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β_2 integrin Mac-1 is a receptor for *Mannheimia haemolytica* leukotoxin on bovine and ovine leukocytes

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Abstract

Pneumonia caused by *Mannheimia haemolytica* is an important disease of cattle (BO), domestic sheep (DS, *Ovis aries*) and bighorn sheep (BHS, *Ovis canadensis*). Leukotoxin (Lkt) produced by *M. haemolytica* is cytolytic to all leukocyte subsets of these three species. Although it is certain that CD18, the β subunit of β_2 integrins, mediates Lkt-induced cytolysis of leukocytes, whether CD18 of all three β_2 integrins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and CR4 (CD11c/CD18), mediates Lkt-induced cytolysis of BO, DS and BHS leukocytes remains a controversy. Based on antibody inhibition experiments, earlier studies suggested that LFA-1, but not Mac-1 and CR-4, serves as a receptor for *M. haemolytica* Lkt. PMNs express all three β_2 integrins, and they are the leukocyte subset that is most susceptible to Lkt. Therefore we hypothesized that all three β_2 integrins serve as the receptor for Lkt. The objective of this study was to determine whether Mac-1 of BO, DS and BHS serves as a receptor for Lkt. cDNAs for CD11b of BO, DS and BHS were transfected into a Lkt-non-susceptible cell line along with cDNAs for CD18 of BO, DS and BHS, respectively. Transfectants stably expressing BO, DS or BHS Mac-1 specifically bound Lkt. These transfectants were lysed by Lkt in a concentration-dependent manner. Increase in intracellular $[Ca^{2+}]_i$ was observed in transfectants following exposure to low concentrations of Lkt indicating signal transduction through secondary messengers. Collectively, these results indicate that Mac-1 from these three species serves as a receptor for *M. haemolytica* Lkt.

Keywords: *Mannheimia haemolytica*; Leukotoxin; Receptor; Mac-1; Cattle; Domestic sheep; Bighorn sheep

1. Introduction

Pneumonia caused by *Mannheimia haemolytica* is an important disease of cattle, domestic sheep and bighorn sheep (Foreyt and Jessup, 1982; Miller et al., 1991; Mosier, 1997; Brogden et al., 1998; Ackermann and Brogden, 2000). Leukotoxin (Lkt) secreted by *M. haemolytica* is the major virulence factor responsible for the pathogenesis of pneumonia caused by this organism. Lkt is a 102 kDa protein belonging to the RTX (repeats in toxin) family of pore-forming toxins produced by several Gram-negative bacteria (Lo et al., 1985; Welch, 1991). At high concentrations the toxin induces trans-membrane pore formation leading to the efflux of K^+, influx of Ca^{2+}, colloidal osmotic swelling, and eventual cytolysis (Clinkenbeard et al., 1989; Jeyaseelan et al., 2001). At sub-lytic concentrations, Lkt activates alveolar macrophages and PMNs resulting in the release of proinflammatory cytokines (Yoo et al., 1995) and induction of apoptosis (Stevens and Czuprynski, 1996).

