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Synthesis and distribution of cytokeratins in healthy and ulcerated bovine claw epidermis

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Summary. Keratinization of the epidermal cells of the bovine claw generates the horn that gives the tissue its mechanical strength. Disruption of keratinization is likely to have a detrimental effect on the strength and integrity of the horn, and could lead to solar lesions and lameness. As part of a wider investigation of the cell biological causes of lameness in dairy animals, we have compared keratin synthesis and distribution in healthy bovine claw tissue with those in hooves with solar ulcers. Protein synthesis was measured by [^{35}S]-labelled amino acid incorporation in claw tissue explant cultures. [^{35}S]-labelled protein synthesis was higher in tissue from diseased claws than in healthy claws, and highest at the ulcer site. The identity of proteins synthesised *in vitro* did not differ between healthy and diseased tissue. DNA synthesis indicative of cell proliferation was also elevated in diseased tissue. Immunoblotting after one- or two-dimensional electrophoresis showed cytokeratins (CK) 4, 5/6, 10 and 14 to be amongst those expressed in healthy claw tissue. The relative abundance of these keratins was not altered in healthy regions of ulcerated hooves, nor at the ulcer site, but CK16, not usually found in healthy tissue, was detected in the sole of diseased claws. CK5/6 and CK14 were shown by immunohistochemistry to be present in the basal epidermis of healthy tissue, whereas CK10 was found in supra-basal layers. In healthy tissue from ulcerated claws, this distribution was unaltered, but at the site of solar ulcers, CK5/6 and CK14 were each found in both basal and supra-basal epidermis. The study suggests that solar ulceration of the bovine claw is not associated with gross alteration in the keratin composition of the tissue, but causes abnormal distribution of cytokeratins, perhaps as a result of loss of positional cues from the basement membrane. Ulceration did, however, stimulate cell repair involving epidermal protein synthesis (including keratins), and keratinocyte proliferation.

Keywords: Hoof, cow, lameness, ulceration, cytokeratins, protein synthesis, cell proliferation.

Lameness is a major problem in the dairy industry, causing serious welfare problems and economic loss. The incidence and prevalence of the diseases contributing to lameness are on the increase (Clarkson *et al.* 1996), but despite the scale of the problem little is known of claw tissue biology in its normal or diseased states. The horny tissue of the bovine claw is the product of keratin synthesis and

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deposition; it is this process which gives the claw its mechanical strength (Fraser & Macrae 1980). Keratinization takes place in epidermal keratinocytes, and involves bundling of keratin filaments into regular macromolecular arrays, co-ordinated arrangement of these structures in adjacent cells through desmosomal tight junctions and, ultimately, enucleation of the keratinized cells (Budras *et al*. 1989; Kempson & Logue 1993; Leach 1993). Other keratinizing epidermal tissues display distinctive patterns of keratin subtype expression which may differ between basal and supra-basal layers (Moll *et al*. 1982). It is presumed that this is also the case in the claw, but the identity of keratins expressed and their distribution is as yet incompletely defined (Hendry *et al*. 1997).

Given its structural importance in the claw, any impairment of keratin deposition would be expected to have a detrimental effect on the strength and integrity of the horny tissue. Accordingly, equine laminitis is associated with alteration in keratin expression and localisation (Grossenbaugh & Hood 1993). Keratin deposition is also altered in human conditions causing pathological cell hyperplasia, with basal cell keratins being expressed in supra-basal locations (Wetzels *et al*. 1991). In this study, we investigated whether ulceration in the bovine claw is accompanied by changes in the rate of synthesis or localisation of keratins that might contribute to the diseased state.

A preliminary communication of this work was presented at the 10th International Symposium on Lameness in Ruminants (Hendry *et al*. 1998).

Materials and methods

Materials

Polyclonal antiserum against fetal bovine hoof keratin, monoclonal antibodies against cytokeratin (CK) 14, CK 1/10/11 (clone K8·6), CK4, FITC- and alkaline phosphatase conjugates of anti-guinea pig IgG, and alkaline phosphatase conjugates of anti-rabbit and anti-mouse IgG were purchased from Sigma Chemical Company (Poole BH12 4QH UK). Monoclonal antibodies to CK10 and CK16 were from Novacastra Laboratories (Newcastle-upon-Tyne NE2 4AA UK). Monoclonal antibody to CK5/6 was from Boehringer-Mannheim (Lewes BN7 1LG UK). Anti-mouse and anti-rabbit IgG-FITC conjugates and non-immune sera were obtained from the Scottish Antibody Production Unit (Carluke ML8 5ES UK). Pronase, 3-aminopropryltriethoxysilane, urea, IGEPAL CA-630, Periodic Acid Schiff stain kit and Kodak XOMAT film were from Sigma. [^{35}S]-amino acid mix (Easy Tag Express Label) was from NEN Life Science Products (Hounslow TW5 9RT UK). [Methyl-^{3}H]-thymidine was purchased from ICN Biomedicals (Thame OX9 3XA UK). Electrophoresis reagents were from Amersham-Pharmacia Biotech UK (Little Chalfont HP7 9NA UK) or Bio-Rad Laboratories (Hemel Hempstead HP2 7TD UK). Immobilon P membrane was from Millipore (UK) (Watford WD1 8YW UK). Two dimensional electrophoresis markers pI range 7·6–3·8 were from Sigma. The sources of other histological reagents, culture media and laboratory reagents were as described previously (Hendry *et al*. 1995, 1999).

