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Bighorn Sheep β_2-Integrin LFA-1 Serves as a Receptor for *Mannheimia haemolytica* Leukotoxin

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ABSTRACT: *Mannheimia haemolytica* is an important cause of pneumonia in bighorn sheep (BHS; *Ovis canadensis*). Leukotoxin (Lkt), the primary virulence determinant of *M. haemolytica*, induces cytolysis of all subsets of leukocytes. Previously, we have shown that CD18, the β subunit of β_2-integrins, mediates Lkt-induced cytolysis. However, it is not clear whether CD18 of all three β_2-integrins, LFA-1, Mac-1, and CR4, mediates Lkt-induced cytolysis. The objective of this study was to determine whether BHS LFA-1 (CD11a/CD18) serves as a receptor for Lkt. Plasmids encoding cDNA for BHS CD11a and CD18 were cotransfected into Lkt-resistant HEK-293 cells. Flow cytometric analysis of transfectants confirmed cell surface expression of BHS LFA-1, Lkt-LFA-1 binding and Lkt-induced intracellular calcium elevation. More importantly, the transfectants were efficiently lysed by Lkt in a concentration-dependent manner. Collectively, these results indicate that BHS LFA-1 serves as a functional receptor for *M. haemolytica* Lkt.

Key words: Bighorn sheep, leukotoxin, LFA-1, *Mannheimia haemolytica*, receptor.

Historically, bighorn sheep (BHS; *Ovis canadensis*) were abundant in North America, but their numbers have dwindled over the years due to a combination of factors including loss of habitat, competition for forage with domestic livestock, and disease (Coggins, 1988). An important factor, which has limited the growth of BHS populations, is respiratory disease (Foreyt, 1989). Bacteria belonging to the genera *Mannheimia* and *Pasteurella* are the major cause of mortality in BHS populations (Foreyt, 1989; Miller, 2001). Investigations of natural outbreaks of pneumonia and experimental infections have identified *Mannheimia haemolytica* as an important etiologic agent of pneumonia in BHS (Foreyt, 1989). *Mannheimia haemolytica* causes severe pneumonia in BHS, domestic sheep (DS), goats, and cattle (Scanlan et al., 1993; Mosier, 1997; Brogden et al., 1998; Ackermann and Brogden, 2000; Miller, 2001). However, BHS are much more susceptible to this disease than the other ruminants (Foreyt, 1989). Twelve different serotypes of *M. haemolytica* have been identified, so far. Studies involving experimental exposure have identified serotype A2 as the primary cause of pneumonia in BHS (Foreyt, 1989). *Mannheimia haemolytica* produces several virulence determinants, of which leukotoxin (Lkt) is the most important one contributing to the development of pneumonia (Highlander et al., 2000). It induces the cytolysis of all subsets of leukocytes, of which polymorphonuclear leukocytes (PMNs) are the most susceptible subset (Silflow and Foreyt, 1994; Liu et al., 2007). In earlier studies, we, and others, have independently shown that the cytotoxic effect of Lkt on ruminant leukocytes is mediated by Lkt-β_2-integrin interactions (Wang et al., 1998; Ambagala et al., 1999; Li et al., 1999; Jeyaseelan et al., 2000; Deshpande, 2002).

β_2-integrins are leukocyte-specific integrins that are expressed on the cell surface as heterodimers composed of the α subunit CD11 and the β subunit CD18. These subunit associations give rise to four different β_2 integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (CR4), and CD11d/CD18 (Gahmberg, et al., 1998). The CD11d/CD18 has not yet been well characterized in the ruminants.

By rendering Lkt-nonsusceptible murine cells susceptible to Lkt by recombinant expression of BHS CD18, we previously demonstrated that CD18 mediates Lkt-induced cytolysis of target cells (Liu et

al., 2007). However, the question as to whether CD18 of all three β_2-integrins, LFA-1, Mac-1, and CR4 of BHS, mediate Lkt-induced cytolysis remains. Development of transfectants expressing the individual β_2-integrins should answer this question. Therefore, the objective of this study was to develop transfectants expressing BHS LFA-1 and to determine the susceptibility of the transfectant to cytolysis by *M. haemolytica* Lkt.

