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# Potential Involvement of Gelatinases and Their Inhibitors in *Mannheimia haemolytica* Pneumonia in Cattle

Amanda E. Starr,[1] Tonima Dan,[1] Kanwal Minhas,[1] Patricia E. Shewen,[2] and Brenda L. Coomber[1]*

*Departments of Biomedical Sciences[1] and Pathobiology,[2] Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada*

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***Mannheimia haemolytica*** **infection of the lower respiratory tract of cattle results in a bronchofibrinous pneumonia characterized by massive cellular influx and lung tissue remodeling and scarring. Since altered levels of gelatinases and their inhibitors have been detected in a variety of inflammatory conditions and are associated with tissue remodeling, we examined the presence of gelatinases in lesional and nonlesional lung tissue obtained from calves experimentally infected with *M. haemolytica*. Lesional tissue had elevated levels of progelatinase A and B and active gelatinase A and B when compared with nonlesional tissue obtained from the same lung lobe. In vitro, *M. haemolytica* products stimulated production of gelatinase B, but not its activation, by bovine monocytes. Alveolar macrophages showed constitutive production of gelatinase B but no change in response to *M. haemolytica* products. Bovine neutrophils exposed to *M. haemolytica* products also released gelatinase B, and there was a significant increase in the activated form of this enzyme. These effects were virtually identical when recombinant *O*-sialoglycoprotease was used to stimulate these cells. *M. haemolytica* products also enhanced the expression by bovine monocytes and alveolar macrophages of the tissue inhibitor of metalloproteinase 1. Our results provide evidence that matrix metalloproteinases are activated in lung lesions from cattle with shipping fever and that *M. haemolytica* virulence products induce production, release, and especially activation of gelatinase B by bovine inflammatory cells in vitro.**

Bronchofibrinous pneumonia (shipping fever) is a respiratory disease in cattle that is characterized by cellular influx and interalveolar and interlobular fibrosis in the lungs of affected animals (3, 7, 18, 20, 64, 65). Lung lesions consistently show areas of coagulation necrosis, extensive fibrin deposits, and intense cellular infiltration into the alveoli (5, 63, 65). Economic losses to the beef industry due to shipping fever are estimated at over one billion dollars per year in North America alone (64). Causes of this pneumonic condition are multifactorial, including a combination of stress or viral infection, with a final acute illness due most often to the bacteria *Mannheimia* (*Pasteurella*) *haemolytica* serotype A1 (16, 39, 64). *M. haemolytica* serotype A1 is a normal resident of the upper respiratory flora of most cattle, but when aspirated into the lower respiratory tract of immunocompromised animals, it is able to colonize the lungs and induce an inflammatory reaction (65).

*M. haemolytica* was originally classified in the genus *Pasteurella* (50), but more recent genetic characterization has resulted in reclassification of 11 serotypes of biotype A in the new genus *Mannheimia* (4, 9). *M. haemolytica* is a trehalose-negative coccobacillus in which heat-stable lipopolysaccharide (LPS) constitutes 12 to 25% of the dried cell wall (66). During logarithmic growth, the bacteria produce and release a heat-labile exotoxin, termed leukotoxin (Lkt), and a heat-labile enzyme, termed *O*-sialoglycoprotease (Gcp) (1, 31). Both LPS and Lkt have been implicated in initiating an inflammatory response in cattle in vivo, likely through their activities on leukocytes (20, 57, 59, 65).

LPS activates bovine alveolar macrophage expression and production of the proinflammatory cytokines tumor necrosis factor alpha, interleukin-1β (IL-1β), and IL-8 (25, 37, 67, 68), all of which are elevated in the lung tissue and bronchoalveolar lavage fluid of cattle affected with shipping fever (8, 33, 46, 68). Moreover, LPS complexes with and seems to act synergistically with Lkt (13, 28). *M. haemolytica* Lkt is a member of the RTX (repeat in toxin) family of toxins, which also includes cytolysins produced by *Escherichia coli* and *Actinobacillus* species (13, 60). The RTX toxins are characterized by the presence of glycine-rich repeats at the C termini, comparable mechanisms of secretion, genetic homology, and similar bioactivities (28, 62). The 105-kDa Lkt protein is produced as an inactive precursor that is activated by posttranslational acylation (28, 31, 62).

Lkt binds target cells via the β2 integrin lymphocyte function-associated antigen 1 (21). High doses of Lkt cause the lysis of ruminant leukocytes by membrane pore formation when paralleled by a rise in intracellular calcium levels (11, 12, 45, 52). Lower doses of Lkt are chemotactic and stimulate active degranulation of bovine neutrophils in vitro (32, 36). Mutation experiments showed that if Lkt is not produced by *M. haemolytica* or if an inactive Lkt is produced, cattle infected with the bacteria have reduced lung pathology, indicating the importance of Lkt in this disease pathogenesis (20, 59). Unlike the information available about LPS and Lkt, there has been little indication to date of the role of Gcp in bronchofibrinous pneumonia, although a recently published study showed that vaccination with a recombinant Gcp fusion protein induced some

* Corresponding author. Mailing address: Department of Biomedical Sciences, OVC, University of Guelph, Guelph, ON N1G 2W1, Canada. Phone: (519) 824-4120, ext. 54922. Fax: (519) 767-1450. E-mail: bcoomber@uoguelph.ca.

protection against experimental challenge with *M. haemolytica* (54). Gcp is a 35-kDa protein that selectively cleaves O-glycosylated glycoproteins from cell surfaces (2, 58). There is evidence that bovine platelets have altered adhesive properties (40) and human platelets have altered aggregation and degranulation responses (23) when incubated in the presence of Gcp. Further work is required to investigate the potential role of Gcp as an *M. haemolytica* virulence factor.

