



In vitro evidence for a bacterial pathogenesis of equine laminitis

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Abstract

Utilizing an in vitro laminitis explant model, we have investigated how bacterial broth cultures and purified bacterial proteases activate matrix metalloproteinases (MMPs) and alter structural integrity of cultured equine lamellar hoof explants. Four Gram-positive *Streptococcus* spp. and three Gram-negative bacteria all induced a dose-dependent activation of MMP-2 and MMP-9 and caused lamellar explants to separate. MMP activation was deemed to have occurred if a specific MMP inhibitor, batimastat, blocked MMP activity and prevented lamellar separation. Thermolysin and streptococcal pyrogenic exotoxin B (SpeB) both separated explants dose-dependently but only thermolysin was inhibitable by batimastat or induced MMP activation equivalent to that seen with bacterial broths. Additionally, thermolysin and broth MMP activation appeared to be cell dependent as MMP activation did not occur in isolation.

These results suggest the rapid increase in streptococcal species in the caecum and colon observed in parallel with carbohydrate induced equine laminitis may directly cause laminitis via production of exotoxin(s) capable of activating resident MMPs within the lamellar structure. Once activated, these MMPs can degrade key components of the basement membrane (BM) hemidesmosome complex, ultimately separating the BM from the epidermal basal cells resulting in the characteristic laminitis histopathology of hoof lamellae. While many different causative agents have been evaluated in the past, the results of this study provide a unifying aetiological mechanism for the development of carbohydrate induced equine laminitis. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Horse matrix metalloproteinase; Laminitis; *Streptococcus* spp.; MMP activation; Thermolysin; SpeB

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1. Introduction

Numerous clinical conditions, seemingly unrelated, have been linked to the pathophysiology of laminitis (Field and Jeffcott, 1989; Baxter, 1994) all of which ultimately result in a characteristic histopathology (Pollitt, 1996). Despite this, no specific aetiological mechanism has been elucidated and a unifying theory, explaining laminitis pathogenesis, has yet to be achieved. The proposal that sublamellar ischaemia is responsible for a cellular failure, that culminates in detachment of the dermal–epidermal junction in the hoof, is widely held (Coffman et al., 1970; Garner et al., 1975; Hood et al., 1978; Hinckley et al., 1996; Hood, 1999). However, several studies have consistently showed increased sublamellar blood flow during the developmental phase of laminitis (Robinson et al., 1976; Trout et al., 1990; Pollitt and Davies, 1998).

Recent investigations by Pollitt and co-workers have shown that matrix metalloproteinases (MMPs), specifically gelatinases A and B, appear to play a key role in a selective degradation of basement membrane (BM) proteins, that leads to a failure of the BM–epidermal attachment apparatus. Progressive degradation of the major structural components of the BM, collagens IV and VII in addition to laminin occurs in acutely laminitic hooves (Pollitt and Daradka, 1998). Additionally, Pollitt et al. (1998) and Johnson et al. (1998) reported increased gelatinase activity in hoof lamellar homogenates from laminitic horses. There is an increase in MMP-2 (62 kDa active form) and MMP-9 (92 kDa latent form) activity in supernatants from explant cultures of laminitic horses, while laminar homogenates showed an increase in MMP-9 (92 kDa latent form) (Pollitt et al., 1998). Johnson et al. (1998) also reported increase in MMP-2 (both 72 kDa latent and 66 kDa active forms) and MMP-9 (92 kDa latent form) activity in hoof lamellar homogenates. RT-PCR analysis of homogenates of hoof lamellae from horses with acute laminitis shows upregulation of the MMP-2 gene (Kyaw-Tanner, unpublished observation).

A phenomenon commonly associated with the onset of laminitis in horses is a rapid alteration in the composition of the hindgut microflora (Garner et al., 1978) that results in a deterioration of the epithelia lining the caecal lumen (Krueger et al., 1986). This is followed by an increase in the circulating plasma levels of endotoxin (Sprouse et al., 1987) indicating an increase in caecal permeability to large molecules. The usual model for inducing experimental laminitis is the deposition of excess starch into the stomach of horses. After a few hours the starch reaches the hindgut and is rapidly fermented to acid, principally L-lactate (Al Jassim and Rowe, 1999). *Streptococcus bovis* is the key bacterium responsible for fermenting starch in the equine hindgut and in the presence of excess substrate and despite a low pH, *S. bovis* rapidly proliferates to become the dominant hindgut microbe.

