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Video Article

Methods for Detecting Cytotoxic Amyloids Following Infection of Pulmonary Endothelial Cells by *Pseudomonas aeruginosa*

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Abstract

Patients who survive pneumonia have elevated death rates in the months following hospital discharge. It has been hypothesized that infection of pulmonary tissue during pneumonia results in the production of long-lived cytotoxins that can lead to subsequent end organ failure. We have developed *in vitro* assays to test the hypothesis that cytotoxins are produced during pulmonary infection. Isolated rat pulmonary endothelial cells and the bacterium *Pseudomonas aeruginosa* are used as model systems, and the production of cytoxins following infection of the endothelial cells by the bacteria is demonstrated using cell culture followed by direct quantitation using lactate dehydrogenase assays and a novel microscopic method utilizing ImageJ technology. The amyloid nature of these cytotoxins was demonstrated by thioflavin T binding assays and by immunoblotting and immunodepletion using A11 anti-amyloid antibody. Further analyses using immunoblotting demonstrated that oligomeric tau and Aβ were produced and released by endothelial cells following infection by *P. aeruginosa*. These methods should be readily adaptable to analyses of human clinical samples.

Video Link

The video component of this article can be found at https://www.jove.com/video/57447/

Introduction

Patients who survive pneumonia have elevated death rates in the months following hospital discharge[1,2,3,4,5,6]. In most cases, death occurs by some type of end-organ failure including renal, pulmonary, cardiac, or liver events, as well as stroke[5,6]. The reason for the elevated death rate in this patient population has never been established.

Pneumonia is classified as being either community-acquired or hospital-acquired (nosocomial), and agents that can cause pneumonia include bacteria, viruses, fungi, and chemicals. One of the major causes of nosocomial pneumonia is the bacterium *Pseudomonas aeruginosa*. *P. aeruginosa* is a gram-negative organism that uses a type III secretion system to transfer various effector molecules, termed exoenzymes, directly to the cytoplasm of target cells[7,8]. During infection of pulmonary endothelial cells, the exoenzymes target various intracellular proteins, including an endothelial form of the microtubule-associated protein tau[9,10,11,12], leading to endothelial barrier breakdown resulting in severe pulmonary edema, decreased pulmonary function and, oftentimes, death.

As stated previously, patients who survive the initial pneumonia have elevated death rates in the first 12 months following hospital discharge. A potential mechanism for explaining this phenomenon is that some type of long-lived toxin is generated during the initial infection that leads to poor long-term outcome. Two observations support this possibility. First, cultured pulmonary endothelial cells that are treated initially with *P. aeruginosa* fail to proliferate for up to a week after the bacteria are killed by antibiotics[13]. Second, long-lived prions and agents with prion characteristics have been demonstrated in various human and animal diseases, particularly diseases associated with the nervous system[14,15].

Methods for examining the potential production of long-lived cytotoxic agents during pulmonary infection have never been described. Here a series of simple *in vitro* assays are outlined that can be used for investigating cytotoxin production and activity following infection using a common pneumonia causing agent, *P. aeruginosa*. These assays should be readily adaptable to investigate possible cytotoxin induction following infection using other agents that cause pneumonia, and the supernatants that are generated also should be useful for investigating effects of the cytotoxins in whole organs or animals. Finally, the assays that are outlined here most likely will be adaptable to test animal and human biological fluids for the production of cytotoxins during pneumonia.

Protocol

All animal procedures were reviewed and approved by the Institutional and Animal Care Committee of the University of South Alabama and were performed in accordance with all federal, state, and local regulations. Primary cultures of rat pulmonary microvascular endothelial cells (PMVECs) were obtained from the Cell Culture Core Facility at the University of South Alabama's Center for Lung Biology. Cells were prepared using previously described procedures[16].

1. Generation of Cytotoxic Supernatants

Note: Here, we use two different strains of *P. aeruginosa*: PA103, which has an intact type III secretion system capable of transferring the exoenzymes ExoU and ExoT to target cells during infection, and ΔPcrV, which lacks a type III secretion system and is incapable of transferring exoenzymes to target cells following inoculation.