An inflammatory response is initiated to combat the activities of virulence factors during *M. haemolytica* infection in the lung. The inflammatory process generally begins when resident macrophages initiate a cascade of events that recruit leukocytes, including neutrophils and monocytes, from the circulation through the endothelium and basement membrane and into infected tissue, an event requiring matrix proteolysis (24, 42). Once in the tissue, leukocytes interact with bacteria and virulence factors and inflammatory mediators, become activated, and produce or release additional enzymes. These are able to, but may do more than, destroy invading pathogens. Damaged areas of tissue are replaced by abnormal scar tissue that in the case of animals affected with *M. haemolytica* leads to chronic health problems. Despite an understanding of the general processes of inflammatory cell recruitment and activation, there has been little work examining how *M. haemolytica* virulence factors are involved in stimulating host cell damage and remodeling tissue within the lungs of affected cattle.

Recent studies in other species have implicated matrix metalloproteinases (MMPs), in inflammatory disease-associated lung tissue damage. The family of MMPs comprises a group of zinc-binding proteases, released in a zymogen form, that are able to degrade at least one component of the extracellular matrix or basement membrane (39). Two MMPs of particular importance to lung tissue damage are gelatinase A (MMP-2; 72-kDa type IV collagenase) and gelatinase B (MMP-9; 92-kDa type IV collagenase), collectively termed the gelatinases. Gelatinase A is constitutively produced (at low levels) by macrophages, lymphocytes, dendritic cells, and fibroblasts, whereas gelatinase B is inducible in these cell types (44). Conversely, neutrophils do not produce gelatinase A but rather store gelatinase B in their granules; in bovine neutrophils gelatinase B is stored in tertiary granules (44, 29). As with other MMPs, the gelatinases are released as progelatinases that are activated after the removal of an 80-amino-acid residue propeptide by proteases such as plasmin or other MMPs (38, 43). Active gelatinases target type IV collagen and destroy basement membrane tissue and are also able to degrade other matrix components (43).

The proteolytic actions of MMPs are inhibited by noncovalent interaction with tissue inhibitors of metalloproteinases (TIMPs) (17). The regulation of gelatinase activity is complex, but generally it has been recognized that an upset in the balance of MMPs with their inhibitors is involved in the pathogenesis of inflammatory, pulmonary, and neoplastic diseases. Therefore, this study was designed to investigate the presence of gelatinases and their inhibitors in the lung tissue of animals infected with *M. haemolytica* and to determine if gelatinase or inhibitor levels are altered by *M. haemolytica* virulence factors in an in vitro model of bovine inflammation.

## MATERIALS AND METHODS

***M. haemolytica* products.** *M. haemolytica* culture supernatant (CS) was prepared as previously described (52, 53). The 50% effective dose of the CS was 3 mg/ml; this amount of CS resulted in lysis of 50% of cells from the bovine lymphocyte line BL3 as determined by neutral red viability (52, 53). Lyophilized CS was resuspended to a concentration of 20 mg of CS per ml of sterile water. Recombinant Gcp (Cederlane Laboratories, Hornby, Ontario, Canada) was resuspended in sterile water to a concentration of 2.4 mg/ml. Heat-labile components of the preparations were inactivated by incubating aliquots of the CS and of Gcp at 55°C for 45 min to result in heat-treated (HT) culture supernatant (HTCS) and HTGcp, respectively. Aliquots of all preparations were stored at −20°C.

**Calf challenge.** The calf challenge protocol was approved by the University of Guelph Local Animal Care Committee, as per guidelines of the Canadian Council on Animal Care. Six male Holstein calves (aged 3 to 4 months) were inoculated by intrabronchial infusion with $4.4 \times 10^{11}$ CFU of *M. haemolytica* as previously described (54) (although high, this dose is necessary to reliably induce disease in healthy, unstressed animals). Six days postinoculation, animals were euthanized with barbiturate overdoses, lungs were scored for lesions according to established criteria (53, 54), and samples were taken from both lesion areas (presence of lesions and necrosis upon gross examination) and nonlesion areas (healthy tissue, no lesions visible upon gross examination) of lungs from each calf. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Frozen lung samples were homogenized in lysis buffer (48 mM Tris-HCl [pH 7.5], 144 mM $NaCl_2$, 0.48 mM $MgCl_2$, 0.19 mM EGTA, 1% Triton X-100, 0.96 mM phenylmethylsulfonyl fluoride, 0.019 mg of aprotinin per ml, 0.29 mg of dithiothreitol per ml). Samples were incubated on ice for 30 min and then centrifuged to remove cellular debris. The protein-containing supernatant was aliquoted and stored at −80°C.

