MARINE BIOTECHNOLOGY

Effects of Arsenic on Zebrafish Innate Immune System

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Abstract

The innate immune response, the first line of defense against invading pathogens, can be perturbed by environmental toxicants such as arsenic. This study reports the effects of arsenic on innate immunity of zebrafish. Respiratory burst activity, messenger RNA expression of tumor necrosis factor α (TNF-α), a primer of the respiratory burst response, and mRNA expression of the antiviral cytokines interferon (IFN) and Mx, before and after viral infection, were examined in arsenic-exposed zebrafish larvae. Respiratory burst activity and TNF-α expression were decreased upon arsenic exposure, indicating inhibition of TNF-α priming of the respiratory burst response. Arsenic enhanced IFN expression slightly over time, but reduced Mx expression. In zebrafish infected with snakehead rhabdovirus, arsenic decreased induction and altered the kinetics of IFN and Mx upon infection. Differences in IFN and Mx expression in arsenic-exposed larvae point toward an interruption of the Janus kinase–signal transducer and activator of transcription (JAK/STAT) pathway.

Key words: arsenic — respiratory burst — zebrafish — interferon — Mx — TNF-α

Introduction

The semimetal arsenic is an important environmental toxicant produced as a byproduct of smelting, fossil fuel combustion, and pesticide production. Arsenic accumulates in soil, water, and air, where it is taken up by various organisms (Bernstam and Nriagu, 2000). Exposure to arsenic can cause certain forms of cancer by enhancing the carcinogenic effects of other substances and affecting metabolic pathways (Huang et al., 2004).

Arsenic also displays toxic effects on a variety of immune factors. Arsenic exposure reduced the delayed-type hypersensitivity reaction, inhibited mitogen-activated T-cell proliferation, increased free intracellular Ca^{2+} production, and induced early cell death in rodent T cells (Goytia-Acevedo et al., 2003; Schulz et al., 2002). Splenic macrophages from arsenic-exposed mice showed a decrease in phagocytosis, a lower chemotactic index, decreased adhesion, and reduced nitric oxide production after lipopolysaccharide (LPS) stimulation (Sengupta and Bishayi, 2002; Bishayi and Sengupta, 2003). When arsenic-exposed mice were infected with *Staphylococcus aureus*, an increase in bacterial blood load and delayed bacterial clearance could be observed (Bishayi and Sengupta, 2003). Arsenic was found to inhibit Janus kinase 1, which relays signals of cytokine receptors, such as interferon-α/β (IFN-α/β), interferon-γ (IFN-γ), and interleukin 6 (IL-6) receptors (Cheng et al., 2004).

Fishes are good indicators of arsenic toxicity (Bosnir et al., 2003); however, little is known about the effects of arsenic on the immune system of fishes. A 96-hour LC_{50} value (28.1 mg/ml) of toxicity of arsenic has been determined for adult zebrafish (Tisler and Zagorc-Koncan, 2002), but effects on specific systems have not yet been characterized. The zebrafish is widely used as a model system for biomedical research, and is a good model system for studying the effects of arsenic on immune function, because of its rapid rate of reproduction, the optical clarity of its larvae, and the wealth of genetic information available from the recent sequencing of its genome. In addition, a wealth of information about the innate immune system of the zebrafish is available, and several components of zebrafish innate immunity have been characterized. Components of the Toll-like receptor (TLR) pathway, a family of

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pattern recognition receptors, have been cloned and characterized in zebrafish (Jault et al., 2004; Meijer et al., 2004; Peter Phelan, personal communication). TLRs recognize patterns specific to bacteria or viruses and transduce signals that stimulate upregulation of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-β (IL-1β). The measurement of cytokines allows monitoring of an immune response. Our laboratory recently characterized the antiviral cytokine IFN, together with its inducible gene *MX* (Altmann et al., 2003, 2004). We also have shown that IFN and MX mRNA expression is upregulated in the zebrafish upon infection with the fish pathogen snakehead rhabdovirus (SHRV) (Phelan et al., 2004).

