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SILKWORM LARVAE PLASMA (SLP) ASSAY FOR DETECTION OF BACTERIA: FALSE POSITIVES SECONDARY TO INFLAMMATION *IN VIVO*

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Abstract

The silkworm larvae plasma (SLP) assay has been developed as a means to detect bacterial peptidoglycan as a surrogate for live bacteria. Here, we present results that indicate that generation of melanin by this assay is not fully reliable as a surrogate marker for bacterial count.

Keywords

Lactobacillus; inflammation; pattern recognition receptors

1.0 Introduction

While not as enriched in microbes as the gut, the lung is not a sterile environment [Dickson *et al.*, 2016]. The large and small airways are conduits for microbes from the exterior as well as those aspirated from oral secretions. As such, the lungs maintain efficient clearance mechanisms in order to prevent undue bacterial colonization and infection [Kaur *et al.*, 2015; Lovewell *et al.*, 2014; Parker *et al.*, 2016].

With this in mind, our studies have focused on cross-protection against respiratory virus infection elicited by the immunobiotic bacterial strain, *Lactobacillus plantarum* when administered directly to the respiratory tract [Gabryszewski *et al.*, 2011; Garcia-Crespo *et al.*, 2013; Percopo *et al.*, 2014a; Percopo *et al.*, 2015; Rice *et al.*, 2016]. As our most recent efforts have included heat-inactivated *L. plantarum*, our intent was to use the Silkworm

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Author Contributions

MM performed all of the experimental work, carried out research for the text, and prepared primary drafts of the Methods and Figure Legends.

TAR contributed Fig. 1A.

CMP assisted MM in developing the SLP assay for use in the lab.

HFR developed the concept and wrote the paper with assistance from MM as above.

Larvae Plasma (SLP) assay to evaluate the relative rates of clearance of live vs. heat-inactivated *L. plantarum* in parallel experiments.

The SLP assay developed by Tsuchiya *et al.* [1996] is based on the pro-phenoloxidase (proPO) cascade which has been characterized as a means of anti-pathogen host-defense in insect species (reviewed in [Cerenius & Soderhall, 2004; Cerenius *et al.*, 2008]). The essential principle as implemented here is that bacterial peptidoglycan (PGN) binds to a species-specific PGN-binding protein (PGRP) which induces a protease-amplification cascade; this ultimately results in the production of melanin, which can be evaluated quantitatively at OD₆₅₀. This method has broad scope, and the potential to facilitate rapid detection of trace quantities of live or inactivated bacteria in a wide variety of settings without the need for complex culture methods [Kobayashi *et al.*, 2000; Cpyrowski *et al.*, 2014; Shibata *et al.*, 2006].

Here, we present results that indicate that generation of melanin is not fully reliable as a surrogate marker for bacterial counts.

2.0. Materials and Methods

2.1. Mice

C57BL/6 mice (females, 6 – 10 weeks of age) were from Charles River Laboratory, Frederick, MD. Mice devoid of pattern recognition receptors NOD2 and TLR2 (NOD2^{-/-}TLR2^{-/-}) on the C57BL/6 background were generated as described [Rice *et al.*, 2016]. All studies were performed in accordance with institutional guidelines provided by the NIAID Animal Care and Use Committee (ASP LAD8E).

2.2. Intranasal administration of *L. plantarum* and CL-429

Lactobacillus plantarum (BAA-793) was grown overnight at 37°C in Mann-Rogosa-Sharpe (MRS) broth. Bacteria were heat-inactivated at 70°C for 30 min prior to washing and storage at 10¹¹ cells/mL in phosphate buffered saline with 0.1% bovine serum albumin (pbs/bsa), heat-inactivated via three cycles of 95°C followed by freezing on dry ice as previously described [Gabryszewski *et al.*, 2011], or administered as live cells. Heat-inactivation results in simultaneous denaturation of multiple targets within bacterial cells and results in rapid loss of viability [Cangelosi and Meschke 2014]. Heat-inactivation by either of the aforementioned methods rendered *L. plantarum* as non-viable and unable to grow and divide. This latter point confirmed by inability to generate turbidity after overnight growth in culture in MRS broth (monitored by OD₆₀₀) and/or inability to generate colony forming units after overnight culture on MRS agar. *L. plantarum* was administered via intranasal inoculation to isoflurane-anaesthetized mice at 10⁸ cells per mouse in 50 µL sterile PBS/BSA. The bifunctional NOD2-TLR2 agonist, CL-429 (Invivogen) was diluted in PBS/BSA and delivered to isoflurane-anaesthetized mice (20 µg/ 50 µL per mouse) in parallel with appropriate diluent controls.

