Bovine CD18 Is Necessary and Sufficient To Mediate *Mannheimia* (*Pasteurella*) *haemolytica* Leukotoxin-Induced Cytolysis†

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Leukotoxin (Lkt) secreted by *Mannheimia* (*Pasteurella*) *haemolytica* is an RTX toxin which is specific for ruminant leukocytes. Lkt binds to β_2 integrins on the surface of bovine leukocytes. β_2 integrins have a common β subunit, CD18, that associates with three distinct α chains, CD11a, CD11b, and CD11c, to give rise to three different β_2 integrins, CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (CR4), respectively. Our earlier studies revealed that Lkt binds to all three β_2 integrins, suggesting that the common β subunit, CD18, may be the receptor for Lkt. In order to unequivocally elucidate the role of bovine CD18 as a receptor for Lkt, a murine cell line nonsusceptible to Lkt (P815) was transfected with cDNA for bovine CD18. One of the transfectants, 2B2, stably expressed bovine CD18 on the cell surface. The 2B2 transfectant was effectively lysed by Lkt in a concentration-dependent manner, whereas the P815 parent cells were not. Immunoprecipitation of cell surface proteins of 2B2 with monoclonal antibodies specific for bovine CD18 or murine CD11a suggested that bovine CD18 was expressed on the cell surface of 2B2 as a heterodimer with murine CD11a. Expression of bovine CD18 and the Lkt-induced cytotoxicity of 2B2 cells were compared with those of bovine polymorphonuclear neutrophils and lymphocytes. There was a strong correlation between cell surface expression of bovine CD18 and percent cytotoxicity induced by Lkt. These results indicate that bovine CD18 is necessary and sufficient to mediate Lkt-induced cytolysis of target cells.

Mannheimia (*Pasteurella*) *haemolytica* serotype 1 is the major bacterial pathogen of bovine pneumonic pasteurellosis, an acute fibrinous pleuropneumonia, which causes extensive economic losses to the cattle industry in North America and other parts of the world (4). *M. haemolytica* A1 is commonly found in the tonsillar crypts and the upper respiratory tracts of healthy cattle (9). In conjunction with active viral infection and stress factors, *M. haemolytica* migrates to the lungs, where it multiplies rapidly (7, 26). *M. haemolytica* produces several virulence factors, of which the extracellular leukotoxin (Lkt) is considered the most important one responsible for leukocyte damage in the lung (3, 27). Lkt-induced neutrophil lysis and degranulation have been implicated as the primary causes of the acute inflammation characteristic of pneumonic pasteurellosis (31).

Leukotoxin (Lkt) is a 102-kDa glycoprotein which is produced during the logarithmic phase of bacterial growth in vitro (2, 28). Lkt belongs to the family of RTX (repeats in toxins) toxins and shares extensive homology with the exotoxins produced by other gram-negative bacteria such as *Escherichia coli* (33), *Actinobacillus pleuropneumoniae* (8), and *Actinobacillus actinomycetemcomitans* (17). Despite the extensive homology

shared by the RTX family, there is a marked dichotomy among the members of the family with respect to target cell specificity. The toxins secreted by *E. coli* and *A. pleuropneumoniae* are lytic to erythrocytes as well as a variety of nucleated cells including the leukocytes from different species (10, 16). In contrast, the toxins secreted by *A. actinomycetemcomitans* and *M. haemolytica* exhibit species and target cell specificity. Lkt secreted by *A. actinomycetemcomitans* is specific for primate leukocytes (30), while Lkt secreted by *M. haemolytica* is specific for ruminant leukocytes (5, 15, 27). Significant progress has been made towards identifying the receptor for the RTX toxins. Lally et al. (18) identified the β_2 integrin LFA-1 as the receptor for Lkt of *A. actinomycetemcomitans* and the α -hemolysin of *E. coli*. Subsequently, β_2 integrins have been identified as the receptors for Lkt of *M. haemolytica* (1, 13, 20, 37). β_2 integrins are leukocyte-specific integrins which have a common β subunit, CD18, that associates with three distinct α chains, CD11a, CD11b, and CD11c, to give rise to three different β_2 integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (CR4), respectively. While there is agreement that β_2 integrins are the receptors for Lkt, there is no consensus on the subunit of the β_2 integrins that serve as the receptor for Lkt. Previous studies in our laboratory revealed that Lkt binds to all three β_2 integrins, suggesting that the β subunit CD18, which is common to all three β_2 integrins, is the subunit that mediates Lkt-induced cytolysis of bovine leukocytes. One of the methods of unequivocal identification of bovine CD18 as a receptor for Lkt is to render Lkt-nonsusceptible cells susceptible to Lkt-induced lysis by recombinant expression of bovine CD18 in Lkt-nonsusceptible cells. There-