1. Streak bacteria onto Vogel-Bonner (VB) agar plates[17] and grow overnight at 37 °C.
    1. To prepare plates, make the following stock solution (Vogel-Bonner salts; 10x stock): measure 2 g $MgSO_4$, 20 g citric acid (free acid), 100 g K_2PO_4, and 35 g NaH_2PO_4. Add ddH_2O to 1 L and autoclave for 15 min with slow exhaust.
    2. Place 7.5 g agar in 450 mL ddH_2O and autoclave as above.
    3. Following autoclaving, place both solutions into a 50 °C water bath to cool.
    4. Add 50 mL of the 10x VB stock salt solution to the agar.
    5. Add carbenicillin to 400 µg/mL (for the strains of *P. aeruginosa* being used) and mix well by swirling the flask.
    6. Place 5 mL of the VB agar solution into individual sterile culture dishes, allow the agar to solidify, and then store at 4 °C until the day of bacterial seeding.
    7. On the day of bacterial seeding, pre-warm a plate to 37 °C and then streak bacteria onto the plate using a sterile loop. Place the plate in a 37 °C incubator overnight.

2. Verify PMVEC purity by positive staining with fluorescent *Griffonia simplicifolia* lectin and negative staining with fluorescent *Helix pomatia* lectin (a marker for arterial endothelial cells). Aliquot cells and freeze in liquid nitrogen.
3. For experiments, maintain cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, and grow cells in a 37 °C incubator containing 5% CO_2. Use cells at passages 5 - 15.
    1. For generation of cytotoxic supernatants, plate 2 x 10^6 cells into individual 150 cm dishes and grow for 3 - 4 days until confluence is achieved. Use two plates per experiment (see below).
    2. For analysis of cytotoxic supernatants, plate 1 x 10^5 cells into individual wells of a 6-well dish and grow for four days until confluence is achieved.

4. To produce supernatant, wash one of the two 150 cm dishes of PMVECs from step 1.3.1 with phosphate-buffered saline, trypsinize, and then count the cells (we use an automated cell counter and follow the manufacturer's protocol; see **Table of Materials**). Wash the other dish of PMVECs with Hank's Balanced Salt Solution (HBSS) and then infect with either strain of *P. aeruginosa* at a multiplicity of infection (MOI) of 20:1. This is achieved as follows:
    1. For *P. aeruginosa*, OD_{540} 0.25 = 2 x 10^8 CFUs/mL. To prepare this dilution, add 1 mL HBSS to a 1 mL disposable cuvette. Scrape enough bacteria off of the plate that was prepared in step 1.1 until an OD_{540} of 0.25 is achieved when measured using a spectrophotometer. The next step will provide a calculation of how much bacteria will be needed.
    2. Once the number of PMVECs in the culture dish is determined (step 1.4 above), determine the appropriate number of bacteria to be added to the second, non-trypsinized culture dish containing the PMVECs. For example, if it is determined that the culture dish of PMVECs contains 1.3 x 10^7 cells, then prepare 1.3 x 10^7 cells x 20 bacteria/cell x 1 mL/2 x 10^8 CFUs = 1.3 mL bacteria.
    3. Dilute the 1.3 mL of bacteria into HBSS to a final volume of 20 mL, so that the entire surface of a 150 cm dish can be covered with fluid, then add the diluted bacteria to the plate of PMVECs.
    4. Incubate the PMVECs inoculated with the bacteria at 37 °C in a 5% CO_2 incubator for 4 - 5 h, until gaps can be seen forming in the cell monolayer when the plate is observed microscopically (See **Figure 1** for proper level of gap formation).
    5. Collect the supernatant, then centrifuge for 10 min at 2,000 x g in a tabletop centrifuge to remove cellular debris. Note: Any table top centrifuge capable of generating sufficient g force to pellet unlysed cells and large cell fragments can be used for this step.
    6. Pour the supernatant into a syringe that has a 0.2 µm filter attached to its end, then pass the supernatant through the filter to remove bacteria.
    7. Use an aliquot of the sterile supernatant to test cytotoxicity (see 2.1) and freeze the rest of the supernatant at -80 °C for future use.

