Microbial biofilms on the surface of intravaginal rings worn in non-human primates

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Millions of intravaginal rings (IVRs) are used by women worldwide for contraception and for the treatment of vaginal atrophy. These devices also are suitable for local and systemic sustained release drug delivery, notably for antiviral agents in human immunodeficiency virus pre-exposure prophylaxis. Despite the widespread use of IVRs, no studies have examined whether surface-attached bacterial biofilms develop in vivo, an important consideration when determining the safety of these devices. The present study used scanning electron microscopy, fluorescence in situ hybridization and confocal laser scanning microscopy to study biofilms that formed on the surface of IVRs worn for 28 days by six female pig-tailed macaques, an excellent model organism for the human vaginal microbiome. Four of the IVRs released the nucleotide analogue reverse transcriptase inhibitor tenofovir at a controlled rate and the remaining two were unmedicated. Large areas of the ring surfaces were covered with monolayers of epithelial cells. Two bacterial biofilm phenotypes were found to develop on these monolayers and both had a broad diversity of bacterial cells closely associated with the extracellular material. Phenotype I, the more common of the two, consisted of tightly packed bacterial mats approximately 5 μm in thickness. Phenotype II was much thicker, typically 40 μm, and had an open architecture containing interwoven networks of uniform fibres. There was no significant difference in biofilm thickness and appearance between medicated and unmedicated IVRs. These preliminary results suggest that bacterial biofilms could be common on intravaginal devices worn for extended periods of time.

INTRODUCTION

Bacteria are known to colonize surfaces and establish a sessile mode of growth in a chemically and morphologically heterogeneous matrix of extracellular polymeric substances (EPS) known as a biofilm. Microbial biofilms are widely recognized as being implicated in chronic infections (Costerton et al., 1999), and these communities have been found to be more resistant to antimicrobial agents and the immune system than their planktonic counterparts (Davies, 2003; Stewart, 2002). Biofilm bacteria are also difficult to cultivate in the laboratory, frequently resulting in misdiagnosis of the associated infections (Costerton et al., 2003).

The formulation of pharmaceutical agents into intravaginal rings (IVRs) represents an attractive approach to achieving sustained release of compounds to the vaginal cavity for...
local or systemic delivery (Valenta, 2005), thereby increasing efficacy and adherence to therapy while potentially decreasing toxic side effects when compared to daily oral administration. This strategy has become popular in contraception and in oestrogen replacement therapy (Yoo & Lee, 2006), and several products are available commercially. Sustained vaginal delivery of antiviral agents from IVRs constitutes a potential route for human immunodeficiency virus (HIV) pre-exposure prophylaxis in women, particularly in the developing world (Nel et al., 2009; Saxena et al., 2009). Given the widespread use of IVRs, it is surprising that few studies have examined how these abiotic surfaces are colonized by micro-organisms in vivo.

Bacterial vaginosis (BV) has been found to be associated with characteristic biofilms adherent to the vaginal epithelium (Swidsinski et al., 2005), which were composed of confluent Gardnerella vaginalis and other bacterial groups. These biofilms persisted on the vaginal epithelium after standard therapy with oral metronidazole (Swidsinski et al., 2008). Staphylococcus aureus infections have been associated with mortality resulting from tampon-related toxic shock syndrome in menstruating women (Begley & Barnes, 2007; Tang et al., 2010). Veeh et al. (2003) used fluorescence in situ hybridization (FISH) to examine 44 paired – tampon and vaginal – wash specimens from 18 pre-screened women and detected S. aureus biofilms in 37 of these specimens. These results suggest that S. aureus colonization of IVRs in the vaginal canal is possible, especially since the devices remain in the vagina for a longer time than tampons.

Scanning electron microscopy (SEM) was used in ex vivo experiments to show that yeast isolates from vaginal exudates of patients with vulvovaginal candidiasis were able to adhere to a combined contraceptive vaginal ring (NuvaRing; Organon Pharmaceuticals) (Camacho et al., 2007) and to contraceptive intrauterine devices (Chassot et al., 2008), with concomitant biofilm formation. Infected intrauterine devices recovered from patients suffering from reproductive tract infections were found to be tainted with Candida biofilm (Lal et al., 2008). Miller et al. (2005) used SEM to examine a NuvaRing worn for 28 days by a healthy female volunteer and did not observe embedded bacteria, erosion or structural changes compared to an unused ring. This is, to the best of our knowledge, the only report on bacterial biofilms and IVRs in an in vivo setting.

