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Mapping of the Binding Site for *Mannheimia haemolytica* Leukotoxin within Bovine CD18

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To map the site involved in *Mannheimia haemolytica* leukotoxin (LktA) binding and biological activity within bovine CD18, bovine × human CD18 chimeric constructs were generated and coexpressed with bovine CD11a in K562 cells. Studies with the chimeric leukocyte function-associated antigen 1 transductants demonstrate that the site required for LktA binding and biological effects resides within amino acid residues 500 and 600 of the extracellular region of bovine CD18.

Mannheimia (*Pasteurella*) *haemolytica* is the causative agent of bovine pneumonic mannheimiosis (1, 7, 15). The leukotoxin (LktA) produced by the bacterium is a key virulence factor contributing to the pathogenesis of lung injury associated with the disease (7, 19). LktA is a member of the repeats-in-toxin (RTX) family of pore-forming cytolytic proteins from gram-negative bacteria and exhibits a unique specificity in that it interacts with only ruminant leukocytes. Previous investigations have shown that LktA binds to the CD18 subunit of the bovine β2 integrin(s), and the cell and species specificity is conferred by binding to CD18 (2, 10). However, the precise region within bovine CD18 (BoCD18) to which LktA binds and causes the biological effects is not known. Human CD18 (HuCD18) and bovine CD18 have 769 amino acids, and the transmembrane and cytoplasmic domains are fairly conserved (5, 8, 17). While the C-terminal region of CD18 comprises the transmembrane and short cytoplasmic domains, the N-terminal portion of the molecule is extracellular and tightly folded and contributes to both ligand binding and interaction between the α and β subunits of β2 integrins. The amino acid residues located between positions 102 and 344 of the N-terminal region of CD18 are highly conserved among different species (5, 8, 20). Domains within the bovine and human CD18s are not well demarcated and defined, unlike those within CD11a. The objective of the present study was to map the site within bovine CD18 that is required for LktA binding and its biological effects. To accomplish this, we created several bovine × human chimeric CD18 constructs by replacing the nucleotide sequences in the cDNA encoding the amino acids in the extracellular portion of human CD18 with corresponding sequences from bovine CD18. The different chimeric bovine × human CD18 cDNA constructs were recombinantly coexpressed with bovine CD11a cDNA in a human K562 cell line that lacks endogenous β2 integrin expression and is resistant to the effects of LktA. The resulting chimeric leukocyte function-associated antigen 1 (LFA-1) transductant cells were subjected to an LktA binding assay and two different functional assays to determine their susceptibilities to LktA.

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Human CD18 (in pOTB7 vector) was purchased from Invitrogen (Mammalian Gene Collection clone identification no. 3532902; Invitrogen Corp., Carlsbad, Calif.) and subcloned into an MigR1 retroviral vector (16). Bovine CD18 cDNA was provided by M. Kehrli (NADC, Ames, Iowa) and subcloned into MigR1. Bovine CD11a cDNA was generated in our laboratory (GenBank accession no. AY382558) and subcloned into a pMSCV-puro (Clontech Laboratories, Inc., Palo Alto, Calif.) retroviral vector. The chimeric constructs generated using the bovine and human CD18s are shown in Fig. 1. Each construct was generated in two steps (3, 11, 13, 14). In the first step, human or bovine CD18 sequences were amplified with high-fidelity Vent polymerase. In the second step, the isolated PCR products were used as megaprimers that were denatured and annealed to bovine CD18 in MigR1 or human CD18 in MigR1 and extended in the replacement amplification reaction. For example, to replace the N-terminal 600 amino acids of the bovine CD18 with the corresponding human sequence in H600B, the bovine cDNA sequence encoding amino acids 601 to 769 was amplified in a PCR (primers used are shown in Table 1). Each primer was designed to have complementary sequences to the bovine CD18 cDNA sequence and the human CD18 cDNA sequence. The PCR product was analyzed on 1.0% agarose gel, stained with ethidium bromide, and visualized using the EagleEye (Stratagene, Inc., La Jolla, Calif.) apparatus. The amplicon band corresponding to the expected size (0.55 kb) was excised from the agarose gel, purified using a MinElute (QIAGEN, Inc., Valencia, Calif.) gel extraction kit, and sequenced to confirm that the correct region was amplified. The purified PCR product was then used as a megaprimer in the replacement amplification of the MigR1-humanCDl8 to replace the corresponding region with the bovine sequences. All PCRs were carried out under the following conditions. For the first-round PCR, 10 μl of 10× ThermoPol reaction buffer (New England BioLabs, Inc., Beverly, Mass.), 2 μl of 10 mM deoxynucleoside triphosphate, 100 pmol primers, 100 ng template DNA, 1 μl Vent polymerase, and H_2O to 100 μl were used with the following PCR conditions: 95°C for 30 seconds, 55°C for 55 seconds, and 72°C for 1 min/kb. For the second round of PCR, 50 μl of 10× *Pfu* ultra buffer, 1 μl of 10 mM deoxynucleoside triphosphate, 50 ng template DNA, 50 pmol of isolated PCR product from the first round, 1 μl PfuUl-

