Lymphocyte Function-Associated Antigen 1 Is a Receptor for Pasteurella haemolytica Leukotoxin in Bovine Leukocytes


Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108; Leon Levy Research Center for Oral Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104; Metabolic Diseases and Immunology Research Unit, National Animal Disease Center, Ames, Iowa 50010; and Departments of Anesthesiology and of Physiology and Biophysics, Mayo Clinic, Rochester, Minnesota 55905

Received 13 August 1999/Returned for modification 4 October 1999/Accepted 14 October 1999

Pasteurella (Mannheimia) haemolytica leukotoxin (Lkt) causes cell type- and species-specific effects in ruminant leukocytes. Recent studies indicate that P. haemolytica Lkt binds to bovine CD18, the common subunit of all β2 integrins. We designed experiments with the following objectives: to identify which member of the β2 integrins is a receptor for Lkt; to determine whether Lkt binding to the receptor is target cell (bovine leukocytes) specific; to define the relationships between Lkt binding to the receptor, calcium elevation, and cytolysis; and to determine whether a correlation exists between Lkt receptor expression and the magnitude of target cell cytolysis. We compared Lkt-induced cytolysis in neutrophils from control calves and from calves with bovine leukocyte adhesion deficiency (BLAD), because neutrophils from BLAD-homologous calves exhibit reduced β2 integrin expression. The results demonstrate for the first time that Lkt binds to bovine CD11a and CD18 (lymphocyte function-associated antigen 1 [LFA-1]). The binding was abolished by anti-CD11a or anti-CD18 monoclonal antibody (MAb). Lkt-induced calcium elevation in bovine alveolar macrophages (BAMs) was inhibited by anti-CD11a or anti-CD18 MAb (65 to 94% and 37 to 98%, respectively, at 5 and 50 Lkt units per ml; P < 0.05). Lkt-induced cytolysis in neutrophils and BAMs was also inhibited by anti-CD11a or anti-CD18 MAb in a concentration-dependent manner. Lkt bound to porcine LFA-1 but did not induce calcium elevation or cytolysis. In neutrophils from BLAD calves, Lkt-induced cytolysis was decreased by 44% compared to that of neutrophils from control calves (P < 0.05). These results indicate that LFA-1 is a Lkt receptor, Lkt binding to LFA-1 is not target cell specific, Lkt binding to bovine LFA-1 correlates with calcium elevation and cytolysis, and bovine LFA-1 expression correlates with the magnitude of Lkt-induced target cell cytolysis.

Leukotoxin (Lkt) and lipopolysaccharide produced by Pasteurella (Mannheimia) haemolytica serotype 1 are considered to be the primary virulence factors contributing to lung injury in bovine pneumoniae (BPP) (33, 36, 38, 40), a disease of substantial economic importance to the beef and dairy cattle industries in North America (7, 28, 39). Lkt is a member of a family of gram-negative bacterial exotoxins termed RTX (for repeats in toxin) cytolysins (3). Although most RTX cytolysins interact with a variety of cell types from many different species (6), cytolysins produced by Actinobacillus actinomycetemcomitans, Actinobacillus pleuropneumoniae (ApxIIIA), and P. haemolytica are known to have cell type- and species-specific effects. The leukotoxin (LtxA) of A. actinomycetemcomitans, a human pathogen, interacts only with cells of the lymphocytic and monomyelocytic lineages of humans and some nonhuman primates (23); the Lkt of P. haemolytica, a ruminant pathogen, interacts only with ruminant leukocytes causing activation and cytolysis (4, 15, 25, 33, 40). A study by Lally et al. (23) has determined that two RTX cytolysins, LtxA of A. actinomycetemcomitans and alpha-hemolysin of Escherichia coli, bind to human myelomonocytic leukemia cell line (HL60) through a β2 integrin lymphocyte function-associated antigen (LFA-1) and cause cytolysis. Moreover, two recent studies have identified CD18 as a receptor for P. haemolytica Lkt (24, 35). Since CD18 is the common subunit of all three bovine β2 integrins CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (π50/95) (1, 18), it is not clear which of the three β2 integrins is a receptor for P. haemolytica Lkt.