Many bacteria rely on virulence factors to facilitate their invasion and pathogenesis within the host. Among these virulence factors are bacterial products which have been shown to activate MMPs (Matsumoto et al., 1992; Okamoto et al., 1997; Maeda et al., 1998). Group A streptococci produce an extracellular cysteine protease identified as streptococcal pyrogenic exotoxin B (SpeB) (Musser, 1997). SpeB has been implicated as a critical virulence factor in both group A streptococcal human infections (Musser, 1997) and mouse models of invasive streptococcal disease (Lukowski et al., 1997; Kuo et al.,

1998). Additionally, it activates MMP-2 (Burns et al., 1996). *Pseudomonas aeruginosa* elastase, a member of the thermolysin (M4) protease family, has been shown to activate human fibroblast type collagenase (MMP-1) and neutrophil procollagenase (MMP-8) (Sorsa et al., 1992) in addition to activating MMP-2 (Matsumoto et al., 1992). Further research by Okamoto et al. (1997) demonstrated similar activation by *Vibrio cholerae* protease and thermolysin, also members of the M4 protease family.

Interestingly, horses can survive alimentary carbohydrate overload without developing laminitis if *S. bovis* has been eliminated from the hindgut microflora by the prior oral administration of the antibiotic virginiamycin (Rowe et al., 1994). We reasoned that if laminitis is prevented by the absence of *S. bovis* then it may be caused by its presence. Perhaps uncontrolled proliferation of *S. bovis* generates a substance that penetrates the mucosal barrier of the hindgut, enters the circulation and reaches the lamellar BM via the increased sublamellar blood flow reported by Pollitt and Davies (1998). Previous studies utilising this model have revealed a good correlation between the histopathology of explant lesions (Pollitt et al., 1998) with those observed after clinical laminitis (Pollitt, 1996). While the specific dermal–epidermal separation is histologically similar, in vivo laminitis is associated with considerably more BM destruction than that seen in this in vitro model. Manipulation of culture conditions have confirmed that explants are viable up to 8 days in culture (Pass et al., 1998) and can be induced to consistently separate if lamellar MMPs are activated (Pollitt et al., 1998). The lamellar explant model is thus a valid technique for screening numerous putative laminitis causative factors, with the added advantage of avoiding live animal experimentation.

In the current study, we use this in vitro laminitis model to investigate the possibility that a bacterially produced substance can activate resident lamellar MMPs and thus play a role in the pathogenesis of laminitis.

2. Materials and methods

2.1. Chemicals

SpeB stabilized with 0.03% sodium tetrathionate (NaTT, Sigma, St. Louis, Mo) was provided by Dr. J. Cooney (Massey University, Palmerston North, New Zealand). SpeB protein concentration was estimated by A_{280} to be 0.5–2 mg/ml (NaTT interferes with sample absorbance), all concentrations used here have assumed an initial stock concentration of 2 mg/ml. Prior to use SpeB was activated with 10 mM dithiotreitol (DTT, Sigma, St. Louis, Mo). Ammonium sulfate was purchased from Thomas Baker Chemicals (Mumbai, India). Batimastat (BB94) was provided by Dr. E. Bone (British Biotech., Oxford, UK). EDTA was purchased from BDH (Poole, UK). Calcium chloride, sodium azide, gelatin, tris base, PMSF, leupeptin, pepstatin A, thermolysin and lipopolysaccharide were purchased from Sigma (St. Louis, Mo). Gentamycin was purchased from GibcoBRL (Grand Island, NY). Acrylamide was purchased from BioRad (Hercules, CA). Coomassie Blue G-250 was purchased from LKB (Villeneuve-la-Garenne, France).

2.2. Bacterial broth cultures

S. bovis (GDS) derived from equine caecum was a gift from Dr. J. Rowe and Dr. R. Al Jassim (University of New England, Armidale, Australia). *Escherichia coli* (*E. coli*), *Enterobacter aerogenes*, *S. pyogenes* (GAS), *S. agalactiae* (GBS), *S. dysgalactiae* (GCS), *S. zooepidemicus* (GCS), and *Pasteurella multocida* were all obtained from the Veterinary Pathology Culture Collection (University of Queensland, St. Lucia, Australia).