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TABLE 1. Description of the antibodies used in this study

Antibody specificity	Clone	Isotype	Source or reference
Mouse CD11a	2D7	IgG1	BDPharmingen, San Diego, Calif.
Bovine CD18	BAQ30A	IgG1	VMRD, Pullman, Wash.
<i>M. haemolytica</i> Lkt	MM601	IgG1	12
BHV-1 gD	MM113	IgG2a	32
BHV-1 gD	9D6	IgG1	35
Bovine CD18	H20A	IgG1	6

fore, the objective of this study was to transfect an Lkt-non-susceptible murine cell-line with cDNA for bovine CD18 and to determine the susceptibility of the transfectant to Lkt-induced cytolysis.

MATERIALS AND METHODS

Cell lines and antibodies. The cell lines P815 (mastocytoma), A-20 (B cell lymphoma), BW5147 (T-cell lymphoma), and EL4 (thymoma; American Type Culture Collection, Manassas, Va.) were propagated in RDG medium (5 g of RPMI 1640 medium/liter, 5 g of Dulbecco's minimum Eagle's medium/liter, 4.5 g of glucose/liter, and 2.85 g of NaHCO₃/liter) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 20 µg of gentamicin/ml. The transfectants were selected and propagated in the above medium together with 500 µg of Geneticin/ml (Life Technologies, Rockville, Md.).

The monoclonal antibodies (MAbs) used in this study and their specificities are presented in Table 1.

Production of *M. haemolytica* Lkt. Production of Lkt from *M. haemolytica* strain A1 has been described previously (12). Briefly, bacteria grown to logarithmic phase (approximately 4.5 h) in brain heart infusion broth (Difco, Detroit, Mich.) were collected by centrifugation (13,500 × g for 20 min) and resuspended in twice the original culture volume of RPMI 1640 medium supplemented with 4 mM L-glutamine (Sigma, St. Louis, Mo.). After an additional 1 to 1.5 h of growth at 37°C in the RPMI medium, the bacteria were removed from the culture by centrifugation (13,500 × g for 30 min) followed by filter sterilization. The crude toxin in the form of culture supernatant was aliquoted and stored at -20°C. All experiments were performed with the same batch of toxin.

Subcloning and expression of bovine CD18. The cloning, sequencing, and characterization of bovine CD18 has been described previously (29). In this study, the cDNA for bovine CD18 was released from pBluescript vector by using *AccI* (New England Biolabs, Beverly, Mass.). The resultant DNA fragment was subcloned into the *AccI* restriction site in the multiple cloning site of eukaryotic expression vector pCI-neo (Promega, Madison, Wis.) to yield the expression vector pMD-1 (Fig. 1). The correct orientation of the bovine CD18 gene in pMD-1 was confirmed by restriction digestion analysis with *NotI*, *SmaI*, and *XhoI* (New England Biolabs). The sequence of the inserted DNA was further confirmed by sequencing pMD-1, using 5'-CGACTCACTATAGGCGAAT-3' (T7 polymerase) and 5'-ATTAACCCCTCACTAAAG-3' (T3 polymerase) forward and reverse primers, respectively. The sequencing was performed by the DNA Sequencing and Synthesis Facility at Iowa State University, Ames. P815 cells were transfected for stable expression of bovine CD18 with pMD-1, using SuperFect transfection reagent (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. Briefly, 5 × 10⁵ cells were incubated with 0.5 µg of pMD-1 and 2.5 µl of the transfection reagent. The cells were resuspended 48 h later in selection medium supplemented with Geneticin (500 µg/ml) and plated into 96-well plates. Two weeks posttransfection, clones that continued to grow in the selection medium were tested for cell surface expression of bovine CD18 molecules, using the anti-bovine CD18 MAb BAQ30A (Table 1), by flow cytometric analysis.