Tissue collection

Holstein-Friesian cows were from the Hannah Research Institute herd, or from the Acrehead herd of the Crichton Royal Farm, Scottish Agricultural College, Dumfries, Scotland. Tissues from a total of 11 animals were collected. Cows were all more than 3-years-old (3–9 years) and at least second parity. Hoof condition was monitored routinely in the two herds, such that all animals had well-documented

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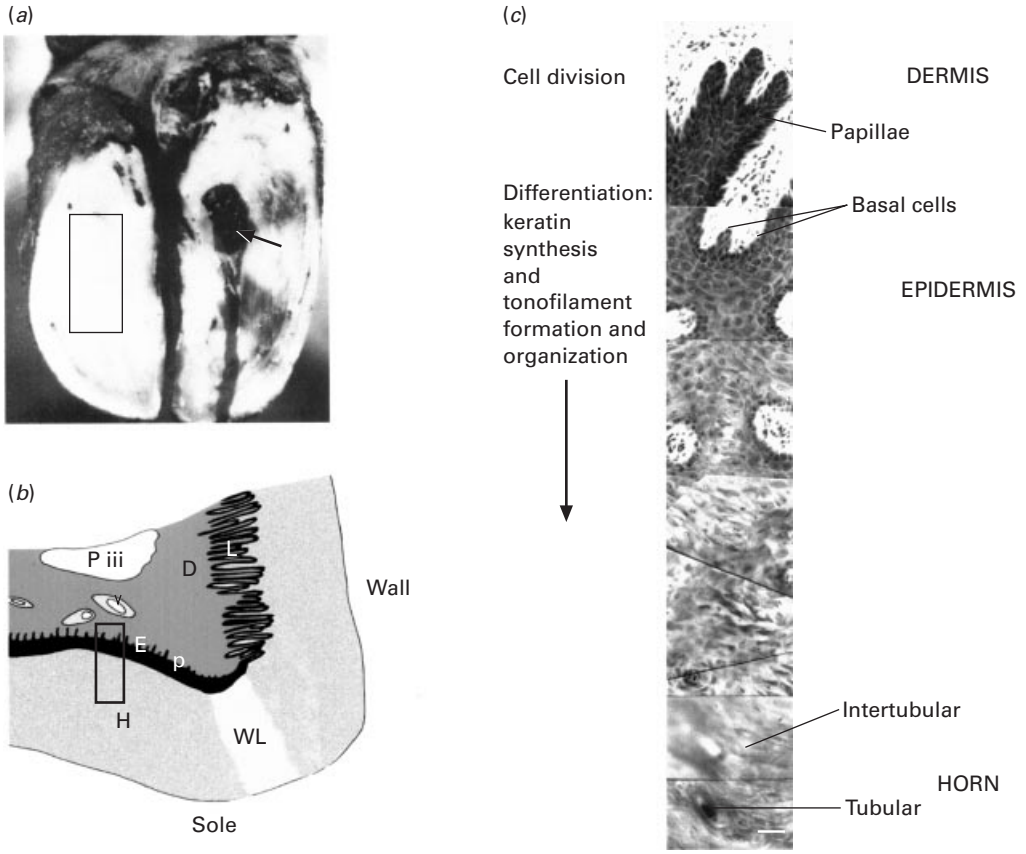

Fig. 1. Anatomy and histology of the bovine claw. (*a*) Sole of bovine claw showing a solar lesion (arrowed) and region tissue collected from (box). (*b*) Diagramatic representation of a cross section of the bovine claw. D, dermis; E, epidermis; H, horn; WL, white line; L, laminae; p, papillae; v, blood vessel; P iii, pedal bone. The boxed area is shown in (*c*) as a micrographic montage of the sole of the bovine claw. Anatomical regions of the epithelium are labelled and the stages of keratinocyte differentiation indicated. Methylene Blue stain; bar = 30 μm. The figures are reprinted from Hendry *et al.* (1997) with modifications.

lameness histories. Decision to cull was in all animals due to lameness, and hoof mapping prior to culling (Logue *et al.* 1994) recorded lesion sites and severity. Animals were killed by captive bolt and exsanguination. Claw tissue was obtained immediately *post mortem*. Claws were separated, and cut into three medial sections using a power saw. Tissue blocks were taken from the sole of the claw and comprised the central portion of zones 4 and 5 (Greenough & Vermunt 1991). Each tissue block comprised dermis, epidermis and horny tissue as described previously (Hendry *et al.* 1995; see Fig. 1), but with most of the hard horn removed. Bovine hoof anatomy and ultrastructure is described in detail elsewhere (Kempson & Logue 1993; Hendry *et al.* 1997), and illustrated in Fig. 1, showing the site of tissue collection. Tissue blocks were processed further for histology, tissue culture or electrophoretic analysis. Paired samples of healthy and diseased tissue were collected from each animal. Tissue designated as healthy was from claws with no documented lesion. Tissue identified as diseased was from claws with documented lesions, but taken distal to an ulcer site. Tissue designated as ulcerated was from an ulcer site, with minimal surrounding tissue such that the ulcer was excised as a block. In the six cases where ulcers were present, these were of similar visual appearance, all being advanced, corium-exposed

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ulcers. An example of the physical manifestation of a solar ulcer similar to those examined in this study is shown in Fig. 1. Most of the ulcers were in outer hind claws, one was in the inner hind claw and another from a toe region of a front inner claw.

