A heat-stable cytotoxic factor produced by *Achromobacter xylosoxidans* isolated from Brazilian patients with CF is associated with in vitro increased proinflammatory cytokines

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Abstract

**Background:** Recently, *Achromobacter xylosoxidans* has been related to chronic lung diseases in patients suffering from cystic fibrosis (CF), but its involvement has not been elucidated. Some virulence properties of *A. xylosoxidans* isolated from Brazilian patients with CF were revealed in this work.

**Methods:** This study examined the production of a cytotoxic factor of *A. xylosoxidans* capable of stimulating the secretion of inflammatory cytokines (IL-6 and IL-8) from lung mucoepidermoid carcinoma cells (NCI-H292). The cytokines were measured using enzyme-linked immunosorbent (ELISA) assays. To investigate whether the cytotoxic factors may be endotoxins, they were treated with polymyxin B.

**Results:** The culture supernatants of all *A. xylosoxidans* produced a heat stable, active cytotoxin in NCI-H292 cells capable of leading to intracellular vacuoles and subsequent cell contact loss, chromatin condensation, a picnotic nucleus and cell death. There was a higher concentration of proinflammatory cytokines in the NCI-H292 cells after 24 h of incubation, with the fraction greater than 50 kDa from the culture supernatant. The cytotoxin activity remained even after treatment with polymyxin B, which suggested that the release of IL-6 and IL-8 was not stimulated by lipopolysaccharide (LPS).

**Conclusion:** The cytotoxic factor produced by *A. xylosoxidans* may represent an important virulence factor, which when associated with CF chronic lung inflammation, may cause tissue damage and decline of lung function.

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Keywords: *A. xylosoxidans*; Cytotoxin; Endotoxin; Cystic fibrosis; Lung inflammation

1. Introduction

Cystic fibrosis (CF) is the most common inherited lethal disorder of homozygous mutant alleles of the CF gene. The mutation is localised at 7q31.2 and causes severe defects in chloride ion transport, leading to a characteristically salty sweat, which is more common among the Caucasian population [1].

In the lungs, mucociliary clearance is impaired, resulting in persistent colonisation with intermittent episodes of debilitating and ultimately fatal infection [2,3]. In particular, the accumulation of proinflammatory cytokines, such as IL-6 and IL-8, results in lung tissue damage [4].

More than 80% of adult CF patients also present chronic pulmonary infection with *Pseudomonas aeruginosa*, a Gram negative bacterium, that is often responsible for morbidity in CF cases [5,6]. However, an increasing number of other Gram-negative microorganisms are capable of causing similar lung chronic infections, like *Burkholderia cepacia* [5,6] mainly due to several virulence factors, such as biofilm formation and proinflammatory cytokine secretion, which allow the strains to remain in the lungs [7,8]. *Achromobacter xylosoxidans* has
been shown to cause inflammation in chronically infected CF patients similarly to *P. aeruginosa* [9].

Although *A. xylosoxidans* is not a common respiratory pathogen, it has been isolated from the lungs of CF patients with increased incidence in recent years [10]. This bacterium is an aerobic, motile, oxidase and catalase positive and nonfermentative Gram-negative bacillus [11]. The colonisation of this pathogen has been associated with the exacerbation of pulmonary symptoms [4]. Additionally, the lung function of patients infected with *A. xylosoxidans* may decrease rapidly compared to the CF patients without this infection [5].

Increased lymphocyte and neutrophil recruitment results from increased levels of the proinflammatory cytokines IL-6 and IL-8. The exaggerated inflammatory response in CF promotes lumen obstruction, which causes the destruction of cells in the airways [4].

The airway epithelial cells, which normally express the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, induce inflammatory responses. Defects in the *CFTR* gene are associated with an increased production of proinflammatory mediators, including IL-6 and IL-8 [4,10]. Studies on epithelial cells have suggested ways in which defects in *CFTR* function may influence the infectious and inflammatory processes in the lungs [12].

Lipopolysaccharides (LPSs) from many Gram-negative bacteria, including *A. xylosoxidans*, release proinflammatory cytokines, such as IL-6 and IL-8 [13], and polymyxin B is used to eliminate endotoxic activity [14]. However, toxic bacterial products other than LPS may contribute to the induction of inflammatory responses in certain infections. For instance, exoproducts from *B. cepacia* were able to stimulate IL-8 release that was not diminished by polymyxin B [15], suggesting the involvement of factors other than LPS.

