

Epidermal cell proliferation in the equine hoof wall

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Summary

Reasons for performing study: Current theories explaining how the hoof wall 'grows' and moves past the stationary distal phalanx are speculative and based on incomplete evidence. Movement in the lamellar region could occur by cell proliferation or an enzyme-based remodelling process. Since laminitis pathogenesis appears to involve increased transcription and activation of enzymes normally involved in tissue remodelling, it is important to know precisely which process dominates the lamellar region of the hoof.

Objectives: To investigate epidermal cell proliferation in the equine hoof wall and calculate a proliferative index (PI) for the coronet, lamellae and toe.

Methods: An analogue of thymidine, 5-bromo-2'-deoxyuridine (BRdU), was infused i.v. into 5 ponies. After tissue harvesting, BRdU (and therefore basal cell proliferation) was detected immunohistochemically using mouse anti-BRdU. PIs were calculated for the coronet and 10 levels of the dorsal hoof wall lamellae.

Results: The highest PIs (mean \pm s.e.) were in the coronet; 12.04% \pm 1.59 and proximal lamellae (7.13% \pm 1.92) and are therefore growth zones of the proximal hoof wall. PIs of more distal lamellae were 0.11% \pm 0.04 to 0.97% \pm 0.29; significantly lower ($P = 0.05$) than the lamellar growth zone.

Conclusions: A 20-fold PI decrease between proximal and more distal lamellae suggests that the majority of the normal lamellae are nonproliferative and their main function is to suspend the distal phalanx within the hoof capsule. Remodelling within the hoof wall epidermal lamellae, which must occur as the hoof wall moves past the stationary distal phalanx, is a process not requiring epidermal cell proliferation.

Potential relevance: A hoof lamellar epidermis that remodels using the same MMPs involved in laminitis pathogenesis implies that laminitis is a normal process out of control. Understanding MMP control and how the normal lamellar epidermis achieves this will help in the development of better laminitis preventative and treatment strategies.

Introduction

The hooves of mature horses 'grow' continuously to replace hoof lost to wear and tear at the ground surface. Continuous cell

proliferation in the coronet is essential for normal hoof maintenance. Mitosis of basal cells of the coronary epidermis (*stratum germinativum*) produces daughter cells that mature and cornify, each generation adding incrementally to the length of the hoof wall (Leach 1980; Leach and Oliphant 1983; Pollitt 1998).

Similarly, continuous basal cell proliferation in the proximal region of the hoof wall lamellae also occurs, presumably causing distal movement of primary epidermal lamellae (PELs). Mitotic figures (MFs) among the basal cells of the proximal lamellar zone are frequent and a steady rate of distal lamellar 'growth' is not contested. However, convincing evidence that lamellar basal cells, in the remaining lamellae, proliferate to the same degree, is lacking.

Indeed, how the PELs remain attached to the stationary secondary epidermal lamellae (SELs), while being pushed distally, by proliferation in the proximal lamellar zone is unresolved (Stump 1967; Leach 1980; Leach and Oliphant 1983; Budras *et al.* 1989; Budras and Huskamp 1994). There is no evidence of progressive keratinisation from the basal cell layer towards the cornified axis of lamellae and most supra basal cells are at a similar stage of keratinisation. Since no MFs were present in normal lamellae Leach supported a 'sterile bed concept': the equine lamellar epidermis was nonproliferative and therefore sterile. However, Budras *et al.* (1989) described MFs in the lamellar region and called the sterile bed concept 'no longer tenable'. By this reckoning, the rate of lamellar basal cell proliferation was sufficient to account for a laminar flow of cells from the proximal lamellae distally, and for the formation of cap horn arcades at the bases of the lamellae.

Counting MFs is however, an inefficient way to investigate proliferation in normal epithelial tissues. When cells are in mitosis, the MF is visible, but only a few proliferating cells are actually accounted for, as the mitosis (M) phase, represents only a small portion of the proliferation cycle (Linden *et al.* 1992). Preceding M phase in the cell cycle is the longer synthesis (S) phase. Incorporation of the thymidine analogue, BRdU, into DNA while it is being synthesised, during the S phase of the proliferating cell cycle, accounts for virtually all cells proliferating during the timespan of BRdU exposure. The presence of BRdU, incorporated in the nuclei of the cells, can be detected in formalin fixed tissue by immunohistochemistry.