**Monocyte assays.** Whole blood from healthy adult Holstein cows was centrifuged, and the buffy coat layer was isolated. Red cells in the buffy coat were lysed with 9 volumes of 4 M ammonium chloride for 2 min. The suspension was centrifuged and the white cell pellet was isolated. After two washes with $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (HBSS), $10^8$ white cells were plated in 100-mm culture dishes in RPMI medium plus 2% fetal bovine serum and cultured for 1 h at 37°C and 5% $CO_2$. Dishes were washed twice to remove nonadherent cells, leaving adherent monocytes. Examination of cytospins from plated material and from culture washes confirmed that greater than 90% of the cells adherent to the plates were monocytes. Monocytes were incubated for 0, 1, 2, 4, and 8 h in fresh medium alone, in medium with CS or HTCS at a final concentration of 0.1 mg/ml, or in medium with 10 μg of Gcp or HTGcp per ml. Following incubation, cell-free monocyte releasate was collected, concentrated five times by lyophilization, resuspended in sterile water, and stored at −80°C.

**Alveolar macrophage assays.** Lungs were obtained from healthy adult cattle and lavaged by using phosphate-buffered saline. The lavage fluid was collected and filtered through a 22-μm-pore-size nylon membrane and centrifuged, and the cell pellet was isolated and resuspended in a smaller volume of $Ca^{2+}$- and $Mg^{2+}$-free HBSS. The cells were separated by using a continuous gradient (5 to 30%) of Optiprep (MJS Biolynx Inc., Brockville, Ontario, Canada). The resuspended cells were applied to the gradient and centrifuged at $800 \times g$ for 20 min, and the cell layer containing alveolar macrophages was isolated. After two washes with $Ca^{2+}$- and $Mg^{2+}$-free HBSS, $10^7$ cells were plated in 100-mm culture dishes in phenol red-free RPMI medium plus 2% fetal bovine serum and cultured for 1 h at 37°C and 5% $CO_2$. Dishes were washed twice to remove nonadherent cells. An examination of cytospins from plated material and from culture washes confirmed that the majority of cells adherent to the plates were alveolar monocytes. Macrophages were incubated for a further 4 h in fresh medium alone or in medium with CS or HTCS at a final concentration of 0.1 mg/ml. Following incubation, cell-free alveolar macrophage releasate was collected, concentrated five times by lyophilization, resuspended in sterile water, and stored at −80°C.

**Neutrophil assays.** Whole blood from healthy adult Holstein cows was centrifuged, and the buffy coat and two-thirds of the red cell layer were removed. Red cells in the remaining layer were lysed with 9 volumes of 4 M ammonium chloride for 2 min. The suspension was centrifuged, and the granulocyte pellet was isolated. After two washes with $Ca^{2+}$- and $Mg^{2+}$-free HBSS, neutrophils were resuspended in HBSS containing 0.1% bovine serum albumin. Cytospins confirmed that isolates contained greater than 95% neutrophils. A total of $2.5 \times 10^6$ cells were added to each reaction tube and incubated in 0.1 mg of CS or HTCS per ml or 10 μg of Gcp or HTGcp per ml for 30 min at 37°C. Tubes were then centrifuged to pellet cells, and cell-free neutrophil releasate was removed, aliquoted, and stored at −80°C.

**Gelatin zymography.** Protein concentrations of inflammatory cell releasates were determined by Bradford assay and confirmed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS–10% PAGE) prior to zymography. Zymography for progelatinase A and B and active gelatinase A and B was performed as described previously (55). Briefly, 15 μg of total lung protein or 45 μg of conditioned-media protein samples from monocytes, alveolar macrophages, or neutrophils was separated by electrophoresis by nonreducing SDS–10% PAGE containing 0.5% gelatin. After electrophoresis, gels were washed twice for 30 min with 2.5% Triton X-100 and then incubated for 18 h at 37°C in buffer (pH 8.0) containing 50 mM Trizma base, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$. Alternatively, the buffer also contained 10 mM EDTA (which chelates zinc ions) to confirm MMP activity (49). After incubation, gels were stained with 0.1% Coomassie blue G-250 and destained in a solution of 5% methanol and 7% acetic acid. The presence of gelatinase was indicated by clear lysis bands on a darkly stained gel. The molecular weights of gelatinolytic bands were estimated by using prestained molecular weight markers (Kaleidoscope; Bio-Rad).