Isolation of polymorphonuclear neutrophils (PMNs) and peripheral blood lymphocytes from cattle. Peripheral blood was collected from healthy cattle by venipuncture and subjected to Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Piscataway, N.J.) centrifugation. PMNs were isolated from the red blood cell pellet by hypotonic lysis and washed three times in phosphate-buffered saline (PBS). Mononuclear cells isolated from Ficoll layer were washed three times in PBS and resuspended in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 20 µg of gentamicin/ml to a final concentration of 2 × 10⁶ cells/ml. Cells were then transferred to a 75-cm² tissue

FIG. 1. Schematic representation of the eukaryotic expression vector pMD-1 expressing bovine CD18. The cDNA for bovine CD18 was subcloned into the mammalian expression vector pCI-neo to yield pMD-1.

culture flask and incubated horizontally for 1 h in a 37°C, 5% CO₂ humidified incubator. After 1 h, nonadherent lymphocytes were harvested and washed once in PBS before use in flow cytometric analysis and cytotoxicity assays.

Flow cytometric analysis for the cell surface expression of bovine CD18. The transfectants, parent cells (P815), bovine PMNs, and bovine lymphocytes were tested for cell surface expression of bovine CD18, using anti-bovine CD18 MAbs (Table 1) in a flow cytometric analysis according to procedures described previously (24). Briefly, 5 × 10⁵ P815 cells, transfectant, bovine PMNs, or lymphocytes were incubated with 50 µl of BAQ30A MAb at 4°C for 1 h. Following three washes in FACS buffer (3% horse serum and 0.01% sodium azide in PBS), the cells were incubated with 50 µl of fluorescein isothiocyanate-labeled goat antibodies specific for mouse Ig (KPL, Gaithersburg, Md.) at 4°C for 30 min. The cells were washed three times with FACS buffer, resuspended, and analyzed by a flow cytometer (Becton-Dickenson, La Jolla, Calif.).

MTT assay for Lkt-induced cytotoxicity and Lkt neutralization. The susceptibility of the different cell types to *M. haemolytica* Lkt-mediated cytolysis was determined by a previously described cytotoxicity assay [MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] dye reduction assay] (1). This assay measures the ability of the endoplasmic reticulum-resident enzymes in viable cells to convert a tetrazolium dye into a purple formazan precipitate, which is later dissolved in acid isopropanol. The optical density (OD) of the end product, representing the intensity of the purple color which developed, is directly proportional to the viability of the cells. Briefly, the cells were resuspended in colorless RPMI 1640 medium (RPMI 1640 medium without neutral red) at a concentration of 1 × 10⁷/ml and seeded into 96-well round-bottomed microtiter plates in duplicates (50 µl per well). Fifty microliters of serially diluted Lkt in colorless RPMI 1640 medium was added into each well, and the plates were incubated at 37°C for 1 h. The cells were centrifuged at 600 × g for 5 min following incubation, and the supernatant fluid was discarded. The cells were resuspended in 100 µl of colorless RPMI 1640 medium, and 20 µl of 0.5% MTT was added to each well. Following incubation at 37°C for 2 h, the cells were centrifuged at 600 × g for 5 min and the supernatant fluid was discarded. The remaining formazan precipitate was thoroughly dissolved in 100 µl of acid isopropanol, and the OD of the samples was measured using an enzyme-linked immunosorbent assay reader. The percent cytotoxicity was calculated as follows: % cytotoxicity = [1 - (OD of toxin-treated cells/OD of toxin-untreated cells)] × 100.

For Lkt neutralization, 50 µl of toxin preparation at a 50% toxicity end point titer of 4 was incubated with 50 µl of Lkt-neutralizing MAb MM601 culture supernatant at 4°C for 30 min, followed by incubation at room temperature for 30 min. 2B2 cells (5 × 10⁵ cells) were added, and the MTT assay was performed as described above. The anti-BHV-1 glycoprotein D MAb MM113 (Table 1) was used as a negative control for neutralization. The percent cytotoxicity was calculated as described above.

