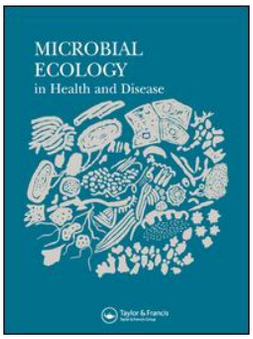

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A Morphological Study of the *in situ* Tissue-Associated Autochthonous Microflora of the Human Vagina

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Direct examination of scrapings from three locations in the vaginas of healthy volunteers, at different times of the menstrual cycle, revealed the presence *in situ* of tissue-associated autochthonous bacterial populations. Most of the bacteria in these scraped samples were intimately associated with the surfaces of epithelial cells, to which they were connected by elements of their exopolysaccharide glycocalyces. Washing of the epithelial sites, before sampling, removed vaginal secretions and their associated bacterial populations but did not remove the tissue-adherent bacterial microcolonies and individual cells. When epithelial cells were exposed to the high shear forces of vortex mixing, centrifugation and sonication, this firmly adherent bacterial population was almost entirely retained at the vaginal cell surface. The nature and strength of this intimate bacteria-tissue association may be important in the colonisation resistance afforded by autochthonous bacteria and in the pathogenesis of vaginal infections, and as such should be a major consideration in future ecological studies.

KEY WORDS—Human vagina; Autochthonous flora; Tissue-association; Ecology.

INTRODUCTION

Although the human vagina constitutes a dynamic microbial ecosystem[1,8,18] the nature and topology of the autochthonous (native) populations of this organ have not been vigorously studied by direct examination. The vagina is lined with a non-secretory stratified squamous epithelium, but the organ receives mucous secretions and periodic menstrual discharges from the cervix and secretions from various lubricating glands. For these reasons, the vagina usually contains both fluid and viscous secretions and large numbers of distal epithelial cells sloughed from its epithelial lining.[11] The vaginal microflora, traditionally sampled by swabbing the vaginal surface, actually represents a composite of organisms from vaginal secretions, sloughed epithelial cells, as well as the surface of the epithelia itself.

Modern microbial ecology[2] asks three questions of an autochthonous tissue population: firstly, what bacterial species are present?, secondly, what cell numbers are attained by various types (e.g. anaerobes) or by individual species? and thirdly what is the topology of these organisms vis-a-vis the tissue surface and other autochthonous species? The first two questions are partially answered by the traditional quantitative microbiological analysis of swab samples, but the third question is left unanswered by such studies. We believe that the location of these autochthonous bacteria may be an equally important determinant of their commensal or pathogenic potential in the genital tract. We have undertaken the present study with the intent of defining these spatial relationships.

The objectives of the present study therefore are to examine the mode of growth of autochthonous bacteria on the human vaginal epithelium, to determine the location of morphologically distinct bacterial sub-populations on these tissues, and to determine the tenacity of the association of tissue-associated organisms with the colonised epithelium. To this end we have developed a scraping technique that has allowed us to study, morphologically, the adhesive relationship within the vaginal flora and between the flora and host tissues.

*Author to whom correspondence should be addressed.

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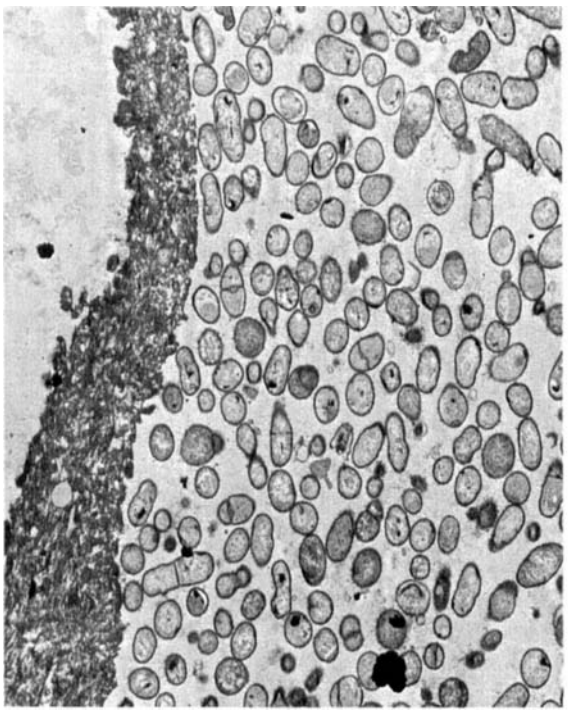

