the infant and in the adult rat.

Cellular mechanisms behind growth retardation during pyelonephritis

Infants with pyelonephritis often develop renal scars or hypoplastic small kidneys post-infectiously. To study the mechanisms behind post-pyelonephritogenic growth retardation we used a rat model that mimicks ascending reflux pyelonephritis in humans. A bacterial suspension of ARD6 was inoculated into the bladder of 20 and 40 d old rats. Four days after infection, histopathological examination showed infiltration of leukocytes in the medullary tissue adjoining the pelvis and calyces. Cortex inflammation was only found in regions adjacent to the pelvis (Paper IV, fig 1 and fig 2). In the 20 d old rat, the tubular cells in the cortex showed marked suppression of DNA synthesis. Immunohistochemistry for PCNA demonstrated a marked decrease in immunoreactivity in RPT cells (Paper IV, fig 4). The mitotic response of RPT cells, assessed by ^{3}H-thymidine autoradiography, showed a highly significant decrease during the first four days after infection (Paper IV, fig 5). Four days after infection, a transient increase in apoptotic cells was observed in cortical cells outside the inflammatory area (Paper IV, fig 3a-c). No increase in apoptotic cells was detected in the cortex 10 days after infection. Pyelonephritis caused a 10-fold increase in apoptotic cells (Paper IV, fig 3d). As apoptotic cells are rapidly digested by adjacent cells, they are only detectable for a short time. A 10-fold increase can thus be considered as a marked apoptotic process. In adult rats, pyelonephritis had no effect on cell proliferation. Four days after infection there was no increase of apoptotic cells in the cortex.

The data indicate that inhibition of cell proliferation and enhancement of apoptosis may contribute to renal parenchymal loss after childhood pyelonephritis, even distant from the site of inflammation.

General discussion

We have identified a novel role for the *E. coli* exotoxin Hly in pyelonephritis, as an inducer of an oscillating second messenger response in target cells sufficient to induce release of pro-inflammatory cytokines. The role of Hly and other pore-forming toxins in disease has long been considered to be through its cytolytic action. Our recent findings suggest that Hly has new clinical implications as a virulence factor.

Here we show that Hly, as the first pathophysiologically relevant protein, induces a constant, low frequency Ca^{2+}_i oscillation to induce a cytokine response. The oscillations were highly regular with a periodicity of approximately 12 min. There are previous reports showing that artificially evoked Ca^{2+}_i oscillations of different amplitude and frequency can induce gene-activation (Dolmetsch et al., 1997; Li et al., 1998). Hly induced Ca^{2+}_i oscillations induced a release of the pro-inflammatory cytokine IL-6 and the chemokine IL-8 in a renal epithelial cell line. Pro-inflammatory cytokines have to be synthesized by gene-activation and are released to elicit an immunological response to invading pathogens. IL-6 and IL-8 are necessary to attract neutrophils to the site of infection for clearance of the invading pathogen (Godaly et al., 1997; Khalil et al., 2000). Clinical studies indicate that the levels of urine IL-6 release may be used a prognostic factor for the long-term outcome pyelonephritis (Jacobson et al., 1994; Tullus et al., 1994b).

We could also show that there has to be a specific interaction between Hly and the epithelial cell. Hly induced Ca^{2+}_i oscillations are achieved by an interaction of calcium influx through VOC in the cell membrane and calcium release from intracellular calcium stores, ER, via IP_3 receptor activation. Calcium in high levels is toxic for the cell and will result in cell death (Berridge et al., 1998). By generating a Ca^{2+}_i oscillation, the toxicity is eliminated, and instead turned into a highly regulated signal. The oscillations disappear when the *E. coli* supernatant is

exchanged to incubation medium or when a defect Hly is added, thereby indicating that Hly has to be present and properly interact with the cell membrane extracellulary. This would implicate a presumed receptor for Hly on the plasma membrane of the target cell. Our present observation that the Ca^{2+}_i oscillations could be blocked by CNQX, add evidence to the concept that Hly may, in sublytical concentrations, act as a ligand to a membrane receptor. There are studies showing that Hly binds to leukocyte function associate antigen, LFA-1 on leukocytes (Lally et al., 1997) and to glycophorin on erythrocytes (Cortajarena et al., 2001). We believe that the essential components necessary to generate an oscillatory signal are clustered by protein-protein-interaction in close proximity to the plasma membrane to increase signal specificity (Davare et al., 2001; Rich et al., 2000) (unpublished observations in our lab). It is possible that Hly-binding to a cellular receptor leads to an increased proximity, via a cytoskeletal protein, of the clustered proteins to interact with the IP_3 receptor. The IP_3 generating PLC is likely part of the clustered proteins. IP_3 is most likely involved as a modulator in Hly-induced Ca^{2+}_i oscillations since previous studies have shown that Hly can induce phosphatidylinositol hydrolysis and IP_3 and diacylglycerol formation in neutrophils and endothelial cells (Grimminger et al., 1997; Grimminger et al., 1991). Activation of VOC can only be speculative, but would in this model be associated with the other regulators. These hypotheses have to be further investigated.

