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Short Communication

Effects of different lipopolysaccharide preparations on neutrophil function in the fathead minnow, *Pimephales promelas* Rafinesque

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The fish innate immune response to pathogens relies on the adequate function of neutrophilic granulocytes (Palić, Andreasen, Herolt, Menzel & Roth 2006). The ability of neutrophils to phagocytose microorganisms and cellular debris is essential for normal development and survival of animal populations (Segal 2005). The evaluation of neutrophil function is valuable for assessing the health status of individuals and fish populations (Smith & Lumsden 1983). Resistance of fishes to septic shock and tolerance to high concentrations of lipopolysaccharide (LPS) was observed as a major difference between mammalian and fish innate immune responses (Berczi, Bertok & Bereznai 1966). This functional difference could be attributed to several costimulatory molecules and intracellular mediators being absent in fish, but active in mammals during response to LPS stimulation (Iliev, Roach, Mackenzie, Planas & Goetz 2005). Most fish do not possess a Toll-like receptor (TLR) with sequence similarity to mammalian TLR4 (Leulier & Lemaitre 2008) and the ones that do have no ability for TLR4 downstream signalling (Sepulcre, Alcaraz-Perez, Lopez-Munoz, Roca, Meseguer, Cayuela & Mulero 2009). Regardless of the absence and functionality of TLR4 and costimulatory molecules, bacterial LPS can induce a robust inflammatory gene response in innate immune fish cells, but at concentrations 1000-fold higher than is commonly observed in mammalian species (μg mL^{-1} vs. ng mL^{-1}) (Palić, Ostojic, Andreasen & Roth 2007; Mackenzie, Roher, Boltaña & Goetz 2010). In contrast, ultrapure LPS preparations are relatively inactive in fish (Iliev *et al.* 2005). Commercially available LPS preparations are often contaminated by other bacterial components, such as lipoproteins or peptidoglycans (PGN), with the potential to activate TLR2 as well as TLR4 signalling, as opposed to ultrapure preparations of LPS that should activate only the TLR4 signalling pathway (Hirschfeld, Ma, Weis, Vogel & Weis 2000). A recent report indicated that pure peptidoglycans were the potent activators of trout macrophages, and it was likely that crude LPS preparations stimulated trout macrophages as a result of peptidoglycan contamination (Mackenzie *et al.* 2010). Crude LPS has been used as standard stimulant in fish neutrophil function assays (Palić *et al.* 2007). In contrast to macrophage activation (Mackenzie *et al.* 2010), stimulation with crude LPS often interferes with the expected stimulative responses of granulocytes (Palić *et al.* 2007; Jovanović, Goetz, Goetz & Palić 2011b). Therefore, we compared the effects of commercially available

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crude and ultrapure LPS preparations as well as PGN products on neutrophil respiratory burst and neutrophil extracellular trap release (NET) in fathead minnow, *Pimephales promelas* Rafinesque.

Neutrophils can be primed or stimulated for respiratory burst with the use of LPS (Forehand, Pabst, Phillips & Johnston 1989). Reactive oxygen species (ROS) generated during respiratory burst facilitate a pH increase in phagocytic vacuoles and the entry of potassium ions, further stimulating the release of the granular enzymes responsible for bacterial killing (Segal 2005). ROS can be further dismutated to hydrogen peroxide that can trigger NETosis, a cell death mechanism characterized by total neutrophil membrane disintegration and release of NETs (composed of DNA, histones and granule proteins) that continue to trap and kill pathogens beyond the neutrophil's lifespan (Fuchs, Abed, Goosmann, Hurwitz, Schulze, Wahn, Weinrauch, Brinkmann & Zychlinsky 2007).

Crude LPS, *Escherichia coli* 0111:B4 (tlrl-eblps); ultrapure LPS, *E. coli* 0111:B4 (tlrl-pelps); PGN from *E. coli* 0111:B4 (tlrl-pgnec); PGN from *Staphylococcus aureus* (tlrl-pgnsa); and PGN from *Bacillus subtilis* (tlrl-pgnbs) were purchased from InvivoGen.

Adult fathead minnows (5 g average weight) were euthanized with an overdose (1 g L^{-1}) of tricaine methane sulphonate (MS-222, Argent Laboratories) according to the protocol approved by the Iowa State University Committee for Animal Care and Use and American Veterinary Medical Association Euthanasia Panel recommendations. Anterior kidneys from eight fish were dissected and pooled as a single sample, and cell suspensions were prepared (Palić, Andreasen, Frank, Menzel & Roth 2005). A total of 24 samples were used per assay, and readings were performed either in duplicate (respiratory burst) or triplicate (NETs release). The final cell isolation was performed as described by Palić *et al.* (2005), with mean neutrophil purity of 72.0 ± 7.3% after gradient purification. The remaining cells were lymphocytes and thrombocytes, and < 5% of isolated cells were monocytes and erythrocytes, with a few kidney epithelial cells. Cell suspensions were adjusted to 2×10^7 cells mL^{-1} and used in all neutrophil function assays. Final concentration of cells inside the well plate during the assays was 0.5×10^7 cells mL^{-1} for respiratory burst and 0.15×10^7 cells mL^{-1} for NETs release as a previously determined standard (Jovanović, Anastasova, Rowe, Zhang, Clapp & Palić 2011a). Cells were exposed to standard phorbol ester stimulant [phorbol myristate acetate (PMA), 1 μg mL^{-1} Sigma-Aldrich Corp.], negative control (Hank's balanced salt solution with Ca, Mg, no Phenol Red, HBSS; Mediatech – CellGro) and the LPS and PGN products at concentrations of 1 and 10 μg mL^{-1}, following the manufacturer's recommended dose guaranteed to stimulate TLR4 and/or TLR2 pathways in vertebrates. Respiratory burst assay was performed according to an established protocol (Hermann, Millard, Blake & Kim 2004) with modification as described by Jovanović *et al.* (2011a). The neutrophil extracellular trap release assay was performed as previously described (Chuammitri, Ostojić, Andreasen, Redmond, Lamont & Palić 2009) with recent modifications (Jovanović *et al.* 2011a). All data were analysed for significance using one-way ANOVA followed by Dunnett's procedure for post hoc comparison of means between single control and multiple experimental groups, and a *P*-value equal to or < 0.05 was considered statistically significant.

