Characterization of Macrophage Activation Induced by Leukotoxin from
Aggregatibacter actinomycetemcomitans

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ABSTRACT

*Aggregatibacter actinomycetemcomitans (Aa)*, is a gram-negative, facultative anaerobe, non-motile coccobacilli colonizing the human oral cavity and is strongly associated with localized aggressive periodontitis (LAP). *Aa* possesses several virulence mechanisms, among others; Lipopolysaccharide (LPS), an endotoxin found in the outer membrane of *Aa* and Leukotoxin (LtxA), an exotoxin attached to the bacterial cell surface or in outer membrane vesicles and which has been correlating with periodontal disease mostly. LtxA specifically targets human leukocytes, causing imbalance in the host inflammatory response. It is involved in the activation of a cellular pathway, starting with ATP release from the cell, possibly through Pannexin-1 (Panx-1) channels, Caspase-1 activation and release of bioactive interleukin-1β (IL-1β) from leukocytes. In the case of periodontitis, inflammatory cytokines like IL-1β will stimulate periodontal tissue breakdown. The aim of the present study was to examine the involvement of Panx-1 in LtxA induced activation and cytotoxicity of human macrophages. To determine viability of the macrophages the release of LDH and the accumulation of neutral red was quantified. The release of IL-1β was analysed by ELISA analyses of the cell culture supernatants. Results showed that macrophages treated with Cbx and exposed to LtxA was still sensitive to LtxA, but showed a decrease in the IL-1β release. In opposite, macrophages exposed to LPS showed increased IL-1β release in presence of Cbx. The conclusion of our study is that Panx-1 channels are partially involved in the cellular pathway leading to IL-1β release on LtxA exposed cells.
INTRODUCTION

Periodontal diseases are widespread, affecting up to 90% of the worldwide population. It is caused by bacterial biofilm accumulating on and around the teeth as a result of inadequate oral hygiene. In addition, there are many risk factors associated with the diseases: genetics, nutritional deficiencies, diabetes, smoking, stress, increased age, osteoporosis, HIV/AIDS and other inflammatory systemic diseases. The milder form of periodontal disease; gingivitis, is an inflammation around the gum and is reversible. The more severe form- periodontitis, is a chronic inflammation with a combination of gingival inflammation, loss of connective tissue and bone structure around the teeth (Pihlstrom et al., 2005).

There are two types of periodontitis: chronic and aggressive periodontitis. Compared to chronic periodontitis, aggressive periodontitis affects younger people (often below 30 years old), is more severe, less prevalent, strongly genetic and has a faster rate of progression (Linde et al., 2005). Even though periodontitis is a polymicrobial infection, there is a strong association of the bacterium Aggregatibacter actinomycetemcomitans (Aa) with aggressive periodontitis, especially in local aggressive periodontitis. Aa is a gram-negative, facultative anaerobe, non-motile coccobacilli, and its potential virulence mechanisms in LAP have been summarized as following: Aa was found in periodontal lesions >90% of LAP patients (Linde et al., 2005). Production of several potentially pathogenic virulence factors by Aa may contribute to the periodontal disease progress. Elevated levels of serum antibodies to Aa were found in LAP patients and treatment with reduction of subgingival load of Aa showed successful clinical response (Socransky, Haffajee, 1992).

This highly virulent bacterium expresses two exotoxins; Cytolethal distending toxin (Cdt) and Leukotoxin (LtxA). Cdt’s are a class of heterotrimeric toxins produced by certain gram-negative bacteria. Its virulence mechanism is based on its cell cycle-inhibitory activity, where it enters eukaryotic cells, breaks the double-stranded DNA, leading to an arrest in the G2/M phase of the host’s cell cycle. Affected cells enlarge until they finally undergo apoptosis (Heywood et al., 2005). While LtxA has been shown to correlate with periodontal disease, the Cdt’s role in the disease is still unclear (Höglund et al., 2014). LtxA is a large pore-forming protein that belongs to the Repeat in Toxin (RTX) family (Linhartová et al., 2010). The LtxA operon consists of four genes (LtxC, LtxA, LtxB, LtxD) and a promotor. The ltxA gene determines the toxin structure, the ltxC gene is responsible for posttranslational modification of the toxin, and the ltxB- and ltxD genes enable transport of toxin to the bacterial outer
membrane (Johansson, 2011). It has been reported that the toxin is attached to the bacteria cell surface and in outer membrane vesicles where it can be released from the bacteria during an infection (Kato et al., 2002).