Cytotoxicity inhibition assay using anti-bovine CD18 MAb. The inhibition of cytotoxicity assay was performed using anti-bovine CD18 MAbs as described previously (1). Briefly, 2B2 cells (5×10^5 cells) were incubated with anti-bovine CD18 MAbs BAQ30A and H20A (Table 1) or control MAbs MM113 and 9D6 (Table 1); the final concentration of each MAb was 100 μ g/ml. After 1.5 h of incubation at 4°C, the cells were washed and the MTT assay was performed as described above. The percent inhibition of cytotoxicity was calculated using the formula: percent inhibition of cytotoxicity = [(percent cytotoxicity in the absence of antibodies – percent cytotoxicity in the presence of antibodies)/percent cytotoxicity in the absence of antibodies] $\times$ 100.

Radiolabeling of cell surface molecules with 125 I. The cell surface proteins of P815 and 2B2 cells were labeled with 125 I (Perkin-Elmer Life Sciences, Boston, Mass.), using the lactoperoxidase method as described previously (34). Briefly, 5×10^7 cells were washed three times in cold PBS and resuspended in 1 ml of 20 mM glucose (Sigma) in PBS (pH 7.4). Ten microliters of 100 mCi of Na 125 I/ml, 30 μ l of lactoperoxidase solution (0.5 mg/ml in distilled water; Sigma), and 10 μ l of glucose oxidase (1,000 U of stock solution/ml in water diluted 1:400 in PBS immediately before use; Sigma) were added in succession to the cell suspension, and the mixture was incubated for 10 min on ice with gentle mixing. The labeled cells were washed three times with PBS to remove unconjugated 125 I. The radiolabeled cells were incubated with 2.5 ml of lysis buffer (0.5% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 10 mM Tris, 151 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 $\times$ protease inhibitor cocktail P-8340; all from Sigma) for 30 min at 4°C. The lysate was centrifuged at 15,000 $\times$ g for 10 min in a microcentrifuge, and the supernatant fluid was passed through a PD-10 column (Sephadex G25; Amersham Pharmacia Biotech) overlaid with anion exchange resin AG1X8 (Bio-Rad, Hercules, Calif.) to remove unbound 125 I. The fall-through fractions were collected, and the radioactivity levels were determined. The fractions with high radioactivity were pooled and used for immunoprecipitation.

Immunoprecipitation of bovine CD18 and mouse CD11a. The radiolabeled cell lysates (750 μ l) were precleared overnight on a shaker at 4°C with 20 μ l (500 μ g/ml) of affinity-purified isotype-matched control 9D6 MAb and protein G Sepharose (Amersham Pharmacia Biotech). The protein G Sepharose beads were removed by centrifugation at 15,000 $\times$ g for 5 min in a microcentrifuge. Immunoprecipitation was performed by incubating 750 μ l of precleared cell lysate with either 20 μ l (500 μ g/ml) of anti-murine CD11a MAb (2D7), anti-bovine CD18 MAb (BAQ30A), or isotype-matched control 9D6 MAb. Following incubation at 4°C for 2 h, the immune complexes were captured using protein G Sepharose beads by incubation for 2 h. The protein G Sepharose beads were washed three times with washing buffer (0.1% CHAPS, 10 mM Tris, 151 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 $\times$ protease inhibitor cocktail P-8340; Sigma), and the immune complexes were eluted by boiling the beads at 95°C for 5 min in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The precipitated proteins were separated by SDS-PAGE, and the gel was dried and subjected to autoradiography.

Statistical analyses. Statistical analyses were performed using Statistical Analysis Software (SAS Institute Inc., Cary, N.C.). Pearson's correlation significance (two-tailed) test was used to determine the correlation between the degree of Lkt-induced cytotoxicity and cell surface expression of CD18.

RESULTS

Transfection of P815 cells with cDNA for bovine CD18 results in cell surface expression of bovine CD18. The murine mastocytoma cell line P815 was chosen for transfection with bovine CD18 because of the nonsusceptibility to *M. haemolytica* Lkt-induced cytotoxicity of the cells. Following transfection, several clones continued to grow in the selection medium. These clones were tested for cell surface expression of bovine CD18 in a flow cytometric analysis using anti-bovine CD18 BAQ30A MAb. One of the clones, 2B2, stably expressed bovine CD18 (Fig. 2).

Bovine CD18 is expressed as a heterodimer with murine CD11a on the surface of transfectants. β_2 -Integrins are expressed as heterodimers of α and β subunits (11). It has been documented that the α subunit (CD11) and the β subunit (CD18) have to associate with each other to be transported to the plasma membrane and expressed on the cell surface (22).