Figure 1. TEM of a section of a ruthenium red stained preparation of material scraped from the vulvar epithelium of a typical healthy volunteer. Note the very extensive colonisation of the free side of an epithelial cell by an adherent microcolony of predominately Gram-positive bacteria. Magnification × 17 500

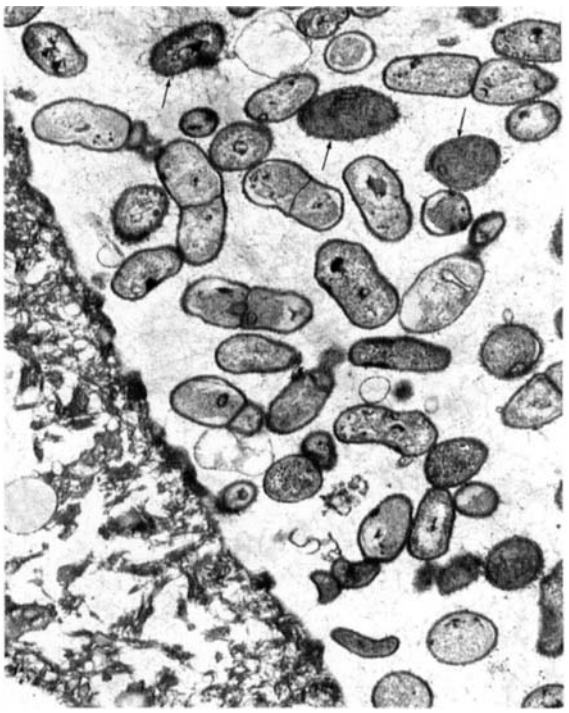

Figure 2. TEM of a section of a ruthenium red stained preparation of material scraped from the exocervical epithelium of the healthy volunteer seen in Figure 1. Note the predominance of Gram-positive cell wall structure in the very extensive bacterial microcolony adherent to this epithelial cell and the presence of occasional Gram-negative cells (arrows) in this mixed autochthonous population. Magnification × 28 800

MATERIALS AND METHODS

Sampling process

Specimens were collected from four age-matched, healthy, sexually-active women by scraping with a sterile tongue depressor placed distal to a vaginal speculum. Three locations, the vulva, posterior lateral third of the vagina, and exocervix were sampled at midcycle (day 15 ± 2) in the first volunteer, and at three stages of the menstrual cycle: menstrual (day 3 ± 2), midcycle (day 15 ± 2), and pre-menstrual (day 25 ± 2) in the remainder. All volunteers were tampon users and one (volunteer three) used a triphasial oral contraceptive. None received antibiotics prior to or during the course of study. Concurrent sampling showed the absence of STD and vaginosis.[10]

Samples from the first volunteer were specially processed (see below) prior to preparation for transmission electron microscopy to provide direct evidence of the strength of host tissue-bacteria interactions.

A first set of scraped specimens (prewash specimens) was taken from each of the three remaining volunteers prior to a vigorous vaginal wash. This was achieved by washing out the vaginal contents with 10 ml of phosphate buffered saline, PBS, pH 7·4 (wash specimens). Then, a second set of scraped specimens (postwash specimens) was taken from the 'mirror sites' of the prewash specimens i.e. from identical but opposite sites.