We have developed IVRs that deliver the nucleotide analogue reverse transcriptase inhibitor tenofovir (TFV) at controlled rates for up to 3 months (Moss et al., 2010), with the goal of preventing HIV infection in women. In the present study six IVRs worn for 28 days by female pig-tailed macaques were examined by SEM, FISH and confocal laser scanning microscopy (CLSM). Four of the IVRs contained and released TFV, while the remaining two rings were non-medicated. The aim of the study was to determine if bacterial biofilms formed on the IVRs in vivo and, if so, whether there would be a difference between the medicated and control rings. This is believed to be the first report of microbial biofilms forming on the surface of IVRs in a model of relevance to humans.

METHODS

Manufacture of silicone IVRs. Silicone IVRs were prepared in a multi-step process from Nusil MED-4840 liquid silicone elastomer (Nusil Silicone Technology) using an injection moulding system developed in house. The ring dimensions (outer diameter 25 mm; inner diameter 15 mm; cross-sectional diameter 5 mm) recommended by Promadej-Lanier et al. (2009) were used. The ring manufacture was accomplished in two separate injection moulding steps. The delivery window (1 mm diameter) was created in a half ring, and four pods consisting of 3 mg each of ((12R)-1-[(6-amino-9H-purin-9-yl)propan-2-yl]oxy)methylphosphonic acid (TFV; Sinoway International) coated with poly-DL-lactide (M ~ 15000, Resomer R 202 S; Boehringer Ingelheim) were placed in pre-moulded, evenly spaced cavities. The second half of the ring then was injection moulded onto the first. Control rings contained poly-DL-lactide-coated silicone plugs of the same dimensions as the drug pods.

Macaques. Six sexually mature female pig-tailed macaques (Macaca nemestrina) were used for this study. All macaques were housed under an approved Centers for Disease Control and Prevention animal care and use protocol and standard guidelines [Department of Health, Education and Welfare (DHEW) no. NIH 86-23] (NIH, 1996). The animal study was approved by the Centers for Disease Control and Prevention IACUC (Institutional Animal Care and Use Committee) (2003DOBMONC-A3). Four macaques (PHD2, PDM2, PID2 and PRB2) received IVRs containing four pods of TFV. IVRs containing blank control pods were placed in two macaques (PZA2 and PVC2). The IVRs were inserted on day 0 into the vaginal vault and retained for a period of 28 days. Vaginal colposcopy was used to confirm placement and retention of the vaginal rings. The animals were placed in ventral recumbency while under anaesthesia. A paediatric speculum was used to open the vaginal vault to visualize the IVR placement. A Rebel Tlii /EOS 500D digital camera (15 megapixels; Canon) was used along with a model LR66238 colposcope (Carl Zeiss). Photographs were taken at ×0.6 and ×1.0 magnification. Plasma samples were analysed for progesterone levels to monitor the menstrual cycle by the Wisconsin National Primate Research Center.

Cytokine analysis. Cervicovaginal lavages (CVLs) and blood were obtained up to 3 weeks prior to the insertion of the ring in order to establish baseline cytokine profiles. PBS solution (4–5 ml) was gently infused into the vaginal vault via a sterile 10 ml syringe attached to a sterile gastric feeding tube (size 5 or 8 French) of adjusted length and CVL was drawn out with the same device. Blood and CVLs were obtained on days −21, −14, −5, 0, 3, 7, 14, 21, 28 and 35. The CVL was observed for the presence of blood or any other discoloration. Amicon Ultra-4 10 kDa concentrators (Millipore) were used to concentrate the CVLs. Induction of mucosal inflammation was monitored by measuring vaginal and systemic cytokines as previously described using fluorescent multiplexed bead-based assays (Invitrogen and Bio-Rad) in accordance with the manufacturer’s instructions (Promadej-Lanier et al., 2009). Interleukin-8 (IL-8), granulocyte colony-stimulating factor, Rantes, IL-1 receptor antagonist (IL-1Ra), macrophage inflammatory protein 1 β (MIP-1β), IL-6, eotaxin, IL-15 and IL-12p40 were analysed by the above methods.