FIG. 2. Cell surface expression of bovine CD18 on P815 cells transfected with bovine CD18. The transfectant expressing bovine CD18 (2B2) and the parent cells (P815) were tested for the cell surface expression of bovine CD18 by flow cytometric analysis using an anti-bovine CD18 BAQ30A MAb. Anti-BHV-1 gD MAb 9D6 (Table 1) was used as an isotype-matched control antibody in the assay. Results of one representative experiment out of three are shown.

Therefore, it was of interest for us to determine whether the bovine CD18 was expressed as a heterodimer with murine CD11a. Lysates of P815 or 2B2 cells labeled with 125 I were immunoprecipitated using MAb specific for bovine CD18 or murine CD11a. Anti-bovine CD18 MAb coprecipitated a protein with an apparent molecular size of 178 kDa (within the anticipated range for murine CD11a) along with a protein with an apparent molecular size of 100 kDa (within the anticipated range for bovine CD18) from 2B2 cells but not from P815 cells (Fig. 3). Anti-murine MAb CD11a, as expected, precipitated two proteins with apparent molecular sizes of 178 kDa and 100 kDa from both P815 and 2B2 cells (suggestive of mouse CD11a and mouse CD18 from P815 cells and mouse CD11a and mouse and/or bovine CD18 from 2B2 cells, respectively). Isotype-matched control antibody 9D6 did not precipitate any protein(s) from either P815 or 2B2 cells. These results suggest that bovine CD18 is expressed as a heterodimer with murine CD11a on the surface of 2B2 cells.

Recombinant expression of bovine CD18 in P815 cells renders them susceptible to Lkt-induced cytotoxicity. Lkt-induced cytotoxicity of the transfectant expressing bovine CD18 (2B2 cells), the parent cell line (P815), and three other murine cell lines of leukocytic origin (A20, BW5147, and EL4) was tested by MTT dye reduction cytotoxicity assay. The 2B2 cells were lysed effectively by Lkt (75% cytotoxicity), while P815 cells were not lysed by Lkt (Fig. 4). The other murine cell lines tested exhibited 5 to 10% lysis, which was within the limits of background lysis that we routinely observe in cytotoxicity assays with some cell types. In order to confirm the susceptibility of the 2B2 cells to Lkt-induced cytotoxicity, the 2B2 cells and the parent cell line P815 were subjected to the cytotoxicity assay with different concentrations of Lkt. As expected, P815 cells were not lysed by Lkt, while the 2B2 cells were lysed by Lkt in a concentration-dependent manner (Fig. 5). Taken together, these results indicate that the susceptibility of the 2B2 cells to

FIG. 3. Bovine CD18 is expressed as a heterodimer with murine CD11a on the surface of bovine CD18 transfectant. ^{125}I -labeled cell surface proteins of transfectant expressing bovine CD18 (2B2) and the parent cells (P815) were subjected to immunoprecipitation with either an anti-bovine CD18 MAb (BAQ30A), anti-murine CD11a MAb (2D7), or an isotype-matched control MAb (9D6). The immunoprecipitated proteins were subjected to SDS-PAGE followed by autoradiography. Lane 1: ^{14}C -molecular weight marker. Lanes 2, 3, and 4 represent 2B2 lysate immunoprecipitated with isotype-matched control MAb, anti-murine CD11a MAb, and anti-bovine CD18 MAb, respectively. Lanes 5, 6, and 7 represent P815 lysate immunoprecipitated with control, anti-murine CD11a, and anti-bovine CD18 MABs, respectively. Results of one representative experiment out of three are shown.

Lkt-induced lysis is mediated by bovine CD18 molecules expressed on the surface of 2B2 cells.