Vortexing, Centrifugation and Sonication prior to transmission electron microscopy (TEM)

This was only applied to specimens from volunteer one and consisted of successive physical steps designed to test the strength of host-bacteria adhesion. Specimens were divided and portions processed for TEM. The remainder were vortexed for 30 sec at medium setting (Vortex-Genie, Scientific Industries, New York, USA) and allowed to settle on the bench for 15 min. They were then centrifuged

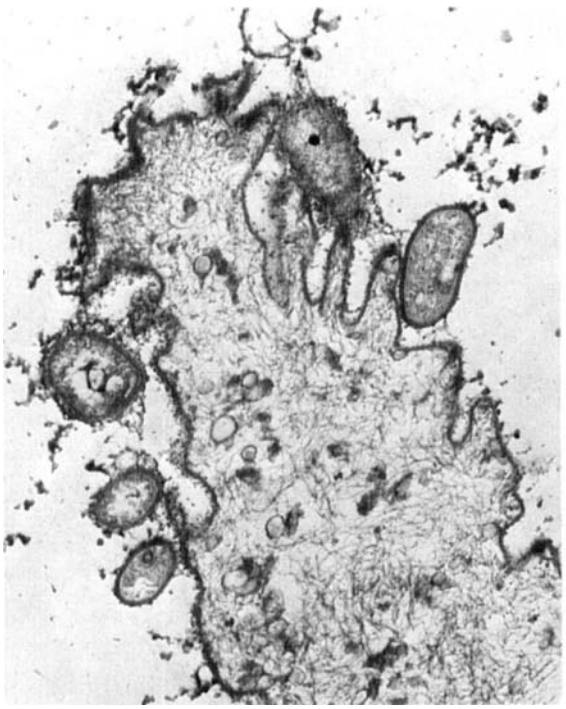

Figure 3. TEM of a section of a ruthenium red stained preparation of material scraped from the exocervical epithelium of the same volunteer seen in Figures 1 and 2. Note the very intimate association of five Gram-positive bacterial cells with this less densely colonised portion of the epithelial cell and the fibrous electron-dense residue of the bacterial glycocalyces that mediate this adhesion. Magnification ×45 000

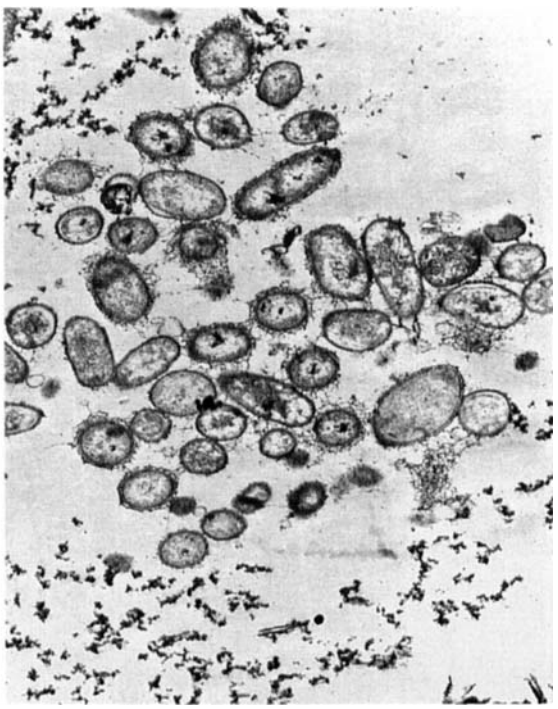

Figure 4. TEM of a section of a ruthenium red stained preparation of material scraped from the exocervical epithelium of the healthy volunteer seen in Figures 1–3. Note the formation of a very extensive glycocalyx-enclosed microcolony that is associated with the viscous secretions of the vagina (electron-dense fibrils) but not with the vaginal epithelium. Magnification ×20 000

at 300 rpm at 4°C for 15 min (Centra 7R; IEC, Needham Heights, USA). The resultant pellet was re-suspended in 10 ml of PBS, pH 7·4, and sonicated at a fixed setting of 50/60 Hz and 1·0 A for 5 min (Model B-220; Bransonic, Shelton, USA). Specimens were allowed to settle out, as above, before sonication was repeated. Finally, after another period of settling, the sediments were processed for TEM.

Portions of scraped specimens (prewash and postwash) and of centrifuged wash specimens from all volunteers were processed as follows for TEM. They were fixed in 5 per cent (v/v) glutaraldehyde in cacodylate buffer (0·067 M, pH 6·2) containing 0·15 per cent (w/v) ruthenium red for 24 h at 4°C. The material was then washed five times in the buffer, postfixed in 2 per cent (v/v) osmium tetroxide in buffer and dehydrated through a series of acetone washes. After further dehydration in propylene oxide, the specimens were embedded in Spurr low viscosity embedding resin[19] (Electron Microscopy Services, Quebec, Canada), sectioned, stained with uranyl acetate and lead citrate,[15] reinforced with evaporated carbon and examined with a Hitachi H-600 (Tokyo, Japan) electron microscope at an acceleration voltage of 60 kV.