**Reverse zymography.** Reverse zymography for TIMPs was performed as described previously (55), utilizing crude gelatinase A and purified mouse TIMPs (Dylan Edwards, University of East Anglia, Norwich, United Kingdom). Samples of 45 μg of total protein in nonreducing buffer were separated by SDS–12.5% PAGE containing 0.2% gelatin and 6.5% crude gelatinase A. Gels were washed twice in 2.5% Triton X for 30 min following electrophoresis and then incubated for 18 h at 37°C in a buffer of 50 mM Tris and 5 mM $CaCl_2$. After incubation gels were stained and destained as for zymography. The activity of TIMPs was identified as dark bands on a light background and corresponded in size to purified mouse TIMPs. Additionally, the molecular weights of the bands were confirmed by using prestained molecular weight markers (Kaleidoscope; Bio-Rad).

**Image processing and densitometric analysis.** Images of zymography and reverse zymography gels were digitized by scanning. Bands corresponding to gelatinases A and B from gelatin zymography and to TIMPs from reverse zymography were analyzed by using Molecular Analyst software (Bio-Rad Laboratories). Background measurements were subtracted from the integration area measured for each band. Thus, results were standardized for each gel and expressed in dimensionless units. Lung tissue protein results were obtained from six separate animals; inflammatory cell results were obtained from three to six separate experiments for each condition and cell type.

**Statistical analysis.** Densitometry results were compared from replicated experiments for inflammatory cell releasates and from replicate animals for lung protein, and means were calculated for each experimental system. All data are presented as the mean ± standard error of the means (SEM). The significance of the differences among the means was determined by one-way analysis of variance, followed by Tukey's multiple comparisons method, and differences were considered significant at a $P$ value of $<0.05$.

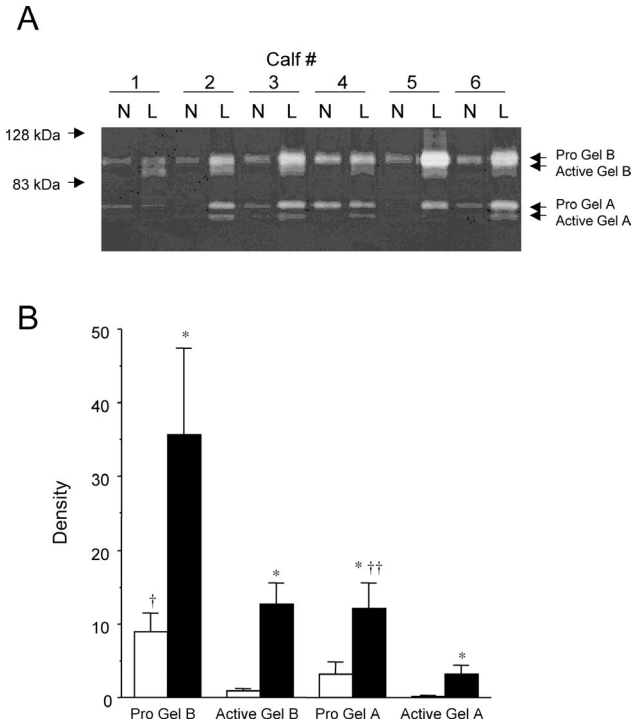


FIG. 1. (A) Zymography showing gelatinase activity in tissue lysates from nonlesional (N) and lesional (L) lung tissue obtained from calves 6 days after challenge with *M. haemolytica*. Lysis bands representing both progelatinase A and B and activated gelatinase A (MMP-2) and B (MMP-9) are more prominent in samples from lesion areas of lung. (B) Densitometric analysis of zymography from lesion and nonlesion areas of lungs from six animals. Open bar, mean density (± SEM) of gelatinases in nonlesional tissue; filled bar, mean density (± SEM) of gelatinases in lesional tissue; *, significantly higher levels compared to nonlesional tissue for each enzyme ($P < 0.05$); †, significantly higher levels of progelatinase B than active gelatinase B in nonlesional tissue ($P < 0.05$); ††, significantly higher levels of progelatinase A than active gelatinase A in lesional tissue ($P < 0.05$). Gel, gelatinase; Pro Gel, progelatinase.

## RESULTS

Levels of progelatinase A and B and active gelatinase A and B were found to be increased in affected areas of lung tissue. All animals challenged with *M. haemolytica* displayed lesions and inflammation that were visible upon gross examination at necropsy. Histological specimens collected from lesion areas indicated extensive mononuclear infiltrate, with regions of necrosis, collagen accumulation, and fibrin deposition, while specimens from nonlesion areas of the same lung did not display evidence of inflammation (results not shown). As detected by zymography (Fig. 1A), bands corresponding to approximately 92, 82, 72, and 68 kDa were evident in samples of lesional and nonlesional lung tissue. These bands were not evident in zymography gels incubated in the presence of EDTA (results not shown), indicating that the gelatin lysis was due to the activities of progelatinase A and B and of active gelatinase A and B (49). The mean levels of progelatinase A and B and active gelatinase A and B in lesional tissue were significantly higher than in nonlesional tissue ($P < 0.05$) (Fig. 1B).