The respiratory burst response constitutes an important method for the measurement of immune health of an organism. Phagocytes engulf invading pathogens and destroy them by producing reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. The amount of ROS produced is an indicator of the intensity of the innate immune response and the general health of the organism. We recently developed a respiratory burst assay for the zebrafish (Hermann et al., 2004). Respiratory burst assays have been used to quantify the effects of exposure to environmental toxins and metals, such as polychlorinated biphenyls (Voie et al., 1998), zinc (Yatsuyanagi and Ogiso, 1988, Ciapetti et al., 1998), and copper (Jacobson et al., 1999). Depending on the system and conditions, arsenic has been found to either decrease or increase the respiratory burst of macrophages. Arsenic decreased the respiratory burst of rat and rabbit alveolar macrophages, but increased ROS production in bovine alveolar macrophages (Lantz et al, 1995; Gulyas et al, 1990; Berg et al., 1993).

This study investigated the effects of arsenic on the innate immune system of zebrafish. The effects of arsenic on respiratory burst activity and messenger RNA expression of the respiratory-burst-priming TNF-α gene were examined in zebrafish larvae. In addition, the expression of the antiviral Mx and IFN-α/β genes was measured before and after infection with a virus in arsenic-exposed larvae.

Materials and Methods

Zebrafish Maintenance. AB inbred strain zebrafish were bred and maintained at the University of Maine Zebrafish Facility in Orono, in an Aquatic Habitats flow-through system at pH 7.6 with a flow rate of 75 L/min. Zebrafish were handled according to Institutional Animal Care and Use Committee guidelines. Adult fish were fed with Hikari Tropical Micropellets (Kyorin Food Ind. Ltd.,) and bred to yield larvae. Larvae were collected, rinsed in egg water (60 μg/ml Instant Ocean, Aquarium Systems), transferred to petri dishes, and held at 28°C until 72 hours after postfertilization (hpf). Larvae were subsequently kept in static 1-L tanks containing water from the flow-through system and fed twice daily.

Respiratory Burst. At the one-cell stage (1 hpf), larvae were exposed to 50 or 100 μM arsenic prepared from sodium arsenite (Sigma) and grown while continuously exposed in static tanks. The respiratory burst of larvae from 4 days postfertilization (dpf) until 11 dpf was measured according to Hermann et al., (2004). Briefly, to each well of a 96-well microplate containing one zebrafish larvae in 100 μl of egg water, 100 μl of 1 μg/ml dihydrodichlorofluorescein diacetate (H_2DCFDA, Molecular Probes) in 0.4% dimethylsulfoxide (DMSO) and 400 ng/ml phorbol myristate acetate (PMA, Molecular Probes) (or DMSO) were added to a final concentration of 500 ng/ml H_2DCFDA, 0.2% DMSO, and 200 ng/ml PMA. Upon production of ROS, H_2DCFDA is oxidized to dichlorofluorescein and emits fluorescent light.

Fluorescence was measured in a Fusion microplate reader (Packard Bioscience Company) with a photomultiplier voltage of 500 mV, a gain of 1, a halogen lamp set at intensity level 7, and excitation and emission filters set at 485 ± 10 and 530 ± 10 nm, respectively. The fluorescence of each sample-containing well was measured immediately after addition of solutions to the wells, and every 2.5 minutes thereafter for 150 minutes. The data from 6 individual larvae per treatment group and time point were averaged and normalized to background fluorescence to permit calculation of fold induction. Error bars were calculated as the square root of the variance. Confidence limits where significant at the 5% level ($P < 0.05$). The data shown are representative of 3 independent experiments.

SHRV Infection and Arsenic Exposure. SHRV at a multiplicity of infection (MOI) of 0.001 was grown in epithelial carp (EPC) cells to yield a viral titer of 10^7 $TCID_{50}$/ml SHRV. At the one-cell stage (1 hpf), larvae were exposed to 50 or 100 μM arsenic prepared from sodium arsenite (or egg water). At 7 dpf the larvae were rinsed in system water and infected with 10^6 $TCID_{50}$/ml SHRV (or mock-infected) in a volume of 2 ml for 5 hours. After incubation with the virus, larvae were transferred to tanks containing 1 L of 50 or 100 μM arsenic or system water.