Anti-bovine CD18 MABs inhibit Lkt-induced cytotoxicity of transfectants expressing bovine CD18. In order to further confirm the fact that bovine CD18 mediates Lkt-induced cytotoxicity, the 2B2 cells were preincubated with anti-bovine CD18 MABs or control MABs prior to the addition of Lkt. Anti-bovine CD18 MABs inhibited the cytotoxicity of 2B2 cells (30%) whereas the control MABs did not have any effect, further confirming

FIG. 4. Lkt lyses bovine CD18 transfectant, but not the parent cells. The transfectant expressing bovine CD18 (2B2), the parent cells (P815), and three murine cell lines of leukocytic origin (A20, a B-cell line; BW5147, a T-cell line; and EL4, a thymoma cell line) were subjected to a MTT dye reduction cytotoxicity assay with a 1:16 dilution of Lkt. The percent cytotoxicity was calculated as follows: percent cytotoxicity = $[1 - (\text{OD of toxin-treated cells} / \text{OD of toxin-untreated cells})] \times 100$. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

FIG. 5. Lkt lyses P815 cells transfected with bovine CD18 in a concentration-dependent manner. The parent cells (P815) and the transfectant expressing bovine CD18 (2B2) were subjected to a MTT dye reduction cytotoxicity assay with twofold dilutions of Lkt. The percent cytotoxicity was calculated as for Fig. 4. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

the fact that Lkt-induced cytotoxicity is mediated by bovine CD18 expressed on the surface of the transfectants (Fig. 6).

Lkt-induced cytotoxicity of transfectants expressing bovine CD18 is abolished by Lkt-neutralizing antibody. In crude toxin preparations, Lkt is associated with lipopolysaccharide, with a molar ratio of lipopolysaccharide/Lkt as high as 60:1 (19). To confirm that the cytotoxicity of 2B2 cells by Lkt was specifically due to Lkt and not due to the components associated with the

FIG. 6. Anti-bovine CD18 MABs inhibit Lkt-induced cytotoxicity of transfectant expressing bovine CD18. Lkt was added to the transfectant expressing bovine CD18 (2B2) that was preincubated with MABs BAQ30A and H20A or MM113 and 9D6. The viability of the cells was determined by a MTT dye reduction cytotoxicity assay. The percent inhibition of cytotoxicity was calculated as follows: percent inhibition of cytotoxicity = $[(\text{percent cytotoxicity in the absence of antibodies} - \text{percent cytotoxicity in the presence of antibodies}) / \text{percent cytotoxicity in the absence of antibodies}] \times 100$. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

FIG. 7. Anti-Lkt neutralizing MAb abolishes Lkt-mediated killing of transfectants expressing bovine CD18. Lkt was incubated with either the neutralizing MAb MM601 or a control MM-113 MAb, and the ability of Lkt to lyse the transfectant expressing bovine CD18 (2B2) was tested in an MTT assay. The percent cytotoxicity was calculated as for Fig. 4. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

toxin, a Lkt neutralization assay was performed using an anti-Lkt MAb MM601 developed in our laboratory (12) which neutralizes the cytotoxic effect of the toxin. Preincubation of Lkt with MM601 completely abolished Lkt-induced cytotoxicity of 2B2 cells, whereas a control antibody did not have any effect on the cytotoxicity of the toxin (Fig. 7), indicating that the lysis of 2B2 cells was specifically due to the cytolytic activity of Lkt.

Lkt-induced cytotoxicity correlates with the expression of bovine CD18. We compared Lkt-induced cytotoxicity of 2B2 cells with that of bovine PMNs and lymphocytes in a MTT dye reduction cytotoxicity assay. Cytotoxicity of 2B2 cells was comparable to that of bovine lymphocytes but lower than that of PMNs (Fig. 8). In order to determine whether there was a correlation between Lkt-induced cytotoxicity and the expression of bovine CD18, we measured the cell surface expression of bovine CD18 on 2B2 cells, bovine lymphocytes, and PMNs by flow cytometric analysis (Fig. 9). Expression of bovine CD18 on the surface of 2B2 was similar to that of bovine lymphocytes but lower than that of bovine PMNs. There was a strong correlation between the degree of Lkt-induced cytotoxicity and cell surface expression of bovine CD18, as revealed by Pearson correlation analysis ($r = 0.99$).