β2 integrins are heterodimeric cell surface glycoproteins composed of a CD11 (α) subunit and a CD18 (β) subunit and are expressed exclusively on leukocytes (5, 9). These leukocyte integrins mediate cell adhesion to endothelial cell ligands such as intracellular adhesion molecules (5, 9). The importance of β2 integrins for host defense against microbial agents is exemplified by leukocyte adhesion deficiency, a rare genetic disease in humans that results in reduced expression of all β2 integrins in leukocytes (21, 22), leading to life-threatening bacterial infections. A similar genetic disorder has been reported for Holstein cattle and termed bovine leukocyte adhesion deficiency (BLAD) syndrome (18, 19). Leukocytes from BLAD-homologous calves are known to have no or reduced expression of these β2 integrins (18, 19). However, the potential role of this reduced β2 integrin expression in the BLAD calf model in Lkt binding and cytolysis has not been examined.

The objectives of the present study are to: (i) identify which member of the β2 integrins is a receptor for P. haemolytica Lkt; (ii) determine whether Lkt binding to the receptor exhibits target cell (bovine leukocytes) specificity; (iii) define the relationship between Lkt binding to the receptor and intracellular ([Ca²⁺]i) elevation and cytolysis; and (iv) determine whether a correlation exists between Lkt receptor expression...
and the magnitude of Lkt-induced target cell cytolyis. We used bovine neutrophils and bovine alveolar macrophages (BAMs) to study Lkt binding and functional effects, since these cells are implicated in the pathophysiology of BPP (2, 30). Porcine alveolar macrophages (PAMs) and HL60 cells are used to demonstrate whether Lkt binding is target cell specific.

MATERIALS AND METHODS

Preparation of P. haemolytica Lkt. Preparation of Lkt from P. haemolytica has been described in a previous publication (25). Briefly, crude Lkt was prepared from logarithmic-phase P. haemolytica (25). Briefly, crude Lkt was prepared from logarithmic-phase P. haemolytica (25), followed by centrifugation, the supernatant from logarithmic-phase P. haemolytica has been described in a previous publication (25). Briefly, crude Lkt was prepared from logarithmic-phase P. haemolytica (25). Following centrifugation, the supernatant from logarithmic-phase P. haemolytica has been described in a previous publication (25). Briefly, crude Lkt was prepared from logarithmic-phase P. haemolytica (25).

Preparation of cell lysates. Lysates were prepared as described by Lally et al. (23). Briefly, 5 × 10^6 cells were suspended in 1 ml of lysis buffer (pH 7.5) (200 mM NaCl, 40 mM NaHCO3, 0.5% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, 5 μg of pepstatin per ml, 0.01% NaN3, incubated on ice, vortexed intermittently for 30 min, and centrifuged at 100,000 × g at 4°C for 1 h, and the lysates were stored at −80°C. Protein concentration of the lysates was measured with the DC-protein assay kit (Bio-Rad, Hercules, Calif.).

Flow cytometry. The expression of β2 integrins on neutrophils, BAMs, and HL60 was assessed by immunofluorescence flow cytometry as described previously (34). Briefly, 10^6 cells were incubated with 1 μg of anti-β2 integrin MAbs or control MAb for 15 min on ice. After the cells were washed, they were incubated with 1:200 diluted phycocyanin-labeled goat anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, Pa.) in fluorescence-activated cell sorting buffer (phosphate-buffered saline [PBS] containing 2% goat serum and 5 mM NaN3) for 15 min on ice. After the cells were washed, they were incubated with 1:200 diluted phycocyanin-labeled goat anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, Pa.) in fluorescence-activated cell sorting buffer (phosphate-buffered saline [PBS] containing 2% goat serum and 5 mM NaN3) for 15 min on ice.

