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The cutaneous biocenosis

The surface of the skin is a critical interface between the highly controlled internal tissues and the external environment, which permits the maintenance of bodily homoeostasis. It is influenced by the external environment but maintains its own distinct set of microenvironments which differ from one another depending on the nature of the stratum corneum, the density of the coat and cutaneous glands, occlusion, wetting and contamination from mucosae, and grooming activities. Within these microenvironments, the cutaneous microbiota and fauna occupy the available residential niches, forming a community that interacts both with the host substrates and amongst its members, thus forming a classical biocenosis.

Given the time available, this review will focus on the bacterial microbiota of the normal canine skin and mucosae, the interactions amongst the microbiota and their relationships with the host cells, particularly the keratinocytes. These interactions and relationships are dynamic and multiplex and the term "crosstalk" has been used to reflect this complexity. As yet, relatively little is known of these interactions *in vivo* and much of our knowledge depends on studies in human or domestic animal models.

Acquisition of the cutaneous microbiota

Colonisation of puppies

In the uterus, the skin is free of bacteria and fungi in healthy individual and microbial colonisation of the newborn occurs as soon as the amniotic membrane has been ruptured. In dogs this occurs when the bitch tears it open, cuts the umbilical cord with her teeth and cleans the puppies by licking. This process, combined with suckling and the occluded environment amongst members of the litter, favours transfer of the maternal oral and cutaneous microbiota and provides an opportunity for attachment and colonisation of the available ecological niches. Licking is important for the transfer of *Staphylococcus intermedius* as this organism is present in the oropharynx and upper respiratory tract of most dogs and the nose and mouth are rapidly colonised in puppies (Saijonmaa-Koulumies and Lloyd, 2002a). The environment of the puppies and the level and frequency of colonisation of the bitches also seem to be important. When Allaker *et al.* (1992) studied colonisation of neonatal puppies in a rigorously clean laboratory unit, they found low numbers and infrequent colonisation by *S. intermedius* amongst the bitches, and colonisation of puppies occurred more gradually over the first week of life.

Adherence to host cells is an important part of the process of colonisation and infection; in human neonates adherence of *S. aureus* to nasal mucosal cells is much lower during the first 4 days but is equivalent to adults at 5 days (Aly and Bibel, 1993; Aly, Shinefield and Maibach, 1980). In contrast, colonisation of infant skin is higher than in adults and occurs before nasal colonisation. (Hurst, 1965). This does not seem to be the case with *S. intermedius* in dogs. Saijonmaa-Koulumies and Lloyd (2002a) showed that one day after birth there was equivalent or higher mucosal colonisation by *S. intermedius* in puppies whilst skin colonisation levels remained higher in bitches until 7 weeks post-partum. Furthermore, at 7 weeks puppies born to bitches that were resident carriers (organism recovered on >75% of sampling occasions) had significantly higher populations. The reason for this is not known but may relate to the level of exposure of the skin of the puppies. Adherence by *S. intermedius* to canine

corneocytes has been shown to be related to bacterial concentration (Saijonmaa-Koulumies and Lloyd, 2002b).

Colonisation of adults

Problems of classification of resident skin microinhabitants and their differentiation from those occupying the skin temporarily have bedevilled the literature on the microbiota of skin. Somerville-Millar and Noble (1974) described three categories of skin microbes: residents, nomads and transients. Residents could be recovered on >75% of sampling occasions, nomads on <75% and >25% and transients on <25% of occasions. However, even accepting such classifications, major differences will be apparent depending on sampling method, sampling intensity, study duration and methods used for quantification and identification. It is likely that methods used in the past have failed to identify some resident organisms and have underestimated the populations present. Molecular methods based on detection of the bacterial 16S rRNA gene are likely to provide the key to greatly increased accuracy in quantitation and assessment of the diversity of the cutaneous microbiota (Fredericks, 2001).

The bacterial colonisation status of canine skin has been reviewed by Saijonmaa-Koulumies and Lloyd (1996). Amongst non-pathogens, the coagulase-negative staphylococci and micrococci seem to be accepted as residents. Harvey and Lloyd (1995) also isolated *Clostridium* spp. and aerobic Gram-negative bacteria in dissected hair follicle contents and Harvey *et al.* (1992) found *Propionibacterium acnes* in canine hair follicles suggesting residency but these findings need to be confirmed.