All bacteria were inoculated on SBA plates and cultured aerobically overnight at 37 °C. After primary inoculation, 20 ml of TSB was inoculated and incubated for 6 h on rollers. This culture was inoculated into 500 ml of Dulbecco's Modified Eagle's Media (D-MEM) and incubated anaerobically at 37 °C for 18 h. Cultures were then centrifuged at 1500g for 15 min, the supernatant was removed and the glucose and pH were adjusted to 25 mM and 7.0, respectively. The supernatant was then 0.22 µm filtered into sterile tubes, supplemented with 0.1 mg/ml gentamycin and stored at 4 °C until used for explant culture. Broth supernatants were cultured with explants as straight broth (10:10), or diluted with D-MEM containing 0.1 mg/ml gentamycin as 2 parts broth with 8 parts D-MEM (2:10), 4 parts broth with 6 parts D-MEM (4:10), 6 parts broth with 4 parts D-MEM (6:10) or 8 parts broth with 2 parts D-MEM (8:10).

Bacterial broth supernatants were concentrated by ammonium sulfate precipitation as follows: Broths grown either with or without 0.03% NaTT were centrifuged to remove cells. The broth supernatant was then centrifuged at 12,000g for 30 min at 4 °C. Supernatant was removed and 70% (sat.) ammonium sulfate was added while stirring until dissolved. Once dissolved, the solution was stored at 4 °C overnight then centrifuged at 1500g for 60 min at 4 °C. The supernatant was then decanted and the precipitate redissolved in D-MEM containing 0.03% NaTT as appropriate. This precipitate was then dialyzed overnight against 5 l dH₂O containing 0.03% NaTT as appropriate. Protein estimation was performed as described by Watanabe et al. (1986) using a protein estimation kit (Randox, Crumlin, UK) run on a Cobas Mira automated analyzer (Roche Diagnostic Systems, Basel, Switzerland). Dialysate was then aliquoted and stored at –80 °C until used for explant culture.

2.3. Explant culture

Hooves from normal horses obtained from a commercial abattoir were transported in ice to the dissection room within 60 min of humane slaughter. Hooves were dissected to obtain pieces of tissue approximately 6 mm by 6 mm extending from the inner hoof wall through the lamellar junction to the dermal connective tissue (Pollitt et al., 1998). Duplicate explants were incubated in D-MEM containing either purified bacterial products SpeB (1–10 mg/ml) or thermolysin (100 ng/ml–100 mg/ml) or concentrated broth supernatants (1–100 mg/ml) prepared as described above. Alternatively, tissues were incubated in spent bacterial broth supernatant with glucose and pH values equivalent to normal D-MEM at 37 °C, 5% CO₂ and 95% relative humidity for 48 h.

One explant from each duplicate was assessed for structural integrity by strain gauge assessment as follows. One end of the explant was immobilized, the other was attached to a calibrated force transducer. Tissues were stretched up to a maximum of 900 g.

Separation forces were averaged over five experiments and statistical analysis performed using a repeated measures analysis of variance procedure (Dunnett's test). Aliquots of all media were stored at -80°C until assessed by SDS-PAGE zymography. Tested explants were pooled with their untested duplicate and either rapidly frozen in liquid nitrogen and stored at -80°C or post-fixed in 1.6% formaldehyde and processed for histological staining (as described below).

Cell free MMP activation was examined using spent explant media subsequently treated with SpeB (1 ng/ml–10 $\mu\text{g/ml}$), thermolysin, concentrated broth supernatants or spent broth media with or without batimastat (100 μM) in a tube assay system (20 μl media, 16 μl treatment compound; 8 μl incubation buffer: 50 mM Tris-HCl, 5 mM calcium chloride and 0.02% sodium azide; final reaction volume 40 μl). Components were added, vortexed briefly, pulse centrifuged and incubated at 37°C for 15, 30, 60 or 120 min. Samples were immediately frozen at -80°C until assessed by SDS-PAGE zymography.

2.4. SDS-PAGE zymography

Samples were electrophoresed for 60 min with 30 mA current/gel at 10°C on 7.5–15% gradient polyacrylamide gels containing 0.1% gelatin under non-reducing conditions as described previously (Pollitt et al., 1998). After 60 min gels were washed for 2×30 min in 2.5% Triton X-100 (Sigma) then incubated for 24 h at 37°C in incubation buffer. Gels were then stained with Coomassie blue G-250 (30 min), destained with 5% acetic acid and 2% glycerol in water (24 h), dried using a BioRad gel dryer and scanned using an HP Scanjet 3C flatbed scanner (Hewlett Packard) into densitometry analysis software GelPro (Media Cybernetics, Silver Springs, CO). Integrated optical density (IOD) measurements were averaged from five experiments and statistical analysis performed using a repeated measures analysis of variance procedure (Dunnett's test).