2.3. Isolation and quantitation of live *L. plantarum* from mouse lung tissue

Live *L. plantarum* was grown overnight as described above [Gabryszewski *et al.*, 2011] and diluted to 2×10^{10} cells/mL in pbs/bsa prior to administration (50 μ L) to isoflurane anaesthetized mice. Mice were sacrificed at time points indicated; whole lungs (one set per mouse) were homogenized in 1 mL sterile pbs/bsa and diluted 100, 1000, 10,000, and 100,000 fold into ice-cold pbs/bsa prior to plating on sterile MRS agar for counts for determination of colony forming units (CFU) at each time point. Each plate was incubated at 37°C for 24 to 30 hrs prior to counting colonies.

2.4. Detection of *L. plantarum* genomic DNA in mouse lung tissue

Lungs from mice inoculated as described above were collected at the time points indicated and stored at -80°C in RNAlater (Ambion). Lung tissue was defrosted and genomic DNA isolated as described [Garcia-Crespo *et al.*, 2013]. *L. plantarum* genomic DNA was evaluated by quantitative PCR using primers and probe designed to detect the *L. plantarum* 16s–23s intergenic region as described [Haarman & Knol, 2006] including forward primer, 5'-TGG ATC ACC TCC TTT CTA AGG AAT -3'; reverse primer, 5'-TGT TCT CGG TTT CAT TAT GAA AAA ATA -3'; and probe, 5'-ACA TTC TTC GAA ACT TTG T -3'-MGB. One microgram of total DNA from mouse lung tissue was added to TaqMan universal PCR master mix and evaluated on an ABI 7500 Real Time PCR system according to manufacturer's instructions. Results were calibrated via interpolation to a standard curve generated from 10 to 10^6 copies of 1.2 kb amplicon of *L. plantarum* gene sequence (nt 504226 to 505452 as per Genbank AL935263.2), amplified by PCR with forward primer (5'-GAA TCG CTA GTA ATC GCG GAT C -3') and reverse primer (5'-CGC GCT CTG ACT TAT TGT AGG C -3'). No *L. plantarum* controls were evaluated and no template controls were included with each experiment. Results are presented as copies *L. plantarum* 16s–23s amplicon per copy cellular GAPDH, the latter determined using primers and probe previously described [Percopo *et al.*, 2014b].

2.5. Silkworm Larva Plasma (SLP) assay

Lungs from mice inoculated as described above were collected at the time points indicated, homogenized in 1 mL and stored at -80°C prior to evaluation. For assay, homogenates and standards (heat-inactivated *L. plantarum*) were diluted 1:10 to a final volume of 70 μ L endotoxin-free water; 50 μ L of diluted material was dispensed into wells of a microtiter plate followed by addition of an equal volume of SLP test solution (Wako). Peptidoglycan (PGN) standards (from *Micrococcus luteus*) were prepared as per manufacturer's instructions (Wako) and are included for comparison. Formation of melanin over time was evaluated in a kinetic assay in which absorbance at 650 nm was measured every 2 min for >90 minutes on a Bio-Rad Benchmark plus reader; readout was determined as time elapsed to half maximum optical density ($\text{OD}_{650} = 0.4$) as per manufacturer's instructions. Values were converted to bacterial equivalents by interpolation from data in the standard curve ($\log [\text{bacterial cells} / \text{mL}]$ vs. $\log [\text{time to half maximum } \text{OD}_{650}]$). Data are presented as bacterial cells per total protein, the latter determined by bicinchoninic acid (BCA) assay (Pierce) with bovine serum albumin standards.

2.6. Statistical Analysis

Data were analyzed by Analysis of Variance (ANOVA; 1-way or 2-way), as appropriate via algorithms within GraphPad Prism 7.0.

3.0. Results and Discussion

3.1. Live *L. plantarum* is cleared from the respiratory tract

In our earlier work, we examined the immunobiotic properties of *L. plantarum* [Gabryszewski *et al.*, 2011; Garcia-Crespo *et al.*, 2013; Percopo *et al.*, 2014a; Percopo *et al.*, 2015; Rice *et al.*, 2016]. Similar to our earlier studies, we show here that *L. plantarum* was cleared rapidly from the lung tissue; here, we observe a 4-log decrease in recovery between 6 - 48 hrs after inoculation with 10^9 colony forming units (CFU) / mL [Fig. 1A]. Likewise, *L. plantarum* genomic DNA was readily detected at 6 and 24 hrs, but dropped to near undetectable at 48 hrs after inoculation; a similar pattern was observed when mice were inoculated with (non-viable) heat-inactivated bacteria [Fig. 1B].