S. intermedius seems to be present sporadically and generally in low numbers on canine skin. It is found particularly in less hairy areas, such as the ventral abdomen, where transfer from the mucosae is likely, and thus it would seem to be a nomad or transient. However, Harvey and Lloyd (1994) found higher numbers in dissected hair follicles and on the distal parts of the hair than at the cutaneous surface and suggested that there might be two distict populations in dogs, one representing contamination from the mucosae and the other resident in the hair follicles.

In dogs, mucosal carriage of *S. intermedius* commonly occurs. Carriage rates of 36-60% for the anus, 36-75% for the nose, 10-75% for the genital tract, 33-46% for the buccal mucosa and 26-65% for the conjunctivae have been reported (see Saijonmaa-Koulumies and Lloyd, 1996). Heaviest populations have generally been reported from the anus but in one report of beagles in a very clean laboratory environment, *S. intermedius* was not isolated from that site (Allaker *et al.*, 1992).

M. pachydermatis is quite readily isolated from the haired skin of the chin and lips, interdigital skin and external ear canal, and less often from other intertriginous areas such as the axilla and groin of dogs. Population sizes in healthy dogs are generally low but are markedly increased, often up to 10,000 fold, in many cases of *Malassezia* dermatitis, although there is overlap in population densities between some healthy and affected dogs (Bond and Lloyd, 1997; Bond, Saijonmaa-Koulumies and Lloyd, 1995). The cutaneous sites most often colonised in healthy animals correlate with regions most often affected in dogs with skin disease caused by the yeast. *Malassezia* can also be isolated from mucosal sites. Bond, Saijonmaa-Koulumies and Lloyd, (1995) showed that it was commonly present at the anus but the nose, mouth, prepuce and

vulva infrequently colonised. Basset hounds have higher populations than mixed breed dogs at sites other than the anus (Bond and Lloyd, 1997).

Effect of climate and other factors external to the skin

The microclimate of the skin and the status of the epidermis are constantly changed by climatic factors in the animal's environment. Temperature and humidity are of special importance. When both are elevated they promote substantial increases in aerobic and anaerobic bacterial populations in animals and man (Lloyd, 1980; McBride, Duncan and Knox, 1977). However, high skin surface temperatures alone do not do this, emphasising the role of moisture. In elevated temperatures, animals that control body temperature by sweating, such as cattle and man, produce wet conditions at the cutaneous surface that are independent of external humidity. In cattle, increased sweat production has been related particularly to elevation of populations of streptococci (Lloyd, 1980). Little is known of the effects of temperature and humidity on the canine cutaneous microbiota although differences in cutaneous humidity exist at different body sites (Chesney, 1995) and occluded skin, at sites such as digital webs and folds tends to have higher populations and is predisposed to surface infections.

Exposure to ultraviolet light is also significant. It can modulate immune responses in both animals and man. These effects may be mediated by many different cutaneous cellular components and effects differ depending on wavelength (reviewed by Beissert and Granstein, 1997). For instance, production of complement component, C_3, by IFN-gamma stimulated keratinocytes is increased on exposure to UVB but reduced by UVA and photochemotherapy (PUVA). There are important effects on both keratinocytes and Langerhans' cells; this aspect has been considered by Dr. Viac earlier in the Symposium. Both local and systemic immunity may be impaired and experimental studies show that resistance to bacteria, fungi, viruses and parasites may be reduced (Sleijffers, Garssen and Van Loveren, 2002). However, little is known of the effects of sunlight on the normal cutaneous microbiota Upregulation of immune responses by low doses of UVB have been shown to promote resistance to leishmaniasis (upregulation of IFN-gamma and TNF-alpha – promotion of the Th1 response) in mice (Khashely *et al.*, 2002) and it is possible that low levels of natural exposure in animals may have beneficial effects at the skin surface.

A variety of other factors can influence the cutaneous microenvironment and the status of organisms colonising the skin. Nutrition is of vital importance. The high metabolic rate and need for constant renewal renders the skin susceptible to suboptimal dietary regimens. Equally, hormonal and metabolic changes, including those associated with puberty and pregnancy can modify keratinisation processes and glandular secretory activity. Such changes may in turn lead to changes in microbial population and the onset of disease both in animals and man. Consideration of these factors is beyond the scope of this presentation.