2.5. Histology

Explants were post-fixed in 4% paraformaldehyde for a minimum of 24 h before processing and embedding in wax. Serial sections of 10 μm were mounted on silanized glass slides, air-dried overnight and stained histochemically with haematoxylin and eosin or periodic acid-Schiff (PAS). Sections were dehydrated using ascending alcohol solutions, cleared with xylene and coverslipped using Depex mounting media (Gurr, BDH, UK). Slides were examined with an Olympus BX50 microscope and images captured and stored on a Pentium II PC (Scientific Instruments and Optical Supplies, Normanby, Australia) via a JVC 3-CCD digital camera (JVC, Yokohama, Japan) utilizing a Flashbus MV Pro frame grabber card (Integral Technologies, Indianapolis) and analyzed using ImagePro software (Media Cybernetics).

2.6. Statistical analyses

All data from explant separation trials and zymographic analysis was analyzed using SigmaStat statistical software (Jandel Scientific Software, San Rafael, CA). Analysis of

variance was performed on all treatment groups and compared pairwise to control groups (Dunnett's test) with significance reported when $p < 0.05$. In all experiments $n = 5$.

3. Results

3.1. Explant separation

Lamellar explants incubated in the presence of SpeB separated dose-dependently (Fig. 1a). This separation was only partially inhibited by the addition of batimastat (Fig. 1a) or EDTA (data not shown), which ultimately had little or no effect at higher concentrations of SpeB. In contrast, explant separation by thermolysin, also dose-dependent, was markedly inhibited by the addition of batimastat or EDTA such that a 2.5- and 5-fold increase in thermolysin concentration was required to produce the same degree of separation in the presence of 50–100 μM batimastat, respectively (Fig. 1b).

Explants separated when exposed to undiluted bacterial broth (straight broth = 10:10) but varied significantly in their ability to separate explants when specific inhibitors were added (Table 1). Explants exposed to GAS broth separated when diluted to 7 parts in 10 (data not shown) and were significantly inhibited by batimastat and pepstatin A (35 and 34%, respectively, Table 1). Both leupeptin and PMSF significantly enhanced explant separation indicating possible toxicity effects. GBS caused explant separation to a similar level as GAS but this effect was not significantly inhibited by any of the inhibitors tested. Again, the serine protease inhibitor PMSF appeared to exacerbate explant separation although this was not significantly different from controls.

GCS separated explants more readily than either GAS or GBS but also appear to be more susceptible to inhibition by a wider range of compounds (Table 1). Batimastat and aprotinin completely abolished separation while pepstatin A was not quite as effective, but still significantly reduced the amount of separation. Additionally, both EDTA and calpain inhibitor II appeared to have considerable inhibitory activity (63 and 59%, respectively) on GCS mediated explant separation (Table 1). GDS was even more potent with respect to explant separation than GCS but in contrast was only significantly inhibited by the specific MMP inhibitor batimastat ($p < 0.001$).

For comparison, several Gram-negative bacterial strains were also tested for explant separation. *E. coli* (Table 1), *E. aerogenes* (data not shown) and *P. multocida* (data not shown) all induced explant separation similar to that observed with the Gram-positive streptococci above. *E. coli* mediated explant separation was only inhibitable by the addition of MMP inhibitors batimastat and EDTA (85 and 78%, respectively, Table 1). Additionally PMSF significantly enhanced explant separation by *E. coli*.

When broths were concentrated by ammonium sulfate precipitation, all broths were completely devoid of any ability to separate explants when tested (data not shown). To ensure that any separations due to bacterial broths were not simply the result of some common artifact, explants were also tested against variations in the media pH and osmolarity, in addition to incubation in the presence of the Gram-negative cell wall component, endotoxin (lipopolysaccharide, LPS). All broths or treatments tested fell within the pH range 6–8.5 both pre- and post-culture. Control media titrated to these

