Published in final edited form as:

Microb Pathog. 2009 March ; 46(3): 159–165. doi:10.1016/j.micpath.2008.12.002.

Brief heat treatment increases cytotoxicity of *Mannheimia haemolytica* leukotoxin in an LFA-1 independent manner

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Abstract

Mannheimia haemolytica is an important respiratory pathogen in cattle. Its predominant virulence factor is a leukotoxin (LKT) that is a member of the RTX family of exotoxins produced by a variety of gram negative bacteria. LKT binds to the CD18 chain of β_2 integrins on bovine leukocytes, resulting in cell death. In this study, we show that brief heat treatment of native LKT (95 °C for 3 min) results in increased cytotoxicity for BL-3 (bovine lymphoblastoid) cells. Similar heat treatment restored the activity of LKT that had been rendered inactive by incubation at 22 °C for 3 days. A hallmark of LKT is that its toxicity is restricted to leukocytes from cattle or other ruminant species. Surprisingly, heat treatment rendered LKT cytotoxic for human, porcine and canine leukocytes. Membrane binding studies suggested that heat-treated LKT binds to membrane proteins other than LFA-1, and is distributed diffusely along the BL3 cell membrane. Circular Dichroism spectroscopy studies indicate that heat treatment induced a small change in the secondary structure of the LKT that was not reversed when the LKT was cooled to room temperature. Thus, we speculate that these structural changes might contribute to the altered biological properties of heat-treated LKT.

Keywords

Leukotoxin; LFA-1; cytotoxicity; bovine leukocytes

1. Introduction

Mannheimia haemolytica, a respiratory pathogen of cattle and other ruminant species, produces a leukotoxin (LKT) that binds to the CD18 chain of the β_2 integrin, LFA-1 (lymphocyte function-associated antigen-1, CD18/CD 11a) on bovine leukocytes [1–5]. LKT binding elicits an array of leukocyte responses ranging from cell activation to cell death. We have shown that membrane bound LKT is internalized into the cell in a lipid raft and clathrin dependent manner [6]. Once internalized, it binds to the mitochondrial outer membrane resulting in collapse of the mitochondrial membrane potential and release of cytochrome c [7]. A number of other Gram negative bacteria, including *Escherichia coli*, *Aggregatibacter*

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actinomycetemcomitans and *Fusobacterium necrophorum*, produce related exotoxins that are members of the RTX toxin (repeats in toxins) family [8,9]. RTX toxins exhibit a shared motif of glycine rich repeat regions, that bear the consensus sequence –G-G-X-G-X-D-X-U-X (U being a hydrophobic residue) distal to the activation region of the toxin molecule. These repeat regions bind calcium (Ca^{2+}) which is required for the biological activity of the toxin [10,11]. Although the precise tertiary structure of RTX toxins is not yet known, studies with an RTX toxin produced by *Pseudomonas aeruginosa* revealed that these glycine repeats form elongated β -roll structures, with β -strands onto which Ca^{2+} ions bind at turns between the strands [8]. Monoclonal antibodies against either CD18/CD11a (i.e. BAT75) or LKT (MM601 and MM605) block the cytotoxic effects of LKT for bovine leukocytes in vitro [12]. It has been hypothesized for a related RTX toxin (*E. coli* haemolysin), that the hydrophobic N-terminal region of the toxin binds to the plasma membrane [8]. This is followed by insertion of the C terminal region with its Ca^{2+} ion bound rigid β barrel into the plasma membrane [10].

It has been shown previously that exposure of LKT to high temperature (90–100 °C) for 30 minutes or more, largely eliminates cytotoxic activity for bovine leukocytes [13]. However, other studies showed that exposure of some bacterial toxins to high temperature has varying bio-physical effects on those toxins [22]. For example, incubation at 80 °C significantly decreased the cytotoxicity of the heat stable enterotoxin of *E. coli*, or *Vibrio cholera* enterotoxin for Vero cells. In contrast, *Bacillus cereus* haemolysin BL demonstrated no change in its activity under the same conditions [14].