The present study aimed to evaluate the capability of *A. xylosoxidans* to produce cytotoxins that stimulate the secretion of inflammatory cytokines (IL-6 and IL-8) in lung mucoepidermoid carcinoma cells (NCI-H292). The results may be helpful to elucidate the mechanism used by *A. xylosoxidans* to develop chronic pulmonary infection in patients with CF.

We studied 1350 samples of sputum and oropharyngeal secretion, from 03/01/2007 to 01/31/2009, that were obtained from 216 patients in our Paediatric CF Reference Centre, UNICAMP—Brazil. Of the 216 patients, 7.41% presented at least one confirmed positive culture of *A. xylosoxidans* and 3.7% fulfilled the criteria of three or more positive cultures, which indicates a high prevalence of these bacteria compared with data from other centres presented in the literature [16–18].

2. Material and methods

2.1. Microorganisms

2.1.1. Bacterial isolation and identification

Seventeen strains of *A. xylosoxidans* were isolated from the sputum and oropharyngeal secretions of 216 cystic fibrosis (CF) patients attended at the Paediatric CF Reference Centre, UNICAMP, São Paulo, Brazil. The LMG® 1863 (*A. xylosoxidans*) was used as previously described by Yabuuchi and Ohyama 1971 [11], as positive control strain of *A. xylosoxidans*. All of the isolates were grown in tryptone soya broth (TSB) (Difco Laboratories, Detroit, MI) at 37 °C, 100 rpm (Incubator shaker, New Brunswick Scientific, USA) for 24 h. The strains were previously identified by biochemical and serial analysis of gender by PCR.

2.1.2. Bacterial culture supernatants

*A. xylosoxidans* strains were grown in TSB medium by shaking at 100 rpm (New Brunswick Scientific Co.) at 37 °C for 24 h. The culture supernatants (CS) were collected by centrifugation at 10,000 rpm for 15 min (Bechman, USA) and were filtered through 0.22 μm membranes (Millipore, USA).

2.2. Cytotoxicity assay

The Human Lung Mucoepidermoid Carcinoma Cell line (NCI-H292) was purchased from the European Collection of Cell Cultures (ECACC) and was maintained in RPMI 1640 (Nutricell, Brazil) supplemented with 10% foetal bovine serum (Nutricell, Brazil) without antibiotics. The cells were grown in 96- (2.5 × 10⁵ cells/ml) or 24- (4 × 10⁵ cells/ml) well tissue microplates and were incubated at 37 °C, 5% CO₂ (Jouan CO₂ Incubator, France) for cytotoxicity or ELISA assays, respectively.

Briefly, NCI-H292 cells were incubated with the bacterial CS and the morphological changes were assessed using an inverted microscope (Nikon Instruments, Japan) with 6, 12, 18, 24 h until 48 h of inoculation was reached.

To analyse the thermostability of the cytotoxins, the bacterial CS were heated at 100 °C for 15 min, cooled to room temperature and assayed for cytotoxicity in NCI-H292 cells. Among all of the strains of *A. xylosoxidans*, only strain A31 was selected for the determination of the approximate molecular mass of the cytotoxic factor present in the cultured supernatants under different dilutions. The culture supernatant was ultrafiltrated using XM-300 and XM-50 cut off Amicon membranes (Millipore, Michigan, MI). The fractions retained in the membrane were subsequently filtered through a 0.22 μm membrane (Millipore, Brazil) prior to the cytotoxicity assay using NCI-H292 cells. TSB was used as a negative control.