Preparation of leukocytes. (i) Bovine neutrophils. Peripheral blood samples were obtained from six healthy age-, breed-, and sex-matched Holstein heifers, using acid-citrate-dextrose as the anticoagulant. Blood samples from three Holstein calves homozygous for BLAD (1) containing the same anticoagulant were used. Neutrophils were isolated by the method described by Neuman et al. (29) and the magnitude of Lkt-induced target cell cytolyis. We used bovine neutrophils and bovine alveolar macrophages (BAMs) to study Lkt binding and functional effects, since these cells are implicated in the pathophysiology of BPP (2, 30). Porcine alveolar macrophages (PAMs) and HL60 cells are used to demonstrate whether Lkt binding is target cell specific.

(ii) BAMs. BAMs were isolated from six 6- to 8-week-old healthy calves as described previously (40). The cells were >98% pure and >98% viable, as determined by nonspecific esterase staining (Sigma Chemical Co., St. Louis, Mo.) and trypan blue exclusion. For [Ca2+]i measurements, BAMs were plated onto round 15-mm-diameter glass coverslips at a density of 7.5 × 10^3 cells/ml in 12-well tissue culture plates. Cells were incubated at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO2. The medium was changed every other day, and the cells were used after 4 days of incubation.

(iii) PAMs and human promyelocytic leukemia cell line (HL60). PAMs were obtained from three 5- to 7-week-old healthy pigs as described previously (16). The HL60 cell line obtained from M. Mellancamp (University of Minnesota, St. Paul) was cultured in RPMI 1640 supplemented with 2 mM l-glutamine and 10% FBS.

Antibodies. Table 1 shows the features and applications of the various antibodies used in this study. Monoclonal antibodies (MAbs) MUC76A, MM12A, BAO153A, BATA5A, and BAO30A were purchased from VMDR, Inc. (Pullman, Wash.). MAbs R15.7 and R3.1 were provided by R. Rothlein (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Conn.). The Lkt-neutralizing MAb (MAB601) was provided by S. Srikumaran (University of Nebraska, Lincoln, NE). The R7928 polyclonal antibody was provided by C. Parkos (Emory University, Atlanta, Ga.).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Species reactivity</th>
<th>Applications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a</td>
<td>R3.1</td>
<td>IgG1</td>
<td>Cat, dogs</td>
<td>FC, BE</td>
</tr>
<tr>
<td>CD11a</td>
<td>MUC76A</td>
<td>IgG2a</td>
<td>Cat, sheep, swine</td>
<td>WB</td>
</tr>
<tr>
<td>CD11b</td>
<td>MM12A</td>
<td>IgG1</td>
<td>Cat, goats, sheep</td>
<td>FC, BE</td>
</tr>
<tr>
<td>CD11b</td>
<td>R7928</td>
<td>Polyclonal</td>
<td>Humans, cattle</td>
<td>WB</td>
</tr>
<tr>
<td>CD11c</td>
<td>BAO153A</td>
<td>IgM</td>
<td>Cat, goats, sheep</td>
<td>FC, BE, FB, CD</td>
</tr>
<tr>
<td>CD18</td>
<td>R15.7</td>
<td>IgG1</td>
<td>Cat, dogs, rabbits</td>
<td>FC, BE</td>
</tr>
<tr>
<td>CD18</td>
<td>BAO30A</td>
<td>IgG1</td>
<td>Cat, goats, sheep, dogs, cats, rabbits</td>
<td>FC, BE</td>
</tr>
<tr>
<td>CD18</td>
<td>BAT77A</td>
<td>IgG1</td>
<td>Cat, goats</td>
<td>FC, BE, FB, CB</td>
</tr>
<tr>
<td>CD18</td>
<td>E-selectin</td>
<td>EL112</td>
<td>IgG1</td>
<td>FC, BE</td>
</tr>
<tr>
<td>CD18</td>
<td>Irrelevant</td>
<td>MOPC21</td>
<td>IgG1</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Lkt</td>
<td>MAB601</td>
<td>IgG1</td>
<td>Not applicable</td>
<td>BE</td>
</tr>
</tbody>
</table>

* Abbreviations: FC, flow cytometry; BE, blocking experiments include blocking of Lkt binding, calcium elevation, and cytolyis; WB, Western blotting.