Table 1. Primers for Quantitative Real-Time PCR

Name	*Sequence (5′–3′)*	*Fragment length (bp)*
β-Actin	ATGGATGAGGAAATCGCTC ATGCCAACCATCACTACTCCCTG	130
IFN	GAATGGCTTGGCCGATACAGGATA TCCTCCACCTTTGACTTGTCCATC	136
Mx	ATAGGAGACCAAAGCCGGGAAAG ATTCTCCCATGCCACCTATCTGG	145
TNF	GCTGGATCTTCAAAGTCGGGTGTA TGTGAGTCTCAGCACACTTCCATC	138

RNA Extraction and Synthesis of cDNA. To obtain total RNA 10 larvae previously exposed to arsenic (or system water) at the one-cell stage and infected with SHRV (or mock-infected) at 7 dpf were collected for each treatment at 12, 24, 48, 72, and 96 hours postinfection (hpi). Fish were preserved in RNA (Epicenter) until further processing. Total RNA was extracted using the Masterpure RNA Purification Kit (Epicenter). Total RNA (400 ng) was included in a reverse-transcription reaction to produce complementary DNA using the ImProm-II™ Reverse Transcription System (Promega, I).

Quantitative Polymerase Chain Reaction. TNF-α, IFN-α/β, and Mx cDNAs were quantitated using SYBR green nucleic acid stain (Bio-Rad). Primers used for the amplification of β-actin (GenBank accession number NM_131031), TNF-α (AY427649), IFN (AY135716), and Mx (AF533769) are listed in Table 1. A standard curve was constructed by serially diluting linearized plasmids containing the TNF-α, IFN, and Mx open reading frames, respectively. Zebrafish β-actin primers (PE Applied Biosystems) were used to normalize for starting quantity of RNA. Reactions were performed in an iCycler iQ Real-Time PCR detection system (Bio-Rad) according to the manufacturer's instructions. Reactions were carried out in a volume of 20 μl containing 10 μl of 2 × iQ-mix (Bio-Rad), 1 μl of 5.0 μM primers, 8 μl nuclease-free water, and 1 μl cDNA. Cycling parameters were 94°C for 4.5 minutes to activate the polymerase followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Fluorescence measurements were taken at each cycle during the 55°C step. RNA levels were determined as copy number based on the standard curve, after which these values were normalized to the corresponding β-actin values. Fold inductions for cytokine levels without viral infection were subsequently calculated by dividing the normalized values of 50 and 100 μM exposed larvae by normalized values of unexposed controls. One was subtracted from all values to yield zero-fold induction for controls. Fold induction for cytokine levels of SHRV-infected larvae was calculated by dividing the normalized values of SHRV-infected larvae by the normalized values of corresponding mock-infected controls. Unit induction was set equal to zero for the control, and induction or suppression was displayed as positive or negative values, respectively. Error bars were calculated as the square root of the variance. Confidence limits were significant at the 5% level ($P < 0.05$).

Results

Respiratory Burst in Arsenic-Exposed Larvae. The development of respiratory burst activity in response to PMA stimulation was measured from 4 to 11 dpf (Figure 1). Groups of larvae were treated as follows: control larvae with DMSO (Control A), control larvae with PMA (Induced A), 50 μM arsenic-exposed larvae with DMSO (Control B), 50 μM arsenic-exposed larvae with PMA (Induced B), 100 μM arsenic-exposed larvae with DMSO (Control C), and 100 μM arsenic-exposed larvae with PMA (Induced C). Induction of ROS production in PMA-induced control larvae (Induced A) compared with DMSO-treated controls (Control A), was 3.3-fold at 4 dpf. Induction gradually increased, reaching a maximum at 6 dpf of 7.3-fold induction of respiratory burst activity in PMA-treated controls (Induced A). A gradual decrease in respiratory burst activity was then observed until 8 dpf, when ROS induction was reduced to 4.8-fold. Respiratory burst activity increased again at 9 dpf to a 5.9-fold induction, followed by a decrease of respiratory burst activity at 10 dpf to a 4.1-fold induction. A slight increase of ROS production could again be observed at 11 dpf, when control larvae (Control A) displayed a 4.7-fold induction. Induction of ROS production between 7 and 11 dpf varied between experiments, but followed a general trend, showing a gradual decrease.