RESULTS

The vaginal tissues (vulva, vagina and exocervix) of all volunteers were very heavily colonised by bacteria at all three locations, throughout the menstrual cycles (menstrual, mid-cycle, pre-menstrual). These organisms, which were predominantly Gram-positive rod-shaped bacteria, formed very extensive adherent microcolonies (Figure 1) on the surfaces of intact and of partially degraded epithelial cells, constituting a sessile bacterial biofilm adherent to the tissue surface. Examination of these adherent populations at greater magnification showed that these microcolonies were largely composed of morphologically similar sister cells embedded in the

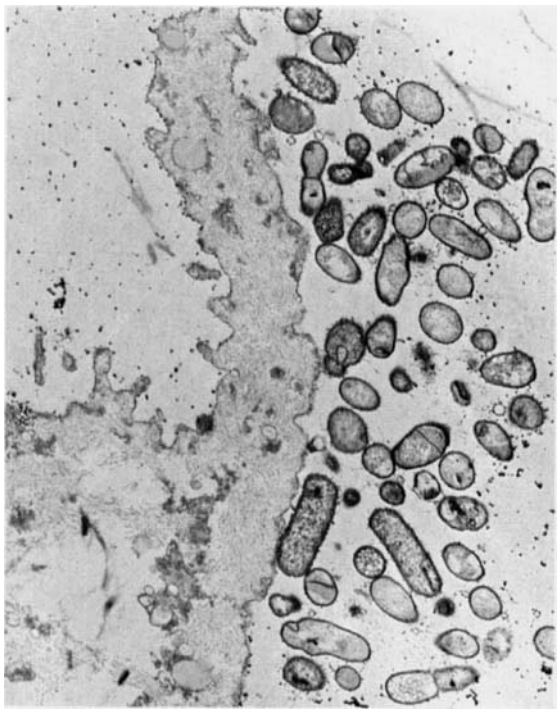

Figure 5. TEM of a section of a ruthenium red stained preparation of material scraped from the exocervical epithelium of the healthy volunteer seen in Figures 1–4 after washing of the sample area with PBS solution. Note the retention by the washed epithelial cell of a very extensive bacterial microcolony composed largely of Gram-positive cells. Magnification × 21 000

fine fibrillar matrix of their dehydration-condensed glycocalyces (Figure 2). Some Gram-negative cells were seen between the microcolonies of Gram-positive cells in these extensive adherent populations (Figure 2, arrows). In more lightly colonised areas of these epithelial cell surfaces, the very intimate spatial relationship between these Gram-positive rods and the surfaces of the vaginal epithelial cells can be more clearly observed as the dehydration-condensed strands of the bacterial glycocalyx can be seen to mediate this association (Figure 3). Morphologically similar Gram-positive rod-shaped bacterial cells are very often seen to form glycocalyx-enclosed microcolonies (Figure 4) that are not directly attached to the epithelial surface but are seen within the amorphous matrix of the viscous secretions of the vagina. After washing with a stream of PBS, these secretions and their associated bacterial populations were removed but vaginal tissues were still colonised by very large numbers of Gram-positive rods in adherent

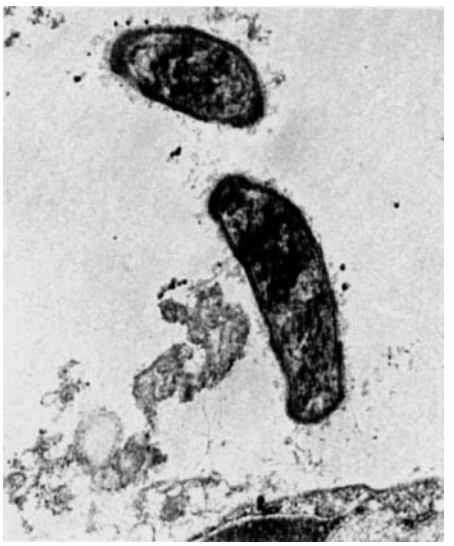

Figure 6. TEM of a section of a ruthenium red stained preparation of material obtained in the PBS vaginal wash from a healthy volunteer. Note the presence of Gram-positive rod-shaped bacteria connected to the epithelial surface by means of their fibrous glycocalyces. Magnification × 31 500