Histology

Tissue was cut into smaller blocks approximately 3 mm^3 (each containing all tissue layers) and fixed in Methacarn for 24 h. Tissue was processed and embedded in fibrowax by conventional methods. Sections (5 μm) were mounted on slides coated with 3-aminopropyltriethoxysilane. Tissue was also processed for cryo-sectioning without fixation by immediate addition of OCT embedding medium, freezing in liquid nitrogen and storage at −70 °C. Frozen sections (5 μm) were mounted on silanized slides, air dried and stored at −20 °C.

Periodic acid Schiff staining

Staining of sections was carried out using the Sigma PAS (Sigma) kit according to manufacturers instructions. Counter staining was by Gill's Heamatoxylin also included in the PAS kit.

Immunofluorescence microscopy

Paraffin sections were dewaxed, rehydrated, and treated for 10 min with 1 mg/ml $NaBH_4$ in PBS (0·14 M-NaCl-9 mM-Na_2HPO_4-1·3 mM-NaH_2PO_4, pH 7·6). Sections were rinsed in PBS and incubated for 10 min at 37 °C in PBS containing 1 g Pronase/l. Cryosections were thawed at room temperature. All sections were incubated with non-immune serum from the same species as that used to raise the secondary antibody, diluted 1:3 in PBS containing 1 g Tween 20/l (PBST). Sections were then treated with primary antibody diluted in PBST (anti-CK14, 1:1000; anti-CK5/6, 1:250 of a 50-μg/ml solution; anti-CK10, 1:100 dilution; anti-CK16, 1:50) and after washing in PBST, incubated in FITC-labelled antibody diluted in PBST. All incubations were carried out at room temperature in a humid chamber. Sections were mounted in Vectashield mounting media containing DAPI and examined using a Lecia DMR microscope with epifluorescence optics viewed with a No. 13 (UV) and A filter. Images were photographed onto Fujichrome Provia 400 ASA film.

Tissue culture

Claw tissue culture was performed as described previously (Hendry *et al.* 1995). Protein synthesis was measured by incorporation of [^{35}S]-labelled amino acids (20 μCi/ml) into explants cultured in Hams F12/DMEM medium (1:1, v/v) for 3 h at 37 °C in an atmosphere of air/CO_2 (95:5, v/v). Other groups of explants were cultured in 50 μCi/ml [^{35}S]-labelled amino acid mix for fluorographic detection of radiolabelled proteins. In some experiments, DNA synthesis was measured by incorporation of [methyl-^{3}H]-thymidine (5 μCi/ml). Cultured explants were rinsed in nonradioactive medium, blotted, snap frozen in liquid nitrogen and stored at −70 °C. Experiments compared triplicate culture wells of solar explants from one healthy claw and one ulcerated claw of the same animal.

Protein and DNA synthesis

Homogenates from the cultured claw tissue were prepared as previously described (Hendry *et al.* 1995). DNA content of the homogenate was measured by a fluorometric method (Labarca & Paigen 1980) using calf thymus DNA as a standard. Radiolabelled protein and DNA were extracted by precipitation with 100 g

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trichloroacetic acid/l as previously described (Hendry *et al.* 1995) and processed for scintillation counting by standard methods. Results were analysed by paired *t* test on Minitab release 8 (Minitab Inc., PA 16801-3008 USA).

Proteins in tissue homogenates were separated by SDS-PAGE in 12 % (w/v) gels under reducing conditions (Laemmli, 1970). Gels were loaded on an equal protein basis and stained with Coomassie blue, or on an equal DPM basis for fluorographic identification of radiolabelled proteins.

Keratin extraction

Tissue blocks or explants, the former dissected to select epidermal tissue, were ground to a powder under liquid nitrogen, and keratins were extracted using the method of Kvedar *et al.* (1986). Powdered tissue was suspended at 50 mg/ml in 50 mM-Tris, pH 8·0 containing 8 M-urea (Tris-urea buffer) and 0·2 mM-PMSF, homogenised as above and centrifuged at 100000 ***g*** for 30 min. The supernatant was collected and protein concentration was determined using bovine serum albumin as a standard (Bradford, 1976). Aliquots were diluted to 1–2 mg/ml and dialysed against 5 mM-Tris buffer with 25 mM-2-mercaptoethanol, pH 8·0 for 24 h at 4 °C. Keratin filaments were collected by centrifugation at 1000 ***g*** for 10 min, and redissolved at 2 mg/ml in Tris-urea buffer.