When larvae were exposed to 50 μM arsenic (Induced B), a 2.6-fold induction of ROS production was observed at 4 dpf. Respiratory burst activity in-

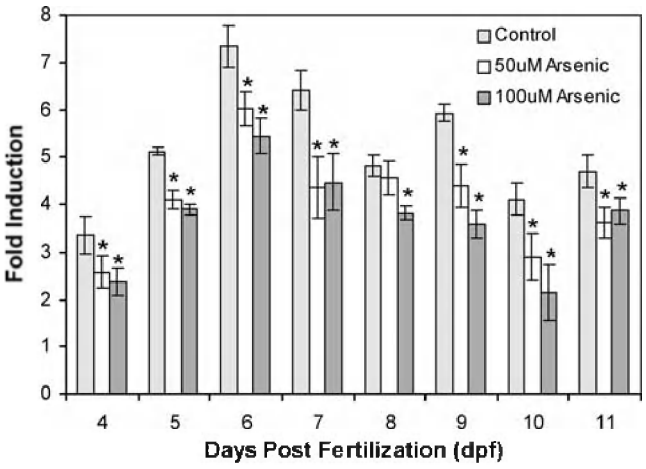

Fig. 1. Respiratory burst activity in arsenic-exposed larvae, measured by oxidation of H_2DCFDA to DCF. Embryos were exposed to 50 or 100 μM arsenic from the one-cell stage. At the indicated time point, larvae were treated with 0.2% DMSO or 400 ng/mL PMA in egg water, in the presence of 1 μg/ml H_2DCFDA. Fluorescence intensity was measured every 2.5 minutes for 150 minutes, using excitation and emission filters of 480 ± 10 and 530 ± 10 nm, respectively. Treatment groups: Control is Induced A; 50 μM arsenic, Induced B; 100 μM arsenic, Induced C. Each bar represents the average of measurements from 6 individual larvae. Error bars represent the square root of the variance. (*Significant differences compared with controls.) Data are representative of 3 independent experiments.

creased gradually until reaching a maximum of 6.0-fold induced ROS production at 6 dpf. ROS production decreased to 4.4-fold at 7 dpf and did not change markedly at 8 and 9 dpf. At 10 dpf a further decrease in respiratory burst activity to a 2.9-fold induction in ROS production was observed. At 11 dpf respiratory burst activity increased again to a 3.6-fold induction. Overall, respiratory burst activity was reduced in 50 μM arsenic-exposed larvae (Induced B) compared with controls (Induced A). This decrease was statistically significant ($P < 0.05$) at all time points except 8 dpf.

In larvae exposed to 100 μM arsenic (Induced C), a 2.4-fold induction was measured at 4 dpf. ROS production increased gradually until reaching a maximum of 5.4-fold induction, compared with DMSO-treated controls (Control C) at 6 dpf. Subsequently, respiratory burst activity decreased until 10 dpf, when a 2.2-fold induction in ROS production was observed. At 11 dpf ROS production increased again to 3.9-fold induction. Overall, exposure of larvae to 100 μM arsenic (Induced C) decreased ROS production compared with controls (Induced A). This reduction in respiratory burst activity was significant at all time points ($P < 0.05$).

These results indicate that the overall immune health of zebrafish larvae, as measured by respiratory burst, was affected by exposure to either 50 or 100 μM arsenic. The data show that arsenic exposed larvae displayed lower ROS production between 4 and 11 dpf, indicating that by arsenic exposure altered the normal development of the larvae's immune system. Variations in fold induction could be observed between different experiments, but overall trends were conserved.

TNF-α Expression after Arsenic Exposure. TNF-α plays an important role in priming the respiratory burst response. Inhibition of TNF-α by a TNF-α-neutralizing antibody led to decreased ROS production in human neutrophils (Niwa et al., 1996). To support our data showing reduction of ROS production after arsenic exposure, we examined the effects of arsenic on TNF-α mRNA expression.

Expression of TNF-α mRNA was measured from 7 to 11 dpf (Figure 2), using quantitative real-time polymerase chain reaction (PCR). TNF-α expression in larvae exposed to 50 μM arsenic (group D) or 100 μM arsenic (group E) was normalized to TNF-α mRNA expression of unexposed controls (group F) as described in "Materials and Methods". At 7 dpf, 50 μM arsenic-exposed larvae (group D) showed 0.68-fold suppression (reduced to 32% of controls) in TNF-α expression compared with control larvae (group F). At 8 dpf, TNF-α expression was suppressed 0.54-fold (reduced to 46% of controls), followed by 0.77-fold suppression (reduced to 23% of controls) at 9 dpf, 0.41-fold suppression at 10 dpf (reduced to 59% of controls) and 0.68-fold suppression at 11 dpf (reduced to 32% of controls). Differences in TNF-α expression were statistically significant ($P < 0.05$) at all time points compared with controls.