We used gelatin zymography to determine the potential of recruited monocytes to contribute to MMP levels and activation in affected areas of lung and found that the predominant gelatinolytic bands were consistently found only at 92 kDa in samples from monocytes stimulated with CS or HTCS (Fig. 2A) or with Gcp or HTGcp (Fig. 2B). Occasional faint bands were also visible at approximately 72 kDa, but this was not reproducible and did not correspond to cell treatments. Releasates from monocytes stimulated with either CS or with HTCS had higher levels of the 92-kDa gelatinolytic protein than did controls at each time point tested (Fig. 2C), and both levels were significantly higher than those of controls at the 2-h time point. Similarly, releasates from monocytes stimulated with Gcp or HTGcp had significantly higher 92-kDa gelatinolytic protein levels than controls at 2 and 4 h (Fig. 2D).

We also analyzed samples from alveolar macrophages stimulated with CS or HTCS and found detectable levels of progelatinase B in all samples, with no evidence that *M. haemolytica* products influenced production of this enzyme (data not shown). However, bovine neutrophils exposed to *M. haemolytica* products in vitro released extensive amounts of gelatinase B (Fig. 3). Cells released equal amounts of progelatinase B when exposed to the control or heat-treated products (or to *E. coli* LPS; data not shown) for 30 min. Over the same time

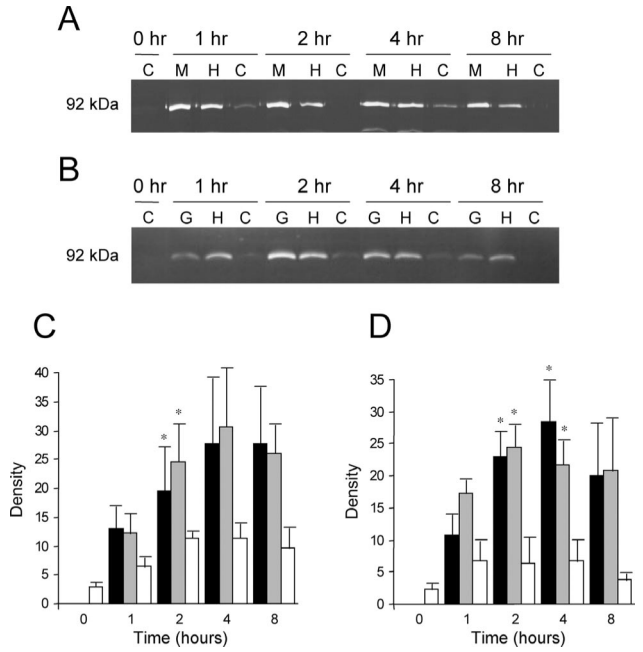


FIG. 2. (A) Representative zymography of gelatinase B activity in monocytes stimulated with *M. haemolytica* products. M, *M. haemolytica* supernatant; H, HTCS; C, control (vehicle only) conditions. (B) Representative zymography of gelatinase B activity in monocytes stimulated with the following: G, *M. haemolytica* recombinant Gcp; H, HTGcp; C, control conditions. (C) Densitometric analysis of zymography gels from four separate experiments, showing the mean density (± SEM) of gelatinase B from monocytes stimulated with CS (black), HTCS (gray), or control (vehicle only) conditions (white). (D) Densitometric analysis of zymography gels from four separate experiments, showing the mean density (± SEM) of gelatinase B from monocytes stimulated with recombinant Gcp (black), HTGcp (gray), or under control conditions (white). *, significant difference from control at the same time point ($P < 0.05$).

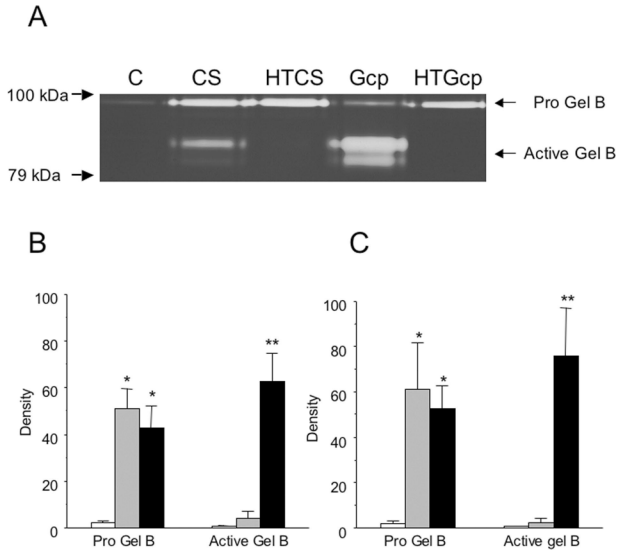


FIG. 3. (A) Representative zymography of gelatinase B activity in bovine neutrophil releasates from cells stimulated with *M. haemolytica* CS, HTCS, Gcp, HTGcp, or control conditions (vehicle only) for 30 min in vitro. Bands of approximately 82 kDa, indicative of activated gelatinase B, were prominent in releasates from cells stimulated with active but not heat-treated *M. hemolytic* products. (B) Densitometric analysis of zymography gels from six separate experiments, showing the mean density (± SEM) of gelatinase B from cells stimulated under control conditions (white bars) or with HTCS (gray bars) or CS (black bars). Stimulation with *M. haemolytica* products induced extensive release of gelatinase B in relation to the level released under control conditions. (C) Densitometric analysis of zymography gels from four separate experiments, showing the mean density (± SEM) of gelatinase B from cells stimulated with control (white bars), HTGcp (gray bars), or Gcp (black bars). *, significantly higher levels of progelatinase B than under control conditions ($P < 0.05$); **, significantly higher levels of active gelatinase B than for control or under heat-treated conditions ($P < 0.05$). Gel, gelatinase; Pro Gel, progelatinase.

