A murine model for catheter-associated candiduria

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Candiduria is a common finding in hospitalized patients with indwelling urine-draining devices. Animal models for candiduria are not well-developed and, despite its prevalence and associated mortality, candiduria is understudied. The presence of \textit{Candida} in urine does not imply disease because it is also a commensal. Biofilm formation on catheters and the host–pathogen interaction are likely to be important factors that contribute to the pathogenesis. The objective of this study was to establish a candiduria model in mice with indwelling catheters. Our data demonstrate that biofilm formation on indwelling catheters and persistent candiduria can be established in mice. The study supports the concept that biofilm formation contributes to persistence. It also outlines differences between catheter-related candiduria in mice and humans. Specifically, mice exhibit higher levels of leukocyturia. In addition, mean daily fungal burden in urine in the murine model is 10-100-fold lower than that in humans. These important findings must be taken into consideration when using this model to study host–pathogen interaction in the setting of candiduria.

\textbf{INTRODUCTION}

\textit{Candida albicans} is the most common pathogen implicated in nosocomial urinary-tract infection (UTI). Studies report that 9–44 \% of nosocomial UTIs are caused by \textit{Candida} spp. (Passos \textit{et al.}, 2005; Richards \textit{et al.}, 1999, 2000). Clinical studies have consistently identified similar predisposing risk factors including old age, female sex, prior antibiotic usage and indwelling urine-draining devices (Alvarez-Lerma \textit{et al.}, 2003; Kauffman \textit{et al.}, 2000). Although the clinical significance of candiduria is disputed (Achkar & Fries, 2010), it is of concern that large studies in hospitalized and renal-transplant patients have reported reduced survival of candiduric patients compared with control populations (Alvarez-Lerma \textit{et al.}, 2003; Bougnoux \textit{et al.}, 2008; Safdar \textit{et al.}, 2005). Studies have reported that 8 \% of candiduric patients developed candidaemia with the same species (Bougnoux \textit{et al.}, 2008).

Despite the high incidence of candiduria, only a few studies on its pathogenesis have been performed because adequate models are lacking. Reconstituted epithelial \textit{in vitro} tissue models, cell lines as well as nematode models, do not mimic the host environment adequately (Means \textit{et al.}, 2009; Pukkila-Worley \textit{et al.}, 2009a, b).

So far, candiduria has been investigated predominantly in mice, rats and rabbits (Chen \textit{et al.}, 2006; Hurley & Winner, 1963; Khan & Owais, 2006; Navarro \textit{et al.}, 1994; Nishiksawa \textit{et al.}, 1997; Silva \textit{et al.}, 2007; Tarry \textit{et al.}, 1989; Toth & Hughes, 2006). Experiments in larger animals are more costly, cannot be done with large numbers and genetic-knockout strains are limited or do not exist. In addition, most published animal experiments examined renal candidiasis, which is achieved by intravenous injection of \textit{Candida}. In that setting, haematogenous candidaemia leads to renal candidiasis, hence candiduria reflects a ‘descending’ infection.

However, molecular typing of \textit{Candida} strains derived from vagina and urine has established a clonal relationship (Daniels \textit{et al.}, 2001; Silva \textit{et al.}, 2007) and supports the notion that candiduria similar to bacterial UTIs is mostly an ‘ascending’ infection and not the result of haematogenous invasion of the urinary-tract system. In hospitalized patients, ascending persistent candiduria in the setting of indwelling urine-draining devices is a very common entity, for which a murine model has not been established to date. The objective of this study was to determine whether a murine model for ascending persistent candiduria could be established and whether it would adequately mimic the pathogenesis of candiduria.