2.2.1. MTT assay

To detect the cellular reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) by the mitochondrial dehydrogenase of viable cells, NCI-H292 cells were grown in 96-well plates for 24 h and were treated with 4 μg of fraction >50 kDa for 12, 24, and 48 h and after the medium was removed. Fresh medium containing MTT solution (0.8 mg MTT/ml) was added to each well and after was incubated for 3 h at 37 °C, in the dark. Subsequently, the medium was removed and the formazan solubilized in extract solution HCl 1 N–isopropanol (1:24 v/v). The plate was shaken for 10 min and the absorbance was measured in a spectrophotometer (Amershan Bioscience, England) at 550 nm (reference to 700 nm).
2.3. Morphological alteration by supernatants of _A. xylosoxidans_

2.3.1. Toluidine blue (TB) dye assays

The bacterial culture of strain A31 was applied to coverslip-grown cells over 6, 12, 18, 24, 40 and 48 h. The coverslips were subsequently washed with phosphate buffered saline (PBS), fixed in a 10% (v/v) formaldehyde solution in PBS for 1 h, washed with distilled water, stained with 0.25% (w/v) toluidine blue (pH 4.0) and washed once more in distilled water. After air drying, the coverslips were cleared in xylene, mounted on slides using Entellan (Merck, Whitehouse Station, NJ) and observed by light microscopy (Zeiss, Germany) [19].

2.3.2. Hematoxylin and eosin (HE) assays

The cells were grown in 24-well plates (4×10^5 cells/ml) and were incubated at 37 °C, 5% CO2 (Jouan CO2 Incubator, France). Bacterial culture supernatant of strain A31 was applied to coverslip-grown cells over 24, 40 and 48 h. The coverslips were subsequently washed with PBS, fixed in a 10% (v/v) formaldehyde solution in PBS for 1 h, washed with distilled water, stained with hematoxylin (2–3 min), washed with distilled water again and destained with 0.3% acid alcohol. After the washing step, the coverslips were stained by an eosin for 12 s, washed with distilled water, dehydrated, cleared, mounted on slides and observed by light microscopy (Zeiss, Germany) [19].

2.4. ELISA assay

After the determination of the molecular size of the cytotoxic fraction of the supernatant of strain A31, the NCI-H292 cells were grown on 24-well plates (4×10^5 cells/ml) at 37 °C for 24 h in 5% CO2 and incubated 1 ml with the supernatant of a sample that had been treated with polymyxin B or not, and with fractions >50 kDa and <50 kDa (containing 40–50 μg of protein) for 24 h. Polymyxin B was used to remove endotoxic activity. The protocol was adopted from Cooperstock et al. [14]. Briefly, the culture supernatants of strain A31 were heated at 100 °C for 15 min and were incubated at 37 °C for 20 min with polymyxin B (INLAB®, Brazil) at 0.1, 10, 20 and 40 μg/ml. The supernatant was subsequently tested for cytotoxicity in NCI-H292 cells. All of the samples and their respective controls were tested in triplicate and 40 μg/ml was selected for ELISA.

Prior to the enzyme-linked immunosorbent assay (ELISA), the supernatants of the cells were removed, filtered using 0.22 μm pore-size membrane and frozen with protease inhibitor cocktail (Sigma®, Germany). The concentrations of IL-6 and IL-8 in the supernatants (diluted 1:10) were determined by measuring an ELISA according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN).

2.5. Statistical analyses

All of the data were expressed as the mean and standard error of the mean (SEM). A comparison of the release of interleukins from the samples and the negative controls (the TSB groups) was conducted using Dunnett’s test for multiple comparisons. Differences were considered statistically significant if P<0.05.

3. Results

3.1. Cytotoxicity assay

All of the samples showed cytotoxic activity in the NCI-H292 cells. Culture supernatant filtrates from all of the isolates of _A. xylosoxidans_ and LMG 1863 caused cell rounding and detachment of cells that were observed after 48 h of incubation. Accordingly, it was observed that the cellular cytotoxic activity was not reduced by heating the bacterial supernatant at 100 °C for 15 min. However, because strain A31 cytotoxicity on NCI-H292 cells was observed till 1:4 dilution, which indicated higher cellular activity, unlike the other strains that were observed till 1:2 dilution, it was chosen to identify the approximate molecular weight of the cytotoxin. When different ultrafiltrated fractions of _A. xylosoxidans_ supernatant were inoculated on NCI-H292 cells, the cytotoxic activity was observed only in the fraction between 300 kDa and 50 kDa (Fig. 1).

Our results indicate loss of cell viability over time. The ability of the mitochondria to reduce MTT to formazan, in relation to time of incubation with the supernatant fraction >50 kDa is shown in Fig. 2. The mitochondrial activity of the strain A31 was lower than strain LMG 1863 and on average 0.6 times smaller than the TSB at all times.