2. Analysis of Cytotoxic Supernatants

1. Cytotoxicity Assay
    1. Grow PMVECs in 6-well dishes until confluence. Wash wells once with HBSS, then add 1.5 mL of filter-sterilized supernatant (collected from either PA103- or ΔPcrV-inoculated cells) to individual wells. Add sterile HBSS to another well as a negative control.
    2. Place the plate into the CO_2 incubator for 21 - 24 h, then observe for cell killing/cytotoxicity (see next step). Use supernatants that exhibit cytotoxicity for further experimentation, and discard those that do not show cell killing.
    3. Quantitation of cell killing
        1. Measure lactate dehydrogenase (LDH) release from dead cells using commercially available LDH Assay Kits and manufacturer's recommended procedures.

2. Alternatively, quantify cell killing microscopically using ImageJ software. For this, record microscopic images and areas of the culture dish containing intact cells, then quantify regions lacking cells (indicative of cell death in a monolayer) using a custom ImageJ (National Institutes of Health) macro (see **Supplementary Coding File**). If desired, carry out the macro steps manually as follows:
    1. Input images (RGB or Tiff format) into the macro and adjust contrast to 15% saturated pixels. Duplicate contrast-adjusted images and perform the "subtract background" command to obtain a high-contrast image of both cell and gap area within the field of view.
    2. Subtract this image from the original image and combine the resulting image with the original image using the image calculator "AND" function. Convert the resultant image to black (gaps) and white (cells) mask with the threshold function (minimum 0, maximum 5), and use the "binary erode" function to remove noise from the image.
    3. Measure the ratio of black to white pixels within the resultant image using the "area fraction" measurement. Plot and express fractional areas for each treatment time point as percent of maximal gap area.
    4. Calculate means and compare using one-way ANOVA with Tukey's post hoc test, with *p* values less than 0.05 considered significant.

4. Use immunoblot analysis to establish the presence of amyloid, oligomeric tau, and Aβ in culture supernatants. Perform immunoblot analysis using standard procedures[10,11,12], except for concentrating culture supernatants at least 10-fold prior to electrophoresis.
    1. To concentrate supernatant, place 1 - 2 mL of supernatant into a centrifugation filter unit whose membrane has a molecular weight cut-off of 10 kDa. Then, place the filter unit into a table top centrifuge and centrifuge at 2,000 x g until the required degree of concentration is achieved (usually 45 - 60 min).
    2. Determine the amount of protein in the concentrated supernatant[11] and load equal amounts of samples into individual wells of the gel.
    3. Run gels, transfer the proteins to nitrocellulose, then probe blots with either A11 anti-amyloid antibody, T22 anti-oligomeric tau antibody, or MOAB2 anti-Aβ antibody followed by appropriate secondary antibodies. Develop the blots using standard chemiluminescence procedures.

5. To do a thioflavin T Assay, use thioflavin T fluorescence (ThT) to quantify amyloids in supernatants. For these measurements, use filter-sterilized supernatants.
    1. Prepare a stock solution (50x) of thioflavin T by suspending 8 mg thioflavin T (ThT) into 10 mL phosphate buffered saline (PBS). After mixing, filter the solution through a 0.22 µm filter to remove particulates.
    2. For measurements, add 20 µL of ThT stock to 1 mL of PBS in a 1 mL spectrophotometer cuvette. Place the diluted sample into a spectrofluorimeter.
    3. Measure the baseline fluorescence emission using 425 nm excitation and scanning the fluorescence emission from 450-575 nm in 2 nm increments.
    4. Perform a time-lapse scan using 425 nm excitation and 482 nm emission, with data acquired every 0.2 s for 60 s.
    5. The initial 20 s of the time-lapse scan measures fluorescence in the blank cuvette. At 20 s, pause the scan and add 10 µL of the filter-sterilized supernatant to the cuvette. Mix the cuvette by inversion and then place back into the spectrofluorimeter.
    6. Resume the time-based scan, acquiring the final 40 s of data.
    7. Upon completion of the time-lapse scan, perform a final fluorescence emission spectrum scan using identical settings, as detailed in 2.1.5.3.