\textbf{METHODS}

\textbf{Candida strains.} \textit{C. albicans} (strain SC5314) was obtained from the ATCC and kept in glycerol stocks at \textdegree{}70 \textdegree{}C. Thawed aliquots of strain SC5314 were streaked on Sabouraud dextrose agar (SDA) before growth in standard Sabouraud dextrose broth. Yeast cells were washed three times with sterile PBS prior to injection.
Mouse strains. C57BL/6 and 129Sv mice (female, 6–8 weeks old) were purchased from the National Cancer Institute, Bethesda, MD, USA. The infection model was tested in lysozyme M-deficient (lysM−/−) mice because they exhibit increased sensitivity to pathogens. Lysozyme is present on mucosal surfaces and expressed by neutrophils and is an important effector in the defence barrier of innate immunity. These mice were a generous gift from Thomas Graf, Center for Genomic Regulation, Barcelona, Spain, and are described elsewhere (Faust et al., 2000). LysM−/− mice are transgenic mice that contain contributions from both C57BL/6 and 129Sv strains. Accordingly, the suggested control mice are the non-transgenic parental strains C57BL/6 and 129Sv. LysM−/− mice express GFP brightly in their phagocytic cells, including the neutrophil [polymorphonuclear neutrophils or white blood cells (WBCs)]. In all experiments, only female mice were used because the anatomy of the male urethra is prohibitive to catheterization. Infection, analgesia, anaesthesia and killing (by CO2) of mice were approved by and performed in accordance with the Animal Institute Committee at Albert Einstein College of Medicine.

Surgical placement of intravesicular catheter. Mice were anaesthetized by intraperitoneal injection of ketamine (37.5 mg ml−1) and xylazine (1.9 mg ml−1). For catheter insertion, published procedures (Hopkins, 1999; Johnson & Lockatell, 1999) were modified and done under a surgical dissection microscope (10×) (Fig. 1a–c). Mice were placed in the supine position and a 5 mm suprapubic incision was done to expose the bladder. A 25 mm long polyethylene catheter threaded onto a guide wire was inserted via the urethra into bladder. The first part (4 mm) of the catheter was cut on the guide wire and released into the bladder lumen. Visualization of this 4 mm sling catheter segment through the translucent bladder wall was assisted by black catheter markings. To secure the catheter segment and prevent it from obstructing the bladder outlet, a 5–0 polypropylene suture was threaded through the lumen of the bladder catheter segment, back through the bladder wall, and a second bladder catheter was placed on the serosal side of the bladder, which prevents erosion of the suture. The abdomen was closed with sutures.

Infection procedure. Mice were infected 5–7 days after catheter placement. Histological analysis of bladder tissue was normal and leukocyturia was minimal prior to infection. Anaesthetized mice were infected intravesically by injection of yeast into the bladder via a polyethylene catheter, which was inserted as described above. The guide wire was removed and a 30G 1/2” hypodermic needle attached to a 5–100 µ Hamilton syringe was inserted into the catheter opening. A 50 µl C. albicans cell suspension in sterile PBS was injected slowly into the bladder lumen. The size of the inoculum (containing 105–107 cells) was confirmed by plating diluted aliquots on SDA. The polyethylene tube was withdrawn and mice were returned to their cages. Sham-infected control mice were injected by the same procedure with PBS only.

Specimen collection and microscopic studies. Urine was collected every third day over a period of 28 days into Eppendorf tubes by gently rubbing the suprapubic area of mice restrained in the supine position. WBCs and yeast cells were counted by haemocytometer, and 10 µl urine was added to 90 µl PBS and plated on SDA supplemented with streptomycin to determine fungal burden. Fungal burden (c.f.u.) varied among mice and also in consecutive collections from individual mice. Therefore, mean daily c.f.u. per mouse was calculated for the time period (28 days) and comparisons of mean daily counts were made between groups of mice (n=5).