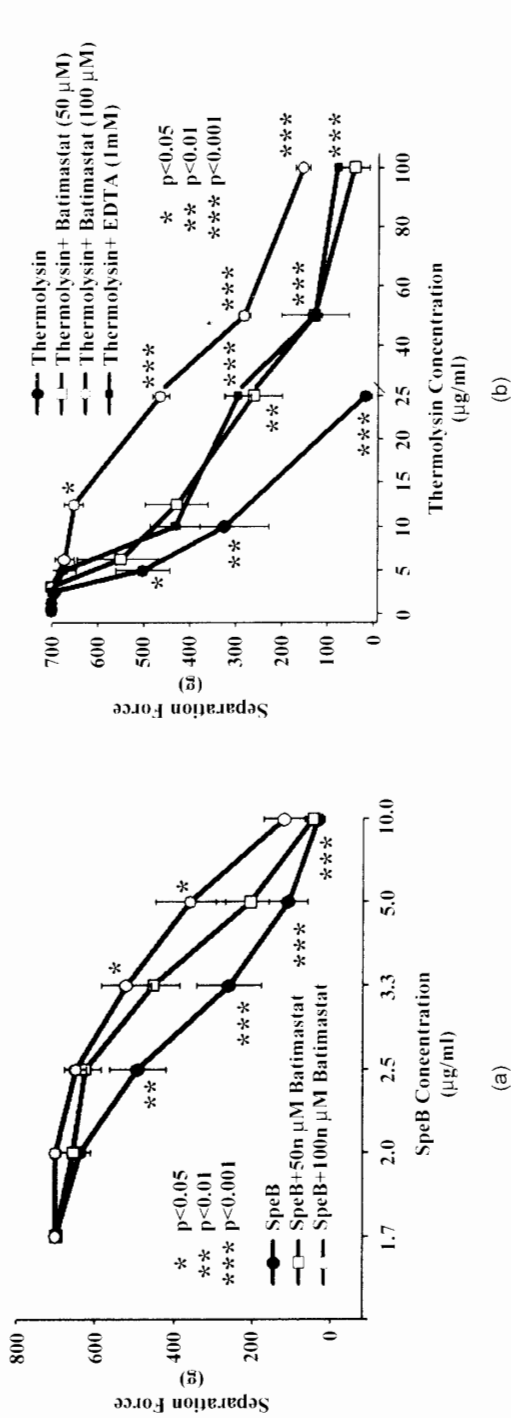


Fig. 1. Dose-response curves for SpeB (a) and thermolysin (b) on explant separation in culture. Inhibition by the specific MMP inhibitor, batimastat, is dose-dependent, confirmed for thermolysin by incubation with a non-specific metalloprotease inhibitor, EDTA (mean) ± (S.E.). For SpeB and thermolysin curves, significance is calculated compared to untreated controls (D-MEM), while significance for inhibitor curves is calculated compared to uninhibited treated explants (SpeB and thermolysin).

Table 1
Inhibition of bacterial broth-mediated explant separation in culture^a

Bacterial strain	Inhibition (%)						
	BB94	EDTA	Aprotonin	Leupeptin	Calpain inhibitor II	Pepstatin A	PMSF
<i>S. pyogenes</i>	35.71 ^{bc}	-10.48	-8.57	-19.52 ^{abc}	12.38	34.29	-66.67 ^{cd}
<i>S. agalactiae</i>	53.91	36.55	26.21	38.16	-12.30	30.00	-229.43
<i>S. dysgalactiae</i>	100.00 ^{cd}	63.08	100.00 ^{cd}	9.94	59.83	78.58 ^c	-16.61
<i>S. bovis</i>	63.41 ^{abc}	9.78	19.35	17.60	15.72	5.85	-6.92
<i>S. zooepidemicus</i>	94.74 ^{cd}	54.39 ^c	56.84	2.63	37.02	26.49 ^{bc}	22.98
<i>E. coli</i>	85.00 ^c	78.33 ^c	43.33	-91.67	30.00	-33.33	-325.00 ^d

^a Values shown are the percentage by which explant separation induced by straight bacterial broth (10:10) is inhibited by the addition of various inhibitors.

^b $p < 0.05$.

^c $p < 0.01$.

^d $p < 0.001$.

extremes pre-culture had no influence on explant integrity after 48 h culture (data not shown). Similarly, explants incubated with either fructose or mannitol (10–30 mM) to increase the media osmolarity showed no difference in separation profiles (data not shown). Addition of LPS alone produced some explant separation but this effect was not consistent and did not appear to be dose-dependent (data not shown).

3.2. SDS-PAGE zymography

SpeB had no effect on gelatinase activation in explant culture (data not shown), whereas thermolysin produced a dose-dependent activation of MMP-2, converting the latent pro-enzyme form (72 kDa) to smaller active forms (66–62 kDa) and degrading the latent pro-enzyme form of MMP-9 (92 kDa), usually producing small amounts of 82 kDa fragments (active MMP-9) detectable by zymography (Fig. 2a). This activation was not affected by addition of any of the inhibitors listed above, including the MMP inhibitors batimastat and EDTA (data not shown).

Similarly, all bacterial broths tested showed a dose-dependent activation of pro-MMP-2 in addition to a potent degradation of pro-MMP-9 (Fig. 2b). Corroborating the separation results, different strains appeared to have different potencies for activating MMPs (data not shown). As with separation, broth concentrates had no effect on MMP activation (data not shown).