Exposure to 100 μM arsenic (group E) resulted in a 0.17-fold reduction (reduced to 83% of controls) of TNF-α expression at 7 dpf. At 8 dpf, TNF-α expression did not change compared to controls (group F) (87 copies in controls). At 9 dpf, however, 100 μM arsenic exposure (group E) resulted in 0.93-fold suppression of TNF-α expression (reduced to 7% of controls). This suppression of TNF-α expression continued through 10 and 11 dpf, with 0.54-fold and 0.51-fold suppression (reduced to 46% and 48% of controls), respectively. Fold reductions were significant for all time points ($P < 0.05$), except at 7 and 8 dpf, TNF-α expression did not differ significantly from controls. Variations in fold induction of TNF-α could be observed between different experiments, but overall trends were conserved.

Expression of IFN and Mx in Arsenic-Exposed Fish Before Infection. The effect of arsenic exposure on induction of an immune response to a viral

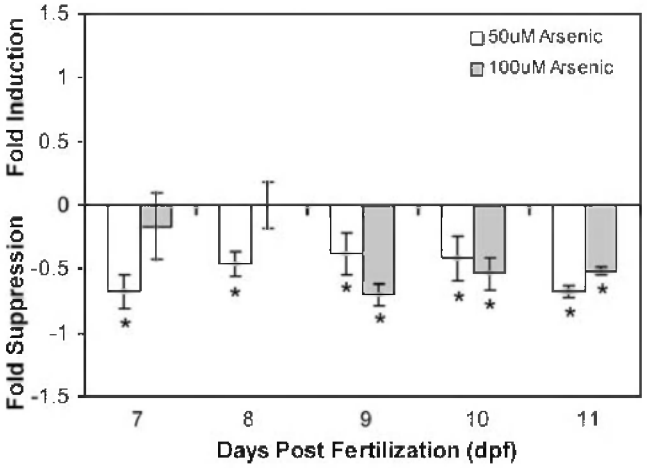

Fig. 2. TNF-α expression of arsenic-exposed larvae measured by quantitative real-time PCR. Larvae were exposed to 50 or 100 μM arsenic from the one-cell stage. Treatment groups: Control is group F; 50 μM arsenic, group D; 100 μM arsenic, group E. Total RNA was harvested at designated time points and reverse transcribed into cDNA. The resultant cDNA was used in a quantitative real-time PCR reaction using primers specific for zebrafish TNF-α. Expression values were normalized to zebrafish β-actin. Fold induction was calculated by dividing copy numbers determined for arsenic-exposed larvae by those for unexposed controls. Unit induction was set equal to zero and induction or suppression was displayed as positive or negative values, respectively. TNF-α expression at each time point was assayed in triplicate. Error bars indicate the square root of the variance. (*Significant differences compared with controls.) These results are representative of 3 independent experiments.