A well-known example of a highly leukotoxic clone of Aa with enhanced leukotoxic activity is the JP2 clone, which has a 530 bp deletion in the promoter region of the leukotoxin operon. Prospective population studies have shown that JP2 carriers have an increased risk of developing aggressive periodontitis (Haubek, 2010). These carriers come from specific regions in the world, particularly from North- and West-Africa (Henderson et al., 2010).

LtxA selectively affects leukocytes in a variety of ways by binding to the cellular receptor/leukocyte’s integrin LFA-1. It causes apoptosis of lymphocytes, degranulation and killing of neutrophils, induces activation and release of the proteolytic enzymes: matrix metalloproteinase and lysosomal enzymes from the neutrophils, activates caspase-1 that induces bio-active IL-1β secretion from macrophages (Johansson, 2011).

The cellular pathway of the inflammatory activation of macrophage by LtxA has been described by Haubek & Johansson (2014). When LtxA binds to the LFA-receptor on the macrophage, it activates an extracellular release of adenosine triphosphate (ATP). The exact mechanics of the ATP-release from the cell when affected by LtxA is unknown. However, Pannexin-1 (Panx-1) channels have been shown to be a possible pathway. Panx-1 channels are hemichannels which have shown to be a possible channel for ATP efflux (Velasquez & Eugenin, 2014). Panx-1 channels have previously been shown to take part in P2X7R activation (Pelegrin, Surprenant, 2006). The now extracellular ATP acts as a ligand on the P2X7-receptor on the macrophage (Kelk et al., 2011). Blocking of the P2X7R with oATP has shown to inhibit the effects of LtxA induced cell death (Kelk et al., 2011). The binding of ATP to the P2X7R leads to a potassium-efflux from the monocyte, which triggers the formation of the inflammasome multimer to a caspase-1 activation leading to IL-1β and IL-18 cytokine release and inflammatory cell death; Pyroptosis (Haubek, Johansson, 2014). Release of IL-1β will attract other immune cells like neutrophils to the infection site, which can cause tissue damage. IL-1β also activates bone-resorbing cells; osteoclasts, leading to alveolar bone loss (Dewhirst et al., 1985; Lorenzo et al., 1987).

Carbenoxolone (Cbx), a derivative of glycyrrhetinic acid is a gap junction blocker known to block Panx-1 channels (Bruzzone et al., 2005). It has been used in treatment of peptic ulcers (Pinder et al. 1976). Blocking of this upstream molecule could possibly be a useful tool to
block caspase-1 cleavage and activation of IL-1β release. The purpose of the present study was to investigate whether Cbx could affect LtxA induced IL-1β release and cell death by blocking Panx-1 channels. Our hypothesis is that Cbx blocks Panx-1 channels, which in turn inhibits the release of ATP from the cell. By blocking Panx-1 channels we could maybe observe an effect similar to oATP blocking of P2X7R, by blocking the release of ATP. We also study the effect of Cbx in LPS induced macrophage activation. LPS is an endotoxin found in the outer membrane of gram-negative bacteria, which can stimulate to an increased storage of pro-IL-1β in macrophages. If the macrophages then are exposed to a secondary factor like LtxA, caspase-1 will be activated and pro-IL-1β will be transformed to IL-1β and secreted from the cell. In this way, macrophages are self-devastating to the tissue (Kelk et al., 2008).