3.2. Morphological alteration by supernatants of _A. xylosoxidans_

NCI-H292 cells did not present morphological changes before 24 h (at 6, 12, 18 h intervals of incubation), but present few morphological changes after 24 h as evidenced by the TB and HE dye assays, at the beginning of vacuolisation in the cytoplasm. Exacerbated cytotoxic effects were observed at 40 h.
and 48 h (Fig. 3). The 40 h of treatment indicated an induced decrease of cellular contacts and junctions, the presence of a pericromatic ring, uniform nuclei and the presence of nuclear bodies with chromatin condensation, which were observed by the affinity of TB dye to acidic substances, such as the nucleic acids [19]. Within 48 h of incubation, the number of large vacuoles increased, indicating a large number of vesicles, the loss of cellular contacts, chromatin condensation and pyknotic nuclei.

3.3. Induction of interleukins 6 and 8

The cytotoxic fraction (>50 kDa) of the \textit{A. xylosoxidans} culture strain A31 stimulated IL-6 and IL-8 production in NCI-H292 cells, as indicated by ELISA. We aimed to determine whether the bacterium cytotoxin had endotoxic activity,
which perhaps during cellular growth plays a role in the extracellular IL-6- and IL-8-inducing activity; however, the cytotoxin in the CS of the bacterium was not inactivated by polymyxin B and did not inhibit the production of the supernatant of bacteria to release interleukins.

Secretion of IL-6 following the stimulation with CS showed a median of 180.62 pg/ml, which was almost 6 fold the cells with TSB (negative control). The fraction >50 kDa showed a median of 281.58 pg/ml, which was almost 9 times more than the negative control (TSB). The CS with polymyxin B showed a median of 136 pg/ml, which was almost 8 times more than the negative control with polymyxin B. The IL-8 concentration following stimulation with CS showed a median of 3795.65 pg/ml, which was almost 6 times more than the negative control (TSB). The fraction >50 kDa showed a median of 3739.85 pg/ml, which was 5 times more than the negative control (TSB). Culture supernatants with polymyxin B showed a median of 3897.5 pg/ml, which was almost 6 times more compared to the negative control with polymyxin B (Fig. 4). The TSB medium did not influence the release of IL-6 and IL-8 compared to the control cell.

4. Discussion

The increase in A. xylosoxidans in the sputum cultures of CF patients is not accompanied by published data on the virulence factors of this bacterium. In this study, we provide evidence that some virulence factors produced by this bacterium may contribute to pathogenesis in the lungs of CF patients.

Culture supernatants of all A. xylosoxidans isolates caused a rounding of NCI-H292 cells by 48 h, which presented membrane alterations followed by detachment. The cells treated with the culture supernatant were also stained with toluidine blue and hematoxylin and eosin dyes, which showed that the cells suffered an increase in their normal volumes, the loss of cellular contacts and the presence of cells with pyknotic nuclei, which probably signal cell death. Kim et al. [20] investigated the ability of other opportunistic pathogen of CF, Burkholderia cenocepacia, to alter the permeability of the respiratory epithelium, but the bacteria did not invade the cell and that in some way promote the loss of cell junctions, but has not been shown, however in our study A. xylosoxidans neither invaded the cell NCI-H292 (data not shown), but the supernatants of these bacteria resulted in the loss of cell junctions, justifying the presence of a cytotoxic factor. This cytotoxic factor can lead to damage in target cells, contributing to the worsening of the disease that could explain cases such as those studied by Romano et al. [21], which show rapid lung function decline after acquiring a chronic A. xylosoxidans infection, leading to death in less than 5 years.

To estimate the molecular weight of the A. xylosoxidans cytotoxin that likely induces the release of interleukins in NCI-H292 lung cells, the culture supernatant of the bacterium was subjected to ultrafiltration using XM 300 and XM 50 membranes successively. We observed that the cytotoxic activity factor was between 50 kDa and 300 kDa, and moreover, it is a heat-stable cytotoxin. The fraction greater than 50 kDa was associated with the highest concentrations of IL-6 and IL-8 release in NCI-H292 cells. Although the cytotoxin is not yet purified, these two effects suggest that the cytotoxic factor in the cells is the same factor that induces the release of interleukins.