LFA-1 is a Leukotoxin Receptor
filter. Images were acquired once every second using a silicon-intensified target video camera (66 Series; DAGE-MTI Inc., Michigan City, Ind.). The integrated [Ca2+]i response, a measure of total [Ca2+]i elevation during the period of stimulation, was calculated (17). To examine the effects of anti-β2 integrin MAbs on Lkt-induced [Ca2+]i elevation, BAMS were preincubated with anti-CD11a, anti-CD11b, anti-CD11c, anti-CD18, or control MAb for 45 min at room temperature prior to Lkt exposure. From each coverslip, 30 cells were sampled and two coverslips were used for each experiment. The percent inhibition of [Ca2+]i elevation was calculated as follows: percent inhibition of [Ca2+]i elevation = ((percent [Ca2+]i elevation in the presence of antibodies)/percent [Ca2+]i elevation in the presence of antibodies) - percent [Ca2+]i elevation.

Measurement of LDH release. Lkt-induced cytolysis was assessed by measuring leakage of LDH activity from cells into supernatant, using a commercial kit purchased from Boehringer Mannheim (Indianapolis, Ind.). One hundred microliters of neutrophils or BAMS at a concentration of 4 × 105 cells/ml was added to each well in a 96-well U-bottom microtiter plate. Spontaneous LDH release was measured by exposing cells to assay medium (phenol red-free RPMI 1640 supplemented with 2 mM L-glutamine and 3% FBS). Total LDH release was measured by lysing the cells with 100 μl of 2% Triton X-100 in assay medium. Experimental LDH release was measured by exposing cells to assay medium containing Lkt. Neutrophils or BAMS were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 90 min. After incubation, the cells were centrifuged at 200 g for 5 min. One hundred microliters of the supernatant from each well was transferred to each well of a 96-well flat-bottom microtiter plate. One hundred microliters of reaction reagent (prepared according to the manufacturer’s recommendations) was added to each well and incubated for 30 min at room temperature in the dark. LDH activity in the supernatants was determined by measuring the optical density at 490 nm with a microplate enzyme-linked immunosorbant assay reader (Molecular Device Corp., Menlo Park, Calif.) with a reference wavelength of 620 nm. Each sample was tested in triplicate, and Lkt-induced cytolysis was calculated by using the following formula: percent cytolysis = [(OD of C − OD of A)/(OD of B − OD of A)] × 100, where OD is the optical density at 490 nm, A is spontaneous LDH release, B is total LDH release, and C is experimental LDH release. The effects of antibodies against the subunits of β2 integrins on Lkt-induced cytolysis were also studied. Cells were incubated with different concentrations of antibodies for 45 min at 37°C in a humidified atmosphere under 5% CO2 prior to incubation with Lkt. Inhibition of Lkt-induced cytolysis was calculated as follows: percent inhibition of cytolysis = [(percent cytolysis − percent cytolysis in the presence of antibodies)/percent cytolysis] × 100.

Reagents. Dulbecco’s modified Eagle’s medium and RPMI 1640 were purchased from Celox Laboratories, Inc. (St. Paul, Minn.). Fura-2/AM was purchased from Molecular Probes (Eugene, Oreg.). Polystyrene beads were obtained from Orange Products Inc. (Allentown, Pa.). Phycoerythrin-labeled goat anti-mouse secondary antibodies were obtained from Jackson Immunoresearch (West Grove, Pa.). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (IgG) were obtained from ICN Biomedial Research Products (Costa Mesa, Calif.). Horseradish peroxidase-conjugated goat antimouse IgM was obtained from Pierce Chemical Co. Other reagents were obtained from Sigma Chemical Co.

Statistical analysis. All results are expressed as means ± standard error of the mean (SEM). Comparisons are made with the unpaired Student t test to determine statistically significant differences. The term significant indicates a P value of less than 0.05.