Vaginal microbiological assessment. Sterile swabs were collected from all six macaques to complete a microbiological characterization on day −14, 0, 7, 21, 28 and 35. Each swab was placed individually in a Port-A-Cul transport tube (Becton Dickinson) and transported on ice packs to Magee-Womens Research Institute within 24 h of collection for quantitative culture analysis. This transport system has
been shown to maintain the viability and quantity of organisms for up to 48 h (Stoner et al., 2008). Quantitative cultures and bacterial identification were performed as previously described (Patton et al., 2006). Lactobacillus and viridans streptococci were tested for hydrogen peroxide production in a qualitative assay on tetrathymethylbenzidine agar plates (Rabe & Hillier, 2003).

IVR processing for biofilm characterization. TFV and blank pod IVRs were removed on day 28 from the macaques. The rings were cut into sections and segments without pods were placed in either 2.5% glutaraldehyde in phosphate buffer, pH 7.2, or 50% ethanol in water and transported on ice to the Oak Crest Institute of Science for biofilm characterization.

SEM. Glutaraldehyde-fixed samples were prepared for SEM as described previously (Webster et al., 2004). Dehydration was carried out in an ethanol line followed by critical point drying. The dried ring segments were cut lengthwise, mounted on metal specimen stubs, coated with a 16 nm thick platinum film, and imaged by an XL-30 S FEG 6 SEM (FEI Company) operating at 5 kV.

Biofilm thickness. Horizontal cross-sections (1 mm) of gluteraldehyde-fixed ring segments were cut at random points using a razor blade under an inspection scope. These samples were cut into four equal-sized quadrants and processed for SEM analysis as described above. Examination of the quadrants under low magnification (×500) by SEM allowed the height of the biofilm from the ring surface to be determined. At least two images were captured at random points for each quadrant representing the inner and outer surface of the ring. A minimum of four images were obtained for each sample. Random measurements of biofilm thickness were made for each image using ImageJ (http://rsweb.nih.gov/ij/), affording means and SDs.

FISH. Samples for FISH were preserved in 50% ethanol in water, as described above, and stored at 4°C prior to processing. Sample preparation was carried out as described by Macalady et al. (2006), except that the paraformaldehyde fixing step was omitted. We have found this approach to afford strong, selective FISH signals with minimal biofilm disruption (Romero et al., 2008). The oligonucleotide probe EUB338 was used to target most bacterial groups and was synthesized and labelled at the 5′ end with the fluorescent dye Cy5 (Integrated DNA Technologies). SEM and FISH/CLSM samples were fixed as described above (SEM, glutaraldehyde; FISH/CLSM, ethanol).

CLSM. Thin (approximately 2 mm) vertical cross-sections of ring segments were incubated in the presence of fluorescent dyes, rinsed, placed in sterile water in a slide chamber (Lab-Tek; Electron Microscopy Sciences) and imaged by confocal microscopy (LSM710; Carl Zeiss MicroImaging). The following fluorescent dyes were used either alone or in conjunction with FISH experiments: 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; MP Biomedicals); concanavalin, FITC (Integrated DNA Technologies); wheatgerm agglutinin (WGA), FITC (Integrated DNA Technologies); Syto60 (Invitrogen).

Image processing and manipulation. All images in this study were digitally captured and manipulated to adjust image size, contrast and brightness. Linear adjustment of size, contrast or brightness was always applied equally to the entire image.

RESULTS

Cytokine analysis

Mucosal and plasma levels of the proinflammatory cytokines IL-6 and IL-8 remained stable throughout the study period in all animals with no significant increases observed as a result of the IVRs. This is typical and has been observed previously in pigtailed macaques in the context of IVRs (Promadej-Lanier et al., 2009). Changes in local mucosal cytokine levels therefore were not a contributing factor to differences observed in the biofilms.