The surface microenvironment

The stratum corneum and hair follicles form the major microbial habitats of the skin (Lloyd, 1980; Lloyd, Dick and Jenkinson, 1979) although substantial numbers of organisms are also found on the hairs, particularly distally, and colonisation may also occur in the sebaceous glands (Saijonmaa-Koulumies and Lloyd, 1996). The populations present are determined by their ability to adhere to the keratinocytes and make use of available nutrients whilst

tolerating the prevailing physical and chemical conditions. Factors such as pH, salinity, nature of the lipids, and the presence of proteins including immunoglobulins, complement and transferrin (e.g. Lloyd, Mabon and Jenkinson, 1977; Garthwaite, Lloyd and Thomsett, 1982; Campbell and Dorn, 1992; Mueller *et al.*, 1997) may be involved. Immunoglobulin A is secreted via the sweat gland and specific antibody levels within surface IgA can be raised by intradermal vaccination (Lloyd and Jenkinson, 1981). More recently the production in the human sweat gland of the antimicrobial peptides, cathelicidin (LL-37) and dermcidin, has been demonstrated and these substances believed to be an important component of innate epidermal immunity (Murakami *et al.,* 2002; Schittek *et al.*, 2001).

The role of keratinocytes

Although the above elements undoubtedly influence microbial colonisation, it is becoming increasingly clear that the keratinocytes also play an important role. Powerful antimicrobial substances released by the keratinocytes that have been recognised recently include the beta-defensins and elafin (skin-derived antileucoproteinase).

Human beta-defensin 3 has particularly strong antimicrobial activity against *S. aureus*. Studies of beta-defensin 2 show that it can be induced in keratinocytes by both gram-positive and gram-negative bacteria and that this action is independent of adherence. *Streptococcus pyogenes* is an exception to this and although highly sensitive is a poor inducer, and it has been suggested that this may help it to evade innate cutaneous defences and induce infection (Dinulos *et al.*, 2003). Human defensin can promote release of IL-1beta from activated monocytes but production of beta-defensin 2 by keratinocytes is also regulated by IL-1 and the state of differentiation of the keratinocytes (Liu *et al.*, 2002). Human beta-defensin has been shown to have synergistic activity with LL-37 against *S. aureus* and, interestingly, expression of these agents appears to be downregulated in lesions of atopic dermatitis possibly acting as a predisposing factor to bacterial infection in atopics (Ong *et al.*, 2002)

Elafin (skin-derived antileucoproteinase) is a potent serine protease inhibitor that is highly induced in inflamed or damaged skin and by serum and TNF-alpha and is active against staphylococci, *Ps. Aeruginosa* and *Candida albicans* (Pol *et al.*, 2003).

Interaction between microbes and the keratinocytes can also have profound effects the epidermis and both innate and specific immune responses. *S. intermedius* isolates can produce the superantigen enterotoxins A, B, C and D and toxic shock syndrome toxin (see Hendricks *et al*, 2002) and both soluble and cell-bound Protein A (Fehrer, Boyle, Halliwell, 1988). Exposure of human keratinocytes to heat-killed *S. aureus, S. epidermidis* and *S. intermedius* promotes release of IL-6 and IL-8 (Sasaki *et al.*, 2003) and it is likely that these cytokines are involved in promoting the vigorous neutrophil response seen in staphylococcal dermatoses. In contrast, exposure of keratinocytes to staphylococcal superantigens induces production of TNF-alpha (Tokura *et al.*, 1994) but not IL-6 or IL-8.

Malassezia yeasts have also been shown to promote cytokine production from keratinocytes in tissue culture. Watanabe *et al.* (2001) showed that exposure of human keratinocytes to the cells of *M. pachydermatis* and the lipid dependent species, *M. slooffiae* and *M. sympodialis* led to secretion of IL-1β, IL-6 and IL-8 but not monocyte chemotactic protein-1. The most potent effects were observed with *M. pachydermatis*. *Malassezia* culture supernatants failed to stimulate release of these cytokines and thus contact between living keratinocytes and the

yeast cells seems necessary. This is unlikely in to occur as a consequence of skin colonisation but may occur following damage or infection leading to destruction of the stratum cornem.

Microbial interaction

The surface microbiota interacts amongst its different components and with the host's cells at many different levels with both competitive and symbiotic relationships (Allaker and Noble, 1993). Resident organisms establish themselves in the available ecological niches of the skin and mucosae and appear to be an important component of cutaneous defence against infection. Residency status also appears to favour transmission from bitches to their puppies. Saijonmaa-Koulumies and Lloyd (2003) studied cutaneous and mucosal isolates of *S. intermedius* collected from three healthy Cavalier King Charles spaniels and their puppies during the immediate prepartum period and after whelping. Using random amplified polymeric DNA-polymerase chain reaction analysis (RAPD-PCR) they identified 17 different genotypes. However, one or two genotypes were dominant in each bitch and that these were the organisms that were transferred and became dominant in their puppies. This may provide a mechanism by which virulent staphylococci could persist from generation to generation within particular lines of dogs. It may also provide a mechanism whereby introduction of avirulent staphylococci could be used to protect lines that are susceptible to canine pyoderma.