RESULTS

Analysis of β2 integrin expression in leukocytes. (i) Flow cytometry. Relative expression of LFA-1, Mac-1, and p150/95 in neutrophils, BAMS, PAMS, and HL60 cells was determined. Neutrophils from control calves expressed CD11a (MFI of 188), CD11b (MFI of 546), and CD11c (MFI of 80). The expression of CD18 in neutrophils from control calves was determined using three different MAbs (R15.7, BAQ30A, and BAT75A) with MFIs of 550, 480, and 1,800, respectively (Fig. 1A). BAMS expressed CD11a (MFI of 41), CD11b (MFI of 43), CD11c (MFI of 1.3), and CD18 (MFIs of 65, 82, and 87 using R15.7, BAQ30A, and BAT75A, respectively), albeit at levels much lower than those observed for neutrophils (Fig. 1B). However, in neutrophils from BLAD calves, very low level expression of CD18 was detected by BAT75A (MFI of 60), but not by the other two anti-CD18 MAbs. Neutrophils from BLAD calves also lacked expression of CD11a, CD11b, or CD11c (Fig. 1C). Expression of CD11a and CD18, comparable to levels in BAMS, was detected in PAMS and HL60 cells using anti-CD11a (R3.1) and anti-CD18 MABS (BAQ30A) (data not shown).

(ii) Western blotting. In lysates of neutrophils from control calves, a 95-kDa CD18 band was detected (Fig. 2A, lane 1). In lysates from neutrophils of BLAD calves, two lower-molecular-mass CD18 bands (90 and 85 kDa) were identified by using the BAT75A MAb (Fig. 2A, lane 3). BAMS from control calves also had two CD18 bands corresponding to 90 and 95 kDa (Fig. 2A, lane 2). A 180-kDa CD11a band was detected in both.

![Image](http://iai.asm.org/DownloadedFromhttp://iai.asm.org/)
neutrophils and BAMs (Fig. 2B, lanes 1 and 2). However, neutrophils from BLAD calves had very low expression of CD11a (Fig. 2B, lane 3). In lysates from neutrophils and BAMs, Western blot analysis also showed two bands, one corresponding to CD11b (170 kDa) (Fig. 3A, lanes 1 and 2) and another band corresponding to CD11c (160 kDa) (Fig. 3B, lanes 1 and 2). However, no CD11b (Fig. 3A, lane 3) or CD11c (Fig. 3B, lane 3) bands were detected in lysates from neutrophils of BLAD calves. Lysates from PAMs had only a 95-kDa CD18 band (Fig. 4A, lane 1). In HL60 cell lysates, three different CD18 bands (95, 100, and 105 kDa) were detected (Fig. 4A, lane 2). A 180-kDa CD11a band was detected in lysates from PAMs and HL60 cells (Fig. 4B, lanes 1 and 2). Table 2 summarizes the molecular masses of the various β2 integrin subunits expressed in the various cell types studied.

Detection of Lkt binding to β2 integrins. Polystyrene beads coated with Lkt or BSA were incubated with lysates from the different cells. Bound proteins eluted from the beads were separated by SDSPAGE under nonreducing conditions, transferred to a polyvinylidene difluoride membrane, and probed with MAbs against CD11a (MUC76A), CD11b (MM122A), CD11c (BAQ153A), and CD18 (BAQ30A and BA775A). Bound proteins in eluants from beads coated with Lkt and incubated with lysates from neutrophils or BAMs from control calves contained a 95-kDa CD18 band (Fig. 2A, lanes 4 and 5). In contrast, bound proteins eluted from beads coated with Lkt and incubated with lysates from neutrophils from BLAD calves contained a 85-kDa CD18 band (Fig. 2A, lane 6). In addition, in the eluants from beads coated with Lkt and incubated with lysates from neutrophils of control or BLAD calves or BAMs, a 180-kDa CD11a band was detected (Fig. 2B, lanes 4 to 6). Although the eluant from Lkt-coated beads incubated with lysates from PAMs showed the 95-kDa CD18 band (Fig. 4A, lane 3) and 180-kDa CD11a bands (Fig. 4B, lane 3), no such bands were detected in the eluant from beads incubated with lysates from HL60 cells (Fig. 4A and 4B, lanes 4). Table 2 summarizes Lkt binding to the various β2 integrin subunits in the different cell types used in this study.