TABLE 1. Primers used for domain swapping[a]

Construct	Forward primer	Reverse primer
B110H	5′-CCTTCCCTCCACAGGACATGCTGCGCCAGC-3′	5′-GTTGAACGCTGCTGCCTGGCCTGGTCTCAGGTAGAGC-3′
B200H	5′-GCCCCAAGAAGGAGAAGGAGTGCCAGCCCCCGTTTGCCTTCAGGC-3′	5′-GTGCAGAAGGCCTTGTCTTCACCGGGCACCCCTAACTCTCAGCAAACTTG-3′
B400H	5′-GACCAGCCCAGAGGGGACTGTGATGGCGTGCAGATC-3′	5′-GTGCAGAAGGCCTTGTCTTCACCGGGCACCCCTAACTCTCAGCAAACTTG-3′
B600H	5′-GGCCGATGCCGCTGCAATGTATGCGAGTGCCATTCAG-3′	5′-GTGCAGAAGGCCTTGTCTTCACCGGGCACCCCTAACTCTCAGCAAACTTG-3′
B700H	5′-GTGAAGGGCCCCAACATCGCCGCCATCGTC-3′	5′-GTGCAGAAGGCCTTGTCTTCACCGGGCACCCCTAACTCTCAGCAAACTTG-3′
H110B	5′-CCAGGGCATCCAGGGGACATGCTGGGCCTGC-3′	5′-TGAACGCAACTGCCTGACCTGGTCGCAGGTAAAGC-3′
H200B	5′-CCAGGGCATCCAGGGGACATGCTGGGCCTGC-3′	5′-CCTGAAGGCGAACGGGGCTGGCACTCTTTC-3′
H400B	5′-CACAGGAACCAGCCCAGAGGTGACTGCGACGGCGTCCAG-3′	5′-CGGCCTTGTCTTCACCAAGTGCTCCTAACTCTCGGCAAACTT-3′
H500B	5′-CAGGAGCTGGAAGGAAGGACAACAGCTCCATC-3′	5′-CGGCCTTGTCTTCACCAAGTGCTCCTAACTCTCGGCAAACTT-3′
H600B	5′-AGTGGTCGTGGCCGGTGCCGCTGCAACGTGTGCCAGTGCCGACC-3′	5′-CGGCCTTGTCTTCACCAAGTGCTCCTAACTCTCGGCAAACTT-3′

[a] Underlines indicate human sequences. Overlapping primers were used to amplify the appropriate regions of human or bovine CD18, and the resultant products were used in the replacement amplification with either MigR1-bovine CD18 or MigR1-human CD18 as the template.