3.3. Histology

Explants incubated in D-MEM alone reveal a tight connection between the epidermal basal cells and the overlying BM (Fig. 3a arrows). Explants cultured with SpeB (Fig. 3b) or thermolysin (Fig. 3c) revealed an extensive separation occurring at the dermal–epidermal junction (Fig. 3b and c, arrows). Additionally, SpeB or thermolysin mediated separation also involved considerable destruction of the BM and adjacent epidermis

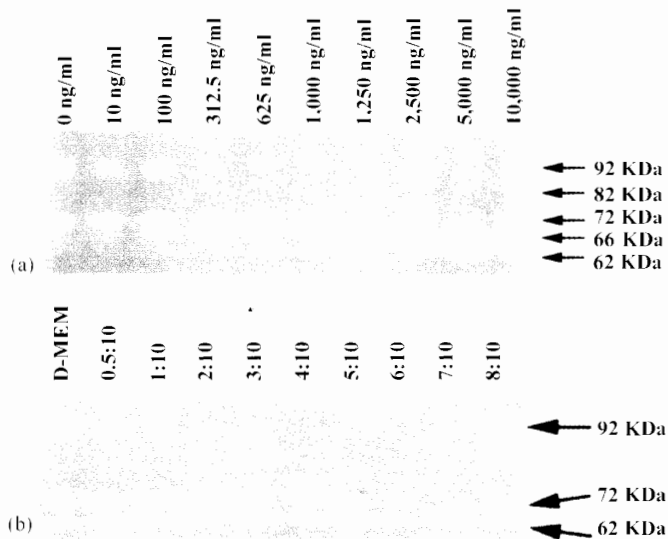


Fig. 2. (a) Representative zymograms of (b) thermolysin and (c) bacterial broth (*S. bovis*) mediated MMP activation in hoof explant culture. Molecular weights are indicated for each figure.

(Fig. 3b, arrowheads). In contrast, explants incubated with *S. bovis* broth showed a clear separation of the BM from the epidermal cells with no evidence of BM proteolysis (Fig. 3d arrows). All broths tested showed a similar BM separation, only *S. bovis* has been shown as a representative case.

4. Discussion

Many bacteria have novel mechanisms to facilitate host invasion. Bacterial invasion may be mediated by the direct activity of one or more proteases produced by the invading bacteria or indirectly via activation of host enzyme cascades (Matsumoto et al., 1992; Maeda and Yamamoto, 1996; Okamoto et al., 1997; Maeda et al., 1998). Alternatively, various pathologies result from the production of superantigens or similar immune system modulating compounds (Musser, 1997). These studies suggest that bacterial exotoxins, commonly proteases, may specifically activate host enzymes, either at the site of invasion or remotely via the circulation thus overwhelming the tight regulation of enzyme activity which normally prevails (Woessner, 1991).

We have demonstrated that the cysteine protease SpeB induces explant separation in culture by direct proteolysis of BM components (Elliot, 1945; Musser, 1997) while the bacterial metalloprotease, thermolysin mediates explant separation via MMP activation. SpeB has been shown to directly degrade many components of the ECM including fibronectin and vitronectin (Kapur et al., 1993b), casein, gelatin, human milk and fibrin (Elliot, 1945). Additionally, SpeB has been shown to have superantigen properties demonstrated by pyrogenicity, enhanced susceptibility to endotoxin-mediated shock,