Microflora

The facultative and anaerobic micro-organisms recovered from vaginal swabs from the six pig-tailed macaques are listed in Table 1. The following organisms were not detected in any of the samples: group B, C, G, and non-group β-haemolytic Streptococcus, Micrococcus spp. and Proteus spp. Fig. 1 compares the mean cell counts for hydrogen peroxide (H2O2) producers and anaerobes recovered from vaginal samples. The microbial production of lactic acid and H2O2 is important to maintain a balanced vaginal microflora ecosystem and to prevent colonization by other pathogenic micro-organisms (Martín & Suárez, 2010). These results indicate a drop in H2O2-producing populations on insertion of the IVRs, but no significant impact on anaerobe populations. One of the control animals (PZA2) developed BV-like flora following insertion of the IVR, evidenced by a drop in H2O2-producing bacterial populations and a concomitant infection by G. vaginalis (Table 1 and Fig. 1), which subsided after the IVR was removed. Escherichia coli and Enterococcus spp., micro-organisms typically found in faeces, were not detected in most samples with the exception of PID2, which had elevated levels of both organisms (E. coli 8.5×10⁵±1.1×10⁶ c.f.u. g⁻¹; Enterococcus spp. 1.1×10⁵±2.0×10⁵ c.f.u. g⁻¹). These results agree with those from other reports that macaque vaginas only contain minor contamination from faecal matter (Patton et al., 1996).

Visual examination of IVRs

Fig. 2 shows colposcopic images taken in vivo with the IVRs (indicated by arrows) in place. The white cylinders, labelled ‘P’ in Fig. 2, are the TFV pods. Visual inspection of the IVRs following the animal study revealed no discoloration of devices recovered from PHD2 (medicated), PMD2 (medicated), PRB2 (medicated) and PVC2 (control). The rings worn by PID2 (medicated), PRB2 (medicated) and PVC2 (control) were covered with low and copious amounts of brown residue, respectively. When examined at low magnification (×20) under the stereo light microscope, a colourless deposit was seen to have accumulated on all rings, mostly located on the inner surface, on the ridge formed by the two ring halves (the IVRs were injection-moulded as two separate halves), and where sprues were cut during the production process.

SEM examination of biofilms

SEM examination of the biological material covering the IVR surface revealed a high degree of heterogeneity that
could be classified in terms of consistently recurring structural features as illustrated by Figs 3 and 4. There was no observable difference between the IVRs containing TFV and the controls, with the exception of the device recovered from experimental animal PZA2 as discussed below. Large areas (>4 mm\(^2\)) of the IVRs were covered in monolayers of epithelial cells (Figs 3a and 4a). In some cases, these monolayers were closely associated with bacterial cells and biofilm EPS (Fig. 3c and Fig. 4b, c). Some regions of the biofilms embedded reticulated structures (Figs 3b and 4b), which were concluded to be the epithelial cell surface. While bacterial biofilms formed on the epithelial cells that covered the silicone IVR surfaces, there were no observed instances of epithelial cells growing on a bacterial mat.

Two biofilm phenotypes were observed in this study and were classified as phenotype I and II. Phenotype I consisted of 2–5 μm thick (Fig. 3e) mats of tightly packed bacteria embedded in an amorphous matrix. The bacterial communities in these structures were highly diverse both in terms of the shape and size of the cells. Biofilm phenotype II was made up of an open, fibrous matrix (Fig. 3f and Fig. 4d, f). These architectures were populated by a high diversity – in terms of cell shape and size – of exposed bacteria and many appeared to be connected by thin fibres of uniform diameter (Fig. 4d). Bacteria within both biofilm phenotypes were a mixture of cocci, rods and spirochaetes (Fig. 3d, e and Fig. 4d, e). Overall, phenotype I was observed more frequently than phenotype II.

### Table 1. Vaginal facultative and anaerobic micro-organisms recovered from six *M. nemestrina* during the course of the study

Data are shown as numbers (%).