We developed BRdU immunohistochemistry in horses to settle the question of proliferative activity in the lamellae of normal dorsal hoof walls. Lamellar basal cell proliferation was compared to proliferation in the coronet and other epithelial

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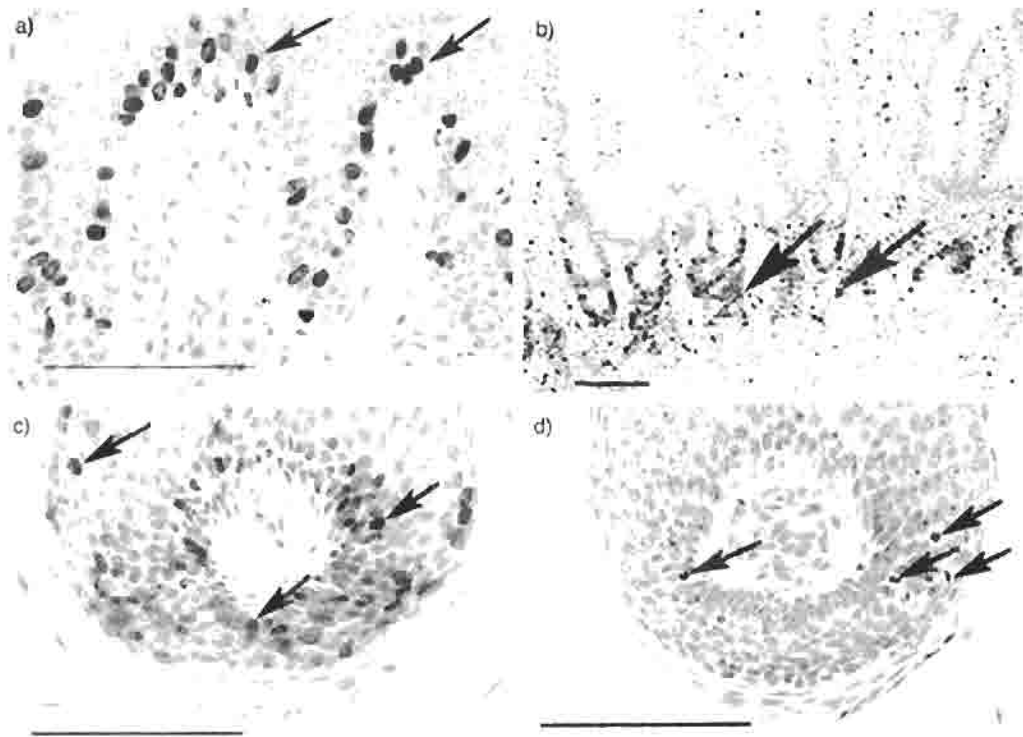


Fig 1: Photomicrographs of sections of tongue (a), small intestine (b) and skin (c, d) from a pony subjected to euthanasia 60 mins after the start of a 15 min i.v. BRdU infusion. The highly proliferative epidermal basal cells (arrowed) of the tongue, the crypts of Lieberkuhn (b) and skin hair follicle (c) show dark brown, BRdU positive, immunostaining (arrowed). A hair follicle bulb (d) from the same tissue block was stained as in Figure (c), but with sheep serum substituted for the primary anti-BRdU antibody and is therefore a negative control (arrows show mitotic figures). Haematoxylin counterstain. Bars = 100 µm.

TABLE 1: Means (\pm s.e.) of cells counted that were BRdU positive, BRdU negative and contained (MFs) in the coronet (COR) and in the 10 lamellar levels (L1–L10) of 5 normal ponies