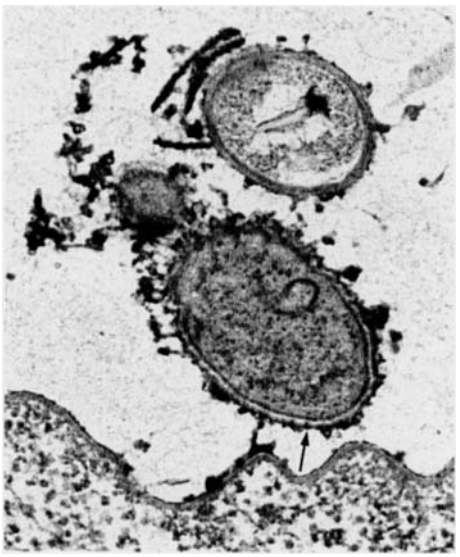

Figure 7. TEM of a section of a ruthenium red stained preparation of material scraped from the exocervical epithelium of a healthy volunteer. Note the presence of bacterial cells with the Gram-negative trilamellar cell wall structure (arrow) adherent to the epithelial surface by means of their fibrous glycocalyces. Magnification × 50 000

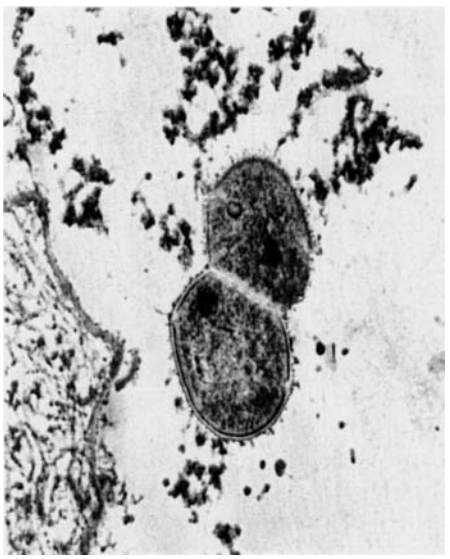

Figure 8. TEM of a section of a ruthenium red stained preparation of material from the PBS vaginal wash of a healthy volunteer showing a dividing Gram-positive coccoid bacterial cell in close association with an epithelial cell. Magnification × 50 000

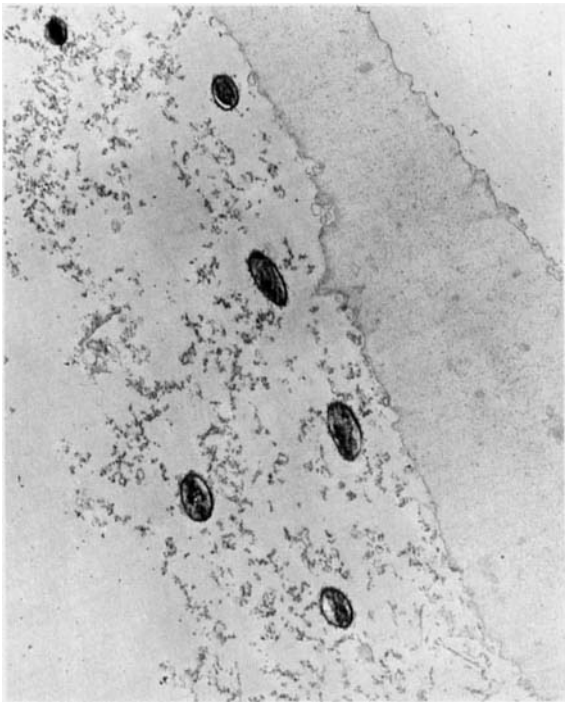

Figure 10. TEM of a section of a ruthenium red stained preparation of material from the vaginal epithelium of a healthy volunteer. Note the growth of Gram-positive rod-shaped bacterial cells within the viscous secretions at the surface of a vaginal epithelial cell. Magnification × 17 400