The aim of the present study was to examine the involvement of Panx-1 in LtxA induced activation and cytotoxicity of human macrophages. This study seeks to evolve the understanding of how LtxA affects human macrophages which in the future may contribute to new medical development against aggressive forms of periodontitis.

MATERIAL AND METHOD

Literature
A literature research was made on the medical database PubMed. Following MeSH-terms were used: (“Periodontitis” [MeSH Term], “etiology” [MeSH Term], “risk factors” [MeSH Term]) for a more general understanding of the disease Periodontitis. (“Aggregatibacter actinomycetemcomitans”[MeSH Term] AND “Leukotoxin” [MeSH Term]), (“Aggregatibacter actinomycetemcomitans”[MeSH Term] AND “Exotoxin” [MeSH Term]) about the relationship between the bacterium and its virulence mechanism. (“Pannexin” [MeSH Term] AND “Connexin” [MeSH Term]), (“Leukotoxin”[MeSH Term] AND “Interleukin-1beta”/”secretion” [MeSH Terms]), (“Neutral-Red” [MeSH Term] uptake assay”), (“Enzyme-Linked Immunosorbent Assay” [MeSH Term]) for a more detailed understanding about the underlying molecular mechanism to our experiment. Some references were supplied by our tutor.
Ethical considerations
This is an experimental study with no human test subjects present. The cells come from a commercially available THP-1 cancer cell line (ATCC®, TIB202™, Manassas, Virginia, USA), taken from individuals who have consented to a scientific use of their cells. These cells cannot be traced back.

Cell culture
The cells used were human THP-1 cells from a monocyte cancer cell line. Cells were counted in a Bürker-chamber, and together with RPMI-1640 10 % Fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA), a suspension with a cell concentration of $10^6$/mL was created. Cells were then differentiated by using Phorbol myristate acetate (PMA) with a concentration of 50 nM. The cell suspension was added to a 96-well plate with 100 µL per well. The plate was then incubated at 37 °C in 5 % CO₂ for 24 hours. During the second day, the PMA containing medium was replaced with 100 µL new RPMI-1640 10 % in each well and then incubated again at 37 °C in 5 % CO₂ for 24 hours. This described cell culture preparation was performed the same way in all following experiments prior to exposure to LtxA and Cbx.

Leukotoxin induced LDH release
Lactate dehydrogenase (LDH) is a cytosolic enzyme that can be released from cells after irreparable membrane damage. After the incubation of the 96-well plate and change of cell-medium during the first two days (see above), LtxA (previously purified according to method described in Johansson et al., 2000) and Cbx (Sigma-Aldrich, St. Louis, USA) were prepared so that they could be added to the cells during the third day. The cells were split into two different groups and 100 µL medium/well was added: RPMI to the first group and RPMI + Cbx 200 µM to the second group. Each group had five different triplicates containing various concentrations of LtxA (0-, 25-, 50-, 100 ng/mL), in the last triplicate, 1 µL Triton was added to find out maximum amount of LDH-release. Six extra wells (containing no cells) were filled with 100 µL RPMI to find out the values of the RPMI in the spectrophotometric analysis (Spectra MAX 340, SOFT max PRO, Molecular Devices, Silicon Valley, CA, USA). This was done to be able to withdraw the RPMIs effect from the values we seek. The cells were then incubated at 37 °C in 5 % CO₂ for two hours in presence of LtxA and Cbx. After the incubation, 25 µL medium from each well were transferred to a new 96-well plate.
To quantify the activity of the released LDH, 180 µl substrate buffer was added to 25 µl medium before analysis in the spectrophotometer. The absorbance was read at 340 nm just after substrate buffer appliance, and then read continuously until a sufficient decrease in the absorbance was seen. The difference between first- and last read were then calculated in Microsoft Excel to compare the LDH-release from LtxA- and Triton treated cells (100 % lysis), and LDH release between LtxA treated cells with- and without Cbx.