2. Results

2.1 Brief heat treatment enhances the cytotoxicity of *M. haemolytica* LKT

It has been reported previously that heat treatment of LKT at 100 °C for 30–60 min inactivates its biological activity. Paradoxically, we found that LKT heated for 1–3 minutes at 95 °C exhibited increased cytotoxic activity as compared to the untreated toxin (Fig. 1). Furthermore, LKT that had become inactive as a result of prolonged incubation at 22 °C for 3 days, regained its biological activity when heated at 95 °C for 3 minutes (Fig. 1). However, a biologically inactive LKT produced by an *M. haemolytica* *lktC* gene mutant unable to acylate the LKT protein did not exhibit cytotoxic activity after the same heat treatment (data not shown). These observations suggest that brief heat treatment (95 °C for 3 minutes) can enhance or restore the biological activity of acylated LKT.

2.2 The biological activity of heat-activated LKT is neutralized by an anti-Lkt mAb, but not by an anti-LFA-1 (BAT-75) antibody

To exclude the possibility that an extraneous substance was responsible for the enhanced leukotoxic activity of heat-treated LKT, we preincubated heat treated and native LKT with a neutralizing anti-LKT mAb (MM601). As expected, the anti-LKT mAb blocked the cytotoxic activity of both heat-treated and native LKT. To our surprise, preincubation of BL-3 cells with anti-LFA-1 mAb (BAT-75) did not block the cytotoxicity of heat treated LKT, although as expected it did so for the native LKT (Fig. 2). These data suggest that brief heat treatment alters LKT in a manner that changes its ability to bind LFA-1, or circumvents the need to bind LFA-1 to exert toxic activity.

2.3 Heat treated LKT is cytotoxic to non-bovine lymphocytes

As reported previously, we found that 1.0 unit of LKT had no cytotoxicity for human lymphocytes (10^6) when incubated at 37 °C for an hour. In contrast, human lymphocytes incubated with heat treated LKT under the same conditions, exhibited 55% cytotoxicity. Likewise, canine and porcine lymphocytes exhibited 43% and 52% cytotoxicity, respectively,

when incubated with heat activated LKT, whereas the native LKT was not cytotoxic for these cells (Fig. 3).

2.4 Binding of heat treated LKT to BL-3 cells

When we used fluorescence microscopy to examine binding of native and heat-treated LKT to BL-3 cells, we observed a diffuse distribution of heat treated LKT and focal distribution of native LKT on the BL-3 cell surface (Fig. 4). Using far-western immunoblotting, we observed that the heat-treated LKT bound to an array of cellular proteins other than LFA-1 (CD18/CD11a), ranging in molecular weight from 15 to 80 kD. In contrast, native LKT bound principally to LFA-1 (Fig. 5).

2.5 CD spectroscopy

The above results suggested that brief heat treatment caused a conformational change in the LKT protein. We used CD spectroscopy to assess changes in LKT structure as a result of heat treatment. We focused on the far UV spectral region which is generally considered to reflect the secondary structure of proteins. At 25 °C, the spectrum of LKT was characterized by a minimum at ~210 nm. Significant changes in spectral features, implying changes in secondary structure, were observed when LKT was heated to 95 °C. The 210 nm minimum appeared to shift to a lower wavelength and, more importantly, there was a significant reduction in magnitude. These changes were complete soon after achieving 95 °C and remained stable at that temperature. When the temperature was returned to 25 °C, the CD signal became more negative and was similar in shape to the original spectrum. However, near 220 nm there was a distinct loss in magnitude, suggesting that the heat treatment had induced a stable change in LKT structure (Fig. 6). The spectra at all temperatures were similar for LKT at protein concentration of 0.5 or 1.0 mg/ml.