Keratin electrophoresis

Samples were diluted to 1 mg/ml in sample buffer (Laemmli, 1970) for one-dimensional SDS-PAGE, or in 9 M-urea containing 50 g 2-mercaptoethanol/l, 20 ml Pharmalyte 3–10/l, 20 g IGEPAL CA-630/l and 1 g bromophenol blue/l for two-dimensional electrophoresis. Keratin extracts (2 μg) were subjected to one-dimensional SDS-PAGE in 10 % (w/v) gels (Laemmli, 1970). Alternatively, keratin proteins (1 μg) were resolved by two-dimensional electrophoresis using the Phast system (Amersham-Pharmacia Biotech) according to the manufacturers instructions or, in larger quantities (5 μg), by the method of O'Farrell (1975). In each case, isoelectric focusing in the first dimension was performed using Pharmalyte 3–10 ampholines. SDS-PAGE in the second dimension was in 20 % (w/v) PhastGels, or 12 % (w/v) homogeneous mini-gels (7 × 9 cm), and proteins were visualised by silver staining.

Immunoblotting

Keratins resolved by one or two dimensional electrophoresis were transferred to Immobilon P membrane by conventional methods (Towbin *et al.* 1979) or, in the case of PhastGels by semi-dry blotting, both using a transfer buffer containing 25 mM-Tris-192 mM-glycine and 200 ml methanol/l. Blotted proteins were stained with 0·1 % Amido Black or probed with monoclonal antibodies to keratins CK4, CK10, CK14, CK5/6, CK16, or antiserum to fetal bovine hoof keratins. Binding was detected using antispecies IgG conjugated to alkaline phosphatase as described previously (Hendry *et al.* 1995). Stained gels and blots were analysed using a Personal Densitometer SI and ImageQuaNT software version 4.1 (Molecular Dynamics, Amersham-Pharmacia Biotech, Little Chalfont HP7 9NA UK).

RESULTS

Protein synthesis

Total protein synthesis was measured by [^{35}S]-labelled amino acid incorporation in claw tissue cultures. Explants prepared from healthy regions of diseased tissue

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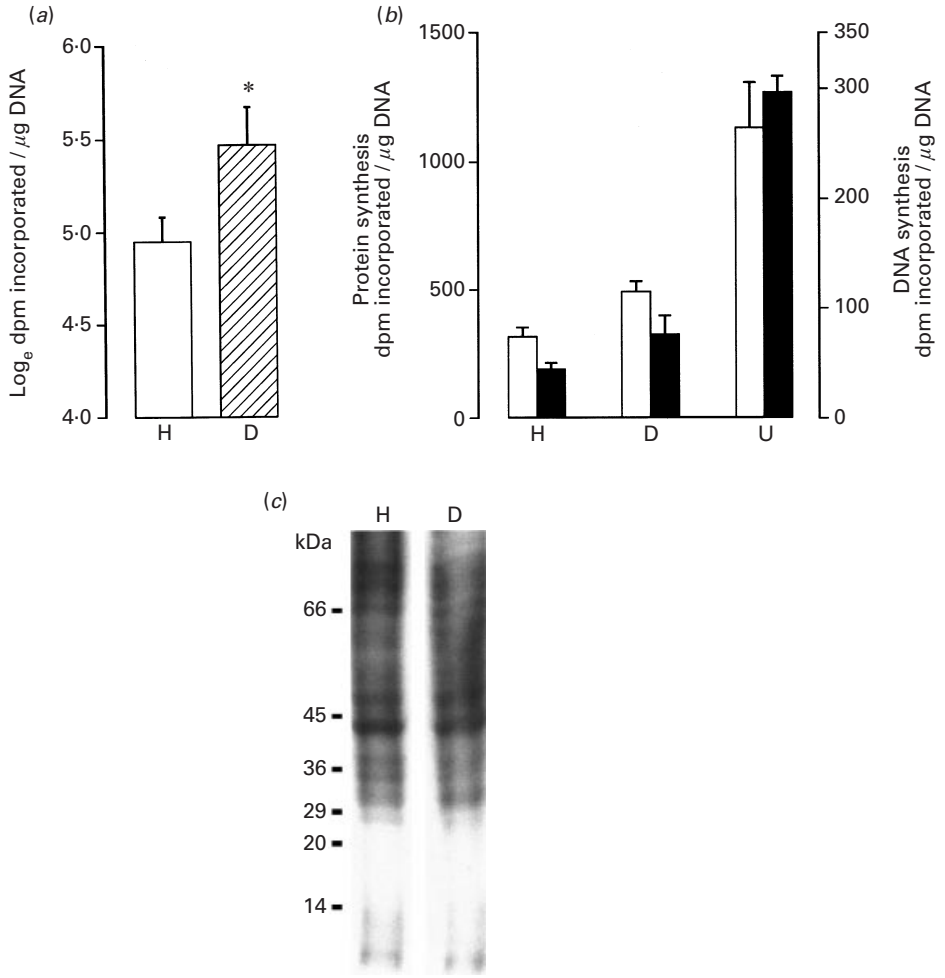

Fig. 2. Protein and DNA synthesis in healthy and diseased bovine solar tissue. (*a*) protein synthesis in healthy (H) and non-ulcerated tissue from diseased claws (D) was measured by incorporation of [^{35}S]-labelled amino acids in short-term explant cultures. Results are the mean ± SEM for five experiments. *$P = 0{\cdot}038$. (*b*) protein synthesis (open bars) and DNA synthesis (filled bars) in explant cultures of healthy tissue (H), non-ulcerated tissue from diseased claws (D), and ulcerated tissue (U) from a single animal. DNA synthesis was measured by incorporation of [^{3}H]-thymidine. Values are the mean ± SEM of three replicates. (*c*) fluorography of proteins synthesised in tissue explants from healthy (H) and non-ulcerated tissue of a diseased claw (D) and resolved by SDS-PAGE.