BHS CD11a was amplified from total RNA, isolated from PMNs by RT-PCR, as we described earlier (Liu et al., 2006) for BHS CD18, but with domestic sheep CD11a-specific primers (Fett et al., 2005). The PCR products obtained were cloned into the mammalian expression vector (pcDNA6.2/GW/D-TOPO) to yield pWL/CD11a and sequenced. The BHS CD11a sequence has been deposited in GenBank (accession no. DQ454072). The mammalian expression vectors carrying BHS CD11a (pWL/CD11a) and CD18 (pWL/CD18, Liu et al., 2006) were cotransfected into human embryonic kidney cells (HEK-293) using PerFectin transfection reagent, as previously described (Dassanayake et al., 2007). The HEK-293 cell line was selected for transfection studies because of their lack of expression of any β_2-integrins and for their resistance to *M. haemolytica* Lkt-induced cytolysis (Lawrence et al., 2007). Two days post-transfection, cells were transferred from the 6-well plate into 75-cm^2 tissue culture flasks containing the selection medium (10 μg/ml blasticidin and 800 μg/ml geneticin). The transfectants were examined for cell surface coexpression of BHS CD11a/ CD18 (LFA-1) by flow cytometry (Dassanayake et al., 2007), using monoclonal antibodies (MAbs) specific for human CD11a (HUH73A, IgG1) and human CD18 (HUH82A, IgG2a), which cross-react with BHS CD11a and CD18, respectively (Saalmuller et al., 2005). With these MAbs, flow cytometric analysis of the transfectants revealed cell surface expression of BHS LFA-1 by the transfectants, but not by the parent cells (data not shown). We have previously evaluated the expression of β_2-integrins on BHS PMNs and peripheral blood mononuclear cells by flow cytometry, after staining these cells with MAbs specific for CD11a, CD11b, CD11c, or CD18, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-murine Ig antibodies. Both cell populations expressed CD11a, CD11b, CD11c, and CD18. However, BHS PMNs expressed lower levels of CD11c compared to that of PBMCs (unpubl. obs.). Expression of all three β_2-integrins by BHS leukocytes suggest that it is likely that *M. haemolytica* Lkt-induced cytolysis of BHS leukocytes is mediated by CD18 of all three β_2-integrins.

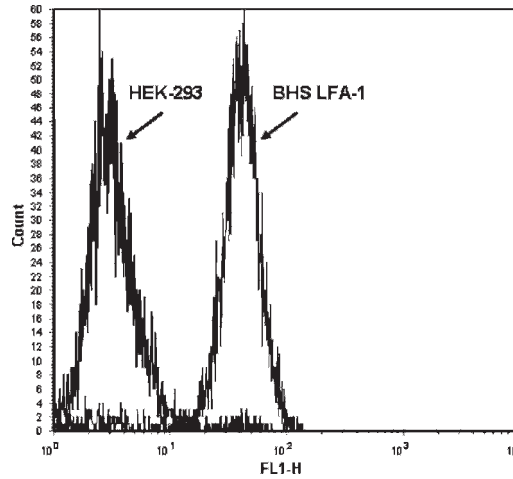

FIGURE 1. *Mannheimia haemolytica* Lkt binds to transfectants expressing bighorn sheep (BHS) LFA-1. The BHS LFA-1 transfectants and the parent HEK-293 cells were incubated with leukotoxin (Lkt), fixed with 2% paraformaldehyde, washed, and incubated with FITC-conjugated Lkt non-neutralizing MAb MM605. Flow cytometric analysis revealed that Lkt bound to BHS LFA-1, but not to HEK-293 cells. Results of one representative experiment out of three are shown.

