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Short communication

Bacterial lipopolysaccharides induce genes involved in the innate immune response in embryos of the zebrafish (*Danio rerio*)

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Abstract

The innate immune response in fish represents an early, rapid defence against pathogens. Environmental contaminants could disturb this defence and negatively influence the ability to protect against infection. However, analysis of immune-modulation has not yet been included in testing strategies for environmental risk assessment of chemicals. In order to establish an efficient, small scale test system, the ability to induce the innate immune response by bacterial lipopolysaccharides in zebrafish embryos was investigated. The level of expression of various genes involved in inflammation was used as the endpoint. We could show that immersion of embryos in LPS induced the gene expression of two key pro-inflammatory cytokines, tumor necrosis factor α and interleukin 1β in 32 h old zebrafish embryos. The gene induction required the removal of the chorion prior to lipopolysaccharide exposure.

Keywords: Innate immunity; Zebrafish; Embryo; TNF-α; IL-1β; NF-κB; Gene expression; Environmental chemicals; Chorion

The innate immune response is important in the early, rapid defence against pathogens. Disturbance of this defence by environmental contaminants could negatively influence the ability to protect against infections and influence the health of an individual or population. Fish can be considered organisms of prime risk for modulation of innate immunity by environmental chemicals. This is because first, mounting an adaptive response takes longer in fish than in higher vertebrates [1]; and second, fish are directly and continuously exposed to chemicals discharged into the environment.

A number of diverse examples for the interference of environmental contaminants with the innate immune response in fish have been reported, ranging from excess mucus production, modulation of humoral factors, cellular defence by macrophages to inflammation (reviewed in ref. [2]). Despite its relevance, however, analysis of potential immune-modulating effects on fish has not yet been implemented in testing strategies for environmental risk assessment of chemicals. An appropriate test system would utilize the immune-modulating effect of a test chemical on the stimulated inflammation as the final component of innate immune response. Therefore, inflammation in fish can be experimentally stimulated by either injection of or immersion in bacteria or bacterial lipopolysaccharides (LPS). These techniques have been applied for adult fish, fish embryos and/or primary and permanent fish cells [3–9].

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With respect to the screening for immune-modulation in fish, an efficient test system for high throughput analysis would be required. Adult fish would not be suitable for such a high throughput analysis because exposure and endpoint measurements would be rather elaborate. Furthermore, there are ethical concerns as there is a strong societal demand for the reduction of animal tests. In contrast, fish embryos, such as from zebrafish (*Danio rerio*) would allow a small scale analysis and are suitable for high throughput testing. Experimentation with fish embryos is considered a pain free in vitro alternative and protocols for the use of up to 48 h-old embryos as a replacement method for acute toxicity testing have already been established [10]. In contrast to primary or permanent fish cells, the embryo model offers a higher degree of complexity, resembling an organismic response. It therefore may also more closely reflect the effects in adult animals. For the current study, we have selected the zebrafish embryo in order to examine components of the pro-inflammatory response. The zebrafish is one of the most widely used vertebrate models and is also considered suitable to study human disease and development [11–14]. Since the innate immune system is highly conserved in vertebrates, the analysis of effects on the innate immune response in zebrafish also provides information relevant to human health [15].

In zebrafish embryos, a pro-inflammatory response has been stimulated by injection with bacteria and the clearance of a bacterial infection was used as a measure of the immune function [8,9]. The immersion of embryos to a bacterial suspension has been shown to induce the innate immune response as well [4]. However, the injection of bacteria is time-consuming and needs appropriate technical practice. In the case of immersion, the use of fish-pathogenic bacteria bears the risk of contaminations of fish culture. Furthermore, the immune response might show a higher variability. In contrast, the stimulation of the innate immune response by bacterial lipopolysaccharides (LPS) could be an alternative approach. In embryos, this approach has thus far only been applied for the analysis of *tnfa*-induction by means of GFP (green fluorescent protein) fluorescence in a transgenic reporter zebrafish harbouring a Japanese flounder *tnfa*-GFP promoter construct [16].

The aim of the present study was to test the capability of commercially available lipopolysaccharides to induce the innate immune reaction by analysis of components of the pro-inflammatory response. Cytokine expression, for example, can be activated by LPS via the Toll-like receptor TLR4 and involves the NF-κB-signalling pathway [17]. The differential expression of genes involved in this signalling cascade has been detected in various fish species (e.g. refs. [4,6,7,18–20]). Furthermore, Toll-like receptors and cytokines have also been shown to be expressed during early stages of teleost development [21,22]. In order to monitor the stimulation of the innate immune response by LPS in zebrafish embryos, the transcription of the cytokines *il1b*, *tnfa*, *il10* (interleukin 10), the cyclooxygenase homologues *ptgs1* and *ptgs2*, the NF-κB-subunits *nfkb2* and *rela* and the NF-κB inhibitor *nfkbiab* were analysed by RT-PCR. We could show that immersion of embryos in LPS indeed induced the expression of two key pro-inflammatory cytokines, *tnfa* (tumor necrosis factor α) and *il1b* (interleukin 1β) in 32 h-old zebrafish embryos.

For the experiments, the zebrafish wildtype strain WiK obtained from the Max Planck Institute for Developmental Biology (Tübingen, Germany) was used. Fish were cultured at 26 ± 1 °C at a 14:10 h light:dark cycle in a recirculating tank system using local tap water (pH 8–8.2, water hardness 1.2 to 2.4 mM bivalent ions, conductivity 520–560 μS/cm). Fish were fed *ad libitum* three times daily, once with *Artemia* and twice with commercial flake food (Tetra, Melle, Germany).

Production of embryos was performed according to Nagel [10]. The embryos were collected one hour after the onset of the light and incubated at 26 °C. At 28 h post-fertilization (hpf) the chorion was removed by forceps and embryos were exposed to 0.1, 1 and 10 ng/ml of lipopolysaccharides (serotypes O55:B5, O111:B4 and O127:B8, purchased from Sigma, Deisenhofen, Germany) until 32 hpf. The LPS concentrations were selected based on a pre-screen for the effective range with up to 10 μg/ml and were well below toxicity in zebrafish embryos (100 μg/ml, [16]). LPS was added by diluting a 1 mg/ml aqueous stock solution, which was stored in aliquots at −20 °C prior to the tests. Later post-hatched embryos have not been considered for LPS treatment since existing standard protocols for the use of zebrafish embryos as a replacement method for animal experiments are limited to a maximum of 48 h post-fertilisation [23,24].

Total RNA was extracted from 32 hpf zebrafish embryo using Trizol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with DNAse I (Roche, Grenzach, Germany) for 15 min at 25 °C (10 U/μg RNA). cDNA was synthesized from 1 μg of total DNAse-treated RNA using the RevAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Target genes were amplified from 1 μl of cDNA using 1 unit of Taq Polymerase (Promega, Mannheim, Germany), 50 mM Tris–HCl, pH 9.0, 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton-X 100, 200 μM dNTPs and 0.5 μM of each primer