FIG. 1. Schematic representation of the different chimeric CD18 constructs. B110H, the N-terminal 110 amino acids of human CD18 were replaced with bovine CD18; B200H, the N-terminal 200 amino acids of human CD18 were replaced with bovine CD18; B400H, the N-terminal 400 amino acids of human CD18 were replaced with bovine CD18; B600H, the N-terminal 600 amino acids of human CD18 were replaced with bovine CD18; B700H, the entire extracellular domain of human CD18 was replaced with bovine CD18; H110B, the N-terminal 110 amino acids of bovine CD18 were replaced with human CD18; H200B, the N-terminal 200 amino acids of bovine CD18 were replaced with human CD18; H400B, the N-terminal 400 amino acids of bovine CD18 were replaced with human CD18; H500B, the N-terminal 500 amino acids of bovine CD18 were replaced with human CD18; H600B, the N-terminal 600 amino acids of bovine CD18 were replaced with human CD18. The figure also shows schematic depiction of the BoCD18 and HuCD18. aa, amino acids.

tra high-fidelity DNA polymerase (Stratagene, Inc., La Jolla, Calif.), and H_2O to 50 μl were used with the following PCR conditions: 95°C for 30 seconds, 60°C for 50 seconds, and 72°C 1 min/kb. This step was followed by DpnI digestion for 1 h to destroy the parental strand, and the construct was transformed into HL-10 Gold Ultra competent cells (Stratagene, Inc., La Jolla, Calif.) and subjected to selection on Luria-Bertani–ampicillin agar plates. Clones generated by domain swapping were then sequence verified at the Advanced Genetic Analysis Center, University of Minnesota. Sequencing results from each construct were aligned with both human CD18 and bovine CD18 using MegAlign (DNAStar, Madison, Wis.). The results indicated that all 10 constructs contained the appropriate swapped regions, with no nonspecific mutations.

Each chimeric CD18 cDNA construct in MigR1 was cotransfected individually with plasmid VSV-G (Clontech Laboratories, Inc., Palo Alto, Calif.) into GP293 packaging cells (Clontech Laboratories, Inc., Palo Alto, Calif.). In a similar way, bovine CD11a cDNA in pMSCV-puro was cotransfected with VSV-G into GP293 cells. Transfection was performed using the Effectene (QIAGEN, Inc., Valencia, Calif.) reagent. Virus collection was performed at 24 and 48 h, and the viruses were sterile filtered with a 0.45-μm filter and stored at −80°C in 1-ml aliquots. For recombinant expression, K562 cells were coated with hexadimethrine bromide (Polybrene; Sigma Chemical Company, St. Louis, Mo.) to prevent charge repulsion during virus infection and added to 24-well plates at a concentration of 1×10^6 cells/ml and 0.5 ml/well. One milliliter of virus (0.5 ml CD11a and 0.5 ml CD18 chimera) was added per well. Plates were spun at 3,000 rpm for 1 h at room tem-

perature and transferred to a 37°C incubator. After 24 h, cells were transferred to fresh medium. Forty-eight hours postinfection, cells were transferred to selection medium containing 2 μg/ml puromycin. At this time, transductants were analyzed for green fluorescent protein expression by using a fluorescence microscope. Transductants expressing chimeric LFA-1 were purified using a magnetic cell sorting column (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were mixed with anti-bovine CD11a-specific monoclonal antibody (MAb) R3.1 and incubated with anti-immunoglobulin G1–coupled magnetic beads. Cells were passed through a magnetic cell sorting column and washed three times, and the column was removed from the magnetic field to collect and expand the positive cell fraction. The resultant transductants were designated with the same abbreviations as those used for the respective chimeric CD18 constructs (e.g., B200H). In addition, BoLFA-1 (BoCD11a/BoCD18), BoCD11a/HuCD18, KL/4 cell (HuLFA-1, HuCD11a/HuCD18) (9), and parent K562 cell transductants were used as controls.