When antibiotics are given which eliminate susceptible species from cutaneous and mucosal sites, this may allow invasion by resistant organisms. This is especially likely in animals with skin infection where susceptibility to bacterial invasion is already established. In dogs, the increasing recognition of infection with methicillin-resistant *S. aureus* (MRSA), in animals under treatment for infections of the skin and other organs may be an example of this phenomenon (Tomlin *et al.*, 1999). Elimination of pathogenic staphylococci from such sites is also used in the treatment of chronic pyoderma and allows for the inoculation and establishment of avirulent organisms that can prevent subsequent colonisation and infection by virulent strains by bacterial interference (Aly and Shinefield, 1982; Lloyd and Noble, 1984; Allaker, Lloyd and Smith, 1988).

Such interfering strains will only persist if they are able to compete effectively with the existing microbiota. Antagonism amongst bacteria commonly involves the production of antibiotics or bacteriocins that are able to disrupt the cell membranes of susceptible organisms. These are usually directed at closely related bacteria, which compete for the same environments and substrates (Hechard and Sahl, 2002; Riley and Wertz, 2002). Saijonmaa-Koulumies and Lloyd (1995) studied antagonism to three indicator strains of *S. intermedius in vitro* amongst 950 bacterial isolates from the skin and mucosae of healthy dogs and those with pyoderma. Fifteen isolates of *S. intermedius*, four coagulase negative staphylococci and one *Micrococcus* sp. were antagonistic to at least one indicator strain. Antagonists were significantly associated with the mucosae and dogs with pyoderma. The authors suggested that this reflected the increased selection pressure associated with elevated populations in diseased animals and in the mucosae.

Bacterial interference can involve a variety of other mechanisms. Ji, Beavis and Novick (1997) described interference mediated by autoinducing peptide variants able to inhibit the synthesis of virulence factors, such as enterotoxins and other extracellular proteins under the control of the agr locus. They studied *S. aureus* strains and were able to divide them into three groups. Within groups, strains showed mutual activation of the agr secretory response but inhibited agr expression of members of the other two groups. Non-*S. aureus* strains generally

inhibited the agr secretory response of all three *S. aureus* groups. Activation of agr is dependent on quorum sensing. In rapidly growing populations agr switches on toxin production whereas under conditions of limited growth this is switched off and cell wall components including Protein A are produced. We have demonstrated recently that exoprotein gene expression in canine isolates of *S. intermedius* is regulated by agr in a quorum sensing manner (Sung, Lloyd and Chantler, 2003).

Microbial interaction on the skin can also involve symbiotic or commensal relationships. Metabolism of nutrients by one set of organisms may change the microenvironment so as to favour survival and colonisation by another (Holland, 1993; Allaker and Noble, 1993). This may explain the existence of obligate anaerobic bacteria in the relatively aerobic conditions within the skin. The intimate relationships of bacteria within the ecological niches of the skin also promote gene transfer. This can happen more rapidly and with higher rates of transfer on skin than *in vitro*. Naidoo and Lloyd (1984) demonstrated that transfer of plasmids coding for resistance to tetracycline, erythromycin and gentamycin from human isolates of *S. epidermidis* to canine *S. aureus* (probably *S. intermedius*) over a six-hour period. Rates of transfer for tetracycline and erythromycin resistance were 100-1000 fold higher on murine skin.

Conclusions

The cutaneous and mucosal microbiota can aid in the prevention of microbial dermatosis but also acts as a reservoir for important potential pathogens such as *S. intermedius* and *M. pachydermatis*. This is particularly important in diseases that predispose to cutaneous infection, such as canine atopy. An understanding of the normal microbiota can aid in the development of methods both for the treatment of such infections and for the maintenance or restoration of the normal cutaneous biocenosis in animals at risk to infection. As we come to understand the factors, which control colonisation and the expression of virulence factors by potential pathogens, it is likely that the possibility of long term disease control by bacterial interference and manipulation of the cutaneous microenvironment will become possible.

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