Crude LPS did not elicit respiratory burst in *P. promelas* neutrophils at any concentration, while the ultrapure LPS demonstrated potent induction of respiratory burst especially at 10 μg L^{-1} (Fig. 1a). The ultrapure LPS induced a significantly stronger response than the PMA, the potent activator of protein kinase C and inducer of respiratory burst (Janeway, Murphy, Travers & Walport 2008). Both LPS products used in the experiment were prepared from *E. coli*, and the only difference is that crude LPS contains impurities in the form of PGNs, as given in the manufacturer's product information.

Peptidoglycans from all bacteria tested induced respiratory burst (Fig. 1a). Interestingly, PGN stimulation was not dose dependent at the concentrations used, while a significant difference in respiratory burst induced by ultrapure LPS was observed between the two concentrations tested. Furthermore, ultrapure LPS induced a dose dependent formation and release of NETs (Fig. 1b). Crude LPS and PGNs did not have any stimulatory effect on NET release as compared to controls, but the 10 μg mL^{-1} dose of PGN *E. coli* suppressed NET formation (Fig. 1b).

The mechanism behind the strong activation of an immune response in fathead minnow neutrophils by ultrapure LPS, as opposed to crude LPS and PGNs, is still unclear. Ultrapure LPS only activates

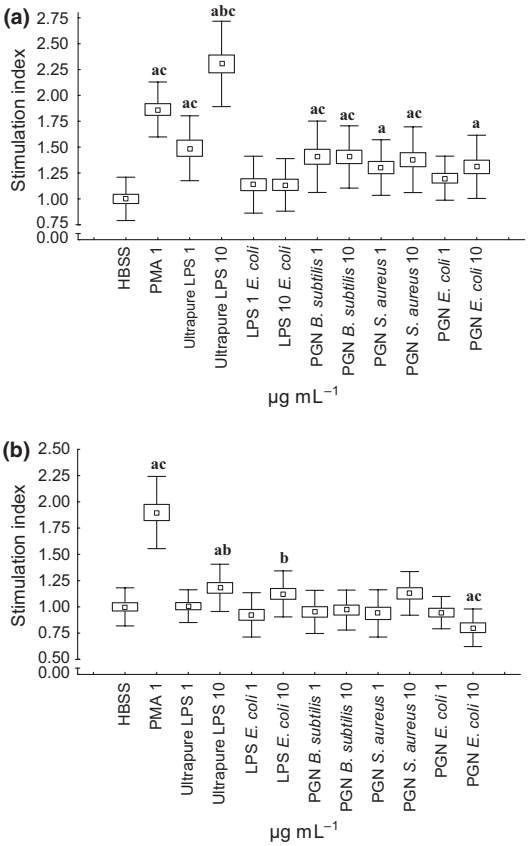

Figure 1 *In vitro* effect of lipopolysaccharides (LPS) and peptidoglycans (PGN) on neutrophil respiratory burst (a) and neutrophil extracellular trap release (b) in fathead minnows. Neutrophils were stimulated with phorbol myristate acetate (PMA, positive control). Background controls were cell suspensions exposed to Hank's balanced salt solution with Ca, Mg, no phenol red (HBSS). Stimulation index was calculated according to the following formula: Stimulation index = compound stimulated cell suspension fluorescence/fluorescence of the same cell suspension exposed to HBSS. Significant difference at $P < 0.05$ was observed between [a]compound and background control; [b]two doses of the same compound; and [c]compound and crude LPS preparation at corresponding concentrations. Empty squares in the centre of the boxes represent mean values; boxes represent standard errors and bars represent standard deviations of the mean.

the TLR4 pathway (Hirschfeld *et al.* 2000) that is absent or non-functional in fish macrophages (Sepulcre *et al.* 2009), and ultrapure LPS does not activate macrophages (Mackenzie *et al.* 2010). Mammalian neutrophils possess TLR4 receptors (Hayashi, Means & Luster 2003; Koller, Kappler, Latzin, Gaggar, Schreiner, Takyar, Kormann, Kabesch, Roos, Griese & Hartl 2008); therefore, it is possible in fish that have a TLR with sequence similarity to mammalian TLR4 that the functional activity of ultrapure LPS is primarily observed in neutrophils rather than macrophages. Zebrafish have been shown to have a TLR with sequence similarity to mammalian TLR4, and because zebrafish and fathead minnows are both cyprinids, it is possible that fatheads have a TLR4 as well. It is also possible that a yet unidentified receptor is responsible for the neutrophil response to ultrapure LPS or that ultrapure LPS can cross-react with TLR receptors other than TLR4 on fish neutrophil membranes and initiates the respiratory burst response. In this regard, it was recently demonstrated in trout macrophages that ultrapure LPS can stimulate the secretion of a TNFα protein even though it cannot stimulate TNFα transcription (Roher, Callol, Planas, Goetz & MacKenzie 2010).

As the effects of crude and ultrapure LPS and PGNs on the immune response in fathead minnow neutrophils were markedly different than that previously reported for trout macrophages, it seems prudent to test various LPS-associated compounds (e.g. crude, ultrapure, PGNs) when looking at the effects of LPS on different fish species and/or cell types.

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