Hoof tissue level	BRdU +ve		BRdU -ve		Mitotic figures		Sum of BRdU +ve and MFs		Total basal cells		% BRdU +ve		% Mitotic figures		% Proliferative index (\pm s.e.)
	Mean	\pm s.e.	Mean	\pm s.e.	Mean	\pm s.e.	Mean	\pm s.e.	Mean	\pm s.e.	Mean	\pm s.e.	Mean	\pm s.e.	
COR	1090.2	251.42	8135.6	954.90	70.8	19.32	1161.0	266.01	9296.6	1167.39	11.30	1.547	0.743	0.131	12.04 (1.587)
L1	109.4	40.24	1713.4	379.45	12.4	4.06	121.8	41.69	1835.2	394.31	6.37	1.805	0.759	0.231	7.13 (1.922)
L2	21.6	5.73	2817.6	503.05	5.2	2.20	26.8	6.87	2844.4	506.01	0.81	0.261	0.161	0.053	0.97 (0.289)
L3	9.4	3.03	2918.4	576.21	1.8	0.58	11.2	3.43	2929.6	579.25	0.30	0.048	0.067	0.028	0.36 (0.066)
L4	9.2	3.18	2798.8	468.95	1.8	1.11	11.0	4.23	2809.8	472.97	0.29	0.075	0.046	0.029	0.34 (0.097)
L5	6.4	2.94	2897.0	423.08	1.2	0.97	7.6	3.83	2904.6	424.86	0.21	0.082	0.036	0.026	0.24 (0.104)
L6	5.0	2.17	2681.0	418.55	0.4	0.40	5.4	2.46	2686.4	418.97	0.18	0.086	0.016	0.016	0.20 (0.097)
L7	5.0	2.68	2395.6	325.28	0.6	0.40	5.6	3.06	2401.2	325.95	0.20	0.115	0.024	0.015	0.23 (0.128)
L8	3.0	1.67	2266.4	368.14	0.2	0.20	3.2	1.77	2269.6	368.20	0.13	0.076	0.009	0.009	0.14 (0.081)
L9	2.4	0.87	2183.8	153.99	0.2	0.20	2.6	1.03	2186.4	154.53	0.11	0.038	0.008	0.008	0.11 (0.043)
L10	10.6	3.39	2223.2	374.02	3.6	1.12	14.2	3.48	2237.4	371.56	0.57	0.228	0.191	0.074	0.76 (0.249)

tissues. More information about the nature of the hoof lamellar epidermis may improve understanding of the pathophysiology of equine laminitis, a disease characterised by disorganisation of lamellar anatomy (Pollitt 1996).

Materials and methods

The feet of 5 normal footed ponies (2 male and 3 female) weight 120–150 kg, age 2–5 years and with at least one white hoof, were studied. The experiments were conducted in winter, spring and summer, at first light in the morning or after dark in the evening. BRdU¹ sufficient to dose the ponies at 35 mg/kg bwt (Holle and Birtles 1990) was dissolved in one litre of normal saline. The

solution was filtered through a 0.2 micron filter² and transferred to a plastic infusion bag³. The ponies were confined in stocks and the solution was infused into the jugular vein of the ponies over a 15 min period using a protocol approved by the Animal Experimentation Ethics Committee of The University of Queensland. Upon completion of infusion each pony was walked to a stable and unrestrained, given free access to food and water. Sixty minutes after commencement of the infusion, the animals were subjected to euthanasia by i.v. sodium pentobarbitone (Lethabarb)⁴. The feet were disarticulated at the fetlock joint and the dorsal hoof wall processed into 10 blocks of tissue (Pollitt 1996) and labelled L1 (the most proximal level) to L10 (the most distal). Each block was 15 x 15 mm and after trimming contained approximately 25 lamellae.