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Previous studies in our laboratory and that of others identified β_2 integrins as the receptor for Lkt on the target cells (Wang et al., 1998; Ambagala et al., 1999; Li et al., 1999; Jeyaseelan et al., 2000). β_2 integrins are leukocyte-specific integrins, and have a common β subunit, CD18, which associates with three distinct α subunits, CD11a, CD11b, and CD11c, giving rise to three well-characterized integrins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and CR4 (CD11c/CD18) (Gahmberg et al., 1997; Hogg et al., 2002). Recently identified β_2 integrin, CD11d/CD18 (Noti et al., 2000), has not been well characterized in ruminants. β_2 integrins are critical for leukocyte homing into areas of inflammation, phagocytosis, antigen presentation and cytotoxicity. Studies in our laboratory involving recombinant expression of CD18 in Lkt-non-susceptible cells have shown that CD18, the β subunit of β_2 integrins, mediates Lkt-induced cytolysis of leukocytes of cattle (Deshpande et al., 2002), BHS (Liu et al., 2007) and DS (Dassanayake et al., 2007). However, it is not clear whether the CD18 of all three β_2 integrins, LFA-1, Mac-1 and CR4, mediates Lkt-induced cytolysis of BO, DS and BHS leukocytes. Since PMNs, which express all three β_2 integrins, are the leukocyte subset that is most susceptible to Lkt, it is logical to hypothesize that all three β_2 integrins serve as the receptor for Lkt. LFA-1 of cattle, DS and BHS has been shown to be a receptor for Lkt (Jeyaseelan et al., 2000; Thumbikat et al., 2005; Lawrence et al., 2007; Dassanayake et al., in press). However, based on the results of antibody inhibition assays, earlier studies reported that Mac-1 does not serve as a receptor for *M. haemolytica* Lkt (Jeyaseelan et al., 2000; Thumbikat et al., 2005). The objective of this study was to determine unambiguously the role of Mac-1 in Lkt-induced cytolysis by developing transfectants expressing BO, DS or BHS Mac-1, and determine their susceptibility to Lkt-induced cytolysis without the confounding effects of LFA-1 and CR4.

2. Materials and methods

2.1. Cell lines and growth conditions

The human embryonic kidney cell line, HEK-293 (ATCC® Number: CRL-1573™) was cultured in complete culture medium (DMEM medium [Invitrogen] supplemented with 10% [v/v] heat-inactivated fetal bovine serum along with L-glutamine 4 mM and gentamicin 50 μg/ml [Sigma]). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. HEK-293 cells stably transfected with BHS and DS Mac-1 (CD11b/CD18) were selected and maintained in complete culture medium containing selection antibiotics: geneticin, 800 μg/ml (G418; Invitrogen) and blasticidin, 30 μg/ml (InvivoGen). BO Mac-1 transfectants were maintained on medium supplemented with 500 μg/ml each of hygromycin (Invitrogen) and geneticin.

2.2. Monoclonal antibodies and leukotoxin

The monoclonal antibodies (MAbs) specific for human CD11b (MM12A, IgG1), and human CD18 (HUH82A, IgG2a), which cross-reacts with BO, DS and BHS CD11b and CD18, respectively (Saalmuller et al., 2005) were obtained from Washington State University Monoclonal Antibody Center. The Lkt-neutralizing MAb MM601 (IgG1), and the FITC-conjugated Lkt-non-neutralizing MAb MM605 (IgG2a) developed earlier in our laboratory (Gentry and Srikumaran, 1991) were used in Lkt-neutralization and Lkt-binding assays, respectively. The MAbs 8G12 (IgG1) specific for bovine respiratory syncytial virus (Klucas and Anderson, 1988) and MM113 (IgG2a) specific for bovine herpesvirus 1 (Srikumaran et al., 1990) were obtained from the Department of Veterinary and Biomedical Sciences at the University of Nebraska-Lincoln, and used as isotype-matched controls. The Lkt from *M. haemolytica* (serotypes A1, A2 and A6) was prepared as described earlier (Gentry and Srikumaran, 1991). The culture supernatant containing Lkt was filter sterilized and stored at −20 °C in aliquots until needed. Same batch of Lkt was used in all the experiments.

2.3. Expression constructs of CD11b and CD18

The cDNA encoding bovine CD11b (Gopinath et al., 2005; GenBank Accession No. AY841169) was subcloned into the eukaryotic expression vector pcDNA3.1/Hygro(+) (Invitrogen) to yield the expression vector pWL/BO CD11b. The cDNA encoding CD11b from DS (GenBank Accession No. EF206308) and BHS (GenBank Accession No. EF206309) were cloned earlier into pUC19 vector (Lawrence and Srikumaran, 2007). In order to make expression constructs for transfection experiments, these genes were subcloned into mammalian expression vector, pcDNA6.2/GW/D-TOPO (Invitrogen) by PCR. All PCR reactions were carried out using PfuUltra™ II Fusion HS (Stratagene), a high fidelity DNA polymerase. The resulting constructs, pKL/DS CD11b and