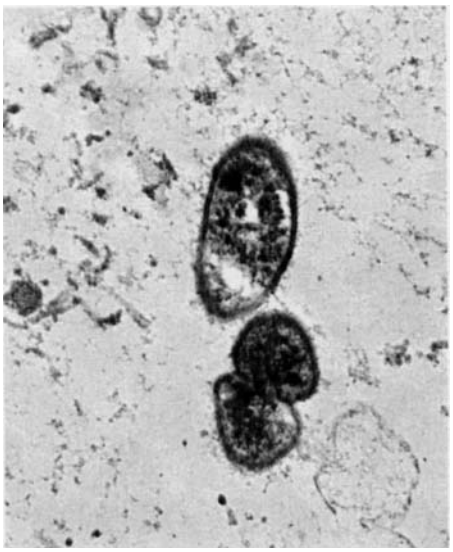

Figure 9. TEM of a section of ruthenium red stained preparation of material scraped from the exocervical epithelium of a healthy volunteer. Note the formation of a small bacterial microcolony of Gram-positive cells enclosed by their fibrous glycocalyces. Magnification × 34 000

microcolonies (Figure 5). Although Gram-positive rod-shaped bacteria were the most common morphotype among these adherent organisms (Figure 6), Gram-negative rods (Figure 7) and Gram-positive cocci (Figure 8) were also observed and they too were intimately associated with epithelial cells. The existence of adherent bacterial microcolonies on the exposed surfaces of vaginal epithelial cells were consistently observed from all sampled sites and at the different times of the menstrual cycle.

Bacterial microcolonies were occasionally seen in the amorphous matrix of viscous vaginal secretions (Figure 9) and dispersed Gram-positive rod-shaped cells were often seen within a layer of viscous material at the surfaces of vaginal epithelial cells (Figure 10). When the vaginal epithelial cells in the PBS wash solution from the first volunteer were examined by TEM they were found to be heavily colonised, and to retain significant amounts of their adherent bacterial population following vortexing, centrifugation and sonication. Examination of epithelial cells by TEM showed that large mixed

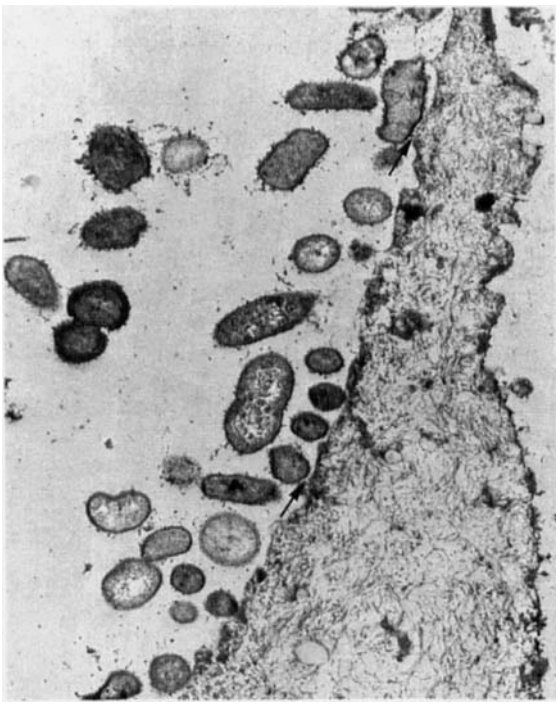

Figure 11. TEM of a ruthenium red stained preparation of epithelial cells obtained from the vagina of a healthy volunteer and exposed to vortex mixing (30 sec), centrifugation (15 min, 300 rpm), and low output sonication (5 min) [details in text]. The predominantly Gram-positive bacteria that form extensive microcolonies on these epithelial cells are retained on their surfaces, despite exposure to very strong shear forces, by the avid adhesion mechanism of their exopolysaccharide glycocalyces. Magnification × 28 000

microcolonies of bacteria were still adherent to their surfaces and that these bacterial cells were immediately juxtaposed to the epithelial surface to which they appeared to be directly connected by means of their dehydration-condensed glycocalyces (Figure 11, arrows).