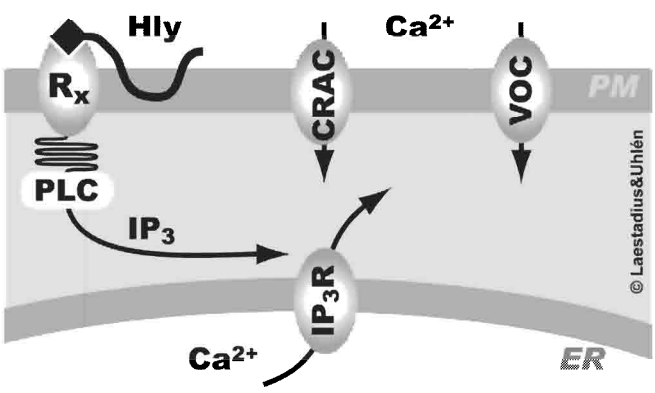

Fig 2. Proposed model for Hly induced Ca^{2+}_I oscillations. R_x = unknown Hly receptor. PM= plasma membrane.

Our studies have shown that Hly has a dual, dose-dependent effect on renal epithelial cells. Low concentrations of Hly induce a Ca^{2+}_i oscillation with no apparent morphological effects, while high doses lead to a sustained increase in the level of Ca^{2+}_i and cell lysis. Concentrations of Hly sufficient to cause cell lysis, can presumably be obtained when the Hly expressing bacteria have invaded the renal tissue and attached to the cells. Bacterial tissue invasion is facilitated by excretion of cytolytic toxins to kill epithelial cells to disrupt the tightly linked epithelial cells in the urinary tract. Since uropathogenic *E. coli* is an extracellular bacterium, it would benefit from the nutrients released from the lysed cells, which will promote bacterial survival and multiplication. Before bacterial invasion into target tissue occurs, secreted Hly should, however, due to the diluting and flushing effect of urine, be present in much lower concentrations. The sublytical concentrations of Hly will induce Ca^{2+}_i oscillations and the release of pro-inflammatory cytokines to serve as a host defense.

Dual activity has recently been observed to be a common phenomenon of other bacterial pore-forming toxins. The *Bordetella pertussis* RTX-toxin, CyaA, harboring an adenylate cyclase and a pore-forming domain, has been shown to exert its cytotoxic action by an uncontrolled cAMP production in low doses, while higher doses lead to lysis of the target cell (Ladant and Ullmann, 1999). Staphylococcal α-toxin has been shown to induce IL-8 production via NF-κB in sublytical concentration, and pore formation and cell lysis in high doses (Dragneva et al., 2001).

Secreted toxins from uropathogenic *E. coli* interact with the renal epithelial cells to induce an innate immune response in a very precise manner. The immunological response, dependent on the host cell intracellular signaling machinery, will decide the outcome of the infection. We speculate that the increased susceptibility to pyelonephritis in infants compared to adults is due to a cellular immaturity in the innate immune response to *E. coli* secreted toxins. In addition to Hly,

uropathogenic *E. coli* sheds LPS. This endotoxin signals via TLR4 and is generally considered to be of major importance for activation of the immune system.

We found that rat infant renal epithelial cells seem to have a well-developed innate immune response system to respond to *E. coli* secreted toxins. Although IL-6 levels released from the infant renal tissue never reach the levels of the adult, they exhibited a relatively larger increase compared to basal secretion. Several studies have shown that monocytes in preterm infants have lower or equal cytokine basal secretion compared to adults and that cytokine release is increased upon pathogenic bacterial stimulation, either proportional to adult or a relative increase (Berner et al., 2002; Elsasser-Beile et al., 1995). Intracellular signaling pathways were equally developed in infant and adult renal cells, as shown by the ability of Hly to induced Ca^{2+}_i oscillations of similar frequency and by the LPS signaling through TLR4 mediated cell activation.

We find it unlikely that the quantitative age-dependent differences in IL-6 response observed in our study is of major importance for the relatively high susceptibility of infants to pyelonephritis. It is more likely that the higher susceptibility is multifactorial and that anatomical immaturity plays a major role. One aspect may be that the epithelial lining in the infant kidney has a higher transepithelial passage than in the adult kidney (Horster and Larsson, 1976). This is due to a less tight epithelial lining in the urinary tract than in the adult. The tightly linked epithelial cells, present in the fully mature kidney, can be considered as a part of the innate immune system, and may be the strongest defense against invading pathogens. To invade the host tissue, the bacteria must disrupt this barrier most certainly by secreting cytolytic toxins. The less tight epithelial cells in the newborn should therefore facilitate transepithelial invasion of bacteria. Another important aspect may be that young children have a high incidence of vesicoureteral reflux (Ditchfield et al., 1994; Lomberg et al., 1989a; Risdon, 1993) and that bacterial deposition in the kidney and exposure to Hly is enhanced. Stagnant urine should increase both the exposure time and the concentration of secreted toxins in the

renal tissue. As the cytolytic action of Hly is both concentration and time dependent (Moayeri and Welch, 1994) this should have clinical implications. The role of Hly in tissue destruction and its contribution to renal scarring in children with severe reflux remains to be elucidated.