course we saw statistically significant ($P < 0.05$) conversion of progelatinase B to its cleaved, active form but only in samples treated with native (versus heat-treated) forms of CS or recombinant Gcp (Fig. 3B and C).

Reverse zymography was employed to investigate the presence of TIMPs in lung tissue isolated from *M. haemolytica*-challenged calves and the production of TIMPs by stimulated monocytes and alveolar macrophages. Only control mouse TIMPs were evident in reverse zymography gels with samples from nonlesional and lesional lung tissue (results not shown). Figure 4A and Fig. 5A are representative gels of CS-stimulated monocytes and macrophages, respectively. While purified mouse TIMP-1 and TIMP-3 standards were detectable by reverse zymography, only a band corresponding to TIMP-1 was evident in monocyte- and macrophage-conditioned media. The band occasionally appeared as a doublet; no detectable levels of TIMP-2 or TIMP-3 were found (Fig. 4A and B). Densitometry revealed that the levels of TIMP-1 from monocytes stimulated with CS were significantly higher at all time points tested when compared with control cells and higher at 1 and 2 h when compared with HTCS-stimulated cells ($P < 0.05$) (Fig. 4C). There was no significant difference in TIMP-1 observed between HTCS-stimulated and control monocyte-cultured media. Similarly, the levels of TIMP-1 produced by monocytes stimulated with Gcp were significantly higher ($P < 0.05$) at all times tested compared to levels in control cells and higher at 1, 2, and 4 h than levels in cells stimulated with HTGcp (Fig. 4D). Significantly increased TIMP-1 levels were also seen in alveolar macrophages stimulated with CS versus HTCS for 4 h ($P < 0.05$) (Fig. 5B).

## DISCUSSION

Here we demonstrate that the MMPs gelatinase A and B are detectable as both progelatinases and active forms in bovine lung tissue lysates and that activities of these gelatinases are increased in lung lesions produced by *M. haemolytica* challenge. Gelatinase B was produced by bovine inflammatory cells exposed to *M. haemolytica* products in vitro, and active gelatinase B was generated by stimulated neutrophils. The expression and production of a gelatinase moderator, TIMP-1, was also altered in bovine monocytes and alveolar macrophages cultured with *M. haemolytica* products.

Several studies have associated MMPs with lung damage observed in other pulmonary infectious and inflammatory conditions (10, 15, 22, 26, 27, 48). The ratio of active gelatinase A to progelatinase A is significantly higher in lung tissue homogenate from human patients suffering from bronchiolitis oblit-

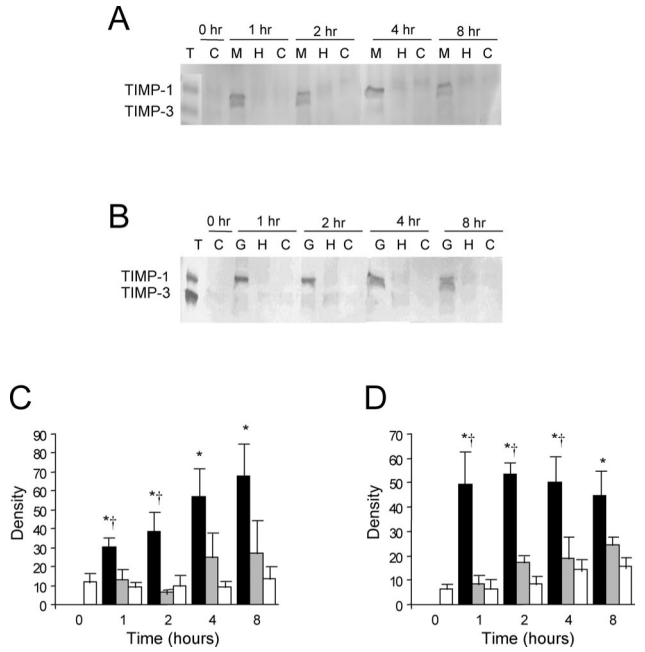


FIG. 4. (A) Composite reverse zymography of TIMP activity in monocytes stimulated with *M. haemolytica* products. M, *M. haemolytica* supernatant; H, HTCS; C, control (vehicle) conditions. Purified mouse TIMPs (T) were run as standards. (B) Composite reverse zymography of TIMP activity in monocytes stimulated with recombinant *M. haemolytica* Gcp (G) or HTGcp (H) or under control (vehicle) conditions (C). Purified mouse TIMPs (T) were run as standards. (C) Densitometric analysis of reverse zymography gels from four separate experiments. Columns represent the mean density (± SEM) of TIMP-1 from monocytes stimulated with CS (black), HTCS (gray), or under control conditions (white). (D) Densitometric analysis of reverse zymography gels from four separate experiments. Columns represent the mean density (± SEM) of TIMP-1 from monocytes stimulated with Gcp (black), HTGcp (gray), or under control conditions (white). *, significant difference from control at the same time point; †, significant difference from heat-treated counterpart at the same time point ($P < 0.05$).