3.0 Discussion

In this study we show that the cytotoxic activity of *Mannheimia haemolytica* leukotoxin can be increased by brief heat treatment (95 °C for 3 minutes). Furthermore, we show that LKT that has lost its cytotoxic activity by standing at room temperature for 3 days can be reactivated by the same short term (95 °C for 3 minutes) heat exposure (Fig. 1). The cytotoxic activity of heat treated LKT was neutralized by an anti-LKT mAb (MM601), indicating that the activity was due to LKT and not a contaminant. In contrast, non-acetylated LKT produced by a biologically inactive *M. haemolytica lktC* mutant (LKTm) exhibited no cytotoxicity either before or after heat treatment (data not shown). These observations suggest that heat treatment caused changes in the structure of acetylated LKT that augments, or restores, its cytotoxic activity for BL-3 cells.

Despite the impressive ability of a brief heat treatment to increase or restore LKT cytotoxicity, heating LKT for more than 3 minutes resulted in a time-dependent decrease in its cytotoxicity. We believe that this is likely due to excessive denaturing of the LKT protein that disrupts its ability to bind to BL-3 cells. One hallmark of native LKT is that its cytotoxicity is limited to ruminant leukocytes, a property that is presumed to be dependent on its ability to bind ruminant LFA-1. One of the most exciting observations of the present study was that the ability of heat-treated LKT to cause cytotoxicity for BL-3 cells was not blocked by a mAb against LFA-1 (CD18/CD11a) that neutralizes the cytotoxicity of native LKT (Fig. 2). These findings suggest that brief heat-treatment caused conformational changes in the LKT molecule that allowed it to bind to the cell membrane in an LFA-1 independent manner. Furthermore, we found that heat-treated LKT was cytotoxic to non-ruminant cells (i.e. human, canine and porcine leukocytes) which are resistant to native LKT (Fig. 3). However, the relatively low levels of cytotoxicity of heat-treated LKT for these cells, as compared to BL-3 cells, may indicate that

the heated LKT protein still binds less efficiently to heterologous leukocytes. Alternatively, perhaps heat treatment alters the structure only of some of the LKT protein molecules, which could then bind to the leukocyte membrane independently of LFA-1 and cause cytolysis. Although heat treatment enhanced cytotoxic activity of LKT for leukocytes, it did not render the LKT hemolytic for bovine erythrocytes, even at higher concentrations of heat-treated LKT (data not shown). Similarly, heat activated LKT had no activity against K-562 cells, a human erythroleukemia cell line [15,16] (data not shown).

Using immunofluorescent microscopy, we observed a substantial difference in the spatial distribution of LKT and heat-treated LKT on BL-3 cells. Binding of native LKT to BL-3 cells was focal and polarized (i.e. capping), whereas, heat-treated LKT bound more diffusely to the BL-3 cell surface (Fig. 4). Consistent with these data, we used Far-Western Immunoblotting to show that heat treated LKT, unlike native LKT, binds to both LFA-1 and other cell membrane proteins (Fig. 5). This observation may help to explain the ability of heat treated LKT to cause LFA-1 independent cytotoxicity to BL-3 cells, human, porcine and canine leukocyte. Taken together, these observations suggest that heat treated LKT, unlike native LKT, binds to BL-3 cell membrane proteins other than LFA-1. We hypothesize that differences in LKT binding and cytotoxicity reflect structural changes that occur during the heating process of the LKT molecule. Our CD spectroscopy analysis of LKT suggests that this is the case. Below 260 nm, the CD spectrum of a protein is generally considered to be dominated by contributions from the elements of secondary structure that are present [17]. For LKT, the low magnitude of the CD signal and the lack of a distinct minimum at ~220 nm suggests that very little, if any, α -helix is present [18]. Although the exact structure of LKT is unknown, studies on another member of the RTX toxin family, *Escherichia coli* hemolysin (HlyA), confirm the presence of an amphipathic α -helix and a charge rich linker followed by another α -helix near the C-terminal end [11]. Perhaps, this unanticipated result explains the structural diversity of RTX toxins and account for functional differences within this toxin family. The strength of CD spectroscopy, however, lies less in its ability to assign structural features than its exquisite sensitivity to detect structural changes arising from perturbations in temperature, pH or ligand binding [19–21]. This strength was exploited here to understand the influence of temperature on the properties of LKT. The spectrum of unheated LKT was characterized by a distinct minimum near 210 nm that was reduced by about one third, and probably shifted slightly to lower wavelength, upon heating to 95 °C. These changes were largely independent of protein concentration (0.5 and 1.0 mg/ml). After the temperature returned to 25 °C, the magnitude of the 210 nm peak did not completely recover to the pre-heated baseline value. The spectroscopic change was most obvious near 220 nm. It should be noted that there was no visible indication of LKT aggregation during or after heating. These studies suggest that conformational changes occur when LKT is heated to 95 °C, and these are not fully reversed upon cooling. We infer that these changes result in a new stable LKT species that's acting in an LFA-1 independent manner (Fig. 7). This is similar to what has been reported with certain other large protein molecules [22,23]. For example comparable structural changes were reported for a different RTX toxin, the *Pseudomonas aeruginosa* protease, which suggested that the glycine repeats form β strands rather than an α strand [24].

