Short communication: Recombinant bacteriophage endolysin PlyC is nontoxic and does not alter blood neutrophil oxidative response in lactating dairy cows

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ABSTRACT

Mastitis is the leading cause of antimicrobial use on dairy farms. The potential for antimicrobial resistance has led to the examination of alternative strategies for controlling mastitis. One such alternative is PlyC, a potent peptidoglycan hydrolase derived from the streptococcal C1 bacteriophage that causes targeted lysis of the cell wall of *Streptococcus uberis*. At a concentration of 1.0 µg/mL, recombinant PlyC can induce lytic activity, suggesting that a low dose may successfully eliminate infection. We evaluated the dose effect of PlyC (1–50 µg/mL) on cytotoxicity and oxidative response on bovine blood polymorphonuclear leukocytes (PMN) obtained from 12 healthy, mid-lactation primiparous dairy cows. Following incubation at 0.5 and 2 h, cytotoxicity was characterized by measuring lactate dehydrogenase release from isolated cells. Oxidative burst response was characterized as the intensity of chemiluminescence produced in the interaction of reactive oxygen species generated in response to 0 or 1.6 µg/mL of phorbol 12-myristate-13-acetate (PMA) with a luminescent substrate with and without addition to PlyC to the incubation matrix. Data were analyzed as a complete randomized block design using mixed model procedures. Cytotoxicity of PlyC was not affected by concentrations up to 50 µg/mL. As expected, PlyC cytotoxicity on PMN varied across incubation time with greater cell toxicity measured at 2 h of incubation as compared with 0.5 h and is primarily attributed to the short life of PMN ex vivo. Concentrations of PlyC up to 50 µg/mL did not affect oxidative response; however, oxidative response was affected by incubation time and PMA concentration. In summary, varying doses

of PlyC are nontoxic as estimated by lactate dehydrogenase release from cells and do not appear to alter PMA-stimulated reactive oxygen species production in bovine PMN. These early observations support continued work on the potential for application of this novel agent in combating mastitis.

Key words: mastitis, antibiotic alternative

Short Communication

With regard to dairy production, mastitis is the most common and economically significant disease. The estimated losses to producers in the United States exceed \$2 billion per year with each clinical case costing approximately \$179 (Cha et al., 2011). A wide variety of pathogens cause bovine mastitis including bacteria, yeast, and algae. Pathogens arising from the environment, such as *Streptococcus uberis* and *Escherichia coli*, are consistently being detected and reported as a leading cause of subclinical and clinical bovine mastitis throughout the world (National Mastitis Council, 2015). *Streptococcus uberis* is the most prevalent environmental streptococci mastitis pathogen (Jayarao et al., 1999). The number of cases caused by *S. uberis* continues to rise even in well-managed herds as typical mastitis control practices, such as teat disinfection and sanitary housing and milking environments, are less effective at preventing *S. uberis*-associated mastitis (Kromker et al., 2014).

The most common treatment strategy for clinical cases of *S. uberis* is the administration of intramammary antibiotics, such as cephalosporin β-lactams or penicillin G (Oliver et al., 2011). Growing concerns regarding antibiotics and the potential for antimicrobial resistance from their use have led to the development of alternative strategies for controlling mastitis (Bannantine et al., 2013). An antimicrobial alternative currently being developed is the bacteriolytic cell wall hydrolases known as lysins (or endolysins). These

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bacteriophage-derived enzymes rapidly and specifically lyse bacteria when applied exogenously (Fischetti, 2006). One of the most potent endolysins to date is the streptococcus-specific recombinant streptococcal C1 phage lysin (**PlyC**). Previous research spanning over 50 yr has shown that PlyC can rapidly lyse streptococci groups A, C, and E in addition to *S. uberis* and *Streptococcus equi* and has been shown to protect mice from streptococcal infection challenge (Krause, 1957; Nelson et al., 2001). Therefore, the use of recombinant PlyC is an appealing choice for use against select streptococcal species in bovine mastitis, such as *S. uberis*.