synthesised protein at a higher rate than those prepared from healthy claws ($P = 0{\cdot}038$; Fig. 2*a*). The difference between healthy and diseased claws was more pronounced when measurements were made at, rather than adjacent to, the ulcer site (Fig. 2*b*). Protein synthesis was 2–4-fold greater in ulcerated tissue, a difference which was not ascribable to microbial contamination (results not shown), nor to a change in the population of proteins synthesised. Fluorography of [^{35}S]-labelled proteins from ulcerated tissue after one-dimensional PAGE produced a banding pattern indistinguishable from that of healthy tissue (Fig. 2*c*), an observation confirmed in keratin extracts of [^{35}S]-labelled proteins (results not shown). DNA synthesis was also increased in explants from diseased claws, and highest at the ulcer site (Fig. 2*b*).

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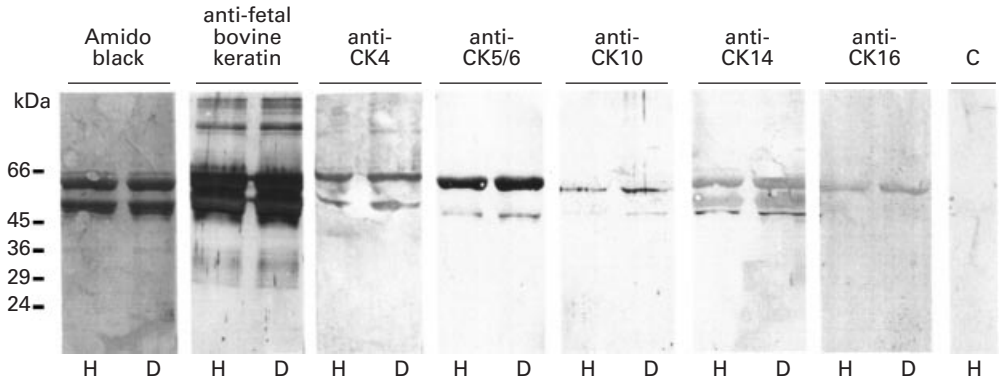

Fig. 3. Cytokeratin content of healthy and diseased bovine solar epidermis. Tissue extracts from healthy (H) and non-diseased areas of ulcerated claws (D) were resolved by single dimension SDS-PAGE, blotted onto PVDF membrane and stained with Amido Black or immunoblotted with antibodies against fetal bovine claw keratins, CK4, CK5/6, CK10, CK14, or CK16. C, anti-mouse IgG-alkaline phosphatase conjugate only. Representative samples from six animals are shown.

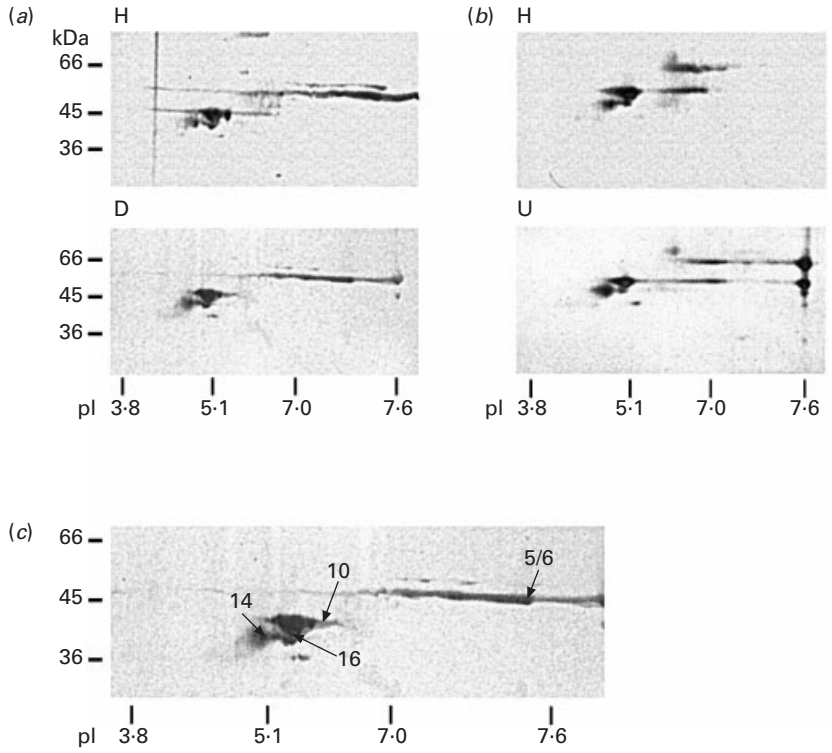

Fig. 4. Two-dimensional electrophoresis of cytokeratins in healthy and diseased bovine sole epidermis. Cytokeratins from healthy claws (H), non-diseased tissue of an ulcerated claw (D) and ulcerated tissue (U) were resolved by two-dimensional electrophoresis using a mini-gel system. Panels *a* and *b* show results for paired samples from two animals (representative of six preparations); (*c*) cytokeratins were located in two-dimensional electrophoretograms by immunoblotting using specific antisera. Cytokeratin subtypes are numbered and indicated by arrows.