<table>
<thead>
<tr>
<th>Organism(s)</th>
<th>Day −14</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
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<tr>
<td></td>
<td>TFV*</td>
<td>Control†</td>
<td>TFV*</td>
<td>Control†</td>
<td>TFV*</td>
<td>Control†</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>4 (100)</td>
<td>1 (50)</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>H(_2)O(_2)+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>2 (50)</td>
<td>1 (50)</td>
<td>3 (75)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>H(_2)O(_2)−</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. vaginalis</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (50)</td>
<td>0 (0)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Diphtheroids</td>
<td>4 (100)</td>
<td>1 (50)</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>4 (100)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E. coli</td>
<td>1 (25)</td>
<td>1 (50)</td>
<td>1 (25)</td>
<td>0 (0)</td>
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<td>0 (0)</td>
</tr>
<tr>
<td>Viridans streptococci</td>
<td>3 (75)</td>
<td>2 (100)</td>
<td>4 (100)</td>
<td>1 (50)</td>
<td>4 (100)</td>
<td>1 (50)</td>
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<tr>
<td>H(_2)O(_2)+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viridans streptococci</td>
<td>4 (100)</td>
<td>2 (50)</td>
<td>3 (75)</td>
<td>2 (100)</td>
<td>4 (100)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>H(_2)O(_2)−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group F</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Streptococcus</td>
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<td>1 (50)</td>
<td>0 (0)</td>
<td>1 (50)</td>
<td>0 (0)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>3 (75)</td>
<td>1 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bacillus spp.</td>
<td>4 (100)</td>
<td>1 (50)</td>
<td>3 (75)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
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<tr>
<td>Aerobic Gram-positive rods</td>
<td>1 (25)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>1 (50)</td>
<td>0 (0)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Aerobic Gram-positive cocci</td>
<td>2 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>0 (0)</td>
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<td>Other Gram-negative rods</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Anaerobic GNR non-pigmented</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Black anaerobic GNR</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
<td>3 (75)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

GNR, Gram-negative rods.

*IVRs containing and releasing TFV (n=4).

†Control IVRs that did not contain TFV (n=2).
Biofilm samples on the IVR recovered from experimental animal PZA2 (control, not exposed to sustained release TFV) were unusual as they contained spherical cells (diameter 4 \( \mu \text{m} \)), which were embedded in biofilm EPS and were surrounded by bacterial cells. This macaque was the only animal in the group to develop BV-like microflora while wearing the IVR (positive for \( \text{G. vaginalis} \), Table 1). Large areas of the IVR also were covered in brown residue as a result of recent menstruation. The spherical cells appeared to be eukaryotic in origin and could have been yeast cells, B-cells or red blood cells; the cell in the centre of Fig. 3(b) appears to have the characteristic dimple of red blood cells. None of these interpretations are fully satisfactory, however, as no budding or other surface features were observed on most of these cells. \( \text{Candida albicans} \) cells isolated from vaginal exudates of patients with vulvovaginal candidiasis were 1–2 \( \mu \text{m} \) in diameter by SEM analysis (Camacho et al., 2007), significantly smaller than the 4 \( \mu \text{m} \) spherical cells seen here, while B-cells and red blood cells are larger than 4 \( \mu \text{m} \). No yeast cells could be cultured from any of the samples suggesting the absence of \( \text{Candida} \) spp.

**Biofilm thickness**

Biofilm measurements of the inner and outer IVR surfaces were carried out using SEM and the results are presented in Table 2. Most of the biofilm mass was located on the inner surface of the rings, presumably due to mechanical removal from the outer surface when the IVRs were worn. This is not surprising given the images shown in Fig. 2. The thickness of biofilm phenotype I was typically ten times less than that of phenotype II, with mean values ranging between 0.5–7.5 \( \mu \text{m} \) and 16.3–59.2 \( \mu \text{m} \) for phenotypes I and II, respectively.

**FISH–CLSM analysis**

Fig. 5 shows IVR samples hybridized with EUB338 (red) and labelled with WGA (green). Yellow areas represent overlap between signals from both probes. EUB338 is a universal FISH oligonucleotide probe designed to exclusively target members of the domain Bacteria (Amann et al., 1995). The fluorescently labelled lectins WGA and concanavalin have been shown to efficiently bind biofilm EPS (Johnsen et al., 2000; Strathmann et al., 2002), and provide useful probes for CLSM examination. Both were evaluated in the present work, and WGA appeared to be superior for the visualization of the studied EPS (data not
shown). Fig. 5 supports the SEM results suggesting that large areas of the IVRs (Fig. 5b) are covered with bacterial biofilms and that these structures are comprised of high densities of bacterial cells attached to one another in a matrix of extracellular material. In some regions, the bacteria were clustered together (red zones) in the absence of material labelled by the WGA (see Fig. 5e). Examination of these systems at higher magnification (Fig. 5a) indicated considerable heterogeneity in the clustering of the bacterial cells and their distribution within the EPS.

