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Research paper

Co-expression of ovine LPS receptor CD14 with *Mannheimia haemolytica* leukotoxin receptor LFA-1 or Mac-1 does not enhance leukotoxin-induced cytotoxicity

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ABSTRACT

Leukotoxin (Lkt) and LPS are the major virulence determinants of *Mannheimia haemolytica* that contribute to the pathogenesis of bovine and ovine pneumonic pasteurellosis. We have previously identified bovine and ovine CD18 as the functional receptor for Lkt. LPS complexes with Lkt resulting in increased thermal stability and enhanced cytotoxic activity of Lkt. Cellular recognition of LPS involves several different molecules including CD14. We hypothesized that expression of ovine CD14 together with LFA-1 or Mac-1 would enhance Lkt-induced cytotoxicity. Ovine cDNA for CD14 was amplified by PCR and cloned into mammalian expression vectors. The 1122 bp cDNAs for bighorn sheep (BHS) and domestic sheep (DS) CD14 encode 373 amino acids which exhibit 99% identity with each other. Ovine CD14 plasmids were transfected either into HEK-293 cells, or previous HEK-293 transfectants stably expressing ovine LFA-1 or Mac-1. Flow cytometric analysis of transfectants confirmed the cell surface expression of CD14. The transfectants expressing LFA-1 or Mac-1 and the transfectants co-expressing CD14 with LFA-1 or Mac-1 did not show any significant difference in Lkt-induced cytotoxicity when incubated with LPS complexed Lkt. In contrast, incubation of the LFA-1 or Mac-1 and LFA-1/CD14 or Mac-1/CD14 transfectants with Lkt which lacks LPS, resulted in reduced cytotoxicity. None of the above transfectants showed any difference in $[Ca^{2+}]_i$ elevation when incubated with both types of Lkt preparations. Lkt did not induce any cytotoxicity or $[Ca^{2+}]_i$ elevation in ovine CD14 transfectants or parent HEK-293 cells. Based on these findings, we conclude that expression of CD14 together with LFA-1 or Mac-1 does not enhance Lkt-induced cytotoxicity, whereas LPS enhances cytotoxicity by complexing with Lkt.

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1. Introduction

Mannheimia (*Pasteurella*) *haemolytica* causes severe pneumonia in cattle, goats, domestic sheep (DS, *Ovis aries*) and bighorn sheep (BHS, *Ovis canadensis*; Mosier, 1997; Brogden et al., 1998; Ackermann and Brogden, 2000; Highlander et al., 2000; Miller, 2001). However, BHS are much more susceptible to *M. haemolytica*-caused pneumonia than other ruminants (Foreyt et al., 1994; Dassanayake

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et al., 2009). This bacterium produces several virulence determinants, of which leukotoxin (Lkt) and LPS are the major ones that contribute to the pathogenesis of pneumonia (Highlander et al., 2000; Hodgson et al., 2003; Dassanayake et al., 2009). Lkt is a member of the repeats-in-toxin (RTX) family of pore-forming cytolysins produced by a group of Gram-negative bacteria. It exhibits cell type – as well as species – specificity. Lkt is cytotoxic to all subsets of leukocytes of domestic and wild ruminants (Sutherland, 1985; Clinkenbeard et al., 1989; Silflow and Foreyt, 1994). However, PMNs are the most susceptible subset. Lkt-induced PMN lysis and degranulation have been implicated as the primary cause of acute inflammation and lung injury characteristic of pneumonia (Slocombe et al., 1985).

Earlier studies performed in our laboratory and that of others have shown that the cytotoxic effect of Lkt on bovine and ovine leukocytes is mediated by Lkt-β_2 integrin interactions (Ambagala et al., 1999; Deshpande et al., 2002; Dassanayake et al., 2007a,b, 2008; Lawrence et al., 2007, 2008). β_2 Integrins are leukocyte-specific integrins expressed on the cell surface as heterodimeric glycoproteins. They have a common β subunit, CD18, which associates with four α subunits to 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). Since Lkt binds to CD18, and the species-specific susceptibility to Lkt-induced effects is resident in the CD18 subunit, it has been accepted as the receptor for Lkt on ruminant leukocytes (Ambagala et al., 1999; Wang et al., 1998). Indeed, in our recent studies, recombinant expression of monomeric bovine CD18 by an Lkt non-susceptible cell line (HEK-293) rendered it susceptible to Lkt-induced cytolysis and $[Ca^{2+}]_i$ confirming that the CD18 is the functional receptor for Lkt (Dassanayake et al., 2007a).