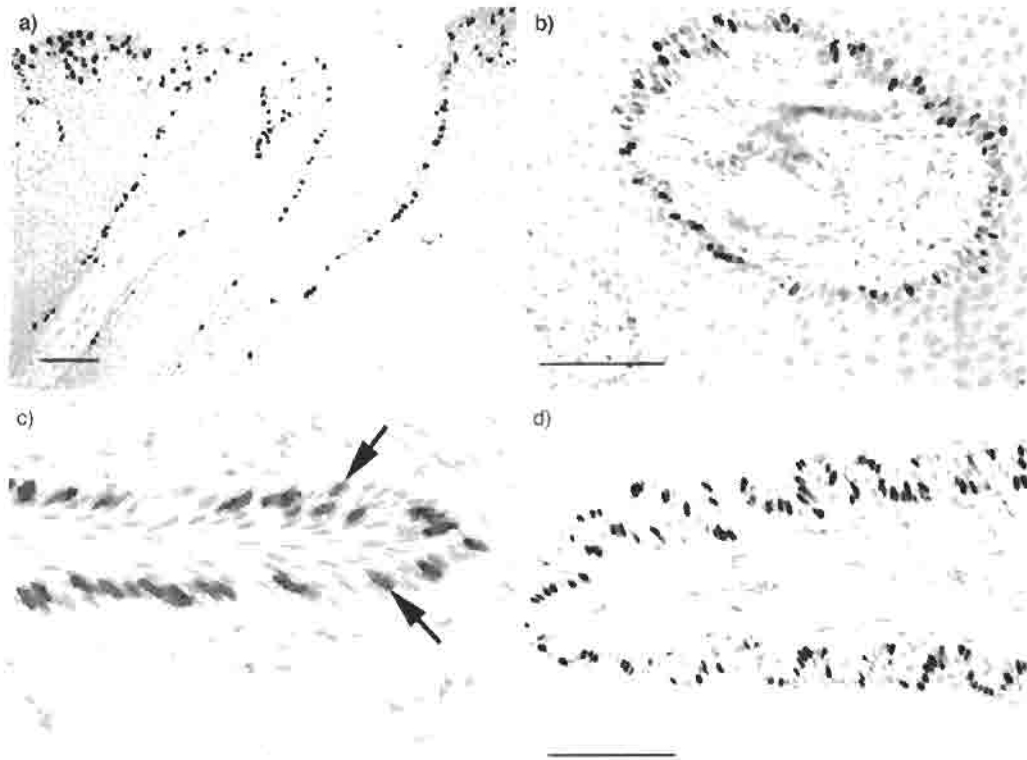


Fig 2: Photomicrographs of LS (a) and TS (b) of coronary band tubular and intertubular epidermis and TS of lamella tip (c) and base (d) at level L1. There are many black-brown, BRdU positive, immunostained epidermal basal cells in the intertubular and proximal tubular epidermis of the coronet and in both the tip (arrowed) and base of the proximal lamellae. Bars = 100 μ m.

Samples of coronet, sole, skin, tongue and small intestine were also harvested. The tissues were fixed in Bouin's fixative for 4 h at 5°C and stored in 80% alcohol solution until processed (Holle and Birtles 1990).

Immunohistochemistry of hoof tissue samples was performed using the method of Pollitt and Daradka (1998). The primary antibody was mouse anti-BRdU containing deoxyribonuclease (Dnase)⁵. Negative controls were tissues processed with sheep serum substituted for the primary and secondary antibody and similar tissues not previously exposed to BRdU. Positive controls were tissues previously established to be BRdU positive in other species (Holle and Birtles 1990; De Fazio *et al.* 1987).

Coronet and lamellar epidermal basal cell nuclei were classified into 3 groups; BRdU negative cells with nuclei stained light blue with haematoxylin, BRdU positive cells with dark brown diaminobenzidine (DAB)⁶ stained nuclei and a third group of cells containing MFs. BRdU positive and negative basal cells in the coronet and 10 lamellar levels (each level containing approximately 25 lamellae) for the 5 ponies were counted using Image-Pro Plus software⁷. MFs were also counted and added to the total of BRdU positive cells to calculate the proliferative index (PI).

The PIs of the dorsal hoof wall of 5 hooves, assumed to be random and independent, were calculated. The percentages were transformed, using natural logarithms, because the means positively correlated with variances. A univariate repeated measures analysis of variance (ANOVA) was used. The Greenhouse-Geisser Epsilon adjustment was applied to F-tests where there was a failure in the assumption of sphericity of orthogonal transformations on the tissue levels. Significant F-test results were followed by *post hoc* tests to compare the levels pairwise and sequentially, as well as to compare each level with

the first level in the series. Analyses were done separately for 3 overlapping blocks of the levels to try to capture changes taking place, because the number of tissue levels ($n = 11$) is greater than the number of hooves examined ($n = 5$). Block I) = Cor L1-L4, block II) = L3-L7, block III) = L6-L10.

A probability value of 0.05 was used as the criterion for significance. SAS 6.12 software (SAS Institute) and the General Linear Models procedure were used to perform the computations.

Results

Intravenous infusion of 35 mg/kg bwt BRdU for 15 mins, with tissues harvested at 60 mins, gave satisfactory immunostaining and specificity. The ponies showed no clinical signs of discomfort or abnormal behaviour during either the *i.v.* infusion of BRdU or the subsequent experimental period. There was no colic or diarrhoea.

Proliferating epidermal basal cells in tongue (Fig 1a), crypts of the small intestine (Fig 1b), hair follicle bulbs (Fig 1c) showed BRdU positive immunostaining. Control sections in which the primary and secondary antibody were withheld failed to show positive staining (Fig 1d), as did similar tissues from horses not treated with BRdU (data not shown).