Following bacteria entry into the mammary gland, circulating PMN (i.e., neutrophils) are the first cells recruited and then represent the most predominant somatic cell type (Burvenich et al., 1994). The initial magnitude of the PMN response will often dictate the overall effect of mastitis on the cow, as increased recruitment of PMN and greater overall PMN activity will decrease mastitis duration and severity (Rainard and Riollet, 2003). Stimuli from mammary cells and invading pathogens activate PMN to eliminate the pathogens through the production of chemical mediators, such as reactive oxygen species (**ROS**), and phagocytosis (Miller et al., 1993). As an alternative to pathogenic bacteria, phorbol 12-myristate-13-acetate (**PMA**) is commonly used to stimulate cellular aspects of oxidative metabolism and structure normally associated with bactericidal activity in vitro (Rinaldi et al., 2007; Freitas et al., 2009).

Although intramammary administration of PlyC is a promising new management strategy to treat *S. uberis* mastitis, the effects of PlyC on the bovine immune response remain unknown. Delayed appearance or decreased immunological activity of PMN can discourage the occurrence of a bacteriological cure. Potential antibiotic alternatives should not interfere with or harm the host immune response if they are to be considered useful. The objective of this study was to evaluate any cytotoxicity effects of PlyC on bovine PMN and on the PMN oxidative response to activation. We hypothesized that various concentrations of PlyC would be nontoxic and not alter the inflammatory response of bovine PMN in vitro.

All procedures involving the use of live animals were approved in accordance with the regulations and guidelines set forth by the USDA-Beltsville Animal Care and Use Committee.

Twelve healthy primiparous Holstein cows were used for this study. To be eligible, cows must have had a composite milk SCC <100,000 cells/mL and cows must not have been treated for clinical signs of mastitis or any other disease during early lactation. Eligible cows were housed in tiestalls and individually fed a standard

TMR to meet the requirement of lactating dairy cows at the USDA-Beltsville Agricultural Research Center. Free choice water was available and cows were fed ad libitum twice daily to ensure 5 to 10% refusals. All cows were milked twice daily at 0730 and 1630 h in a double-8 herring bone parlor with milk records recorded monthly. Cows averaged 178 ± 4 DIM and 40.7 ± 1.3 kg of milk/d at the time of sampling.

Recombinant PlyC was expressed and purified as previously described (Nelson et al., 2001, 2006). Briefly, *E. coli* were grown at 37°C in baffled flasks to an optical density of 1 at 600 nm in LB-Miller medium (Luria broth containing 10 g/L of sodium chloride) supplemented with 100 µg/mL of ampicillin. Expression of PlyC was induced by the addition of arabinose (0.25%). After growth for an additional 4 h at 37°C, cells were harvested, lysed by sonication, and crude phage extracts were purified by ceramic hydroxyapatite resin (Bio-Rad Laboratories Inc., Hercules, CA) to remove cellular debris. As approximately 80 to 90% pure protein was eluted by 1 M sodium phosphate at pH 7.2, a second round of purification by S200 size exclusion chromatography was performed to remove endotoxin. All PlyC used in experiments was produced in a single batch.

Approximately 200 mL of blood was collected via jugular venipuncture from each cow into 15-mL centrifuge tubes with conical bottoms (VWR, Radnor, PA) containing 2.5 mL of acid-citrate dextrose solution (Sigma-Aldrich, St. Louis, MO) and stored on ice. Blood samples were processed within 1 h of collection. Isolation of PMN, viability, and cell differentiation were performed using endotoxin-free materials and reagents as previously described (Garcia et al., 2015). Viability of PMN was 96% with a cell differential of 91% PMN. Cells were counted by a TC20 Automated Cell Counter (Bio-Rad Laboratories) then diluted to concentrations appropriate for each assay. From each cow, 800 µL of PMN were placed in 24-well cell culture plates and treated with 200 µL of 0, 1, 10, or 50 µg of PlyC in 1× phosphate buffer solution. Plates were incubated for 0.5 or 2 h at 37°C, 95% relative humidity, and 5% CO₂.