Representative Results

A simple *in vitro* assay has been developed to assay for the presence of cytotoxins in supernatants of cells infected with the bacterium *P. aeruginosa*. Basically, culture medium from infected cells is collected 4 h after bacterial addition, the bacteria are removed by filter sterilization of the culture supernatant, and then the sterile supernatant is added to a new population of cells. The cells are then observed 21 - 24 h after the addition of supernatant and cell killing is quantified.

Addition of *P. aeruginosa* to confluent layers of PMVECs induces formation of gaps between the cells. *In vivo*, gap formation leads to pulmonary edema and decreased pulmonary function. In the described assay, gap formation is used as the time-point for screening for cytotoxin release into cell culture supernatants and sufficient duration of incubation with PA103 is indicated by the presence of gaps that begin to form in the cell monolayer as shown in **Figure 1**. ΔPcrV bacteria do not induce cell gaps under these incubation conditions. Once gaps are detected, the supernatant is collected, filter sterilized, and then added to naïve PMVECs to assess cytotoxic activity. The PMVECs in their respective supernatants then are placed in an incubator for 21 - 24 h, and then observed and photographed. As shown in **Figure 2**, supernatant collected from PMVECs that had been infected with *P. aeruginosa* strain PA103 contained cytotoxins that induced death of cultured cells by 21 h after the addition of the supernatant. In contrast, no cell death was observed in control cells treated with either HBSS medium or with supernatant collected from the ΔPcrV strain of Pseudomonas. Immunoblot analysis demonstrated that amyloid molecules, including oligomeric tau and Aβ, are generated during infection with the PA103 strain, while no amyloids are generated by the ΔPcrV strain following inoculation of PMVECs (**Figure 2**). The role of amyloids as the cytotoxins in this assay has been confirmed by immunodepletion of amyloids from the supernatant prior to addition to cultured cells[12]. Although not shown here, immunodepletion of cytotoxic supernatant using A11 anti-amyloid antibody and T22 anti-tau oligomer antibody completely depletes cytotoxic activity from supernatants produced following PA103 infection (Balczon *et al.* 2017[12] and Balczon *et al.*, in preparation).

A novel, inexpensive, and simple method of quantification of cell killing has been developed. For this assay, ImageJ software has been adapted to allow direct measurement of areas in a microscopic field that lack cells (indicative of cell killing). The reliability of this method has been verified by direct comparison to the well-established method of measuring cell killing, which is LDH release from cells. As shown in **Figure 3**, ImageJ software can be used to convert regions of a microscopic field that contain cells to white and regions that lack cells to black. Then, a simple ratio of white areas to black can be used to measure cell killing. When starting with a confluent monolayer of cells, all regions of the microscopic field are white. However, by 18 h after the addition of supernatants containing cytotoxins, gaps in the monolayer could be detected. The amount of area of the culture dish devoid of cells then increased in a linear manner until 36 h post-addition of the cytotoxic supernatant. When measuring LDH release, identical results were obtained, with LDH release being first detected at 18 h after the addition of cytotoxic supernatant. LDH release then increased in a linear manner until maximal cell killing was measured at 36 h after the addition of supernatant. Little cell death was measured in cells treated with supernatant collected from ΔPcrV inoculated cells (**Figure 3**) or in cultures treated with HBSS for 36 h.

The amount of amyloid released from cells treated with bacteria can be quantified by measuring ThT fluorescence. Binding of amyloids to ThT causes a conformational shift in the molecule and an increase in fluorescence intensity. For this assay, thioflavin T is added to a cuvette and baseline fluorescence is measured. The fluorescence recording is then stopped while a small aliquot of sample is added. The cuvette then is returned to the fluorimeter and the change is fluorescence emission is recorded. As shown in **Figure 4**, supernatant collected from cells that were incubated with PA103 strain of *P. aeruginosa* contained amyloid as indicated by the increase in fluorescence emission. In contrast, supernatant from PMVECs inoculated with ΔPcrV strain lacked amyloids, as indicated by an absence of increased fluorescence.