Coronet basal and suprabasal cells of the intertubular and tubular epidermis showed many BRdU positive cells (Figs 2a,b). The percentage of BRdU positive cells in the coronet was (mean \pm s.e.) 11.30% \pm 1.55 (Table 1). The PI was 12.04% \pm 1.59.

Many basal cells of the most proximal lamellae (L1) showed positive BRdU immunostaining (Figs 2b,c) and, in this regard, the proximal lamellae closely resembled the coronet. Proliferating cells were evenly distributed throughout the basal cell layer, at the bases of the lamellae (Fig 2d) as well as the tips (Fig 2c).

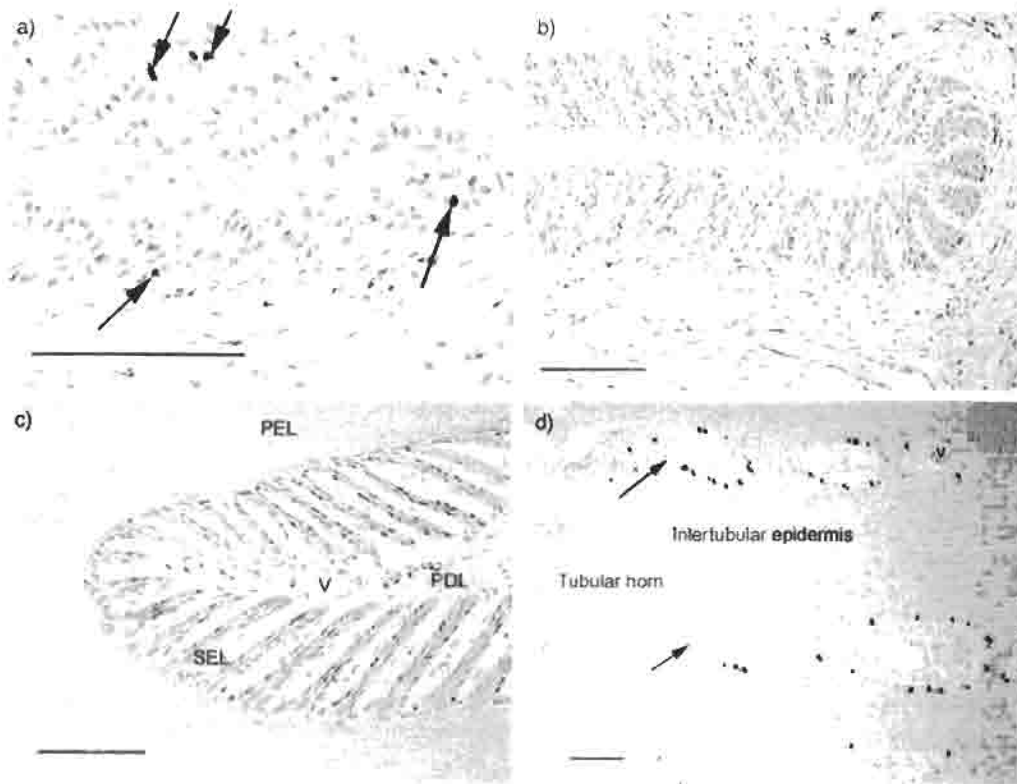


Fig 3: Photomicrograph of lamellae (a,b,c) and terminal papillae (d). In (a), a lamellar tip from L2, a few basal cells show positive BRdU immunostaining (arrows). In (b), a lamella tip from L3 and in (c), a lamellar base from L8, there are no BRdU positive cells. In (d), the L10 epidermis of terminal papillae (arrowed) there are numerous BRdU positive basal cells. Bars = 100 µm.

The percentage of BRdU positive cells in the proximal lamellar layer (L1) was (mean ± s.e.) 6.37% ± 1.81. The PI was 7.13% ± 1.92. The lamellae in L2 (Fig 3a) displayed some BRdU positive cells (0.81% ± 0.26), but less than L1. L3 (Fig 3b) had even fewer BRdU positive cells (0.30% ± 0.05) and a PI of 0.36% ± 0.07. Between L3 and L9 there were very few BRdU positive cells (Fig 4 and Table 1). Particular attention was paid to the cells at the bases of the PELs. There was no evidence of basal cell proliferation in these cap horn arcades (Fig 3c). L10 showed some