The cytotoxicity of PlyC on PMN was determined in vitro. Prior to incubation with PlyC, PMN were diluted to $\sim 3.5 \times 10^5$ per mL for analysis of PMN cytotoxicity using the CytoTox 96 nonradioactive, colorimetric assay (Promega, Madison, WI) to quantify lactate dehydrogenase production. Relative cytotoxicity was calculated per instructions provided by the manufacturer (experimental divided by maximum lysis). Within incubation period and cow, each PlyC treatment was performed in triplicate (CV = 2.66%).

The oxidative response of PMN was determined by luminol chemiluminescence assay to quantify ROS pro-

duction. Neutrophils were diluted to $\sim 3.0 \times 10^6$ per mL before incubation with PlyC. Following incubation with PlyC for 0.5 or 2 h, 0.5 mL of medium was transferred into round-bottomed, glass 7-mL tubes. Tubes were placed into a heating block and incubated at 37°C for 10 min. In a separate reaction tube (2 per each medium tube), 900 μ L of assay buffer (0.5 mM CaCl_2 in Hanks' balanced salt solution) and 100 μ L of a 5 mM luminol solution in dimethyl sulfoxide (Sigma-Aldrich) were added and placed in the heating block. At the end of the incubation, either 40 μ L of sterile water or 1.6 μ g/mL of PMA (Sigma-Aldrich) solution was added to each medium tube. Tubes were gently vortexed and returned to the heating block. After heating, 40 μ L from the medium tube was transferred to the reaction tube. Tubes were again lightly vortexed and immediately placed in the luminometer (AutoLumat LB 953, Berthold Technologies, Germany) to measure relative chemiluminescence for 20 min. Within incubation period, PMA concentration, and cow, each PlyC treatment was performed in duplicate (CV = 11.96%).

Experimental units and the corresponding sample size of cows that were used in this study have been determined using the POWER procedure of SAS (SAS/STAT version 9.3, SAS Institute Inc., Cary, NC) for a 1-way ANOVA. Means and average standard deviation values for PMN oxidative response and PlyC cytotoxicity were calculated from a previously conducted pilot study. The following are parameters included in the procedure: (1) an α set at 0.05 to declare significance, (2) an 80% probability of detecting a difference in treatment means, (3) a 15,000 luminescence unit difference between treatment means, and (4) an average standard deviation value of 6,400. The procedure was repeated for PlyC cytotoxicity with a 15 unit difference between means and an average standard deviation value of 7. Based on the results of the power analyses, it was concluded that at least 10 cows are required for this study.

Response variables were analyzed as a complete randomized block design using the PROC MIXED procedure of SAS. Sources of variation within the model included effects of PlyC concentration, PMA concentration (oxidative response model only), and incubation time as well as 2- and 3-way interaction among the main effects. Cow was designated as a random effect in the model. Separation of least squares means for significant effects was accomplished using the PDIF option in the MIXED procedure. Significance was declared at $P \leq 0.05$. Data are presented as least squares means $\pm$ standard errors of the mean.

In the current study, concentrations of purified PlyC at 1, 10, and 50 μ g/mL did not have a cytotoxic effect on PMN relative to cells incubated in buffered solution ($P = 0.41$) as cytotoxicity remained constant across

Figure 1. Least squares means and SE for bovine neutrophil cytotoxicity of neutrophils incubated in vitro for 0.5 and 2 h in various concentrations of recombinant streptococcal C1 phage lysin (PlyC).

concentrations (47–55%). These results indicate that PlyC, even at high doses, is not toxic to bovine PMN in vitro. Incubation time increased PlyC cytotoxicity by 25% from 0.5 to 2 h (44.6 vs. $55.0 \pm 2.8\%$; $P = 0.01$) as expected, given that the relatively short half-life of circulating PMN is 6 to 8 h (Summers et al., 2010). However, incubation time across PlyC concentrations did not affect cytotoxicity ($P = 0.78$; Figure 1).