Our results are similar to those described by Palfreyman et al. [15] in bacterial supernatants of some strains of B. cepacia, which, after boiling, showed little or no reduction in the induction of IL-8 release in A549 (human lung epithelial adenocarcinoma) cells. However, we found that the cytotoxic fraction of all of the A. xylosoxidans samples analysed were thermostable. Massion et al. [22] revealed that P. aeruginosa supernatant also induced IL-8 production after heating, using primary bronchial epithelial cells, a human bronchial epithelial cell line (16-HBE cells) and monocytes. In their study, the fraction that stimulated the release of interleukin was approximately 1 kDa, which

![Fig. 4. Secretion of proinflammatory cytokines, A—IL-6 and B—IL-8 from NCI-H292 cells following stimulation with culture supernatant (CS), the >50 kDa fraction of the CS, the <50 kDa fraction of the CS, CS with polymyxin B cells, cell controls with and without polymyxin B and cells with TSB (negative control). Concentrations of cytokines were measured by ELISA and subjected to analysis of variance (ANOVA), followed by Dunnett’s test for multiple comparisons, the results were expressed as median±SD of two independent experiments performed in duplicate. CS, CS with polymyxin B cells and the >50 kDa fraction of the CS, showed significant difference (*P<0.05) compared with the negative control. polB=40 μg/ml polymyxin B.](image-url)
suggested that the activity product was not a protein, differing from the data obtained for the cytotoxic factor of *A. xylosoxidans* in our work.

Conversely, Bonfield et al. [23] and Palfreyman et al. [15] observed an increase in the release of IL-6 and IL-8 in the sputum of CF patients infected with *P. aeruginosa* and *B. cepacia* as further amplification of the inflammatory responses in the lungs of CF patients was observed. Hansen et al. [9] analysed the inflammatory responses induced by *A. xylosoxidans* infections and observed higher concentrations of IL-6 (39.2 pg/ml) in the serum of chronically infected patients compared to non-infected patients and healthy controls, not specifying whether this interleukin release was due to bacteria or deliverable by it.

However, in our study, the levels of IL-6 produced by the NCI-H292 cells due to the action of the culture supernatant fraction >50 kDa were up to 281 pg/ml, which is more than the IL-6 found in the serum of patients in the study by Hansen et al. [9]. Our results indicate the action of a cytotoxic factor of *A. xylosoxidans*, elucidating its ability to induce the release of high levels of IL-6 and IL-8 in human lung cells, which leads to an exacerbated inflammatory response because these cytokines are potent lymphocytes and neutrophil chemo-attractants that stimulate the influx of massive numbers of neutrophils into the airways [10]. These neutrophils are the primary effector cells responsible for the pathological manifestations of CF lung disease [4,10]. Inflammation is a vital process needed to counteract infection, but it is increasingly thought that the normal inflammatory process is disrupted in CF early in the course of the disease [24].

A study by Hutchison et al. [13] quantified the inflammatory potency of lipopolysaccharides (LPSs) from eight species of Gram-negative organisms, among them *A. xylosoxidans*, and demonstrated that the LPS of *A. xylosoxidans* also expressed these proinflammatory cytokines (IL-6, IL-8 and TNF). Because LPSs are heat stable and have molecular weights between 10 kDa and 118 kDa [25,26], we assessed whether the cytotoxic activity produced by *A. xylosoxidans* could be attributed to the action of LPSs. The action of polymyxin-B did not inhibit the cytotoxic activity of the bacterial culture supernatant, no cell changes were observed and the release of interleukins did not decrease compared with the positive control. Our results suggested that this cytotoxin is not an endotoxin.

5. Conclusion

In conclusion, we observed that the bacterial culture supernatant contains a cytotoxic factor able to induce lung cell alterations, such as vacuoles in the cytoplasm and cell death (chromatin condensation and pyknotic nuclei). The main effect of this cytotoxic factor was the stimulation of proinflammatory cytokine release (IL-6 and IL-8), which is involved in the inflammatory processes observed in CF patients and attracts a large number of neutrophils, which cannot be cleared from the lungs because of the physical and chemical changes of the mucus, leading to airway obstruction and tissue damage [20] and worsening the CF disease. Currently, we are working towards purifying and characterising this toxin for future descriptions of molecular mechanisms in action.

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