Fig. 3. Scanning electron micrographs of the surface features on IVRs containing blank (control) pods after 28 days implantation. The corresponding macaque ID and scale bar dimensions for each frame are given in parentheses below. (a) Portions of the ring surface were covered with a monolayer of epithelial cells along with EPS (PZA2; bar, 1 mm). (b) Amorphous biofilms contained numerous bacterial cells and thin fibres (right side). Other regions (left side) of the biofilm had a reticulated appearance. Spherical cells (diameter 4 μm) were associated with the biofilms (PZA2; bar, 10 μm). (c) The monolayer of epithelial cells was covered with bacteria and biofilm EPS. Bacterial cells with different sizes and shapes (see inset) were associated with the epithelial cell surface (PVC2; bar, 20 μm). (d) Some regions of the biofilms contained dense clusters of bacteria with variable size and shape. Long (>2 μm) rod-shaped bacteria were the most abundant and were interconnected by thin fibres (PVC2; bar, 2 μm). (e) Other regions of the biofilms appeared to have more associated extracellular material (PVC2; bar 2 μm). (f) Side view of bacterial biofilm cross-section used in thickness measurements. The clear material at the bottom of the image corresponds to the ring surface (PZA2; bar, 50 μm).

DISCUSSION

Several commercial IVR products are available for contraception and for the treatment of vaginal atrophy (Dezarnaulds & Fraser, 2003). Millions of these devices are used by women worldwide. Additionally, the CAPRISA 004 trial represents a recent demonstration of prophylaxis against HIV and herpes simplex virus using a topical microbicide (Abdool Karim et al., 2010). Protection was based on the antiviral drug TFV and was greater with
increased adherence. Since IVRs are believed to increase adherence, the development of ring formulations of antiviral drugs is an urgent global priority and likely will lead to increased use of IVR products in the future (Geonnotti & Katz, 2010; Promadej-Lanier et al., 2009; Romano et al., 2009; Saxena et al., 2009; Smith et al., 2008). The potential for microbial biofilms to develop on the surfaces of these devices in vivo therefore needs to be better understood.

In this study we have conducted a safety study of silicone IVRs developed in house using a pig-tailed macaque model (Promadej-Lanier et al., 2009). Patton et al. (1996) have demonstrated that the vaginal microflora of the pig-tailed macaque is a useful model in the evaluation of intravaginal devices prior to widespread intravaginal use in women. Like humans, M. nemestrina typically has a 28 day menstrual cycle and no significant changes in microflora have been observed over that period (Patton et al., 1996). More recent studies using culture-independent techniques confirmed the usefulness of the macaque model in studies involving the vaginal microbiota (Spear et al., 2010; Yu et al., 2009). In the present study, the vaginal microflora of

**Fig. 4.** Scanning electron micrographs of surface features on IVRs containing TFV after 28 days implantation. The corresponding macaque ID and scale bar dimensions for each frame are given in parentheses below. (a) Portions of the ring surface were covered with a monolayer of epithelial cells and EPS (PRB2; bar, 1 mm). (b) Biofilms, consisting of bacterial cells linked by thin fibres and amorphous extracellular material, were observed on the surface of epithelial cells (PID2; bar, 10 μm). (c) In other regions, bacterial mats (see inset) were associated with the epithelial cell monolayer (PMD2; bar, 20 μm). (d) Rod-shaped bacteria were interconnected by thin fibres on an amorphous extracellular structure (PID2; bar, 2 μm). (e) At higher magnification, the bacterial mat consisted of bacteria with a variety of shapes. In this image, which shows a disruption at the edge of the biofilm, the bacteria can be clearly observed embedded in the extracellular matrix (PMD2; bar, 2 μm). (f) Side view of bacterial biofilm cross-section used in thickness measurements. The dark stripe at the bottom of the image corresponds to the ring surface (PRB2; bar, 50 μm).
### Table 2. Biofilm thickness measurements on the inner and outer surfaces of the IVRs

Measurements were made on cross-sections of glutaraldehyde-fixed ring segments using SEM.