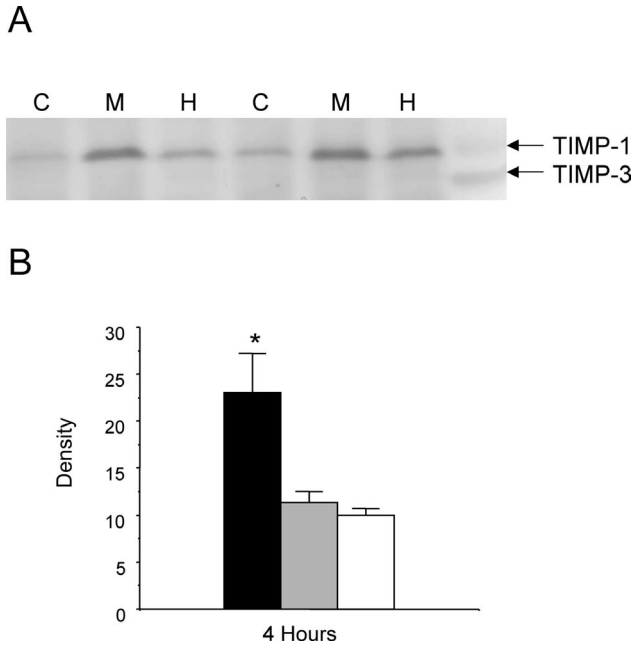


FIG. 5. (A) Representative reverse zymography of TIMP activity in alveolar macrophages stimulated with *M. haemolytica* products for 4 h. M, *M. haemolytica* supernatant; H, HTCS; C, control conditions. Purified mouse TIMPs (T) were run on the gel as standards. (B) Densitometric analysis of reverse zymography gels from four separate experiments. Columns represent the mean density (± SEM) of TIMP-1 from alveolar macrophages stimulated with CS (black), HTCS (gray), or under control conditions (white). *, significant difference from control and heat-treated sample at the same time point ($P < 0.05$).

erans/organizing pneumonia or from idiopathic pulmonary fibrosis when compared with control lungs (14). Similarly, increased levels of gelatinase A and B were observed in bronchoalveolar lavage fluid obtained from human patients with adult respiratory distress syndrome (60). In a gene knockout study, immune complex-induced lung injury resulted in less severe damage to lungs of gelatinase B-deficient mice than in the control counterparts (61).

In our study, zinc-dependent gelatinolytic activity in lung tissue was observed at approximately 92, 82, 72, and 68 kDa, which suggests that these bands correspond to progelatinase B, active gelatinase B, progelatinase A, and active gelatinase A, respectively (29, 55). Gelatinase A is produced by alveolar epithelial cells (6, 14), as well as lung vascular endothelial cells (14). Levels of both gelatinase A and B were higher in lesional tissue from our study compared to levels in nonlesional tissue. Moreover, the amount of active gelatinase B was also significantly higher than that of progelatinase B in lesional tissue, suggesting that enzyme activation, as well as enhanced synthesis, is occurring in lesion areas. Gelatinase B activation follows cleavage of the propeptide by the enzymatic activities of other MMPs, such as gelatinase A and stromelysin 1 (MMP-3), and also by hypochlorous acid, a product of the respiratory burst of activated neutrophils (43). Gelatinase B, in addition to degrading components of the basement membrane, is also able to activate the cytokines IL-1 and IL-8, thereby enhancing the inflammatory response (44).

Our lung tissue homogenate contains both resident and recruited cells such as alveolar cells, macrophages, endothelial cells, monocytes, neutrophils, and lymphocytes. Studies in other species have implicated the alveolar macrophage production of gelatinases in pulmonary disease pathogenesis. Human alveolar macrophages isolated from patients suffering from pulmonary sarcoidosis or pneumonia produce gelatinase A at levels exceeding that from healthy counterparts (22). Similarly, gelatinase B production is elevated in human alveolar macrophages obtained from patients with idiopathic pulmonary fibrosis (28). Gibbs and coworkers (15) investigated the potential of rat lung fibroblasts, endothelial cells, type II epithelial cells, neutrophils, and alveolar macrophages as sources of MMPs identified in bronchoalveolar lavage fluid from injured rat lungs. Only alveolar macrophages had a profile of activity similar to that seen in the lavage fluid (15). Thus, in our study, bovine alveolar macrophages and blood monocytes seemed a likely source of the gelatinases observed in the lung tissue, and so we applied an in vitro model system to investigate this possibility.