M. haemolytica LPS is the major constituent of the cell-wall, as with other Gram negative bacteria. *M. haemolytica* LPS is similar to that of other bacteria and consists of the innermost lipid A (endotoxin), the core oligosaccharide, and the outer most polysaccharide O-side chain (O-antigen; Richards and Leitch, 1989; Davis et al., 1991). *M. haemolytica* LPS is known to complex with Lkt at molar ratios as high as 60:1 which results in enhanced stability and cytotoxic activity of Lkt (Li and Clinkenbeard, 1999). However, the molecular basis underlying the enhanced cytotoxicity of LPS-complexed Lkt is not clear. We hypothesize that the Lkt-complexed LPS binding to its receptor CD14 on the PMNs enhances the binding of Lkt to its receptor CD18 and enhances the cytotoxicity of Lkt. Therefore, the objective of this study was to determine whether co-transfection of ovine CD14 with LFA-1 or Mac-1 enhances the Lkt-induced cytotoxicity of the transfectants.

2. Materials and methods

2.1. Cell lines and growth conditions

The human embryonic kidney cell line 293 (HEK-293; ATCC CRL-1573) was maintained in complete growth medium (Dulbecco's modified Eagle's growth medium) containing 4 mM L-glutamine and 50 μg/ml gentamicin (Invitrogen) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals) at 37 °C in a humidified atmosphere of 5% CO_2. Transfectants stably expressing ovine LFA-1 and Mac-1 were maintained in the complete growth medium supplemented with 10 μg/ml blasticidin and 800 μg/ml G418 (Invivogen).

2.2. Monoclonal antibodies and leukotoxin

Anti-human CD11a (HUH73A, IgG1), CD11b (MM12A, IgG1), CD14 (CAM36A, IgG1) and CD18 (HUH82A, IgG2a) mouse MAbs that cross-react with ovine CD11a, CD11b, CD14, and CD18, respectively were obtained from Washington State University Monoclonal Antibody Center. MAbs 8G12 (IgG1; Klucas and Anderson, 1988) and MM113 (IgG2a; 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. FITC-conjugated goat anti-mouse Ig (Caltech Laboratories) was used as the secondary Ab in flow cytometry. The Lkt from logarithmic phase cultures of *M. haemolytica* serotype A1 was prepared as previously described and stored at −80 °C until used (Gentry and Srikumaran, 1991). All the experiments were performed with the same batch of Lkt. In order to remove LPS from Lkt preparations, culture supernatants were incubated with polymyxin B (Sigma) at 10 μg/ml for 30 min on ice. Chromogenic *Limulus* amebocyte lysate assay (LAL, QCL-1000) was performed to quantify LPS concentrations in Lkt preparations according to the manufacturer's instruction (Lonza).

2.3. Molecular cloning of cDNA encoding ovine CD14

Peripheral blood from healthy BHS and DS were collected by venipuncture and subjected to Ficoll-Paque (Amersham Pharmacia Biotech) density gradient centrifugation, and PMNs were isolated from the erythrocyte pellet by hypotonic lysis. Total cellular RNA was extracted from 5×10^6 ovine PMNs using TRIzol reagent (Invitrogen). cDNA synthesis was performed using superscript III first-strand cDNA synthesis system for RT-PCR using oligo $d(T)_{20}$ primers according to manufacturer's instructions (Invitrogen). Forward primer with CACC overhang for directional TOPO cloning (5′-CACCATGGTGTGCGTGCCCTACCTG-3′) and reverse primer (5′-TTACGCGAAGCCTCGGGCTCCTTG-3′) were designed based on available bovine CD14 sequence (GenBank accession no. NM_174008). PCR was carried out in a 50 μl volume containing 1× *PfuUltra*™ reaction buffer, 0.25 mM each dNTPs, 0.2 μM each primer, 2 μl cDNA template, 1 U of *PfuUltra*™ II Fusion HS DNA polymerase (Stratagene) and distilled water (to 50 μl) as described by the manufacturer. A single band with an approximate size of 1.2 kb was purified from agarose gel and cloned into the mammalian expression vector, pcDNA3.2/Hygro (Invitrogen) and transformed into *Escherichia coli* TOP10 chemically competent cells. Plasmid DNA was extracted from ampicillin-resistant clones and sequenced to identify intact ovine CD14. The full length BHS- and DS-CD14 gene sequences (1122 bp) are available in the GenBank under accession numbers DQ917484 and DQ917485.

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