DISCUSSION

β_2 integrins have been identified as the receptors for Lkt secreted by *M. haemolytica* (1, 13, 20, 37). Based on their observation that MAbs specific for CD11a/CD18 and CD18 inhibited Lkt-mediated apoptosis, Wang et al. (37) were the first to report identification of β_2 integrins as a receptor for Lkt. Identification of a 95-kDa protein in the eluant from Lkt-beads, preincubated with lysate from BL-3 cells (bovine lymphoma) by a MAb specific for bovine CD18, prompted these workers to suggest that bovine CD18 is involved in Lkt binding to bovine leukocytes. Binding of bovine CD18 isolated from BL3 cells by Lkt in ligand blotting experiments, and

FIG. 8. Cytotoxicity of bovine PMNs, lymphocytes, and murine transfectant expressing bovine CD18. Lkt-induced cytotoxicity of bovine CD18 transfectant (2B2), bovine PMNs, and lymphocytes was measured by an MTT dye reduction cytotoxicity assay. The percent cytotoxicity was calculated as for Fig. 4. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

partial inhibition of Lkt-induced cytotoxicity by anti-CD18 or anti-CD11a/CD18 MAbs, prompted Li et al. (20) to propose bovine CD18 as a species-specific receptor for Lkt. In earlier studies (1), SDS-PAGE of proteins isolated from PMN lysate on Lkt affinity columns revealed four bands with apparent molecular sizes of 180, 170, 150, and 95 kDa, in addition to the 102 kDa Lkt band. The amino acid sequence data clearly identified the 170-kDa band as CD11b. Furthermore, the PMN proteins eluted from the Lkt-bound column reacted with MAbs specific for CD11a, CD11b, CD11c, and CD18 in a radioimmunoassay, indicating that the proteins contained all three α subunits (CD11a, CD11b, and CD11c) and the β subunit (CD18) of β_2 integrins. These results suggested that the CD18 subunit brought down all three α subunits along with it when the cell lysate was passed through a Lkt prebound column. In addition, in a cytotoxicity inhibition assay, an anti-CD18 MAb reduced Lkt-induced cytotoxicity of bovine PMNs by more than 50%. Taken together, these results indicated that Lkt binds to the β_2 integrins, most likely via CD18 (1). However, a later study by Jeyaseelan et al. (13), based on Western blot analysis of proteins eluted from Lkt beads and blocking experiments with integrin subunit-specific MAbs, concluded that Lkt binds to CD11a and not to CD18. Therefore, our study was designed to resolve the discrepancy on the role of bovine CD18 in Lkt-induced cytotoxicity as reported in the literature. We reasoned that recombinant expression of bovine CD18 on Lkt-nonsusceptible cells and examination of their susceptibility to Lkt-induced cytotoxicity would unequivocally determine the role of bovine CD18 in Lkt-induced cytotoxicity. We chose to examine Lkt-induced lysis of the cells rather than the binding of Lkt to the cells, since binding of Lkt to bovine leukocytes is not

FIG. 9. Expression of bovine CD18 by the murine transfectant expressing bovine CD18, bovine PMNs, and lymphocytes. The transfectant (2B2), parent cell line (P815), bovine PMNs, and lymphocytes were analyzed for the degree of cell surface expression of bovine CD18, using an anti-bovine CD18 MAb, by flow cytometry. Results of one representative experiment out of three are shown.

specific. It has been demonstrated that Lkt also binds to non-ruminant leukocytes (13) without eliciting any effects.

Transfection of the murine mastocytoma cell line P815, which is not susceptible to Lkt-induced cytotoxicity, resulted in a stable transfectant, 2B2, that expressed bovine CD18 on the cell surface (Fig. 2). It is a well-established fact that the α subunit (CD11) and the β subunit (CD18) have to associate with each other in order to be transported to the plasma membrane and expressed on the cell surface (22). Hence, it is very likely that the transfected bovine CD18 molecule is expressed on the surface of 2B2 cells as a heterodimer with the murine CD11a molecules of the P815 parent cells (CD11b and CD11c molecules are not expressed on P815 cells; data not shown). Coprecipitation of a 178-kDa protein (likely to be murine CD11a), along with the 100-kDa bovine CD18 from ^{125}I -labeled 2B2 cell surface proteins by the anti-bovine CD18 MAb, supports this hypothesis (Fig. 3).