Respiratory oxidative burst activity of PMN incubated with purified PlyC was determined by luminol chemiluminescence following 0.5 or 2 h of incubation and stimulation by 0 or 1.6 μ g/mL of PMA. Similar to cytotoxicity, respiratory burst was not affected by PlyC concentrations up to 50 μ g/mL ($P = 0.52$) and relative luminescence remained constant ($29,838 \pm 3,315$ relative luminescence units), indicating that PlyC may not interfere with the function of PMN during the inflammatory response in vivo. As expected, increasing PMA concentration from 0 to 1.6 μ g/mL increased PMN respiratory burst response ($21,514$ vs. $38,162 \pm 4,585$ relative luminescence units; $P < 0.01$) reflecting the activation of NADPH oxidase and production of ROS, superoxide anions, and hydrogen peroxide, similarly to that of an invading pathogen (White et al., 1984; Kobayashi et al., 2005). However, PMN respiratory response to PMA was dependent upon incubation time with longer incubation decreasing respiratory burst of

Figure 2. Least squares means and SE for the bovine neutrophil oxidative response as determined by luminol chemiluminescence at 0.5 or 2 h of incubation and stimulated by 0 or 1.6 µg/mL of phorbol 12-myristate-13-acetate (PMA) solution in various concentrations of recombinant streptococcal C1 phage lysin (PlyC). (A) Oxidative response and incubation time across PMA concentration; (B) oxidative response and incubation time across PlyC concentration. Means with different letters (a–c) differ ($P < 0.05$).

PMN incubated with 1.6 µg/mL of PMA ($P < 0.01$; Figure 2A). The overall decreased respiratory burst response across incubation time reflects the increased cell deterioration and death observed in the PMN cytotoxicity analysis. The relative oxidative burst of PMN was not affected by PlyC concentration across incubation time ($P = 0.45$), although relative luminescence was numerically lower following 2 h incubation (Figure 2B).

The growing concern for antibiotic resistance and improved stewardship has fueled the drive to find antibiotic and antimicrobial alternatives. Bacteriophage lysins have been used successfully in a variety of models to control pathogenic antibiotic resistant and tolerant bacteria found on mucosal surfaces and infected tissues. The streptococcal-specific endolysin PlyC is unique among the lysins and has rapid lethal effect in vitro (McGowan et al., 2012) and in vivo (Nelson et al., 2001). Relatively low concentrations of PlyC have successfully been used for disinfection of *S. equi* (1 µg/mL; Hoopes et al., 2009) and degradation of *Streptococcus pyogenes* biofilms (5 µg/mL; Shen et al., 2013) in addition to the prevention and elimination of *S. pyogenes* in mice (0.01 µg; Nelson et al., 2001). These effective concentrations were substantially lower than those examined in the current study. Concentrations of PlyC up to 50 µg/mL were nontoxic on bovine PMN. These results demonstrate that the lytic activity of PlyC does not target bovine PMN and that effective concentrations of

PlyC would likely be nontoxic to recruited neutrophils during mastitis.

Recruited neutrophils use many techniques to destroy pathogenic bacteria that have entered the mammary gland, such as phagocytosis, extracellular traps, and degranulation (Summers et al., 2010). Reactive oxidative species produced and released during the respiratory burst response aid in bacterial destruction and clearance of the infection. Again, high concentrations of PlyC (up to 50 µg/mL) did not affect the respiratory burst response of bovine PMN. Effective lower concentrations of PlyC would likely not affect the respiratory response of PMN during the immunological response to infection.

To our knowledge, this study is the first to characterize the effects of bacteriophage-based lytic enzyme PlyC on bovine PMN. Results show that high concentrations of PlyC are nontoxic and do not impede the oxidative response of bovine PMN. Future studies investigating the use of PlyC as an alternative therapy for treatment of mastitis are warranted.

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