Our data are consistent with a temperature-sensitive unfolding, and incomplete refolding, of LKT to form a thermodynamically stable intermediate, perhaps a molten globule state [25, 26]. Formation of a molten globular state is achieved by loosening or unfolding non-polar side chains, while retaining principal secondary structures such as α and β helices [27,28]. Protein binding to receptors, pH changes, and changes in temperature, also can result in a molten globular state (5). It has been proposed that protein toxins do not insert into cell membranes in their native secondary structure, but instead do so after being transformed into a non-native form [26,29]. Based on our results, we suggest heat treatment of LKT simulates structural changes that might be similar to those that occur when LKT undergoes the transition from a

receptor bound to a membrane inserted form. Based on CD spectroscopic analysis, the heat treated form of LKT appears to be stable and structurally distinct from both the native LKT and LKT held at 95 °C. Cytotoxicity of some RTX toxins is species specific (e.g. LKT and *Aggregatibacter actinomycetemcomitans* leukotoxin) while other RTX toxins are more promiscuous in their cytotoxicity for target cells (e.g. *E.coli* hemolysin). Perhaps, these differences reflect subtle structural variations of these RTX proteins. Our study shows that heat can convert LKT protein from a species-specific form to a more promiscuous and receptor independent toxin. We speculate that this form of the LKT may be structurally related to the membrane inserted form of the native toxin.

4.0 Conclusions

In this study we demonstrate that brief heat treatment of *Mannheimia haemolytica* leukotoxin increases its cytotoxicity for BL-3 cells and restores the cytotoxicity of inactive LKT. The cytotoxicity of heated LKT was LFA-1 independent and promiscuous, extending to non-bovine cells. Immunofluorescence studies and far-western blotting indicate that heat-activated LKT binds more diffusely on BL-3 cells, and binds to cell membrane proteins other than LFA-1. CD spectroscopy showed changes in LKT secondary structure during heating which did not revert completely following cooling. Besides casting new light on structure-function relationships of LKT cytotoxicity, perhaps these findings will also prove useful to modify LKT in vaccines designed to protect cattle from *M. haemolytica* infections.

5.0 Material and methods

5.1 LKT production and purification

Crude LKT was produced and purified as described previously [30]. For all experiments, one unit of LKT activity was defined as the LKT dilution causing 50% killing of BL-3 cells (10^6 cells suspended in 1 ml of RPMI 1640 medium) when incubated at 37°C for 1 h, as determined by Trypan blue exclusion. LKT was stored at -70°C until used in an experiment. An *lktC* mutant of *M. haemolytica* (SH 1562, generously provided by Dr. S.V. Highlander, Houston, TX), that produces a LKT protein (LKTm) with no biological activity, was prepared in a similar manner as that described for the wild type LKT from *M. haemolytica* strain A1.

Heat treated LKT was prepared by incubating LKT at 95 °C in a water bath for the designated time periods, and then kept on ice until used in an experiment. Biologically inactive LKT (LKTinac) was prepared by allowing native LKT to stand at room temperature for 4 to 5 days before use in an experiment.