Surface expression of chimeric CD18 dimerized with bovine CD11a in the transductants was confirmed by fluorescence-activated cell sorting (FACS) analysis using anti-bovine CD11a-specific MAb R3.1 (data not shown) and CD18-specific MAb BAQ30A (Fig. 2). BAQ30A cross-reacts with both bovine and human CD18 (data not shown). Briefly, 10^7 transductant cells were incubated with 1 μg of anti-bovine CD11a or CD18 MAb in FACS buffer (phosphate-buffered saline containing 2% goat serum and 5 mM NaN_3) for 15 min on ice. Cells were washed using FACS buffer and incubated with a 1:200 dilution of phycoerythrin-labeled goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, Pa.) in FACS buffer for 15 min on ice. Cells were washed and resuspended in 100 μl of FACS buffer, and fluorescence was analyzed by a FACS Calibur flow cytometry system using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and expressed as mean fluorescence intensity. All chimeric CD18 antigens expressed on the cell surfaces of the transductants were recognized by the CD18-specific MAb BAQ30A (Fig. 2). Since studies by others (2, 12) have established unequivocally that the CD11 and CD18 subunits have to associate with each other as a heterodimer to be transported and expressed on the surface of a cell, it is very likely that the various chimeric LFA-1 transductant cells used in the present study express these subunits as a heterodimer.

Native LktA from the wild-type strain 153 of *M. haemolytica* was produced, purified, and quantified as described previously (6). All studies were done with the same batch of LktA, which was incubated with 10 μg/ml of polymyxin B for 30 min on ice prior to use to exclude the effect of postpurification contamination with lipopolysaccharide.

A modified affinity chromatography assay was used to demonstrate LktA binding to immobilized chimeric CD18. Lysates (6) from the various chimeric LFA-1 transductant cells (1×10^8 cells) were precleared using isotype-matched control MAb (MOPC21) and sequentially depleted of bovine CD11a and residual heterodimers of CD11a/CD18 three times by immunoprecipitation with anti-CD11a MAb R3.1 coupled to Sepharose CL4B beads (Amersham Biosciences, Piscataway, NJ). Depleted lysates were incubated with anti-CD18 MAb BAQ30A coupled to Sepharose CL4B beads to immunopre-