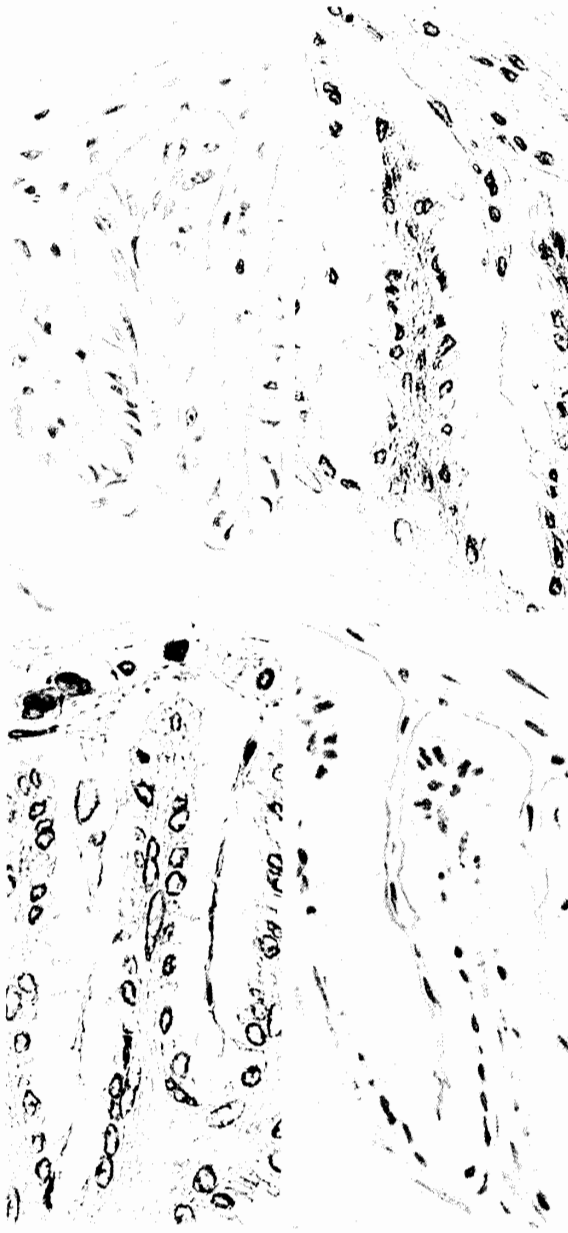


Fig. 3. Histological evaluation of explants after culture. Untreated explants (a) exhibit intimate attachment between the BM (arrows) and the epidermal basal cells (EBC), while explants incubated with SpeB (b) show extensive BM–epidermal separation (arrows). Additionally, SpeB mediated separation also involved considerable destruction of the BM and adjacent epidermis (arrowheads). In contrast, explants incubated with *S. bovis* broth (c) showed a clear separation of the BM from the epidermal cells with no evidence of BM proteolysis (arrows). All broths tested showed a similar BM separation, only *S. bovis* has been shown as a representative case. Scale bar represents 20 μ m.

nonspecific mitogen activity and lymphocyte proliferative activity (Musser, 1997). However, superantigen functions are unlikely to be effective in an isolated in vitro system. SpeB has previously been shown to cleave the IL-1 β precursor to active IL-1 β , thus producing a major mediator of inflammation (Kapur et al., 1993a) although again, in an isolated in vitro system this is unlikely to play a role in dermal–epidermal separation.

Previously, SpeB has been reported to produce a time and concentration dependent activation of MMP-2 in culture (Burns et al., 1996). We found no such activation of MMPs in hoof explant culture with SpeB although it had a potent proteolytic effect on BM and dermal–epidermal integrity.

Thermolysin, in explant culture, was a potent activator of MMP-2 and MMP-9. Thermolysin has an inherent gelatinase activity of its own, however, this was only apparent at considerably higher thermolysin concentrations than that required to activate lamellar MMPs (Mungall and Pollitt, unpublished data). Thermolysin mediated explant separation was inhibitable by MMP inhibitors indicating either a direct inhibition of thermolysin itself, or an inhibition of the lamellar MMPs activated by thermolysin. However, we have also shown in the current study that MMP inhibitors do not prevent thermolysin from activating MMPs. Thus we conclude that the prevention of explant separation by batimastat is due, not to inhibition of thermolysin, but inhibition of lamellar MMP activity.

To elucidate a link between the pathophysiology of equine laminitis and bacterial trigger factors, we tested the ability of streptococci from four Lancefield groups, two Gram-negative gastro-intestinal tract (GIT) resident strains, in addition to one strain of *Pasteurella* to cause separation of cultured explants. All bacterial strains tested caused MMP-2 activation and MMP-9 degradation similar to that caused by thermolysin. This activation occurred in parallel with separation of BM from the epidermis of hoof explants, ultimately resulting in complete separation of the dermis from the epidermis. For most strains, the separation was partially inhibitable by addition of batimastat, suggesting a role for MMPs in this process. *S. pyogenes* mediated separation was equally inhibited by batimastat and pepstatin A indicating a broader mechanism of action. Given that SpeB is produced by *S. pyogenes* (confirmed by PCR results, Kyaw-Tanner, personal communication), it is likely that *S. pyogenes* mediated separation is occurring as a result of either direct protease activity (possibly due to SpeB) or indirectly via MMP activation (via some other exotoxin produced).