The 2B2 cells, but not the parent cells, were lysed by Lkt (Fig. 4). Three other murine cell lines of leukocytic origin were not lysed by Lkt above the background levels routinely observed by us in cytotoxicity assays with some cell types. Furthermore, in a cytotoxicity assay with different concentrations of Lkt, the 2B2 cells were lysed by Lkt in a concentration-dependent manner (Fig. 5). Taken together, these results indicate that the susceptibility of the 2B2 cells to Lkt-induced lysis is mediated by bovine CD18 molecules expressed on the surface of 2B2 cells. The reason that the percent cytotoxicity of 2B2 cells in the cytotoxicity assay in Fig. 5 was lower than that in the cytotoxicity assay in Fig. 4 was that the assay in Fig. 5 was

conducted at a later time and with a different clone of the 2B2 cells that expressed lower levels of bovine CD18.

Preincubation of 2B2 cells with anti-bovine CD18 MAbs partially inhibited the cytotoxicity of these cells (Fig. 6). Lack of complete inhibition of Lkt-induced cytotoxicity by these MAbs could be explainable if these MAbs do not bind to the same site on the CD18 molecule as Lkt does. Partial inhibition of Lkt-induced cytotoxicity by anti-bovine CD18 MAbs have been reported by other workers also (13, 20). Preincubation of Lkt with an Lkt-neutralizing MAb, MM601, abolished the lysis of 2B2 cells, indicating the specificity of lysis (Fig. 7). Next, we compared Lkt-induced cytotoxicity of 2B2 cells with that of bovine PMNs and lymphocytes (Fig. 8). Cytotoxicity levels of 2B2 cells were comparable to those of bovine lymphocytes but lower than those of PMNs. This result is to be expected, since PMNs express all three β_2 integrins (LFA-1, Mac-1, and CR4), while the majority of lymphocytes express only LFA-1 (11). 2B2 cells express a chimeric LFA-1 (murine CD11a/bovine CD18) only, because the parent P815 cells do not express CD11b or CD11c. Thus, the amount of bovine CD18 expressed on PMNs is much greater than that expressed on bovine lymphocytes and 2B2 cells. This differential expression is very likely to be responsible for the higher level of cytotoxicity of PMNs by Lkt. In order to test this hypothesis, we measured cell surface expression of bovine CD18 on PMNs, lymphocytes, and 2B2 cells by flow cytometric analysis with a MAb specific for bovine CD18. There was a strong correlation between the degree of cell surface expression of bovine CD18 and the degree of cytotoxicity of PMNs, lymphocytes, and 2B2 cells. This correlation was statistically significant ($r = 0.99$). In other words, the higher the cell surface expression of bovine CD18, the higher the degree of cytotoxicity. Taken together, these results clearly indicate that Lkt-induced cytotoxicity is mediated by bovine CD18.

Comparison of the amino acid sequence of bovine CD18 with those of murine and human CD18 reveals 86 and 87% sequence homology, respectively. Thus, it is conceivable that the binding site(s) of Lkt on bovine CD18 resides outside these regions of homology and that the specificity of Lkt to ruminant leukocytes is dictated by the domains formed by the remaining 15% of nonhomologous regions on bovine CD18.

The inhibition of binding of Lkt to, and lysis of, bovine PMNs by an anti-bovine CD11a MAb observed by Jeyaseelan et al. (13) could be due to physical obstruction to the Lkt binding of CD18 by binding of the anti-CD11a MAb to CD11a. Several examples of inhibition of binding of a ligand to its receptor by a MAb binding to a closely juxtaposed molecule have been reported in the literature (21, 23, 25, 36). Our finding that bovine CD18 mediates Lkt-induced lysis is further supported by a recent study from the same laboratory (14). This study, which examined the activation of signaling pathways following Lkt binding of bovine leukocytes, concluded that Lkt induces tyrosine phosphorylation of the CD18 tail of LFA-1 in bovine leukocytes.

In summary, inhibition of Lkt-mediated apoptosis studies of Wang et al. (37), ligand blotting studies of Li et al. (20), and Lkt affinity chromatography and immunochemical characterization studies from our laboratory (1) indicated that bovine CD18 is the subunit of β_2 integrins that interacts with Lkt of *M. haemolytica*. In this study, by rendering Lkt-nonsusceptible murine cells susceptible to lysis by Lkt by recombinant expres-

sion of bovine CD18, we have clearly demonstrated in a biologically relevant system that bovine CD18 is necessary and sufficient for Lkt-induced lysis of target cells. However, the CD11 molecule may play a role in stabilization of the proper conformation of the CD18 molecule on the cell surface.

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