3.2. Use of the Silkworm Larvae Plasma (SLP) Assay to detect *Lp* in mouse lung tissue

The SLP assay has been developed and commercialized as a means to detect bacterial peptidoglycan as a surrogate for live bacteria or bacterial contamination [Kobayashi *et al.*, 2000; Cpyrowski *et al.*, 2014; Shibata *et al.*, 2006]. The assay is based on the phenoloxidase - melanisation mechanism common to many species of insects that has been broadly characterized as a host defense mechanism (reviewed in [Cerenius & Soderhall, 2004; Cerenius *et al.*, 2008]). The SLP assay focuses specifically on the binding of PGN to the silkworm (*Bombyx mori*)-derived PGN-binding protein (PGRP) that initiates a protease-amplification cascade [Fig. 2A]; this ultimately results in the formation of melanin, which is measured spectrophotometrically at OD₆₅₀. For the purposes of this work, we utilized whole cell heat-inactivated bacterial standards as shown [Fig. 2B]; this adaptation yielded the standard sigmoidal curves, and permitted us to generate the log (time to the midpoint, 0.4 OD₆₅₀) vs. log (bacterial cells/mL), [Fig. 2C], which yielded the appropriate linear plot over a significant range of concentrations. As shown in Fig. 2D, the standard curve log-log transformation obtained using commercially-prepared PGN is analogous to that obtained using bacterial cells as standards.

3.3. Melanin detection does not correlate with bacterial cell counts

The initial results obtained using this assay were the reverse of what was anticipated. In an experiment analogous to that shown in Fig. 1A, mice were inoculated with live or heat-inactivated *L. plantarum*, and lung tissue was evaluated using the SLP assay [Fig. 3A]. As early as 24 hrs, we observed amplification over baseline levels in response to the heat-inactivated *L. plantarum*, rather than clearance. By 48 hrs, the response had increased to $21 \pm 13 \times 10^4$ and $23 \pm 8.8 \times 10^4$ bacterial equivalents per μ g lung protein from live and heat-inactivated *L. plantarum*, respectively, both values which exceeded the size of the inoculating dose, assuming a lung weight of approximately 10 mg per mouse (** $p < 0.01$).

Furthermore, as shown in Fig. 3B, elevated levels of melanin were detected in lung tissue of mice inoculated with heat-inactivated *L. plantarum* for many days thereafter, up to and

including day 6 after inoculation. Of particular note were levels of melanin detected in parallel experiment carried out in mice devoid of PRRs TLR2 and NOD2. These PRRs mediate clearance of Gram-positive bacteria from lung tissue [Davis *et al.*, 2011] and are the main receptors mediating inflammatory responses from *L. plantarum* both *in vitro* and *in vivo* [Percopo *et al.*, 2015; Rice *et al.*, 2016]. Although melanin responses to *L. plantarum* are persistent in both wild-type and NOD2^{-/-}TLR2^{-/-} mice, those from the gene-deleted mice are significantly lower than those observed from the wild-type counterparts, a finding that is the opposite of what would be expected from a mouse strain with defective bacterial clearance. These results suggest that a significant component of the SLP cascade may be activated by PRR-mediated endogenous inflammation elicited by *L. plantarum* in the lung microenvironment.

3.4. Melanin is generated in response to a biochemical pattern recognition receptor (PRR) agonist

In order to explore these unanticipated results further, we inoculated mice with CL-429, bi-functional PRR agonist that activates TLR2 and NOD2. CL-429 is a soluble adjuvant that promotes inflammatory responses from dendritic cells and alveolar macrophages *ex vivo* [Pavot *et al.*, 2014; Rice *et al.*, 2016]. As shown in Fig. 3C, intranasal inoculation of mice with CL-429 also results production of melanin. Similar to our findings with live and heat-inactivated *L. plantarum*, melanin was detected in increasing concentrations also beginning at time points long after inoculation, as shown here at 72 hrs. Most intriguing, no such escalation was detected when CL-429 was used to challenge the NOD2^{-/-}TLR2^{-/-} mice (data not shown) further suggesting that the responses we observe may be more related to innate PRR signaling and subsequent inflammatory activation rather than direct activation of the SLP assay by bacteria or their byproducts

Although we have not fully explored the mechanism via which endogenous responses, might be activating the SLP assay, previous data permits us to generate some hypotheses. Among the steps leading to melanin formation (Fig. 2A), the protease cascade would be remarkably sensitive to interference from exogenous enzymes; indeed, *B. mori*, similar to other insects, maintains specific protease inhibitors in order to exert control against uncontrolled proteolytic activation *in situ* [Liu *et al.*, 2015] as well as inhibitors that function at other points in the cascade [Liu *et al.*, 2012]. In an earlier study, [Percopo *et al.*, 2015] we determined that *L. plantarum* administered to the respiratory mucosa resulted in significant up-regulation of several proteases, including matrix metalloproteinase 12 (29-fold), and cysteine proteases cathepsins B, S and Z (2-, 4-, and 3- fold, respectively; GSE66721). It is not known whether any or all of these enzymes have the capacity to augment the conversion of the pro-enzymes (pro-PPAE, pro-PO, others) to their active forms, as none of these four enzymes are serine proteases; however, given the nature of enzyme amplification, even minimal cross-activation (coupled with a relatively large amount of protease) might explain the findings reported here.