To assess whether dissemination occurred, fungal burden in spleen, bladder and one kidney was determined. Mice were killed with CO2 and organs were removed, homogenized in PBS and plated on SDA. Fungal burden (c.f.u.) was determined after incubation at 37 °C for 48 h and expressed as either c.f.u. ml−1 (for urine) or c.f.u. per total organ. For urine, the lower cut-off of detection was 100 c.f.u. (ml urine)−1, and for organs, it was 20 c.f.u. per total organ. For histological analysis, bladders were fixed in 10% formalin, paraffin-embedded and sectioned at a thickness of 8 µm for histological study. Bladder tissue was stained by a standard haematoxylin and eosin staining protocol. To test for biofilm formation on indwelling catheters, scanning electron microscopy (SEM) was performed. Post-mortem, the catheter was removed from the bladder and placed in vials that contained cold (4 °C), buffered glutaraldehyde (2.5%). The glutaraldehyde-fixed samples were then rinsed in 0.1 M cacodylate buffer [Na(CH3)2AsO2·3H2O], post-fixed in osmium tetroxide, rinsed again in cacodylate buffer and dehydrated in water/ethanol or ethanol/anyyl acetate. Samples were critical point-dried, fixed to aluminium stubs, coated with gold–palladium and examined under a JEM U3 scanning electron microscope at 15–20 kV.

Fig. 1. Murine model for catheter-associated candiduria. (a) Schematic of the secured indwelling catheter piece; (b) polyethylene tube in the urethra; (c) open abdominal catheter with secured catheter.

Statistical analysis. Fungal burden data (c.f.u.) were converted into log10 units. Differences were calculated by t-test, Mann–Whitney and ANOVA, with PASWStatistics v. 18.0 (IBM) and Excel 2008 v. 12.2 (Microsoft).
RESULTS

Model development

Impact of inoculum. Different inocula (10^5–10^7) of C. albicans were tested to determine the number of yeast cells required to establish persistent infection. Fungal burden (c.f.u.) could only be documented in the urine of mice injected with an inoculum of 10^7 yeast cells delivered in a 50 μl volume of a yeast suspension of 2 x 10^6 ml^-1 in PBS. Lower-dose inocula did not consistently result in persistent candiduria beyond 6 days. More concentrated yeast suspensions were too viscous to pass through the barrel of the syringe and higher volumes leaked out of the urethra.

Impact of mouse strain. Infections were performed with LysM^-/- mice and their control parent strains, C57BL/6 and 129Sv. LysM^-/- mice allow easy distinction of yeast in urine from GFP-expressing WBCs. Mean daily fungal burden in 28 days was significantly higher in LysM^-/- mice with an indwelling catheter than in their respective wild-type control mice, 129Sv and C57BL/6 (P<0.001; ANOVA). In mice without an indwelling catheter, mean daily fungal burden was significantly lower, and lowest in LysM^-/- mice (P=0.01; ANOVA) (Fig. 2a).

Impact of indwelling catheter. Persistent urinary fungal burdens were documented in mice with indwelling urinary catheters, whereas mice without indwelling urinary catheters barely sustained candiduria beyond detection level. Maximum fungal burden was documented early after infection and then had a downward trend over 2 weeks. In LysM^-/- and C57BL/6 mice, c.f.u. in urine increased by 1–2 log_10 after 15 days infection (Fig. 2b). On day 28, candiduria was still detectable in 75% of LysM^-/- mice and C57BL/6 mice, but in only 20% of 129Sv mice with a catheter. In mice without an indwelling catheter, candiduria decreased and became undetectable in 93% of control mice (14 of 15) by day 28. In six of nine mice with persistent candiduria, Candida was grown from bladder tissue, with fungal burden ranging from 2.0 to 4.5 log_10(c.f.u.) per bladder (Fig. 2b). No yeast was grown from spleen or kidney. Yeast was not grown from organs of mice that did not have indwelling catheters.

Biofilm formation in vivo

It was apparent that fungal burden in urine increased again after 15 days, but only in catheterized LysM^-/- and C57BL/6 mice. We examined nine catheters from both C57BL/6 control mice and LysM^-/- mice that manifested persistent candiduria for 28 days. Biofilm formation occurred on all indwelling polyethylene tubes. Cells scratched off the polyethylene tubes were viable and grew on SDA (data not shown). SEM analysis of embedded catheters (removed on day 28) demonstrated thick biofilm formation on the outside and inside of the tubes. In vivo-generated biofilms were composed of yeast cells and hyphae that were adherent to the surfaces of the catheter (Fig. 3a–c).