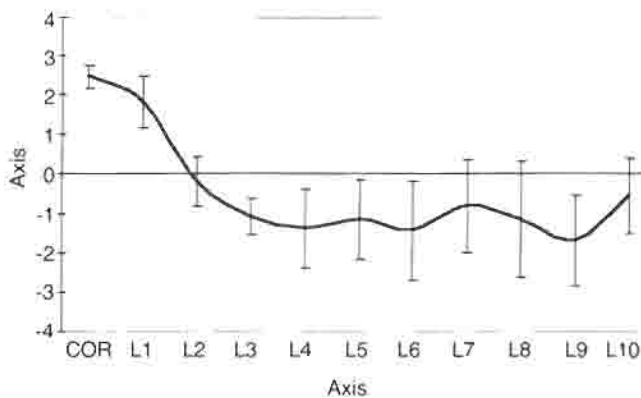


Fig 4: Graph of the means (± s.e.) of natural logarithms of the percentages (Ln %) of proliferative indices for the coronet and lamellae of 5 normal ponies. COR and L1 were not significantly different. There was a significant drop in the mean Ln% from L1 to L2 and from L2 to L3. Thereafter the lamellar levels were not significantly different from each other until L10.

proliferating cells near the terminal papillae (Fig 3d). No season nor time of day differences were detectable. The means of natural logarithms of the percentages (Ln %) of the PIs indices for the coronet and papillae was graphed (Fig 4). COR and L1 were not significantly different (F = 6.42 1,4df P = 0.0644). There was a significant drop in the mean Ln% from L1 to L2 (F = 33.99 1,4df P = 0.0043), and from L2 to L3 (F = 13.77 1,4df P = 0.0206). Thereafter, the lamellar levels were not significantly different from each other until Level 10. There was a significant increase in mean Ln% from level L9 to L10 (F = 20.58 1,4df P = 0.0105) but L10 did not reach the same level of proliferation that was found in the coronet, levels L1 or L2.

Discussion

BRdU immunostaining has been compared with ³H-thymidine labelling in spermatogonial stem cells in the testes and small intestinal crypt cells of adult mice by Thoolen (1990). That author found 90% of the BRdU-labelled cells were also identified by ³H-thymidine labelling autoradiography. Identical patterns of positive cell staining with both histone (H3) *in situ* hybridisation and BRdU immunocytochemistry, in rat tissue, have also been described (Smith *et al.* 1995; Gown *et al.* 1996). Therefore, BRdU immunostaining, for the detection of basal cell proliferation, has been validated against other more traditional techniques, long accepted as markers of proliferative activity.

In the ponies used in this trial, positive BRdU immunostaining of tongue, intestine and hair epithelia was similar to that reported in other mammals (Cairnie 1967; De Fazio *et al.* 1987; Holle and

Birtles 1990; Holle *et al.* 1994; Hynd and Everett 1990). Since continual basal cell proliferation in these tissues is accepted (Wheater *et al.* 1979), the BRdU technique as a marker for epidermal basal cell proliferation in ponies is valid. Toxic side effects occur with BRdU dose rates higher than those used here. The epithelial lining of the digestive tract can be damaged resulting in severe diarrhoea (Holle *et al.* 1994) but no clinical signs or histopathology of intestinal damage were observed in the 5 ponies dosed at 35 mg/kg. The proliferation studies were conducted in winter, spring and summer in Brisbane, Australia (Latitude 27°S) on only 5 ponies. No seasonal differences were detected and none were expected since Buffa *et al.* (1992) showed that the hoof growth of 24 horses measured over 10 months in Pretoria, South Africa (latitude 27°S) also showed no seasonal differences.

The proportion of proliferating cells in the basal cells of the proximal (L1) lamellae was similar to the coronet and decreased significantly ($P < 0.05$) in the more distal lamellar level. Proliferation was virtually nonexistent in lamellar levels L4, L5, L6, L7, L8, L9. This confirms the conclusions of previous studies employing other techniques. Leach (1980), Stump (1967) and Budras *et al.* (1989) counted MFs and also concluded that cell proliferation occurred in these areas.

BRdU immunostaining of coronary and lamellar tissue prompts a reassessment of current hoof growth theory. On the basis of detailed light and electron microscopical studies of the normal horse hooves, Budras *et al.* (1989), claimed constant germinative activity of SEL basal cells accounted for the increase in both length and thickness of the more distal PELs. The increase in length of the more distal PELs was obscured, and had gone unrecognised, because they were buried in 'cap horn' that spanned the arcades at the bases of adjacent PELs. The production of cap horn required constant basal cell proliferation. Our study showed no basal cell proliferation or MFs in any of the cap horn arcades between PELs at any lamellar level more distal than L2. Instead, proliferative activity was almost entirely confined to basal cells of the most proximal (L1 and L2) and distal lamellae (L10) suggesting that lamellar basal cells do not produce cap horn by basal cell proliferation.