4.0. Conclusions

We conclude that findings from the SLP assay must be interpreted with caution. One cannot assume that production of melanin unequivocally correlates with bacterial counts in all environments tested. This is particularly important given recent interest in this assay for use in clinical settings, including primary diagnosis and detection of postoperative infections [Inada et al., 2003; Shimizu *et al.*, 2005], conditions that may be associated with local inflammation.

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Abbreviations

PGN	peptidoglycan
PRR	pattern recognition receptor
SLP	silkworm larvae plasma

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Highlights

- The SLP assay detects bacterial peptidoglycan in biological fluids and tissues.
- Generation of melanin by this assay is not reliable as a surrogate for bacteria.
- Endogenous inflammation may activate the protease cascade leading to melanin.

Figure 1. (A) Detection of live *Lactobacillus plantarum* (*Lp*) and *Lp* genomic DNA from mouse lung tissue

(A) Live *L. plantarum* (colony forming units (CFU) / mL) detected in mouse lung tissue homogenates at time points indicated after intranasal inoculation with diluent only (-) or 10^9 cells in 50 μ L at $t = 0$; nd, none detected, $n = 3 - 6$ mice per group. (B) *L. plantarum* genomic DNA (16s–23s intergenic region, [copies per copies cellular GAPDH] $\times 10^8$) detected in mouse lung tissue at time points indicated after inoculation with live (black

squares) or heat-inactivated (grey squares) *L. plantarum* at 10^9 cells in 50 μ L; * $p < 0.01$, n = 3 mice per group, ** $p < 0.001$ by ANOVA.

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Figure 2. Silkworm Larvae Plasma (SLP) Assay

(A) Schematic of the amplification reaction. Peptidoglycan (PGN) is detected by the silkworm (*Bombyx mori*) peptidoglycan binding protein (PGRP), which initiates proteolytic conversion of Proproteins (ProPPAE, ProPO) to active proteins (PPAE, PO), ultimately resulting in the production of melanin (black symbol). PPAE (prophenoloxidase - activating enzyme; dark blue symbol) is a serine protease; see also Cerenius and Soderhall, 2004; Cerenius *et al.*, 2008. (B) Sigmoidal curves (OD₆₅₀ vs. time) generated in response to challenge with decreasing concentrations of heat-inactivated *L. plantarum*. All curves demonstrate appropriate OD₆₅₀ max = 0.8; OD₆₅₀ = 0.4 (midpoint of log phase) is indicated by the dotted line. (C) Plot of log [time in min to OD₆₅₀ = 0.4] vs. log [bacterial cells / mL] demonstrates appropriate inverse correlation; $r^2 = 0.92$. (D) Plot of log [time in min to OD₆₅₀ = 0.4] vs. log [*M. luteus* peptidoglycan (PGN; ng/mL)] demonstrates an analogous inverse correlation; $r^2 = 0.97$.

Figure 3. Application of SLP Assay for detection of *L. plantarum* in lung tissue homogenates

(A) Intranasal inoculation of wild-type mice with live (black bars) or heat-inactivated (grey-bars) of *L. plantarum* followed by evaluation of lung tissue at time points as in Fig. 1; no *L. plantarum* control is shown in blue at $t = 0$; $n = 3$ mice per group. SLP assay as described in Methods, interpolated to a standard curve as in Fig. 2 is generated for each experiment. (B) Intranasal inoculation of wild-type (blue bars) or NOD2-TLR2 gene-deleted mice (NOD2^{-/-}TLR2^{-/-}; grey bars) with heat-inactivated *L. plantarum* followed by evaluation of lung tissue at time points indicate; $n = 3$ mice per group. (C) Inoculation of wild-type mice with soluble bi-functional pattern-recognition receptor agonist CL-429 (20 μg per mouse in 50 μL pbs/bsa) followed by evaluation of mouse lung tissue at time points indicated; $n = 3 - 4$ mice per group, * $p < 0.01$, ** $p < 0.001$ by ANOVA.