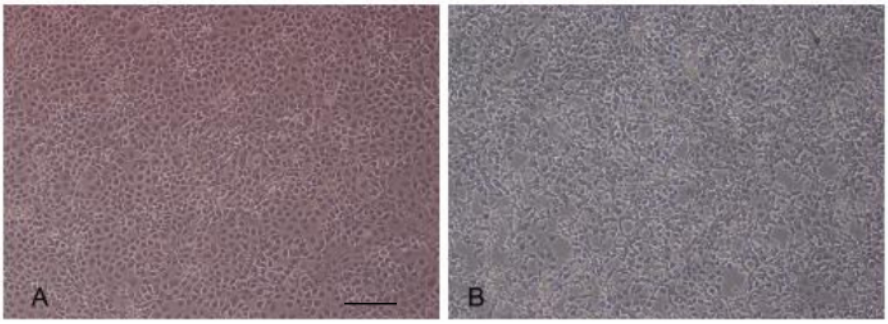

Figure 1. Effects of *P. aeruginosa* inolculation on PMVEC morphology. A confluent monolayer of PMVECs before **(A)** and four hours after the addition **(B)** of PA103 strain of *P. aeruginosa*. Gaps can clearly be seen in the cell monolayer in the infected cells. Scale bar = 100 µm. Please click here to view a larger version of this figure.

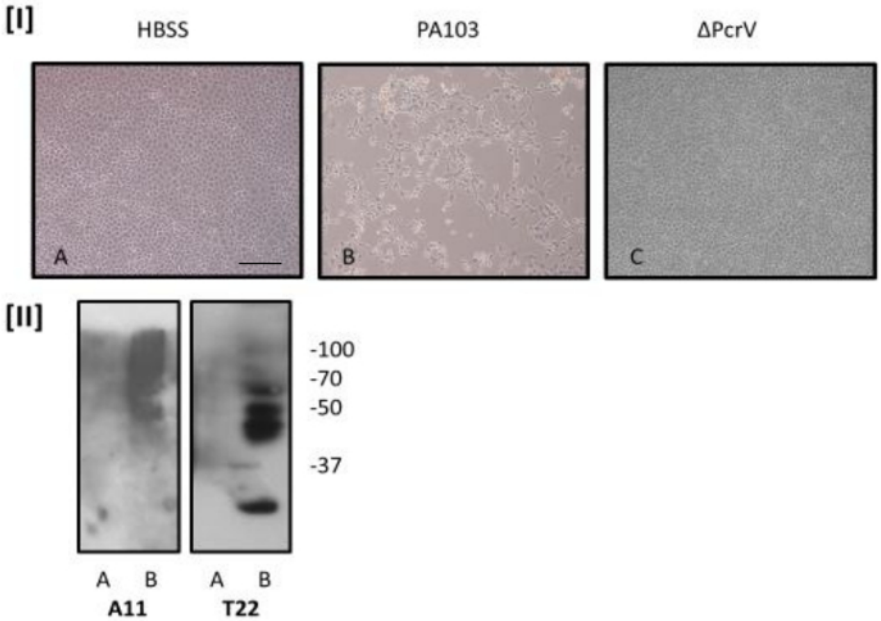

Figure 2. Analyses of cytotoxic supernatants. Part [I]. Representative images of control and treated cells. PMVECs were treated for 21 h with supernatant obtained from cells inoculated with either PA103 bacteria **(B)** or strain ΔPcrV **(C)**. Control cells were also incubated for 21 h with HBSS buffer **(A)**. Scale bar = 100 µm. Part [II]. Representative immunoblots of supernatants collected from either ΔPcrV **(A)** or PA103 **(B)** infected PMVECs. Blots were probed with antibodies against amyloid (A11) or oligomeric tau (T22). M_r in kDa. Please click here to view a larger version of this figure.