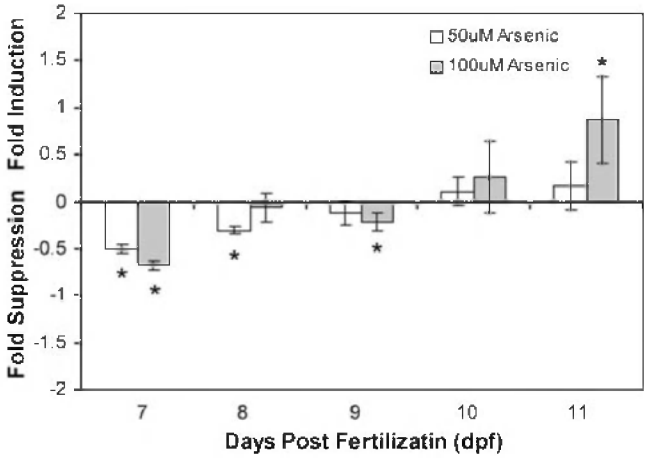

Fig. 3. IFN expression of arsenic-exposed larvae, measured by quantitative real-time PCR. Larvae were exposed to 50 or 100 μM arsenic from the one-cell stage. Treatment groups: Control is group F; 50 μM arsenic, group D; 100 μM arsenic, group E. Total RNA was harvested at designated time points and reverse transcribed into cDNA. The resultant cDNA was used in a quantitative real-time PCR reaction using primers specific for zebrafish IFN. Expression values were normalized to zebrafish β-actin. Fold induction was calculated by dividing copy numbers determined for arsenic-exposed larvae by those for unexposed controls. Unit induction was set equal to zero and induction or suppression was displayed as positive or negative values, respectively. IFN expression at each time point was assayed in triplicate. Error bars indicate the square root of the variance. (*Significant differences compared with controls.) These results are representative of 3 independent experiments.

pathogen was examined to further assess the effects of arsenic on zebrafish immunocompetence. The levels of uninduced expression of the antiviral cytokines IFN and Mx after exposure with arsenic were established first.

IFN expression was measured from 7 to 11 dpf (Figure 3). Larvae exposed to 50 μM arsenic (group D) showed 0.50-fold reduction of IFN expression at 7 dpf (reduced to 50% of controls). At 8 dpf, IFN expression was reduced 0.31-fold (reduced to 69% of controls). IFN expression in 50 μM arsenic-exposed larvae (group D) was further reduced to 0.12-fold 9 dpf (reduced to 88% of controls). At 10 dpf, a 0.11-fold induction of IFN expression (increased to 111% of controls), followed by 0.16-fold induction at 11 dpf (increased to 116% of controls) was observed in 50 μM arsenic exposed larvae (group D). IFN induction and reduction were significant at 7 and 8 dpf ($P < 0.05$), but did not differ significantly from controls (group F) at 9, 10, and 11 dpf (565, 678, and 157 IFN copies in controls). Over the observed time frame, 50 μM arsenic-exposed larvae (group D) first showed a decrease at 7 and 8 dpf, then returned to expression levels at controls in the later time points measured.

Larvae exposed to 100 μM arsenic (group E) showed 0.68-fold reduction in IFN expression at 7 dpf (reduced to 32%). At 8 dpf, an IFN reduction of 0.06-fold was observed (reduced to 94% of controls). At 9 dpf, larvae showed a 0.21-fold reduction (reduced to 79% of controls). At 10 dpf, the reduction was followed by a 0.26-fold induction of IFN in 100 μM arsenic-exposed larvae (group E) (increase to 126% of controls), which continued at 11 dpf, when IFN expression was 0.87-fold induced compared with controls (group F) (increase to 0.87% of controls). Induction and suppression of IFN mRNA expression were significantly different from controls at all time points ($P < 0.05$) except 8 and dpf. Variations in fold induction of IFN could be observed between different experiments, but overall trends were conserved.

Mx Expression Before SHRV Infection. Mx expression was examined from 7 to 11 dpf (Figure 4). In larvae exposed to 50 μM arsenic (group D), Mx was reduced 0.35-fold at 7 dpf (reduced to 65% of con-

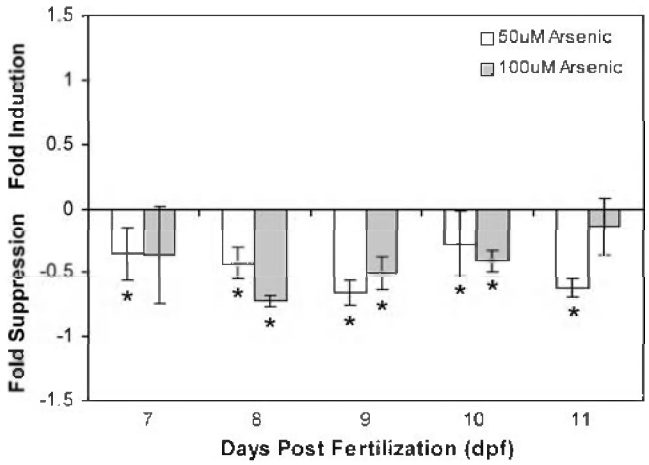

Fig. 4. Mx expression of arsenic-exposed larvae, measured by quantitative real-time PCR. Larvae were exposed to 50 or 100 μM arsenic from the one-cell stage. Treatment groups: Control is group F; 50 μM arsenic, group D; 100 μM arsenic, group E. Total RNA was harvested at designated time points and reverse transcribed into cDNA. The resultant cDNA was used in a quantitative real-time PCR reaction using primers specific for zebrafish Mx. Expression values were normalized to zebrafish β-actin. Fold induction was calculated by dividing copy numbers determined for arsenic-exposed larvae by those for unexposed controls. Unit induction was set equal to zero and induction or suppression was displayed as positive or negative values, respectively. Mx expression at each time point was assayed in triplicate. Error bars indicate the square root of the variance. (*Significant differences compared with controls.) These results are representative of 3 independent experiments.