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Table 1. *Keratin abundance in solar tissue of healthy and ulcerated bovine claws*

(Values in arbitrary units, are the mean ± SEM, number of determinations in parentheses)

Keratin subtype/group	Healthy	Diseased
5/6	1043 ± 235 (6)	919 ± 199 (6)
14	77·1 ± 85·9 (3)	79·9 ± 37·8 (4)
10	2509 ± 1101 (6)	1667 ± 2033 (6)
16	7·28 ± 4·2 (5)	72·2 ± 29·3* (6)

Keratins were resolved by two-dimensional electrophoresis on Pharmacia PhastGels and measured by densitometry of immunoblots of individual keratins. Differences between healthy and diseased tissues were not significant (* $P = 0{\cdot}08$, Student's paired t test).

Identification of claw keratins

Keratins extracted from solar epidermis were analysed by one-dimensional SDS-PAGE. Proteins migrating at molecular mass 40–66 kDa were confirmed as keratins using antiserum against fetal bovine claw keratins (Fig. 3). Screening with antibodies against human CK demonstrated the presence of CK4 (bands at 43 and 52 kDa), CK5/6 (64 and 50 kDa), CK10 (56 kDa), CK14 (49 kDa) and CK16 (59 kDa) subtypes (Fig. 3). Comparison of healthy tissue and non-ulcerated epidermis of diseased claws using SDS-PAGE revealed no difference in electrophoretic banding nor in the identity of keratins present (Fig. 3).

Resolution of keratin extracts by two-dimensional electrophoresis in PhastGel and mini-gel systems produced comparable patterns of protein staining. In each system, the high resolution afforded by two-dimensional electrophoresis still revealed no differences in the protein compositions of healthy tissue, non-ulcerated tissue from diseased hooves and tissue from ulcer sites. Immunoblotting for fetal bovine hoof keratins confirmed these to be the major components of the extracts, and antisera against the main keratin subtypes identified CK5/6 as basic (pI 7·5) and CK10, 14 and 16 as acidic constituents (pI 5·5, 4·85, and 5·1 respectively). Again, the immunoblot patterns of keratins from healthy, non-ulcerated areas of diseased claws and from ulcer sites were generally similar to those of healthy claws (Fig. 4). Densitometric comparison of PhastGel immunoblots for individual keratins found no differences in the intensity of staining for CK5/6, CK10 or CK14 (Table 1). The exception was CK16, which was more abundant, albeit not consistently ($P = 0{\cdot}08$, Student's paired t test), in ulcerated tissue (Table 1) compared with healthy tissue from the same animals (Fig. 5).

Histology

Tissue structure in healthy and diseased claws of the bovine claw was revealed by Periodic Acid Schiff's (PAS) staining. In healthy claws, the basement membrane was visualised as a magenta-coloured line separating the epidermis from the dermis of the claw (Fig. 6*a*). In diseased claws, at areas of the sole distal from ulcer sites, tissue organisation and basement membrane integrity appeared as in the healthy claw (Fig. 6*b*). In damaged tissue remaining at the edges of a solar ulcer, PAS staining for basement membrane was reduced or absent, such that the epidermal-dermal junction was indistinct, but the morphology of papillary extensions of the dermis was maintained (Fig. 6*c*).

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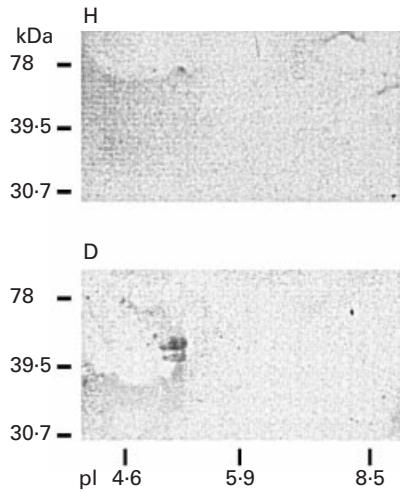

Fig. 5. Cytokeratin 16 in healthy and diseased bovine sole epidermis. Cytokeratins from healthy claws (H) and non-diseased tissue of an ulcerated claw (D) were resolved by two-dimensional electrophoresis on Pharmacia PhastGels and immunoblotted for CK16.

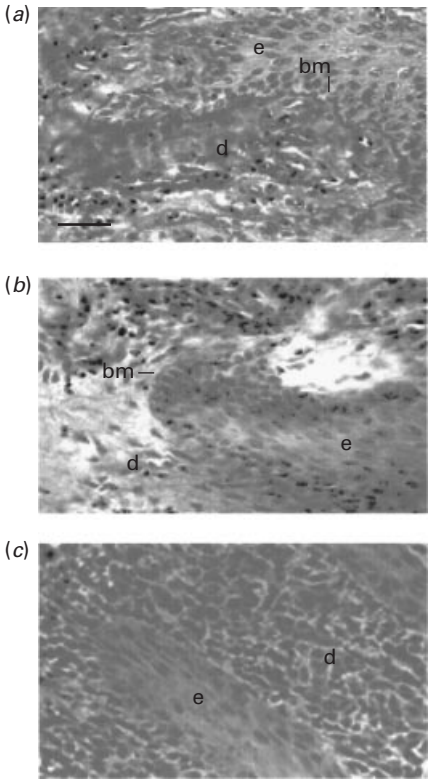

Fig. 6. Periodic acid Schiff staining of the epidermal-dermal junction in healthy and diseased bovine claw soles. (*a*) healthy tissue; (*b*) tissue from a diseased claw distal from the ulcer site; (*c*) tissue from an ulcer site. Key features: e, epidermis; d, dermis; bm-, basement membrane. Bar = 47 μm.