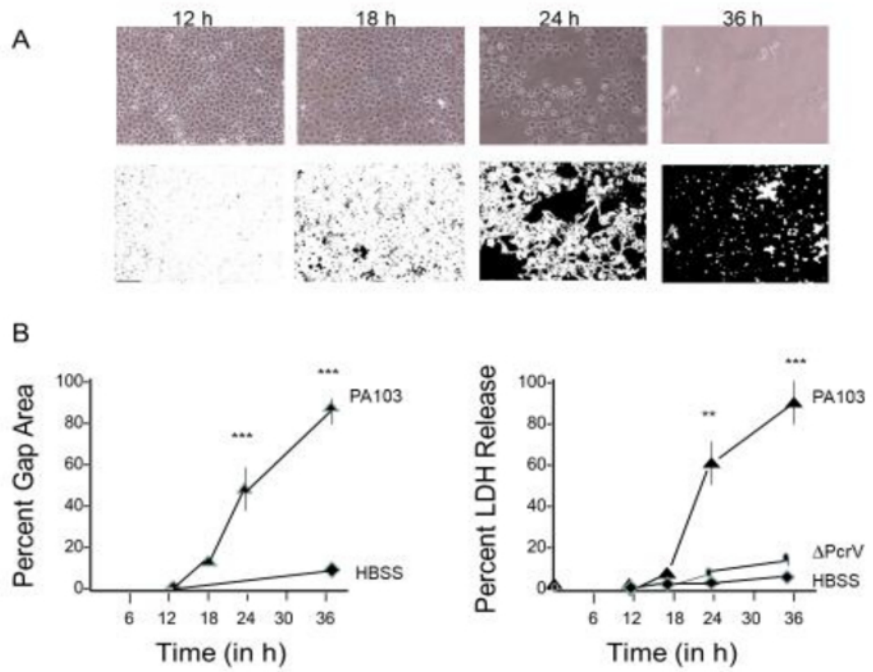

Figure 3. Quantitation of cell killing. (A) Representative microscope fields from different time points (in h after supernatant addition) in which regions of the field containing and lacking cells were converted either to white or black, respectively, using ImageJ software. The same phase contrast image before conversion is also shown. Bar = 100 µm. **(B)** Comparison of quantitation of cell killing using standard LDH release assay and the ImageJ assay. N = 4, $^{**}p$ <0.05, $^{***}p$ <0.01. Please click here to view a larger version of this figure.

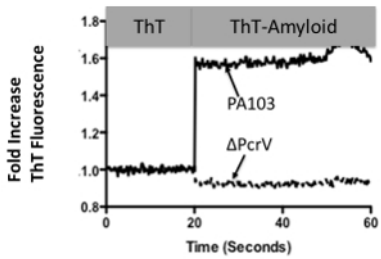

Figure 4. Representative ThT fluorescence assay data. Blank cuvettes containing ThT were excited at 425 nm and then emission fluorescence was measured at 482 nm. Background fluorescence was collected for 20 s, and then supernatant from either PA103- or ΔPcrV-inoculated PMVECs was added to the cuvettes. Fluorescence emission was then recorded for 40 sec.

Discussion

Here, simple *in vitro* methods are outlined which allow demonstration of the generation of cytotoxic amyloids during infection with a pneumonia causing organism. These methods include a cell culture cytotoxicity assay, immunoblotting, quantitation of cell killing using a novel microscopic method, and ThT binding. Analyses of the cytotoxic agents have demonstrated that they are amyloid in nature (**Figure 2 and Figure 4**) and exhibit characteristics of a prion[12]. The generation of cytotoxic prion molecules during pneumonia provides a potential explanation for the elevated death rates observed in pneumonia survivors following their hospital discharge. Experiments in progress are testing this possibility.

The key event for all of the described studies is the generation of the cytotoxic supernatant, and two important variables need to be considered. First, in the outlined experiments rat PMVECs were used for both the infection step and for the cytotoxicity assay. Whether other cell types could be used for production of cytotoxins has not been investigated in detail, and it is possible that other cells may be able to generate cytotoxic supernatants following treatment with various strains of *P. aeruginosa*. As shown, the cytotoxins are amyloid in nature and include oligomeric tau and Aβ. It might be reasonable to expect that other cell types that express tau and/or beta amyloid protein might be induced to generate cytotoxins following bacterial inoculation, although this possibility remains to be tested. The outlined procedures could be used for examining whether other cell types can be induced to produce cytotoxic amyloids following different insults.