All attempts at concentrating the factor(s) in bacterial broths responsible for explant separation by ammonium sulfate precipitation resulted in complete loss of activity. It is possible that the compound responsible for explant separation is highly unstable although, the fact that broths retain considerable explant separation activity after long-term (>1 month) storage at 4 C (Mungall, unpublished observations) indicates this possibility is remote. More likely, the soluble factor(s) initiating explant separation may be very small peptides, un-extracted by ammonium sulfate precipitation, or alternatively, not of protein origin at all. The release of lipopolysaccharide (LPS, endotoxin) components from the cell wall of Gram-negative bacteria as a result of cell lysis is well established (Moore et al., 1979; Sprouse et al., 1987). While a Gram-positive equivalent to endotoxin has not been formally identified, the duration of broth growth will influence the production of cell lysis components. All cultures were incubated for 18 h placing

them in late log phase growth perhaps initiating considerable cell death and thus release of such compounds.

As explants could be separated by all the bacterial strains tested, we carried out auxillary studies to confirm the separation was not the result of artifact such as modification of the media due to bacterial growth. Media titrated to extreme pH (both low and high pH) had no effect on explant separation above pH 4 (data not shown) as did manipulating the osmolarity of the media prior to culture (data not shown).

Endotoxin (LPS) concentrations increase in the circulation during the developmental phase of carbohydrate induced laminitis (Sprouse et al., 1987). Clinically, laminitis is commonly associated with GIT insults, either directly via excessive carbohydrate intake (Baxter, 1994; Slater et al., 1995) or secondary to other GIT disturbances such as colic (Slater et al., 1995). In the carbohydrate overload situation there is a proliferation of lactic acid producing anaerobic streptococci within the equine caecum (Garner et al., 1978; Moore et al., 1979) and an associated demise of Gram-negative species that produces a rapid increase in both caecal (Moore et al., 1979) and plasma (Sprouse et al., 1987) endotoxin concentrations. The endotoxemia accompanying laminitis induction has been thoroughly investigated as a putative trigger factor for equine laminitis without success (Moore et al., 1986, 1989). The role of Gram-positive bacteria that are responsible for most of the fermentation of the carbohydrate in the equine bowel has been largely overlooked other than as a source of lactic acid production (Garner et al., 1978).

A natural variant of SpeB contains an integrin binding sequence, RGD, commonly recognized by integrin receptors (Stockbauer et al., 1999). This enables SpeB to attach at integrin containing sites and promote proteolysis, thus contributing to the increased virulence of *S. pyogenes* strains that produce RGD containing SpeB. A synthetic platelet aggregation inhibitor, which competes for RGD binding sites on platelet integrins, prevented laminitis caused by alimentary starch overload (Weiss et al., 1998). The authors concluded that laminitis may have been prevented because of inhibition of platelet aggregation. We propose the alternative that laminitis may have been prevented by the RGD containing peptide competing with bacterial proteases for integrin receptors within the lamellae. The possibility of an RGD bearing *S. bovis* exotoxin contributing to equine laminitis has not previously been considered. An RGD sequence is critical for ligand recognition by many integrins (Wickham et al., 1993). The presence of this sequence in bacterial proteases would greatly enhance its virulence by enabling it to physically bind to cellular and BM surfaces.

If *S. bovis* contains an RGD sequence this may explain why an *S. bovis* exotoxin can attach to the β -integrin of hemidesmosomes and, over time, if the circulation is vasodilated, cause disruption of lamellar BM. This possibility is worthy of further investigation. A correlation between sublamellar vasodilation and laminitis has been established (Pollitt and Davies, 1998).

In conclusion, we propose that Gram-positive bacterial proliferation in the equine hindgut may be the source of the circulating trigger factors that cause equine laminitis. We have demonstrated that "in vitro laminitis" is triggered by spent bacterial broths in association with the activation of lamellar MMP-2 and MMP-9. We suggest that the rapid proliferation of *S. bovis* (or some other GIT resident Gram-positive species) in response to favorable changes in the GIT nutrient environment (induced by carbohydrate

overload), results in a massive increase in the circulating concentration of *S. bovis* trigger factor(s). These trigger factors probably adhere to BM components, specifically β -integrins (possibly via an RGD motif), where they are free to activate locally produced gelatinases. Once activated, these gelatinases may degrade crucial structural elements of the hemidesmosome–BM complex, ultimately resulting in separation of the BM from the epidermal basal cells thus producing the characteristic histopathology associated with equine laminitis.

The results of the current study indicate a primary role for bacterial exotoxins in the development of equine laminitis. Elucidation of the specific exotoxins involved may lead to the development of effective prophylactic treatment for equine laminitis.

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