Post-pyelonephritogenic renal scarring or hypoplastic small kidneys also accompany the higher susceptibility to pyelonephritis during infancy.

We show that pyelonephritis in infant rats causes renal cortical growth retardation. The cortex in the infected infant kidney was absent of inflammatory cells. This area did however show a decrease in cell proliferation and a marked increase in apoptosis. In the infant, kidney development mainly depends on cell proliferation (Larsson and Aperia, 1991), and influencing factors will subsequently have a large impact on kidney growth. In the adult kidney, where cell proliferation is of little importance, the rate of proliferation was not affected. Post-pyelonephritogenic renal growth retardation in infants is probably due to a direct effect of bacterial toxins or inflammatory mediators, which in a paracrine fashion influences fundamental cell processes distant from the site of infection.

Approximately 50% of all clinical isolates of uropathogenic *E. coli* expresses Hly. Several studies have been performed to characterize uropathogenic strains and establish differences between *E. coli* strains causing cystitis or pyelonephritis and their relationship with renal scarring. These studies are difficult to evaluate since bacterial virulence factors are often co-expressed in pathogenicity islands (Hacker et al., 1997). The role of Hly in pyelonphritis has been debated. Hly is however often associated with pyelonephritogenic isolates and there are studies showing that Hly-expressing strains cause a more severe disease than non-Hly-expressing strains in both humans and mice (Jacobson et al., 1994; O'Hanley et al., 1991). Our recent findings suggest that Hly has new clinical implications as a virulence factor. The role of pore-forming exotoxins in pathogenesis has to be revised. The classical picture of a pore-forming protein, punching holes in target cell, has turned into an intimate interaction with the target cell to induce intracellular signaling pathways.

Secreted toxins can in a paracrine fashion interact with the invaded tissue inducing cellular effects distant from the bacteria. This is a phenomenon known for other bacteria e.g. *Bacillus anthracis* or *Bordetella pertussis* where the secreted toxins are the effectors causing disease (Ladant and Ullmann, 1999; O'Brien et al., 1985).

Conclusions

The findings presented in this thesis show that:

We have identified a novel role for the *E. coli* exotoxin, Hly in pyelonephritis as an inducer of an oscillating second messenger response in target cells that induces release of the pro-inflammatory cytokines.

Hly has a dual role in the interaction with the renal epithelial cell. Sublytic concentrations of Hly induce release of pro-inflammatory cytokines that may serve a role in host-cell defense, and high concentrations of Hly result in sustained elevation in intracellular calcium and irreversible cell damage.

Several signaling pathways in the innate immune system are well developed in the infant rat. Higher susceptibility to *E. coli* pyelonephritis is probably due to cell structural immaturity and a higher incidence of reflux among children than adults.

Pyelonephritis in infant rats induces apoptosis and decreases cell proliferation in the renal cortex distant from the site of infection. This can explain post-pyelonephritogenic growth retardation seen in infants and may be due to bacterial secreted toxins.

Future perspectives

This study demonstrates a novel role for the *E. coli* exotoxin Hly as a virulence factor in pyelonephritis. We have already initiated studies to identify the cellular target receptor for Hly on renal epithelial cells and to further characterize the intracellular signaling pathway. Identification of an Hly receptor should make it pharmacologically possible to prevent the lytic action of high concentrations of Hly and provide a beneficial addition to antibiotics in the treatment of childhood pyelonephritis.

The role of Hly in tissue destruction and its contribution to renal scarring in children with severe reflux has not been fully investigated. There could be a sub-group of patients that would benefit from increased knowledge and additional therapeutic possibilities may be realized in the future.

Uropathogenic *E. coli* secretes other factors, not considered as virulence factors. One such factor is cAMP, now generally believed to be a waste-product from dying bacteria. From own observations we know that large amounts are synthesized by the bacteria. It is now believed that endogenous cAMP can act not only as an intracellular second messenger, but also as a first messenger known to bind to renal epithelial cells. No receptor is identified in mammals but is already well-characterized in lower eucaryotes (Bankir et al., 2002). It would be an interesting topic to investigate if bacterial cAMP has any effects in renal epithelial cells during pyelonephritis.

The classical role of pore-forming toxins in disease has been to cause cell lysis. The concentrations needed to mediate this effect are high, and maybe often unphysiological. The role of many of these toxins in disease may have to be revised. Interaction with the target cell in lower, maybe, physiological concentrations might induce other cellular effects not previously thought. Cytolytic toxins are known to alter intracellular ion homeostasis. It would be interesting to

study the effect of cytotoxins on cell volume regulation and the effect on oedema. This is an important issue regarding e.g. pneumolysin, which is secreted during pneumococcal pneumonia or meningitis.

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