<table>
<thead>
<tr>
<th>Test animal ID and analysed ring surface</th>
<th>Structure thickness [mean ± SD (μm)]</th>
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<tbody>
<tr>
<td></td>
<td>Biofilm phenotype I*</td>
</tr>
<tr>
<td>PHD2 inner§</td>
<td>6.48 ± 4.11</td>
</tr>
<tr>
<td>PHD2 outer</td>
<td></td>
</tr>
<tr>
<td>PID2 inner§</td>
<td>7.50 ± 5.17</td>
</tr>
<tr>
<td>PID2 outer</td>
<td>2.37 ± 0.78</td>
</tr>
<tr>
<td>PID2 inner§</td>
<td>4.72 ± 1.98</td>
</tr>
<tr>
<td>PID2 outer</td>
<td>4.72 ± 1.98</td>
</tr>
<tr>
<td>PHD2 outer</td>
<td></td>
</tr>
<tr>
<td>PID2 inner§</td>
<td>0.47 ± 0.11</td>
</tr>
<tr>
<td>PID2 outer</td>
<td>2.18 ± 0.59</td>
</tr>
<tr>
<td>PID2 inner§</td>
<td>2.18 ± 0.59</td>
</tr>
<tr>
<td>PID2 outer</td>
<td>2.18 ± 0.59</td>
</tr>
<tr>
<td>PZA2 inner</td>
<td></td>
</tr>
<tr>
<td>PZA2 outer</td>
<td>5.17 ± 2.05</td>
</tr>
<tr>
<td>PZA2 outer</td>
<td>4.77 ± 1.77</td>
</tr>
</tbody>
</table>

* Dense bacterial mat, usually on the surface of epithelial cells.
† Fibrous and defined by highly porous, open architecture.
‡ Resembles monolayer of epithelial cells, not a biofilm.
§ IVR containing and releasing TFV.
|| Control IVR that did not contain TFV.

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**Fig. 5.** FISH micrographs of IVR biofilm samples (test animal PZA2) hybridized with EUB338 (red, labels bacteria) and labelled with WGA (green, labels biofilm). Yellow areas represent overlap between signals from both probes. (a) Inner surface of IVR showing the distribution of biofilm and bacteria. Bar, 100 μm. (b) Low magnification view of biofilm and bacteria on an IVR segment, visible as the grey rectangular object. Bar, 1 mm. (c) Imaged ring surface showing dense biofilm. (d) Imaged ring surface showing the location of bacteria. (e) Superimposed image of (c) and (d). Bar, 100 μm.
five of the six macaques was normal and was not significantly affected by retaining the IVR for 28 days. One of the control animals developed BV-like flora after insertion of the ring that resolved spontaneously without treatment when the ring was removed.

Bacterial biofilms formed on all six rings at 28 days, with most of the material accumulating on the inner surfaces. Epithelial cells also were observed covering large areas of the IVRs. In an SEM study on a contraceptive IVR worn for 28 days by a human volunteer, Miller et al. (2005) observed ‘visible mucus and vaginal discharge’ on the ring surface, but did not recognize that this material likely was a monolayer of epithelial cells. Most of the bacterial biofilms observed here formed on the surface of the epithelial cell monolayer, an unexpected result. It appears that epithelial cells colonize the surface of the ring followed by bacterial attachment and subsequent adherent biofilm formation.

No significant difference in biofilm architecture and thickness was observed between the animal positive for _G. vaginalis_ and the remaining five healthy animals. These results suggest that multispecies biofilms form on IVRs coated with epithelial cells, and likely also on the vaginal epithelium, independent of the presence of _G. vaginalis_. No other studies have examined the characteristics of bacterial biofilms in healthy vaginal ecosystems (Witkin et al., 2007).

Two biofilm phenotypes were observed in most of the IVR samples and a broad diversity of bacterial cells were closely associated with the extracellular material. Phenotype I, the more common of the two, consisted of tightly packed bacterial mats approximately 5 μm in thickness. Phenotype II was much thicker, typically 40 μm, and had an open architecture containing interwoven networks of uniform fibres reminiscent of structures observed in monospecies _Pseudomonas_ spp. laboratory cultures (Baum et al., 2009). These so-called nanowires have been observed as a consistent feature of bacterial biofilms (Baum et al., 2009; Gorby et al., 2006; Schaudinn et al., 2007). There was no significant difference in biofilm thickness and appearance between medicated and unmedicated IVRs.

More research is required to better understand the microbial ecology of biofilms developing on the surface of IVRs and the implications to the user. These preliminary results suggest that bacterial biofilms could be common on intravaginal devices worn for extended periods of time.

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