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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 BL-3 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.

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/CD11a) 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 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. BAT-75) 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* hemolysin), 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 min 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* hemolysin BL demonstrated no change in its activity under the same conditions [14].

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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 min 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 min (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 min) 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 U 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 kDa. 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

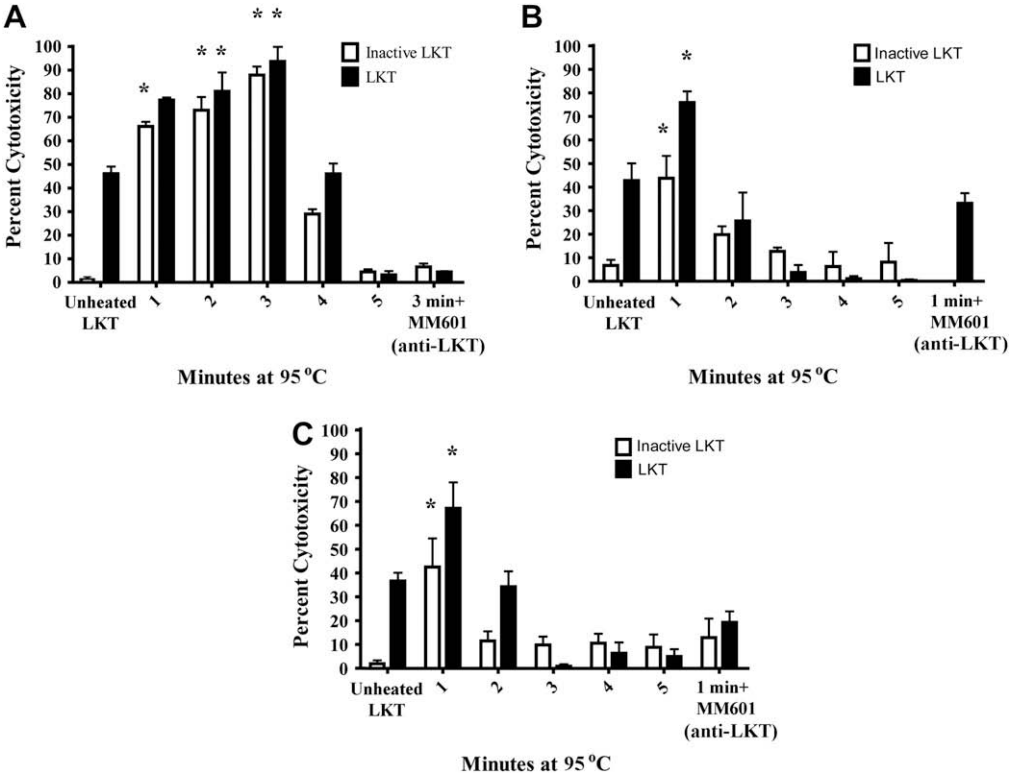

Fig. 1. Brief heat treatment restores the cytotoxicity of inactivated LKT and enhances the cytotoxicity of native LKT. Inactive and native LKT were heated in a 95 °C water bath for the designated time periods and then incubated with (A) BL-3 cells, (B) bovine neutrophils, or (C) bovine macrophages (10^6) in RPMI medium at 37 °C for 1 h. 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 an *lktC* mutant of *M. haemolytica* did not demonstrate any cytotoxicity before and after heat treatment (data not shown). These data illustrate the mean + SEM for 5 separate experiments (* = $p < 0.05$).