Several controls were included in this study to ascertain the specificity of Lkt binding to LFA-1 in bovine leukocytes and PAMs. (i) The binding was abolished by preincubating Lkt-coated beads with a neutralizing anti-Lkt MAb (MAb601) (Fig. 2A and B, lanes 7 to 9 and Fig. 4A and B, lanes 5 and 6). (ii) No evidence of binding was observed in BSA-coated beads (Fig. 2A and B, lanes 10 to 12 and Fig. 4A and B, lanes 7 and 8). (iii) Preincubating BAM lysates with anti-CD18 (Fig. 5A, lanes 4 to 6) resulted in no detectable CD11b or CD11c bands (panels A and B, lanes 1 and 2). In contrast, preincubating Lkt-coated beads with anti-CD18 (Fig. 5A, lanes 3) resulted in a 90- and 85-kDa CD18 band (lane 3). In addition, the eluant from Lkt-coated beads incubated with lysates from control or BLAD calves or BAMs showed specific binding to CD11a (MUC76A) and CD11c (BAQ153A), and CD18 (BAQ30A and BA775A).
lanes 5 and 6) or anti-CD11a (Fig. 5B, lanes 5 and 6) MAb abolished the binding.

**Effects of anti-β2 integrin antibodies on Lkt-induced [Ca2+]i elevation.** MABs against CD11 or CD18 subunits of β2 integrins were used to examine the correlation between Lkt binding to the receptor and [Ca2+]i elevation in BAMs. Cells were preincubated with the MABs (25 μg/ml for 90 min at 37°C) prior to addition of Lkt (5 and 50 LU/ml). Anti-CD11a and anti-CD18 MABs significantly (P < 0.05) inhibited Lkt-induced [Ca2+]i elevation in BAMs (65 to 94% inhibition in cells stimulated with 5 LU/ml and 37 to 98% inhibition in cells stimulated with 50 LU/ml [Fig. 6]). Anti-CD11b, anti-CD11c, or the control MAB (MOPC21) had no significant effects on Lkt-induced [Ca2+]i elevation (Fig. 6). Lkt did not induce [Ca2+]i elevation in PAMs, a finding consistent with our previous study (16) (data not shown).

**Effects of anti-β2 integrin antibodies on Lkt-induced cytolysis.** To examine the correlation between Lkt binding to the receptor and cytolysis, neutrophils or BAMs were preincubated with anti-β2 integrin MAbs (1 to 100 μg/ml for 90 min at 37°C) prior to addition of Lkt (50 LU/ml). Anti-CD11a and anti-CD18 MAbs inhibited Lkt-induced cytolysis in neutrophils and BAMs in a concentration-dependent manner (43% inhibition of cytolysis in neutrophils and 64% inhibition of cytolysis at 100 μg/ml in BAMs [P < 0.05] [Fig. 7]). Anti-CD11b, anti-CD11c MAbs, or the control MAB had no significant effects on Lkt-induced cytolysis (Fig. 7). Inhibition of Lkt-induced cytolysis by anti-β2 integrin MAbs was not studied in PAMs and HL60 cells, since Lkt did not induce cytolysis even at a concentration of 500 LU/ml (data not shown).

**Comparison of Lkt-induced cytolysis in neutrophils from control and BLAD calves.** To determine whether there is a correlation between Lkt receptor expression and magnitude of Lkt-induced cytolysis, cytolysis was compared in neutrophils from control and BLAD calves. Lkt caused a reduced level of cytolysis in BLAD neutrophils as compared to controls (data not shown).

**TABLE 2. Molecular masses of β2 integrin subunits in the various cell types and Lkt binding to the various β2 integrin subunits**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>β2 integrin subunit</th>
<th>Lkt binding to β2 integrin subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD11a</td>
<td>CD11b</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>180</td>
<td>170</td>
</tr>
<tr>
<td>BAMs</td>
<td>180</td>
<td>170</td>
</tr>
<tr>
<td>BLAD neutrophils</td>
<td>180</td>
<td>None</td>
</tr>
<tr>
<td>PAMs</td>
<td>180</td>
<td>NP</td>
</tr>
<tr>
<td>HL60</td>
<td>180</td>
<td>NP</td>
</tr>
</tbody>
</table>

- The values are the molecular masses (in kilodaltons) of the β2 integrin subunits. NP, not performed; NA, not applicable.