Inflammatory parameters in candiduric mice

Urinalysis demonstrated 1–5 WBCs per high-power field (400× magnification) in sham-infected mice, and in infected mice 5 days after they were operated on prior to infection. We did not find baseline differences in leukocyturia. Leukocyturia was higher than in humans, who commonly are asymptomatic and do not manifest leukocyturia (defined as >2 x 10^4 ml^-1), in LysM^-/- and C57BL/6 mice with indwelling catheters and persistent
candiduria beyond 14 days infection. Early increases in leukocyturia may be a result of the infection procedure and were observed in most mice with and without catheters, but subsided (Fig. 4a), and a statistically significant increase in leukocyturia ($P=0.012$ for day 7 versus days 14 and 21; Mann–Whitney) was observed in LysM$^{-/-}$ mice with persistent candiduria. The same trend was seen in C57BL/6 mice. Microscopic analysis of urine from LysM$^{-/-}$ mice, which express GFP in their WBCs, confirmed that counted leukocytes were fluorescent and not dead yeast cells (Fig. 4b). Histological analysis of bladder tissue (Fig. 4c–e) was performed on candiduric LysM$^{-/-}$ and C57BL/6 mice and showed chronic inflammation of submucosal bladder tissue. Large numbers of infiltrating lymphocytes, monocytes and eosinophils were detected. Chronic inflammation was detected both in mice with and in those without an indwelling catheter and did not appear different in C57BL/6 and LysM$^{-/-}$ mice (data not shown). No adherence to or invasion of mucosal membranes by Candida was seen.

**DISCUSSION**

A model for persistent candiduria by ascending transurethral infection in mice with indwelling catheters was established. This model mimics the presentation of catheter-associated UTI in humans, where indwelling urine-draining devices constitute a major risk factor to develop persistent candiduria (Kauffman et al., 2000). The model cannot be established in male mice, but candiduria predominantly occurs in elderly female patients (Kauffman, 2005).

The only trauma to the bladder consists of two punctures when suturing the catheter to the bladder wall (Hopkins, 1999; Johnson & Lockatell, 1999). This prevents urethral or ureteral obstruction. Candida biofilms form in the lumen of the catheter. As a consequence, the tube does not drain the urine from the bladder lumen as draining devices in patients do. The foreign body in this model is polyethylene tubing with luminal and external surfaces similar to those of urinary catheters. Latex, the material of which most urine-draining devices are made, is not available in small lumen sizes. Although biofilm formation varies on different plastic surfaces and may be dependent on growth conditions (Hawser & Douglas, 1994; Jain et al., 2007), SEM analysis of indwelling catheters established that thick biofilms are formed in vivo on the indwelling tube. *C. albicans* biofilms are investigated here because they are resistant to antifungals (Blankenship & Mitchell, 2006; Chandra et al., 2001; Hasan et al., 2009; Kauffman et al., 2000; Pierce et al., 2008) and interfere with successful treatment in patients. Furthermore, in vitro biofilm formation of clinical *C. albicans* strains is highly variable and may be associated with higher potential to cause disease (Hasan et al., 2009; Jain et al., 2007; Shin et al., 2002). This model provides a means to study such clinically relevant hypotheses systematically in vivo. Of note is that retrograde ascent of *C. albicans* cannot be studied in standard mice because they are not colonized by *C. albicans*. This could, however, be achieved with 17β-oestradiol injections (Fidel et al., 1999) and/or gnotobiotic mice, which can be colonized from birth (Balish et al., 1990).