**Exposure of cells with LtxA or E. coli LPS**

Day three after the THP-1 cells had been incubated (see Cell culture section) on the 96-well plate, RPMI was removed from the wells. The cells were then split into three different groups and a total of 100 µL medium per well was added: pure RPMI to the first group, RPMI+100 µM Cbx to the second group, and RPMI+200 µM Cbx to the third group. Each of the three groups had three different triplicates containing either LtxA 25 ng/mL, LPS 100 ng/mL (Sigma-Aldrich, St. Louis, USA) or pure RPMI. The cells were then incubated at 37 °C in 5 % CO₂ for 24 hours. After incubation, the medium was transferred to Eppendorf tubes marked with numbers corresponding to the well numbers and were frozen until ELISA analysis was performed.

**Neutral red uptake assay (NRU-test)**

A NRU-test was performed on the remaining THP-1 cells in the wells from the previous experiment (see above) in order to determine cell viability of the exposed cells (Repetto *et al.*, 2008). After 24 h of incubation, neutral red was added to the wells and incubated for 2 hours. After incubation, the wells were washed with PBS and solved in 150 µL destain solution (50 % EtOH and 1 % acetic acid in water) analysis. Neutral red is accumulated in the lysosomes of viable cells and if quantified represents the proportion of viable cells in a cell culture. Spectrometric analysis was performed by reading the wavelength of 540 nm. The values received were then calculated in Microsoft Excel to find out the cell viability percentage in relation to the control group that was set to 100 % viability.

**Analyses of IL-1β by ELISA**

The concentration of IL-1β in the exposed samples was analyzed in an enzyme linked immune sorbent assay (ELISA) kit in accordance to the manufacturer protocol (R&D systems, Minneapolis, USA). On the first day, 96-well microplate was coated with 100 µL antibody/well, sealed and incubated overnight at room temperature. The rest of the ELISA
procedure took place on the second day. On the second day, 100 µL washing buffer (PBS+0,05 % Tween) was added to each well and was then washed away, this was repeated three times. The plates were then blocked with PBS-FBS, 75 µL/well, incubated for 90 minutes at room temperature. After incubation, a new washing of the plate with washing buffer was repeated three times. 100 µL detection antibody was added to each well before the plate was sealed and incubated for two hours at room temperature. After the incubation, a new washing of the plate with washing buffer was performed three times. After washing, 100 µL Streptavin HRP was added to each well before the plate was incubated for 20 minutes, avoiding light, at room temperature. After that, a new washing of the plate with washing buffer was repeated three times. Substrate solution: 100 µL/well was added, and the plate was incubated for 20 minutes, avoiding light, at room temperature. Lastly, to stop the ELISA-reaction, 50 µL Stop solution (2N H₂SO₄) was added to each well, which reacted with the substrate solution to produce a detectable signal in shape of a color change of the samples from blue to yellow. The ELISA-plate was then analyzed in a spectrophotometer (450 nm) and read in comparison to the standards with known concentrations of recombinant IL-1β. Results were then analyzed in Microsoft Excel and tables were made with normalized values.

**Statistical analysis**

Statistical analysis was performed on raw data from the ELISA analysis in Microsoft Excel. A paired T-test was chosen to find out the statistical differences between the different groups in the experiment. A p-value of <0.05 was determined as significant. Over 12-15 different samples from each group were analyzed: 15 samples in the Cbx 200 µM group and the untreated group, and 12 in the Cbx 100 µM group. This was done because the 100 µM group was added during the second time the experiment was performed to explore a dose-response effect.