**FIG. 5. Western blots probed with anti-CD18 (A) or anti-CD11a (B) showing the effects of anti-β2 integrin antibodies on direct binding of *P. haemolytica* Lkt to bovine LFA-1. (A) Eluants from Lkt-coated beads reacted with BAM lysates and probed with anti-CD18 MAB (BAT75A; lane 1) contain a 95-kDa band; BAM lysates that had been preincubated with anti-CD11b (lane 3), anti-CD11c (lane 4), or MOPC21 (lane 7) MAb, before the Lkt-coated beads had been added and that were probed with anti-CD18 MAb contain a 95-kDa band. By contrast, for lysates preincubated with anti-CD11a (lane 2) or anti-CD18 MAB (lanes 5 and 6) before Lkt-coated beads were added, no 95-kDa band is seen. (B) Eluants from Lkt-coated beads reacted with BAM lysates and probed with anti-CD11a MAB (MUC76A; lane 1) contain a 180-kDa band; BAM lysates that had been preincubated with anti-CD11b (lane 3), anti-CD11c (lane 4), or MOPC21 (lane 7) MAb, before the Lkt-coated beads had been added and that were probed with anti-CD11a MAb contain a 180-kDa band. By contrast, lysates preincubated with anti-CD11a (lane 2) or anti-CD18 (lanes 5 and 6) MAb before Lkt-coated beads were added, no 180-kDa band is seen. Results are representative of three independent experiments. MW, molecular mass (in kilodaltons).

**FIG. 6. Effects of anti-β2 integrin antibodies on Lkt-induced [Ca2+]i elevation.** In BAMs treated with anti-CD11a or anti-CD18 MABs, there is significant inhibition of [Ca2+]i response to 5 (A) and 50 (B) LU per ml. There is no inhibition of [Ca2+]i response in cells treated with anti-CD11b, anti-CD11c, or the control MAB in response to 5 or 50 LU/ml. The results are from four independent experiments (~120 cells) and expressed as means ± SEMs. Values that are significantly different from the control value (P < 0.05) are indicated by asterisks.
cytolysis in neutrophils from BLAD calves (18% in BLAD neutrophils versus 62% in control neutrophils [P, 0.05] [Fig. 8]). Lkt-induced cytolysis was abolished by the Lkt-neutralizing MAb (Fig. 8). The isotype-matched control MAb had no effect on Lkt-induced cytolysis (Fig. 8).

**DISCUSSION**

In the present study, we sought to determine whether a member of the β2 integrins is a receptor for *P. haemolytica* Lkt, whether Lkt binding is target (bovine leukocytes) cell specific, whether Lkt binding to the receptor is required for [Ca²⁺]i elevation and cytolysis, and whether a correlation exists between Lkt receptor expression and magnitude of Lkt-induced cytolysis. The results indicate the following. (i) LFA-1 (CD11a/CD18) is a receptor for *P. haemolytica* Lkt. (ii) Lkt binding to CD11a is not target cell specific, since Lkt binding is observed in PAMs. (iii) Lkt binding to bovine LFA-1 correlates with [Ca²⁺]i elevation and cytolysis, since anti-CD11a and anti-CD18 MAbs, but not anti-CD11b and anti-CD11c MAbs, inhibit these responses. (iv) A reduced LFA-1 expression in the neutrophils from BLAD calves correlates with reduced magnitude of Lkt-induced cytolysis. However, the presence of any additional Lkt receptor in bovine leukocytes cannot be ruled out by our studies.