The transfectants expressing BHS LFA-1 were enriched by using a fluorescence-activated cell sorter (FACSVantage SE, Becton-Dickinson, San Jose, CA, USA), after staining with anti-CD11a and anti-CD18 MAbs. Transfectants stably express-

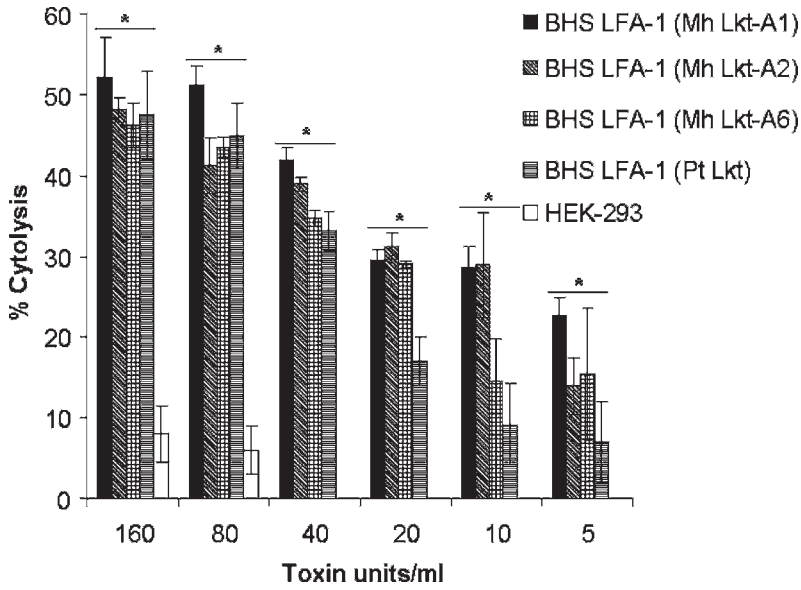

FIGURE 2. *Mannheimia haemolytica* Lkt induces cytolysis of the transfectants expressing bighorn sheep (BHS) LFA-1. The transfectants (BHS LFA-1) and parent HEK-293 cells were incubated with leukotoxin (Lkt) from *M. haemolytica* (Mh, serotype A1, A2, A6) and *P. trehalosi* (Pt), and the percent cytolysis was evaluated by MTT dye reduction cytotoxicity assay. Lkt-induced cytolysis was observed in BHS LFA-1 transfectants, but not in HEK-293 cells, confirming LFA-1 as an Lkt receptor. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means (*$P<0.01$).

ing BHS LFA-1, and the parent HEK-293 cells, were tested for Lkt-binding by flow cytometry, according to previously published procedures (Dassanayake et al., 2007). Flow cytometric analysis of BHS LFA-1 transfectants, after incubation with Lkt followed by FITC-conjugated MAb MM605 (Lkt-non-neutralizing MAb, Gentry and Srikumaran, 1991), revealed that Lkt specifically bound to BHS LFA-1 transfectants but not to the parent HEK-293 cells (Fig. 1). These results indicated that Lkt-binding to transfectants is mediated by the recombinantly expressed BHS LFA-1 on HEK-293 cells. Next, by a previously described MTT dye reduction cytotoxicity assay (Gentry and Srikumaran, 1991), we determined the susceptibility of BHS LFA-1 transfectants to *M. haemolytica* Lkt-induced cytolysis. The parent HEK-293 cells and BHS leukocytes were used as the negative and positive controls, respectively. In this assay, Lkt from *M. haemolytica* serotypes A1, A2, A6, and *P. trehalosi* induced cytolysis of BHS LFA-1 transfectants in a concentration-dependent manner (Fig. 2). The parent HEK-293 cells were not lysed by Lkt. These results confirmed that BHS LFA-1 serves as a receptor for Lkt.