It is undeniable that epidermal proliferation at the coronet constantly adds to the proximal *stratum medium* of the hoof wall causing it to move downwards, past the distal phalanx. The PELs are attached and blend into the inner wall of the *stratum medium* and presumably move downwards with it. However, it is not likely that the SEL basal cells, firmly attached to their underlying basement membrane, move at all, so considerable sliding and relocation of the cells between the basal cell layer and the more keratinised axis of the PEL must occur. Budras *et al.* (1989) acknowledged this and described a laminar flow of cells, in 'sliding contact' with each other, keeping pace with the downward movement of the PELs. The suggestion that basal cells of the SEL multiply at a rate sufficient to keep up with the downward growth of the PEL horny lamellae was also suggested by Stump (1967). Their argument requires a constant supply of new cells to generate the downward laminar flow but evidence that this originates from SEL basal cell proliferation is not provided by this study. The few BRdU positive cells that were detected between L2 and L9 (around 0.20%) had a patchy distribution and were usually located at the PEL tips. The large difference in the rates of proliferation between the proximal ($PI = 7.13\% \pm 1.92$) and mid-lamellar regions of the hoof wall ($PI = 0.24\% \pm 0.10$) suggests that the mid-lamellar region has insufficient basal cell proliferative capacity to account for a laminar flow of cells.

Our results do not entirely substantiate the sterile bed concept. The rate of proliferation is very much higher in the coronet and L1, decreases rapidly at L2 and L3 and thereafter remains consistently low, increasing only again at L10. Therefore, approximately 80% of the lamellar inner hoof wall, while not completely sterile, appears not to contribute significantly to the lamellar regrowth by proliferation. Leach and Oliphant's (1983) proposal that remodelling of existing populations of lamellar cells, by the breaking and reforming of desmosomes in a staggered ratchet like manner, was responsible for the distal movement of PELs past stationary nonproliferative SELs appears to be borne out by the results of this study.

Since Leach and Oliphant's (1983) publication, remodelling of epidermis and extracellular matrix has been shown to be due to the controlled release of activated matrix metalloproteinases (MMPs) and their subsequent inhibition by tissue inhibitors of metalloproteinases (TIMPs). MMPs have been shown to exist in lamellar hoof and their uncontrolled activation has been proposed as a mechanism for the pathogenesis of laminitis (Pollitt *et al.* 1998). The molecular components of desmosomes, hemidesmosomes and basement membranes are substrates for MMP activity and, therefore, Leach and Oliphant's (1983) mechanistic concept of 'formation and destruction of desmosomes in a staggered ratchet-like manner' now has a well referenced, biological explanation (Birkedal-Hansen *et al.* 1993; Woessner 1991). The SEL epidermal cells and their adjacent basement membrane are constantly responding to the stresses and strains of growth and locomotion and the harmonious release of MMPs and TIMPs accomplishes the 'nips and tucks' required.

Since the cells composing horse hoof lamellar tissue appear to remodel using enzymes capable of destroying key components of the very extracellular matrix involved in the apparatus suspending the distal phalanx from the inner hoof wall this may explain why, when lamellar MMPs are inadvertently activated *en masse*, horses are peculiarly susceptible to the destructive effects of laminitis. Good evidence that over expression of MMP-2 occurs in laminitis affected inner hoof wall tissue (Kyaw-Tanner and Pollitt 2004) puts laminitis into the disease category of a normal process gone wrong. Understanding this process offers hope that laminitis may yet be preventable if not curable.

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Manufacturers' addresses

¹Sigma Chemical Co., St Louis, Missouri, USA.

²Milipore Corporation, Bedford, Massachusetts, USA.

³Baxter Healthcare, Old Toongabbie, New South Wales, Australia.

⁴Virbac (Australia), Peakhurst, New South Wales, Australia.

⁵Amersham (Australia), Castle Hill, New South Wales, Australia.

⁶Dako Corporation, Carpinteria, California, USA.

⁷Media Cybernetics, Silver Spring, Maryland, USA.

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