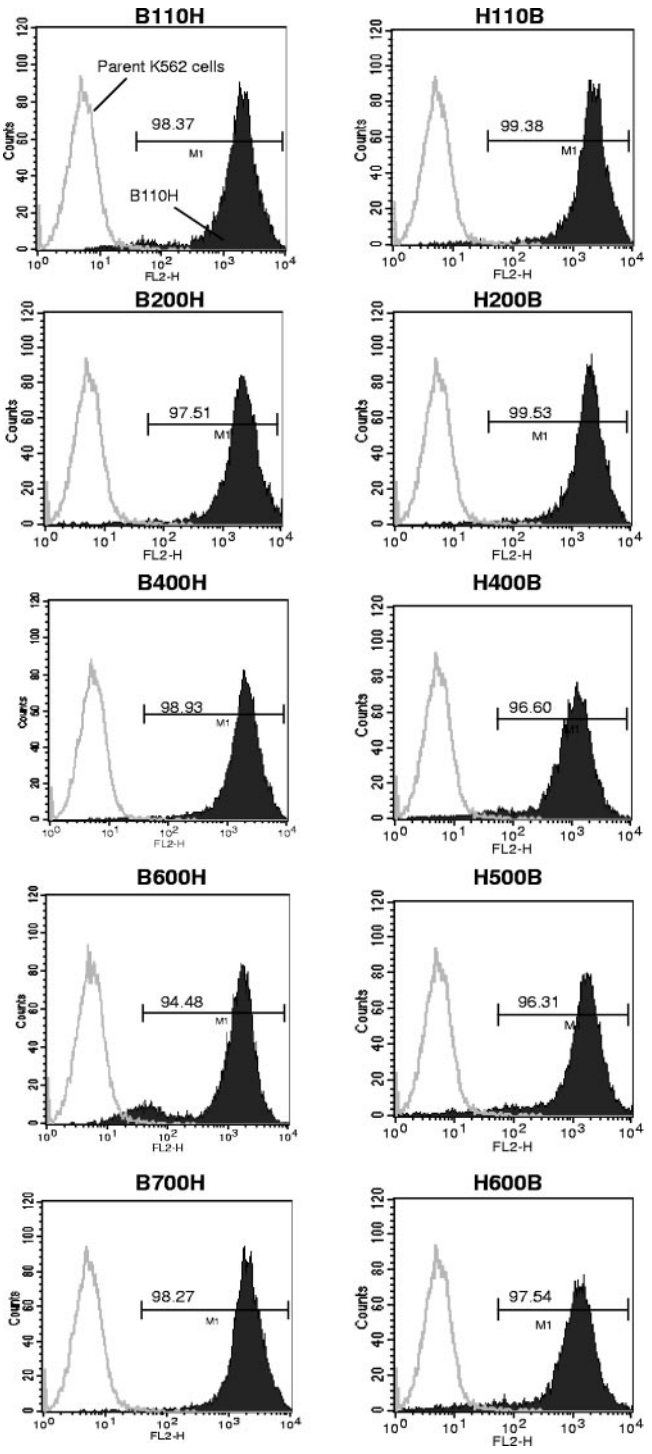

FIG. 2. Demonstration of surface expression of bovine CD18 chimeric antigens in various transductants. Surface expression of chimeric CD18 antigens in the various transductants was confirmed by FACS using anti-CD18-specific MAb BAQ30A. The parent cell line K562 cells (open trace) which do not express CD18 antigen were used as a negative control. The *x* axis shows fluorescence intensity (FL2-H), the *y* axis shows cell numbers (counts), and the numbers shown within the panels are the percentages of positive CD18 cells. Results show high levels of expression of CD18 antigen in all chimeric transductants (filled traces). Data presented are representative of one of three experiments performed. FL2-H, height for phycoerythrin; M1, marker 1.

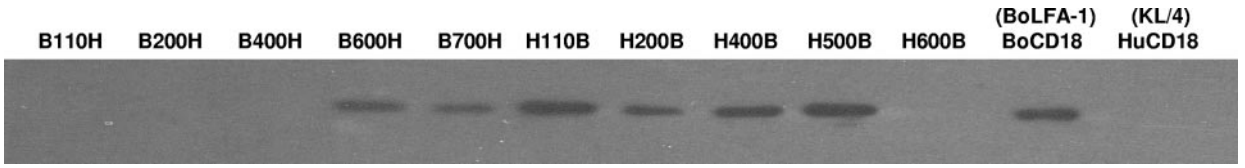

FIG. 3. Western blot demonstrating binding of LktA to immobilized chimeric CD18 from various transductants. Cell lysates from various transductants were processed as described in the text. The different chimeric CD18s were immunoprecipitated from cell lysates with MAb BAQ30A coupled to Sepharose CL-4B beads and incubated separately with LktA, followed by detection of bound LktA with an anti-LktA MAb. LktA binds to chimeric transductants B600H, B700H, H110B, H200B, H400B, and H500B but not to B110H, B200H, B400H, and H600B. Bovine CD18 immunoprecipitated from BoLFA-1 cell lysate and human CD18 immunoprecipitated from KL/4 cell lysate served as controls. Data shown are from one representative experiment of three experiments performed.

cipitate the chimeric CD18. The beads bound to the chimeric CD18 were blocked with 1% bovine serum albumin for 1 h and incubated overnight at 4°C with LktA (50 leukotoxin units [LU]/ml) in Hanks balanced salt solution containing 1 mM $CaCl_2$ and $MgCl_2$. The bound LktA was cross-linked with a fresh solution of 1% paraformaldehyde for 10 min to enhance stability. The beads were washed four times with Hanks balanced salt solution, and the bound proteins were eluted from the beads by boiling the beads with 50 μl of Laemmli sample buffer containing β-mercaptoethanol. Eluted proteins were separated on 4 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gels and subjected to Western blotting. Thereafter, the membrane was incubated with a 1:20,000 dilution of MAb601 (anti-LktA) antibody for 1 h at room temperature, followed by washes and incubation with a 1:50,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blots were developed using the SuperSignal ULTRA chemiluminescence detection system (Pierce Chemical Co.).