The second important variable in the generation of the cytotoxic supernatants is the duration of treatment with bacteria. It is essential that the incubation of the PMVECs be allowed to progress until gaps can be seen in the monolayer. The identification of gaps in the cell monolayer is an indication exoenzymes have been injected by the Pseudomonas bacteria into the host cell cytoplasm and that the exoenzymes are active, resulting in retraction of cell borders (**Figure 1**). Our experience is that the time point of the retraction of cell borders is a reliable indicator of cytotoxin release into the supernatant. It is tempting to speculate that the two events are related as one of the cytotoxic amyloids being released

is oligomeric tau, and disruption of tau activity results in microtubule depolymerization which contributes to cell shape changes. In experiments where treatments have been less than the amount of time required to induce gap formation, little mature cytotoxic amyloid has been detected.

The cytotoxicity assay described in this manuscript is simple and reliable. Cell death can be observed easily by microscopic examination and quantified directly from recorded microscopic images. The methods that are described here have been used exclusively for assessing cytotoxin production following infection of rat pulmonary endothelial cells with *P. aeruginosa*. It seems likely that the procedures could be readily adapted to assess whether cytotoxic amyloids are produced following infection with other pneumonia causing agents, including both nosocomial-associated bacteria and bacteria and viruses responsible for community-acquired types of pneumonia. Moreover, the reported procedures have only been used to examine cytotoxic amyloid generation during infection of cultured cells. Cell culture is an artificial system, and the procedures outlined here most likely could be adapted to assess the generation of cytotoxins during pulmonary infection of both animal models and human patients. Collection of biological fluids, such as bronchoalveolar lavage, from either infected animals or patients could be used in the same types of assays reported here to assess whether cytotoxic amyloids are generated during infection processes in vivo. Obviously, the durations of incubation with bacteria might be quite different when inoculating into animals, and bronchoalveolar lavage would have to be collected at multiple time point to determine ultimate duration of treatment.

Immunoblot analyses have demonstrated that amyloids are present in the supernatants, including oligomeric tau and Aβ (**Figure 3**). Although the immunoblot procedures are standard, there is a key step that must be performed prior to initiating immunoblot analyses. Specifically, it is critical that the supernatants be concentrated at least ten-fold prior to immunoblotting as the amount of amyloid in the un-concentrated supernatant is below the limit of detection by immunoblot analysis. As reported here, centrifugation using tubes equipped with a filter is the method routinely used for this concentration step, but it seems reasonable that other methods, such as TCA precipitation, would be equally as useful for this step.

The quantitation of cell killing using images represents an important advance. Presently available procedures for quantifying cell death rely on the purchase of fairly expensive kits that allow measurement of LDH release from dead cells. The assay described here involves no cost as ImageJ programs are free to download from the NIH website. The simple adaptation of ImageJ programs allows fairly accurate measurement of cell killing. However, it is worth pointing out that a couple of minor artifacts are encountered during performance of the outlined protocol. The major issue is that cell fragments, such as areas of focal contact, may remain associated with the culture dish as cells die. These residual cell fragments can be detected by the camera and give an artificial indication that cells remain on the dish. As such, it is rare that a value of 100% cell killing is obtained using this procedure.

A weakness of the reported assay system is that the cytotoxin amyloids in the preparation have never been completely defined. As such, quantitation is difficult. ELISA assays can be used to provide total levels of different amyloids, without providing any detailed information about the individual species in each preparation. Until more precise assays, such as mass-spec or *in vitro* synthesized amyloid oligomers, can be developed, the best that one can do at present is to start each experiment with the same number of cells, incubate for the same duration, and verify that cytotoxicity occurs at the same rates as previous preparations that have been used. Variation from any of the well-established norms introduces additional uncertainty.

In summary, simple *in vitro* methods are outlined for demonstrating the production of toxic amyloid molecules by pulmonary endothelial cells following infection with a pneumonia-causing agent. The assays are reliable and likely can be adapted for other organisms and for use using biological fluids obtained from infected animals and human patients. These methods will be useful for studies investigating the long-term consequences of pneumonia and for establishing the mechanisms leading to end-organ failure in patients following hospital discharge.

Disclosures

None to report.

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