5.2 Cell cultures

The bovine lymphoblastoid cell line, BL-3 cells (kindly provided by Dr. Ronald Schultz, Madison, WI) was used as a target for LKT-mediated cytotoxicity. These non-adherent cells were grown at 37°C with 5% CO₂ in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Burlington, VT). Human, canine and porcine peripheral blood mononuclear cells were isolated as described previously, and LKT cytotoxicity assays were performed in a similar manner to that described for BL-3 cells [31].

5.3 Viable cell proliferation assay

To quantify cytotoxicity, BL-3 cells (10^6 cells per ml) were washed, resuspended in antibiotic free RPMI-1640 with 10% FBS and incubated with 1 unit of LKT at 37 °C for 60 minutes. Cell viability was then quantified using the Cell Titre 96 AQ One assay system (Promega Cooperation, Madison, WI). Optical density was measured at 450 nm using an automated ELISA reader (Mini plate EL 312, BIO TEK Instruments Inc. Winooski, VT). Monoclonal

antibodies against LKT (MM601) and bovine LFA-1 (BAT75) were used to block LKT mediated cytotoxicity.

5.4 Immunofluorescence studies

BL-3 cells (10^6 cells per ml) were incubated with either 1 unit of LKT, or similar amount of heat treated LKT, for 30 minutes at 37°C. The cells were washed 3X in PBS, and then fixed in 4% paraformaldehyde for 10 min. The fixed cells were washed three times in PBS and then incubated in PBS with 5% bovine serum albumin for 20 min at room temperature. Cells were then incubated with an anti-LKT mAb (MM601) for 60 min at room temperature, washed 3X with PBS and incubated at room temperature for 60 min with FITC labeled rabbit anti mouse IgG. Cells were washed three 3X with PBS and examined by fluorescent microscopy.

5.5 Far-western immunoblotting

Cell membrane lysates from BL-3 cells (100 µg) were prepared as described previously [32]. They were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated for 1 hour at room temperature with renaturing buffers A and B (1X stock buffer: 200mM Hepes, pH 7.7, 250mM NaCl, 50mM MgCl₂, 10mM DTT) supplemented with first 6M (A) and then 0.187M (B) guanidine. The membranes were incubated a second time overnight. Membrane were soaked in 1X stock buffer supplemented with 5% milk for 1 hour, and incubated overnight at 4 °C with LKT or heat treated LKT (5 units/20ml/blot). Membranes were washed with blotting buffer (20 mM HEPES, 150mM KCl, 150 mM sodium chloride, 1 mM dithiothreitol, 1 mM EDTA, 0.05% NP- 40, 1% milk) and probed with anti-LKT (MM106)-HRP conjugated antibody and an ECL chemiluminescence substrate (Pierce).

5.6 Circular Dichroism (CD) Spectroscopy

CD spectra were recorded from 260 to ~205 nm using an Aviv Model 202SF circular dichroism spectrophotometer equipped with thermoelectric temperature control system (Aviv Biomedical, Lakewood, NJ). Quartz cuvettes with a 0.1 cm pathlength were used for all spectra. Samples were prepared in PBS and protein concentrations were estimated based on BCA assay kit (Pierce, Rockford, Illinois) and spectra were recorded for LKT samples both at 0.5 and 1.0 mg/mL (corresponds to ~0.25–0.5 units). Data were corrected for baseline by subtraction of a 25 °C buffer scan, and converted to molar ellipticity based on the measured protein concentration and a mean residue weight of 109. Initial spectra were recorded at 25 °C. After the cuvette was removed from the instrument, the temperature in the instrument was raised to 95 °C, the cuvette reinserted and changes in spectra monitored over time. Finally spectral changes were monitored as the temperature returned to 25 °C.

5.7 Statistical analysis

Group means were compared by ANOVA. Turkey-Kramer pairwise comparison test with the Instat statistical package was used (GraphPad, San Diego, CA). The level of statistical significance was set at $P < 0.05$.