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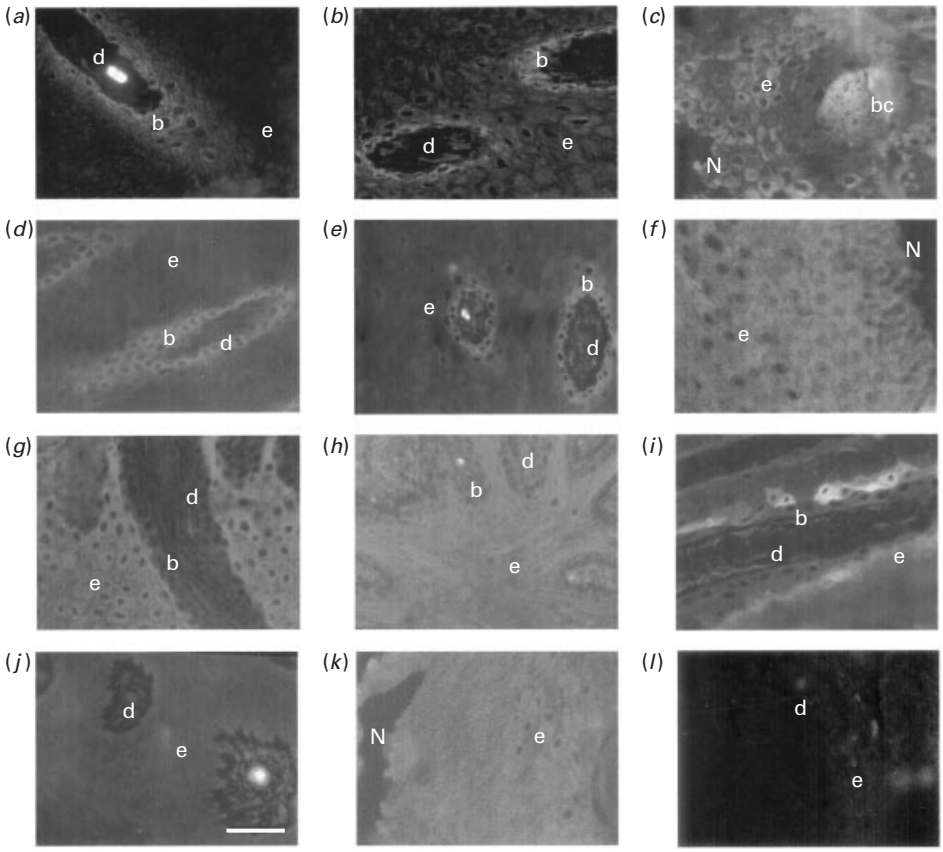

Fig. 7. Immunofluorescent detection of cytokeratins (CK) in healthy and diseased bovine sole epidermis. Healthy (*a*, *d*, *g*, *j*, *i*), non-ulcerated areas of diseased claws (*b*, *e*, *h*) and ulcerated regions (*c*, *f*, *k*) were screened with antibodies to CK14 (*a*, *b*, *c*), CK5/6 (*d*, *e*, *f*), CK10 (*g*, *h*, *i*) and CK16 (*j*, *k*). *l*, anti-mouse IgG control. Bar = 47 μm. Key features: e, epidermis; d, dermis; b, basal cells; N, necrotic area; bc, blood clot. Images are representative of samples from eight healthy, seven diseased and four ulcerated tissues.

Immunohistochemical localisation of keratins

Keratin localisation was compared in healthy claws, non-ulcerated areas of diseased claws and at solar ulcer sites using antibodies against human CK 14, CK 10, CK 5/6 and CK 16. In the sole of healthy claws, CK14 and CK5/6 were localised in the basal cell layers (Fig. 7*a*). CK10, on the other hand, was located in the supra-basal layers of the epidermis, with some cells, particularly in the outer layer of tubule-forming keratinocytes, showing intense fluorescence (Fig. 7*g*, *i*). Similar patterns were observed with CK10 antibodies from two different sources. CK16 was also detected in epidermis rather than dermis, but specific localisation was prevented by the weakness of the signal, which was not improved by antigen unmasking techniques (Fig. 7*j*). CK4 was detected throughout the epidermis in both basal and suprabasal cells (results not shown).

Keratin distribution in non-ulcerated tissue from diseased claws was similar to that of healthy claws of the same animals (Fig. 7*b*, *e*, *h*), but was altered at the actual ulcer site. CK14 and CK 5/6 in ulcerated tissue were found in supra-basal layers of the epidermis immediately adjacent to the area of necrosis, instead of the basal layers (Fig. 7*c*, *f*). The pattern of CK4 and CK10 fluorescence was unchanged (results not shown).