We demonstrate that persistent low-level candiduria in mice required a high inoculum of yeast and the presence of an indwelling catheter. Others have also reported requirement of high inocula ($5 \times 10^6$) for *Escherichia coli* and, even there, significant variability of urine bacterial burden, ranging from $10^5$ to $10^7$ c.f.u., was reported after 72 h (Jakobsen et al., 2010). It is noteworthy that other commonly investigated infection models, e.g. with the highly virulent human pathogen *Staphylococcus aureus*, also require high inocula and the bacteria are still cleared rapidly (Yao et al., 1997). In mice without an indwelling
catheter, funguria was documented mostly around the level of detection, but the level of urine fungal burden rose and stayed above the NIH-defined threshold for candiduria \([10^3\text{ c.f.u. (ml urine)}]^{-1}\); Kauffman et al., 2000) in mice with catheters. Based on our results, it is advisable not to rely on one time point, but rather to measure persistent candiduria in urine over time, as day-to-day variation is present, but trends are stable. Most patients with candiduria have fungal burdens between 5 and 6 \(\log_{10}\text{(c.f.u.)}\) (Jain et al., 2007). They mostly have urine-draining devices in place, removal of which can result in spontaneous resolution of candiduria (Kauffman et al., 2000). In a non-catheter-associated murine UTI model, others (Domergue et al., 2005) have reported higher fungal burdens \([3–4\ \log_{10}\text{(c.f.u.)}]\) in bladder and also in kidney. These studies were done with \(Candida\) \(gablara\) and cultures were obtained only early after infection (days 4 and 7) from bladder and kidney, and not from urine.

Our studies indicate that some mouse strains, such as 129Sv, may be less susceptible than C57BL/6 mice. C57BL/6 mice are the standard parent strain of knockout mice, which allows a systematic approach to studying the contribution of individual host genes to the pathogenesis of candiduria. In contrast to oropharyngeal candidiasis, candiduria is not diagnosed predominantly in patients with deficiencies in T-cell-mediated immunity, but occurs more often in elderly patients. Thus, commercially available colonies of old C57BL/6 mice could be used to study the contribution of age and the resulting altered immunity to host–pathogen interaction.

In vitro lysozyme inhibits \(Candida\) and has been proposed to be a relevant effector molecule against \(Candida\) overgrowth in the oral cavity (Hibino et al., 2009; Lee et al., 2010; Samaranayake et al., 1993, 2009). Although increased susceptibility of \(\text{LysM}^{-/-}\) mice to \(Microcococcus\) \(luteus\) and other pathogens has been reported (Ganz et al., 2003; Markart et al., 2004; Shimada et al., 2008), \(\text{LysM}^{-/-}\) mice are only mildly immunosuppressed and hence it was reasonable to test whether they constitute a good mouse strain to establish candiduria. In addition, the fluorescent
WBCs allow easy differentiation of yeast and WBCs in urine. Our observation that these mice exhibit higher leukocyturia is consistent with observations from other infection models, which have shown enhanced inflammation. At the time of our study, the LysM−/− mice were not entirely back-crossed to the C57BL/6 mice, which limited our ability to draw definitive conclusions on the importance of lysozyme as an effector molecule. Other LysM−/− mice have also been cloned (Sinha et al., 2004) and could potentially be used in future experiments.

Significant leukocyturia (defined as >20 WBCs μl−1) (Stamm, 1983) is absent in most candiduric patients (Kaufman et al., 2000) and this would have to be taken into account when analysing the host response in mice. Histological analysis of bladders demonstrated that Candida did not adhere to or invade the bladder surface and is in accordance with rat experiments that report lack of adherence of Candida to bladder mucosa (Levison & Pitsakis, 1987). The level of invasion and extent of bladder inflammation in human Candida infection have not been studied systematically; mostly extreme cases of emphysematous candidiasis have been reported (Bartkowski & Lanesky, 1988; Comer et al., 1996).

In summary, we describe a murine model for catheter-associated candiduria that is suited to investigations of the pathogenesis of candiduria, including the role of in vivo biofilm formation and its contribution to persistence. Mice mount a pronounced inflammatory response to Candida spp. that may limit the fungal burden. In that respect, even the ascending catheter model will have some limitations in deciphering the pathogenesis of candiduria.

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