**RESULTS**

**Effect of Cbx on LtxA induced LDH release of THP-1 cells**

THP-1 cells incubated for two hours with LtxA (0-,25-,50-,100 ng/mL) showed substantial cell lysis already at LtxA concentration of 25 ng/mL. The cells treated with Cbx 200 µM did not affect the activity of LDH-release in any of the Cbx-treated cell culture samples compared to untreated cells. (Fig. 1)
Effect of Cbx on viability and IL-1β release from LtxA exposed THP-1 cells

THP-1 cells incubated with LtxA 25 ng/mL for 24 h showed a decrease in cell viability in all LtxA treated cells compared to the untreated cells. When comparing the different concentrations of Cbx in the untreated cells, Cbx 200 μM showed a decrease in cell viability while Cbx 100 μM did not. Cbx in any concentration had no apparent effect on the cell viability on the LtxA treated cells. (Fig 2a.) The results from the ELISA-analysis showed a significant increase in IL-1β release in LtxA 25 ng/mL treated cells compared to untreated cells after 24-hour incubation. Cbx 200 μM without LtxA showed a statistically significant increase in IL-1β release (p = 0.00101) compared to untreated cells. However, when comparing Cbx concentrations in the LtxA treated cells, Cbx 200 μM showed a statistically significant decrease in IL-1β release compared to the untreated cells (p = 0.00178). (Fig 2b)

Effect of Cbx on viability and IL-1β from LPS exposed THP-1 cells

THP-1 cells incubated for 24 hours with LPS 100 ng/mL showed no decrease in cell viability compared to untreated cells. As previously mentioned Cbx 200 μM caused a decrease in cell viability in the untreated control group. A similar effect was observed on the LPS treated cells with an even larger decrease in cell viability on cells treated with Cbx 200 μM. (Fig. 3a) Results from the ELISA analysis showed a great difference between IL-1β release between untreated- and LPS treated cells. As mentioned before, Cbx 200 μM caused an increase of IL-1β release in the control group without LPS. The LPS treated THP-1 cells showed a statistically significant increase in IL-1β release when exposed to Cbx 200 μM compared to the LPS treated cells without Cbx (p≈0.00126). (Fig. 3b)

Concentrations of IL-1β released from THP-1 cells exposed for LtxA and LPS

Here we show the actual concentrations of IL-1β caused by the different mixtures added to cultures of THP-1 cells for 24 h. To better compare, our previous data presentations have used normalized data to compare the different groups of samples statistically. Both LtxA 25 ng/mL and LPS 100 ng/mL induced IL-1β release from the exposed macrophages after incubation for 24 hours. (Fig. 4). While the presence of Cbx significantly inhibited the IL-1β induced by LtxA, it stimulated the release induced by LPS.
DISCUSSION

To determine the role of Panx-1 channels for LtxA induced macrophage cell death and IL-1β release, the Panx-1 blocker Cbx was added to the experimental set-up. The measuring of LDH-release from LtxA induced THP-1 cells showed no statistical difference between the cells treated with- or without Cbx. This might indicate that blocking of Panx-1 channels with Cbx had no role in the LtxA’s potential of inducing cell lysis. When measuring extracellular LDH, we measured the cell lysis since LDH is localized inside the cell membrane of viable cells. Our results showed slightly higher cell lysis in Cbx treated cells, however more experimental data is required to draw any conclusions.

Neutral red is another way of measuring cell viability (Repetto et al., 2008). We used this method to measure the cell-viability after 24 h incubation as earlier described. The results surprisingly showed a decrease in cell-viability in cells treated with Cbx 200 μM in both the LPS- and the control group, which could indicate that blocking of gap-junctions slowly strangle the cells. In addition, decrease in cell viability was greater in the LPS group which could indicate that a greater cell-stress accelerates the process.

In the LtxA group of cells, Cbx showed no difference compared to untreated cells in regard to viability of the exposed cells. This indicates that Cbx probably did not have any effect on the activity of LtxA’s ability to cause cell death.