As shown in Fig. 3, LktA binding was demonstrated in chimeric CD18 in transductants B600H, B700H, H110B, H200B, H400B, and H500B but not in the B110H, B200H, B400H, and H600B. These results indicate that LktA binds to a site between amino acid residues 500 and 600 within the bovine CD18 extracellular region.

LktA-induced elevation of intracellular calcium $[Ca^{2+}]_i$ in the transductants was measured by video fluorescence imaging using the ratiometric dye fura-2-acetoxymethyl (AM) ester, as previously described (4). Briefly, fura-2/AM ester–loaded cells attached to poly-L-lysine-coated glass coverslips were placed on the stage of a Nikon Diaphot inverted microscope (Nikon, Inc., Garden City, NY) and viewed using a 40×-fluor objective.

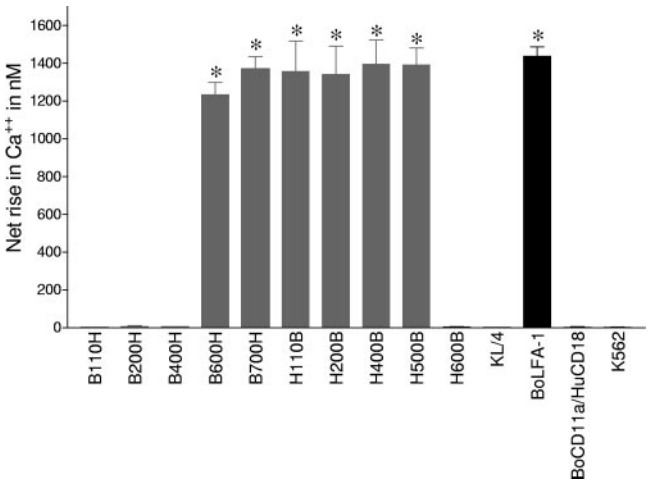

FIG. 4. LktA induces $[Ca^{2+}]_i$ elevation in the B600H, B700H, H110B, H200B, H400B, and H500B but not in the B110H, B200H, B400H, and H600B transductants. Measurement of the $[Ca^{2+}]_i$ level was done using the cell-permeable fluorescent dye fura-2/AM ester. The net $[Ca^{2+}]_i$ response (peak response subtracted from basal values) was measured as described in the text. Results are expressed as means ± standard errors of the means for three separate experiments. The BoLFA-1 (BoCD11a/BoCD18), KL/4 line (HuLFA-1; HuCD11a/HuCD18), and parent K562 cell transductants served as controls. Values that are significantly different from the negative control value ($P < 0.05$) are indicated by asterisks.