Cultured media from monocytes stimulated with CS, HTCS, Gcp, or HTGcp demonstrated zinc-dependent gelatinolytic activity at approximately 92 kDa, indicating that the enzyme

present in the cultured media was progelatinase B (29). In addition, these findings indicate that it is a heat-stable product of *M. haemolytica* CS and of the Gcp preparation that stimulates progelatinase B release by bovine monocytes. Through preincubation with polymyxin B agarose beads, we estimate that LPS composes approximately 75% of the heat-stable portion of our CS preparation. Thus, the heat-stable component of the CS responsible for stimulating progelatinase B production is likely *M. haemolytica* LPS. In the case of monocytes stimulated with recombinant Gcp or HTGcp, the heat-stable factor is likely *E. coli* LPS. This finding is consistent with previous studies that found that bacterial LPS stimulates gelatinase B production by macrophages and monocytes (44).

We found no detectable progelatinase A or active gelatinase A produced by the monocytes and macrophages stimulated with *M. haemolytica* factors within the 8-h time period of our study. Additionally, the lack of active gelatinase B in the monocyte- and macrophage-conditioned media indicates that, while stimulation of these cells with *M. haemolytica* products induces progelatinase B release, enzymes capable of cleaving the propeptide to produce active gelatinase B are not functional or not present in our in vitro model.

Li and coworkers showed that gelatinase B is released (but not activated) by bovine neutrophils in a degranulation response to phorbol myristate acetate (29), indicating that neutrophils may also be a source of the gelatinase B observed in our lung tissue. Of the cells we examined, only neutrophils seemed capable of activating this MMP upon exposure to *M. haemolytica* products. Moreover, unlike the stimulation of progelatinase B production, a heat-labile component of the CS and of the recombinant Gcp appears to participate in neutrophil-associated activation of gelatinase B. Exactly what is involved in activation of gelatinase B once the progelatinase form is secreted is an area of active research. There is evidence in other systems that tissue-associated proteases (19) and matrix adhesion (30) are both required for the activation of gelatinase B. It is also possible that protein complexes (composed of progelatinase B, membrane type MMPs, and TIMPs) associated with the surfaces of cells are responsible for activation of this protease (41, 56).

Elevated levels of gelatinases and other MMPs are frequently associated with the concomitant production of their inhibitors (43); both pro-MMPs and active MMPs are inhibited by TIMPs in a 1:1 ratio (41) TIMP-1 is constitutively produced by macrophages, though production can also be induced (44). In our study, conditioned media from monocytes and alveolar macrophages stimulated with CS or with Gcp contained significantly more TIMP-1 activity than their counterparts exposed to HTCS or HTGcp or than cells incubated under control conditions, an expected result given the increased gelatinase production in stimulated cells.

Surprisingly, despite maximal loading of protein, TIMP activity was not detected by reverse zymography in any of the lung tissue samples. Elevated TIMP-1 levels have been associated with numerous pulmonary diseases in other species, including adult respiratory distress syndrome (60), cryptogenic organizing pneumonia (10), idiopathic pulmonary fibrosis (34, 35, 51), and experimentally induced lung diseases (47, 48). It is therefore difficult to ascertain why there was no evidence of TIMP-1 in lesional or nonlesional lung tissue from calves challenged with *M. haemolytica*. We are unaware of any studies that have investigated TIMP levels in the bovine lung; further work must be completed to determine whether the results obtained herein are specific to *M. haemolytica* infection or are a feature of the bovine lung itself.

To our knowledge, this study is the first to investigate gelatinases and TIMPs in the context of bovine bronchofibrinous pneumonia. We have identified increased levels of gelatinase A and B in lesional lung tissue from calves after *M. haemolytica* challenge and were able to replicate enhanced gelatinase B production in an in vitro model. During inflammation, MMPs may act directly to damage alveolar epithelial and vascular endothelial cells, and MMP destruction of the basement membrane enables enhanced inflammatory cell recruitment (15). Enzymes, free radicals, and other products released by these recruited cells subsequently damage host tissue. There is significant cellular influx into the lungs of cattle suffering from shipping fever (18, 64–66). Bovine neutrophils degranulate and generate free radicals in response to *M. haemolytica* factors in vitro (32), and our experiments provide evidence that heat-stable *M. haemolytica* products stimulate gelatinase production and release by bovine monocytes, macrophages, and neutrophils and that heat-labile factors are involved in neutrophil-associated gelatinase activation. Therefore, all three cell types investigated likely contribute to the tissue damage seen in shipping fever. Moreover, our experiments indicate that *M. haemolytica* heat-labile products alter TIMP-1 production in vitro. The lack of TIMP-1 in bovine lung tissue suggests a possible inability of the *M. haemolytica*-challenged bovine lung to compensate for increased MMP production. An imbalance between MMPs and their inhibitors is involved in the progression of other inflammatory pulmonary diseases (35), consistent with the possibility that gelatinases and their inhibitors are also involved in shipping fever pathogenesis.

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