When testing for IL-1β release, our results showed a decrease in IL-1β release when Cbx 200 μM was used on cells together with LtxA. This seems to correlate well with previous theories that Cbx in this concentration blocks the Panx-1 channels and therefore has an inhibiting effect on the pro-inflammatory response causing release of IL-1β. Our experiments have given us a greater understanding how Cbx interacts with LtxA induced IL-1β release. Since the lower concentration of Cbx showed no statistical effect on IL-1β release, the possibility of further experiments should be performed to show a dose-response correlation between Cbx and IL-1β release. Although Cbx seems to have an inhibitory effect on LtxA induced IL-1β release, the opposite was observed in cells treated with LPS and in the control group. This indicates that different cellular pathways are involved for LPS induced IL-1β release, and that Cbx has no apparent effect on those. Further experiments could show how Cbx affects cells treated with LPS and LtxA in combination.
Human errors should be considered in our experiments, especially when none of the people performing the experiments have any laboratory experience from the past. This could include pipetting, counting before the cells were diluted, counting cells in the microscope or contamination of the cells in some way. There could also be environmental errors that interrupt with the growing cells, for example, if the incubator was not working correctly, the optimal cell incubation environment would not be maintained. Another aspect could be an instrumental error, for example if the Spectrophotometer we used was not calibrated before use. The sample size should also be taken into account. For example, our first experiment-LtxA-induced LDH-release, was performed two times and our second experiment-quantifying LtxA induced IL-1β release, was performed four times and the data from each experiment were later put together into a mean value. The more experiments we do, the more data we will collect and the more precise our results will be.

The possibility of using Cbx as a treatment of aggressive forms of periodontitis does not seem promising. As our results indicate an increased cell toxicity in Cbx treated cells and an increase in IL-1β release in combination with LPS. Since both LtxA and LPS are present in Aa, where they have opposite effects, it is difficult to see any benefits from using Cbx as a therapeutic agent. However, Cbx has since several decades been used in treatment of other inflammatory induced diseases, such as gastric ulcer (Pinder et al., 1976). We propose that highly-leukotoxic Aa where both LtxA and LPS are naturally represented, should be tested on macrophages in further experiments. This could change the view regarding Cbx as a therapeutic agent.

**Conclusions**

Our results could indicate that Panx-1 channels are involved in the ATP release induced by LtxA on macrophages and contribute to the activation of IL-1β release.

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REFERENCES


Fig. 1: LDH-release from LtxA exposed THP-1 cells in presence- or absence of Cbx.

PMA differentiated THP-1 cells with- and without Cbx 200 μM, exposed for different concentrations of LtxA for two hours. Cell lysis was then recorded by measuring the activity of released LDH. Value is presented as a percentage of maximum LDH release from Triton treated cells (100 % cell lysis). Mean values from two different experiments with triplicates ± SD is shown.
Fig. 2: Effect of Cbx on viability and IL-1β release from LtxA exposed THP-1 cells.
PMA differentiated cells were exposed to LtxA for 24 hours in presence or absence of Cbx. Mean ± SD of 3-4 experiments are shown. A) Viability of the exposed cells presented as proportion of neutral red uptake in relation to an untreated control sample. B) IL-1β release in relation to percentage of the release (100 %) caused by LtxA without Cbx. Values marked with an * are statistically different from the RPMI group (p ≤ 0.05).
Fig. 3: Effect of Cbx on viability and IL-1β release from LPS exposed THP-1 cells.
PMA differentiated cells were exposed to *E. coli* LPS for 24 hours in presence or absence of Cbx. Mean ± SD of 3-4 experiments are shown. A) Viability of the exposed cells presented as proportion of neutral red uptake in relation to an untreated control sample. B) IL-1β release in relation to percentage of the maximal release (100 %) caused by LPS without Cbx. Values marked with an asterisk are statistically different from the RPMI group (p ≤ 0.05).
Fig 4. Mean values of actual concentration of IL-1β quantified by ELISA.

Values of IL-1β concentrations before normalization that has been presented in the previous figures (Fig. 2b and fig. 3b). Mean values of 3-4 experiments are shown.