Intracellular calcium ($[Ca^{2+}]_i$) elevation in target cells, following exposure to low concentrations of Lkt, has been accepted as an indication of Lkt-receptor interaction. This elevation of $[Ca^{2+}]_i$ is primarily due to the influx of extracellaular $[Ca^{2+}]$ through voltage-gated channels (Ortiz-Carranza and Czuprinski, 1992). Therefore, we measured $[Ca^{2+}]_i$ elevation in BHS LFA-1 transfectants exposed to Lkt by using a fluorescent calcium indicator (Fluo-4-AM, Invitrogen, Carlsbad, CA, USA) as previously described (Lawrence et al., 2007). As little as 10 U of Lkt was sufficient to elevate the $[Ca^{2+}]_i$ level in BHS LFA-1 transfectants (Fig. 3), whereas incubation of parent HEK-293 cells with either 10 U of Lkt (Fig. 3) or higher Lkt concentrations (640U) failed to induce $[Ca^{2+}]_i$ elevation in these cells. Incubation of BHS LFA-1

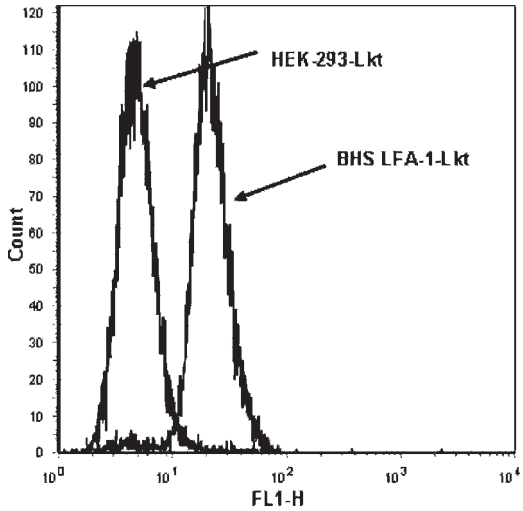

FIGURE 3. *Mannheimia haemolytica* Lkt induces $[Ca^{2+}]_i$ elevation in transfectants expressing bighorn sheep (BHS) LFA-1. The BHS LFA-1 transfectants and parent HEK-293 cells were incubated with fluorescent calcium indicator (Fluo-4-AM) and exposed to 10 U of leukotoxin (Lkt), and $[Ca^{2+}]_i$ elevation was analyzed by flow cytometry. Lkt from *M. haemolytica* induced $[Ca^{2+}]_i$ elevation in BHS LFA-1, but not in HEK-293 cells, confirming $[Ca^{2+}]_i$ elevation is mediated by BHS-LFA-1-Lkt interaction. Results of one representative experiment out of three are shown.

transfectants and HEK-293 cells, with culture supernatant from an Lkt deletion mutant of *M. haemolytica* (Murphy et al., 1995), did not result in $[Ca^{2+}]_i$ elevation (data not shown), indicating that influx of $[Ca^{2+}]_i$ is specifically mediated by Lkt. Previously, we transfected BHS CD18 alone into the murine mastocytoma cell-line P815, and the transfectants expressed BHS CD18, along with murine CD11a innately expressed by P815 cells (Liu et al., 2007). Interestingly, the percent cytolysis and $[Ca^{2+}]_i$ elevation were similar in the P815 transfectants and the HEK-293 transfectants developed in this study, suggesting that either BHS or murine CD11a subunit can associate with BHS CD18 and stabilize it on the cell surface.

In summary, we previously demonstrated that CD18 mediates *M. haemolytica* Lkt-induced cytolysis of BHS leukocytes. However, it was not clear whether CD18 of all three β_2-integrins was involved in Lkt-induced cytolysis. In this study, we have clearly demonstrated that BHS LFA-1 serves as a functional receptor for Lkt. Experiments to determine the involvement of BHS Mac-1 and CR4 in Lkt-induced cytolysis are currently underway in our laboratory.

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