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Figure 1. Brief heat treatment restores the cytotoxicity of inactivated LKT and enhances the cytotoxicity of native LKT

Inactive and native LKT were heated in a 95 °C water bath for the designated time periods and then incubated with (A) BL-3 cells, (B) bovine neutrophils, or (C) bovine macrophages (10^6) in RPMI medium at 37 °C for 1 hour. As a control, heated LKT was preincubated with the LKT neutralizing mAb, MM601 (designated as 1/3 min+MM601), before being added to the cells. Cell viability was measured using the Cell Titre 96 AQ colorimetric assay. Biologically inactive toxin produced by a *lktC* mutant of *M. haemolytica* did not demonstrate any cytotoxicity before and after heat treatment (data not shown). These data illustrate the mean $\pm$ SEM for 5 separate experiments. (* = $p < 0.05$)

Figure 2. Anti-LKT mAb, but not anti-LFA-1 mAb, blocks the cytotoxicity of heat-treated LKT for BL-3 cells, bovine neutrophils and bovine macrophages

Native or heat-treated LKT (95 °C for 3 min) was pre-incubated with anti-LKT mAb for 30 minutes on ice before incubating with (A) BL-3 cells, (B) bovine neutrophils or (C) bovine macrophages. Some cells were pre-incubated with anti-LFA-1 mAb before LKT was added (30 minutes at room temperature). Cell viability was measured using the Cell Titre 96 AQ colorimetric assay. These data illustrate the mean $\pm$ SEM for 5 separate experiments. (* = $p < 0.05$)

Figure 3. Heat treated LKT(LKTh), but not native LKT, is cytotoxic for human, canine and porcine white blood cells

LKT or heat-treated LKT (concentration equivalent to 1.0 U of native LKT) was incubated with human, canine or porcine white blood cells (10^6) at 37 °C for 1 hour. As a control LKT was preincubated with anti-LKT mAb for 30 minutes on ice before incubating with BL-3 cells. Cell viability was measured using the Cell Titre 96 AQ colorimetric assay and the figure illustrate the mean $\pm$ SEM for 5 separate experiments (* = $p < 0.05$).

Figure 4. Heat-treated LKT binds diffusely on the BL-3 cell and bovine neutrophil surfaces
Native LKT (A & C) or heat-treated LKT (B & D) were incubated with BL-3 cells (A & B) or bovine neutrophils (C & D) at 37 °C for 30 min. Cells were fixed with 2% paraformaldehyde, stained with anti-LKT mAb and FITC-labeled anti-mouse IgG, and examined by fluorescent microscopy at an emission wavelength of 448 nm.

Figure 5. Far western immunoblotting demonstrates that heat-treated LKT binds to multiple BL-3 cell membrane proteins

BL-3 cell membrane lysates were prepared, separated by SDS-PAGE, and far western immunoblotting performed with LKT or LKTh as described in the Material and Methods. Native LKT predominantly binds to a 98 kD band of CD18 molecule, (β_2 chain of LFA-1, arrow) while heat-treated LKT binds to numerous other membrane proteins. These data are representative of 3 such far western immunoblotting experiments that were performed.

Figure 6. Circular dichroism (CD) spectroscopy demonstrated a change in the secondary structure of LKT after a brief heat treatment (3 minutes at ~ 95 °C), which persists even after the LKT is cooled to room temperature

As the LKT was heated (95 °C), its ellipticity significantly increased at the wave length of ~210 nm (X) and did not revert to its original level (Y) after the LKT was cooled to room temperature (25 °C). Similar results were obtained with 0.5 and 1.0 mg/ml LKT concentrations. These data are representative of 3 separate experiments that were performed.

The proposed forms of LKT and their interactions

Figure 7. Proposed model demonstrating structural changes that may take place during heat treatment of LKT

We propose that heat treatment of LKT results in a stable intermediate form which enables LKT to bind to the cell membrane in an LFA-1 independent manner.