Previous studies have shown that β2 integrins are receptors for a variety of microbial virulence determinants such as fimbriae of *Porphyromonas gingivalis* (32), cryptococcal polysaccharide (10), and endotoxin (11). A study by Lally et al. (23) has shown that LFA-1 is a cell surface receptor for Lkt of *A. actinomycetemcomitans* and alpha-hemolysin of *E. coli* on human (HL60) target cells. Since *P. haemolytica* Lkt exhibits cell type- and species-specific functional effects on ruminant leukocytes (4, 13, 16, 40), it has been hypothesized that specific receptors are present on ruminant leukocytes (3, 37). In this context, two recent studies have indicated that CD18 is the receptor for *P. haemolytica* Lkt (24, 35). The evidence has been based on the fact that MAbs detected a CD18 band in BL3 cell lysates reacted with Lkt. In addition, both studies showed that anti-CD18 MAbs inhibited Lkt-induced apoptosis or cytolysis in BL3 cells. However, a specific β2 integrin was not identified as the Lkt receptor by these studies. Therefore, we have extended these observations and show that LFA-1, a specific member of the β2 integrins, is a receptor for *P. haemolytica* Lkt.

The Lkt affinity chromatography results of the present study with neutrophils and BAMS and of other investigators (24, 35) with BL3 cells demonstrate an interaction of Lkt with CD18. In addition, we provide evidence that Lkt interacts with CD11a, but not with CD11b or CD11c. Therefore, we propose that the CD18 band identified in the Western blots is the β subunit of LFA-1 but not of Mac-1 or p150/95. In this regard, a previous study by Lally et al. (23) showed that Lkt of *A. actinomycetemcomitans* binds to the LFA-1 heterodimer in HL60 cell lysates. Results from our laboratory (S. L. Hsuan, S. Jeyaseelan, M. S. Kannan, and S. K. Maheswaran, unpublished data) using immunoprecipitation of lysates from bovine leukocytes show the existence of LFA-1 as a heterodimer, rather than as dissociated CD11a and CD18 subunits. This finding, along with the finding that Lkt does not bind to CD11b or CD11c subunits, suggests...
that the binding site for Lkt is the CD11a, but not CD18, subunit of the Lkt receptor LFA-1.

We have demonstrated that Lkt binding to LFA-1 in target cells (bovine leukocytes) is associated with [Ca$^{2+}$]$^i$ elevation and cytolyis. However, Lkt binding by itself is not sufficient for the functional effects, since a nontarget cell (PAMs) used in this study exhibits Lkt binding with no evidence of [Ca$^{2+}$]$^i$ elevation or cytolyis and shows NF-$\kappa$B activation (shown in a previous study [16]). Thus, the cell type- and species-specific effects of Lkt must entail both binding to LFA-1 and activation of LFA-1-associated intracellular pathways, which are present only in bovine leukocytes. Studies are in progress to further elucidate this phenomenon.

Previous investigations have provided evidence by flow cytometric analysis that neutrophils from BLAD calves have no or weak expression of all $\beta$2 integrins (18). Our studies using flow cytometry and Western blot analysis confirm these findings. Consistent with the diminished expression of LFA-1, there is reduced Lkt binding. It is of interest to note that the CD18 bands in neutrophils from BLAD calves had molecular weights lower than those from control calves. In leukocytes from human leukocyte adhesion deficiency patients, the different size of the CD18 protein is reported to result from aberrant splicing (22). It is likely that the low-molecular-weight CD18 proteins in neutrophils from BLAD calves may also be the result of aberrant splicing. In addition, we have established a correlation between LFA-1 expression and the magnitude of Lkt-induced cytolyis, supporting the hypothesis that LFA-1 activation is required for cytolyis.

On the basis of our findings, we speculate that Lkt of $P$. haemolytica utilizes the cell adhesion molecule LFA-1 to cause activation and cytolyis, particularly in the neutrophils and macrophages in the alveolar spaces, leading to production and accumulation of a myriad of proinflammatory mediators and continuous colonization of $P$. haemolytica in the alveolar space. These events result in an uncontrollable inflammatory response leading to lung injury that is characteristic of BPP.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Minnesota Agricultural Experimental Station (to M.S.K. and S.K.M.), NIH-Agricultural Research Service (to G.C.S.), and NIH-Divich for help in collecting alveolar macrophages and critically reading the manuscript. We thank Charles Parkos of Emory University for providing the bovine alveolar macrophage. II in vitro studies with $P$. haemolytica. Vet. Immunol. & Vet. Immunopathol. 39:5–10.


REFERENCES