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DISCUSSION

The cytokeratin subtypes identified in the solar epidermis of the bovine claw were similar to those found in healthy equine hoof using the same antibodies (Pellman *et al.* 1993; Wattle, 1998) and to those found in other regions of the bovine claw (Kvedar *et al.* 1986; Kitahara & Ogawa, 1994). Apparent differences between the present study and the cytokeratin content of bovine claw reported previously (Kvedar *et al.* 1986) are in fact related to the source of the antibody, and the species (human or bovine) from which the antigen was prepared. Thus, CK14 described in this study corresponds to the CK16 described previously (Kvedar *et al.* 1986) which is not the CK16 associated with hyperplasia. The precise size and isoelectric point of the proteins identified by anti-cytokeratin antibodies in the bovine claw differed from those of equine keratins, as has been reported previously (Cooper & Sun, 1986; Wattle, 1998). On the other hand, the tissue distribution of bovine epidermal cytokeratins was similar to that in the equine hoof. Accordingly, equine CK14 (Grossenbaugh & Hood, 1992, 1993; Wattle, 1998) and CK5/6 (Wattle, 1998) were located by immunocytochemistry in basal epidermal cells at several claw locations, and CK10 was found suprabasally in the equine hoof epidermis (Pellman *et al.* 1993; Wattle, 1998). The study was not designed to examine differences in keratin distribution with age or parity. However, recent histological examination of healthy heifer claw tissue suggests that this is unlikely to differ significantly between primiparous and multiparous cows.

Immunoblotting of keratins resolved by one- or two-dimensional electrophoresis showed that cytokeratin composition of the epidermis was not altered by ulceration. Comparison of acidic and basic cytokeratin composition and CK9 content of healthy and diseased bovine solar tissue supports this conclusion (Meyer *et al.* 1998). The difference between healthy and diseased tissue lay instead in the higher overall rate of protein synthesis exhibited by the latter, and in the altered distribution of some cytokeratins between basal and supra-basal layers of the epidermis. The higher rate of protein synthesis measured by incorporation of [^{35}S]-labelled amino acids was not specific to the cytokeratins, nor indeed, to any of the keratin subtypes examined in this study, since fluorography of [^{35}S]-labelled proteins showed no difference in band identity or relative intensity. Abnormal keratinization observed during laminitis (Kempson & Logue, 1993) appears not, on this evidence, to be due to altered expression of individual keratin subtypes nor, conversely, are specific keratins induced in regenerating new, healthy horn. Instead, the biochemical analyses performed in this study suggest that synthesis of claw cytokeratins is co-ordinately regulated, such that there is negligible change in cytokeratin subtype composition in diseased or ulcerated tissue. One exception may be CK16, which was detected predominantly in damaged sole tissue. CK16 is expressed in highly proliferating supra-basal keratinocytes in pathological disease (Weiss *et al.* 1984) and during wound healing (Mansbridge & Knapp, 1987).

The apparently similar synthesis rates of keratin subtypes in healthy and diseased tissue (see below) suggests that supra-basal expression of CK5 and CK14 is due to loss of positional cues to the basal cells, rather than to aberrant CK5 or CK14 synthesis by a population of supra-basal cells which would not normally do so. Supra-basal relocation of basal cytokeratins has also been observed in equine laminitis (Grossenbaugh & Hood, 1993) and may be due to basement membrane damage, an early feature of the disease process in both the horse (Pollitt, 1996) and cow (Kempson & Logue, 1993). Loss of basement membrane interaction is implicated

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in the aberrant cellular differentiation associated with a number of pathological epidermal conditions (Furness, 1997; Suter *et al.* 1997). Similarly, cytokeratin subtype expression is altered in bovine claw cells cultured in the absence of a basement membrane (Kitahara & Ogawa, 1994). Loss of basement membrane integrity may in turn be the result of extracellular protease activity: basement membrane damage at ulcer sites is associated with gelatinase expression in both equine (Johnson *et al.* 1998; Pollitt & Daradka, 1998; Pollitt *et al.* 1998) and bovine hooves (KAK Hendry, unpublished results). It should, however, be remembered that protein synthesis measured biochemically by incorporation of [^{35}S]-labelled amino acids is an average for all the tissue types represented in explant cultures. In such circumstances, a localised increase in the synthesis of specific cytokeratins (in suprabasal cells, for example) could be masked if the proteins are expressed more generally in other cell types.

This study suggests that keratin synthesis rate is indeed altered in the diseased bovine claw, but that this is part of a co-ordinated regulation of epidermal protein synthesis associated with development of or, as in the present case, regeneration of normal keratinized horn. On the other hand, appearance of CK5 and CK14 in suprabasal epidermal tissue indicates a cytokeratin imbalance at ulcer sites, and suggests that a loss of keratinocyte topology contributes to the structural deterioration associated with these lesions. The changes found in keratin distribution are subtle but the potentially important impact of altered protein synthesis on keratin deposition and claw integrity provide an incentive to define the factors that control both protein synthesis and keratinocyte proliferation in the bovine claw. Elucidation of the nature of the biochemical lesions that precipitate abnormal keratinization and therefore horn deterioration should, in turn, contribute to the design of measures for preventing or treating lameness.

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