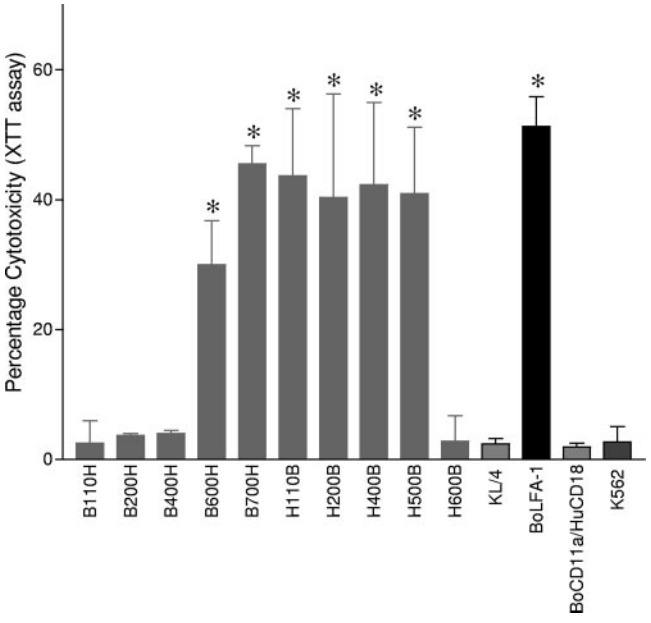

FIG. 5. LktA-induced cytolysis in the chimeric transductants. Cytolysis of various cell lines exposed to 100 units of LktA (100 LU/ml) was measured by the XTT assay as described in the text. Transductants B600H, B700H, H110B, H200B, H400B, and H500B showed marked cytotoxicity compared to that obtained with the parent cell line. By contrast, transductants B110H, B200H, B400H, and H600B showed lower cytotoxicity upon exposure to LktA, comparable to that obtained with parent cell line. The BoLFA-1 (BoCD11a/BoCD18), KL/4 line (HuLFA-1, HuCD11a/HuCD18), and parent K562 cell transductants served as controls. Results are expressed as means ± standard errors of the means for three separate experiments. Values that are significantly different from the negative control value ($P < 0.05$) are indicated by asterisks.

$[Ca^{2+}]_i$ concentrations were determined using a calibration method as described previously (18). From each coverslip, ~50 cells were sampled, and two coverslips were used for each experiment. Significant elevation of $[Ca^{2+}]_i$ levels following exposure to LktA (50 LU/ml) was observed in the same transductants that showed binding to LktA, namely, the B600H, B700H, H110B, H200B, H400B, and H500B transductants but not in the B110H, B200H, B400H, and H600B transductants (Fig. 4).

LktA-induced cytolysis was determined using a previously described XTT dye reduction assay, and the percentage of cytotoxicity was calculated as described previously (6). Exposure to 100 LU/ml LktA resulted in significant cytotoxicity in the B600H, B700H, H110B, H200B, H400B, and H500B transductants similar to that observed in the transductants expressing bovine LFA-1. In the B110H, B200H, B400H, and H600B transductants, where LktA binding does not occur, the magnitude of LktA-induced cytotoxicity was significantly lower and comparable only to that observed in the parent K562 cells (Fig. 5). Statistical analysis of data was performed using Graphpad Prism (version 3.02; San Diego, CA). The term significant indicates a P value of less than 0.05.

In this study, we used an LktA binding assay and two different functional assays, namely, LktA-induced $[Ca^{2+}]$ elevation and cytolysis, as criteria to identify the region in the extracellular domain of CD18 that carries the LktA binding site. The results from studies with the various chimeric LFA-1 transductants clearly demonstrate that the binding site for LktA is contained within 500 to 600 amino acid residues of the extracellular region of bovine CD18. Furthermore, LktA-induced $[Ca^{2+}]_i$ elevation and cytotoxicity were observed only in the transductants where LktA binding occurred. Previous reports have shown that N-terminal amino acid residues 1 to 101 in the CD18 are highly varied among the different species (5, 8), thus raising the possibility that the critical LktA binding site may reside within this region in bovine CD18. However, a more recent study has described 16 potential amino acid residues scattered in the ruminant CD18 that are different from nonruminant CD18 and suggested that some of them may be responsible for the species-specific virulence of *M. haemolytica* LktA (20). Our studies showed that the critical amino acid residues responsible for LktA binding and its biological effects may be confined within a narrower region of bovine CD18. This interpretation is supported by the finding that only in the H500B, not in the H600B, transductant could LktA binding and its biological effects be demonstrated. Of the 16 variable amino acid residues described to occur in the ruminant CD18 (20), 2 amino acid residues specific to ruminant CD18 at positions 561 and 533 are within this region. Whether these two specific residues, by providing the correct conformation, or the entire 100-amino-acid region is responsible for the ruminant specificity of LktA effects remains to be determined. This would help in the design of small-molecule inhibitors or peptidomimetics capable of preventing LktA binding and the ensuing signaling leading to leukocyte activation and inflammatory response in the lung.

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