DISCUSSION

Transmission electron microscopy (TEM) permits distinction between the thick fibrillar cell walls of Gram-positive bacteria from the much thinner trilamellar cell walls of Gram-negative organisms[4] and to distinguish different morphological forms. The spatial relationship of bacteria with cells of the colonised tissue would only be retained throughout the 22 washes involved in specimen processing for TEM if these organisms were truly adherent. The dehydrated residue of the exopolysaccharides that often mediate the adhesion of bacteria to tissue cells are often seen to be attached but fragmentary, although they are much more extensive in their original, hydrated state.

Studies of the autochthonous microflora on mucosal surfaces in different organ systems, notably the bovine digestive tract, have produced an integrated concept of mutual co-operativity between autochthonous bacteria and their host.[2] New studies reveal large populations of bacteria and protozoa in the intestinal lumen and within the highly structured mucous 'blanket' overlying gut tissues,[16] but the actual epithelial surfaces are only colonised by a very small proportion of these organisms that possess highly specialised adhesion mechanisms.[17] Many bacteria display positive chemotaxis to mucus components[6] and this autochthonous mucous blanket population is believed to contribute significantly to colonisation resistance to bacterial infections. Similar approaches to studies of microbial ecology have been very effectively applied to the human mouth[7] and initial morphological studies of these autochthonous populations have given rise to examinations of pathogenic overgrowths of normally autochthonous species and the biochemical details of species–species co-operation.[3] The microbial ecology of the skin has received much less attention, but its autochthonous bacterial population has been described in connection with studies of the bacterial colonisation of sutures[9] and of occlusive bandages.[5] Ecological studies of the microbial colonisation of ileal conduits, which are portions of the intestine connected to elements of the urinary tract that exit via the skin, have established that natural or induced colonisation by autochthonous urinary tract organisms (notably lactobacilli) may preclude upstream bacterial infections.[14] The easily accessible human genito-urinary tract has been the subject of very few definitive ecological studies but we have established that the distal human female urethra is colonised by a tissue-adherent bacterial population[12] and that this autochthonous population may play a role in preventing ascending infections of the urinary tract. Whole cells and cell wall fragments of one of these autochthonous urethral organisms (lactobacillus) have been shown to prevent pathogenic colonisation of the bladders of female experimental animals challenged with uropathogenic strains of *E. coli*.[13]

The human vagina differs profoundly from all of the organs discussed above in that it is lined by a nonsecretory stratified squamous epithelium but is kept generally moist by secretions from the cervix

and from associated glands. In this study, morphologically similar autochthonous bacteria formed very extensive adherent microcolonies within a bacterial biofilm on the surface of epithelial cells in all of the three regions sampled (vulva, vagina, exocervix), and viscous secretions in the vagina were also heavily colonised by these organisms. This consistency of bacterial colonisation between the three different sampling sites was also seen at the three different sampling times. Gentle washing of the area to be sampled removed the secretions and their associated bacterial microorganisms but had very little effect on the numbers or on the predominant morphotypes of tissue-associated bacteria (Domingue, Sadhu, Chow, Bartlett and Costerton, unpublished observations). When colonised epithelial cells were recovered from three sampling sites of the lower female genital tract of a volunteer and subjected to the very strong shear forces of vortex mixing, centrifugation, and low-output sonication these autochthonous bacteria remained firmly adherent to the epithelial cells. This avid adherence appears to be mediated by the bacterial glycocalyx, whose dehydration-condensed residue is seen to connect autochthonous bacteria to epithelial cell surfaces, and to sister cells within adherent microcolonies. The nature of this tissue-associated bacterial population, and its tendency to proliferate to form extensive adherent microcolonies varies between individuals (Domingue *et al.*, unpublished observations). We conclude here that autochthonous bacteria which are predominantly Gram-positive bacillary morphotypes are firmly and directly adherent to epithelial cells in the human vagina and often exist in an extensive microcolony mode of growth. The nature of this intimate bacteria-tissue association may be important in the colonisation resistance afforded by autochthonous bacteria and in the pathogenesis of infection in the lower female genital tract.

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