trols). At 8 dpf, Mx expression was reduced 0.43-fold (57% of controls), and at 9 dpf, it was reduced 0.66-fold (reduced to 34% of controls) in 50 μM arsenic-exposed larvae (group D). At 10 dpf, larvae showed a 0.27-fold reduction in Mx expression (reduced to 63% of controls). At 11 dpf, Mx expression was reduced 0.63-fold in 50 μM arsenic-exposed larvae (group D) (reduced to 37%). During the observed time period, larvae exposed to 50 μM arsenic (group D) showed a reduction in Mx expression compared with controls (group F). Mx reduction was significant at all time points ($P < 0.05$), except 10 dpf, when Mx expression did not differ significantly from control values.

Larvae exposed to 100 μM arsenic (group E) showed a 0.36-fold reduction in Mx expression at 7 dpf (reduced to 64 % of controls). At 8 dpf, a 0.72-fold reduction of Mx expression was observed (reduced to 28% of controls). Mx expression was also decreased at 9 dpf with a 0.51-fold reduction (reduced to 49% of controls) and at 10 dpf with a 0.41-fold reduction (reduced to 59% of controls). At 11 dpf, Mx expression was reduced 0.14-fold in 100μM arsenic-exposed larvae (group E) (reduced to 86% of controls). Over the time period examined, 100 μM arsenic-exposed larvae (group E) showed a reduction of Mx expression compared to controls (group F). Mx suppression was significant at all time points ($P < 0.05$) except 7 and 11 dpf, when Mx expression did not differ significantly from the controls. Variations in suppression of Mx could be observed between different experiments, but overall trends were conserved.

Expression of IFN and Mx in Arsenic-Exposed Larvae After Infection with SHRV. After establishing Mx and IFN mRNA expression levels in arsenic-exposed larvae prior to viral infection, the effect of arsenic exposure on the induction of these antiviral cytokines in response to a viral infection was examined. Fold induction of Mx and IFN expression in SHRV-infected control and arsenic-exposed larvae, compared with mock-infected larvae, was calculated as described in the "Materials and Methods". Larvae were infected with SHRV at 7 dpf. Time points for IFN and Mx expression of 12, 24, 48, 72, and 96 hpi corresponded with larvae, aged 7.5, 8, 9, 10, and 11 dpf, respectively.

IFN mRNA expression was measured from 12 to 96 hpi after SHRV infection (Figure 5). Groups of larvae were treated as follows: Control (group F), control infected with SHRV (group G), 50 μM arsenic-exposed (group D), 50 μM arsenic-exposed, infected with SHRV (group H), 100 μM arsenic-exposed (group E), 100 μM arsenic-exposed, infected with SHRV (group I). Control larvae infected with SHRV (group G) showed a 0.11-fold reduction in IFN expression at 12 hpi. Expression levels increased to 1.3-fold induction at 24 hpi, and 1.4-fold induction at 48 hpi. At 72 hpi, a peak in IFN expression was observed, when infected control larvae (group G) showed an 8.2-fold induction. At 96 hpi, IFN expression was decreased to 1.5-fold induction in infected controls (group G).

Larvae infected with SHRV and previously exposed to 50 μM arsenic (group H) showed 1.3-fold induction of IFN expression at 12 hpi and 0.81-fold induction at 24 hpi. At 48 hpi, IFN expression reached a maximum of 5.9-fold induction. At 72 hpi, IFN expression was reduced to the levels of controls (group D). At 96 hpi, IFN expression returned to baseline levels (group D) of 0.49-fold induction. IFN expression was significant at all time points ($P < 0.05$) except 96 hpi. Over the time frame examined, 50 μM arsenic-exposed larvae, when infected with SHRV (group H), showed an increase in IFN expression at 12 and 48 hpi, compared with infected controls (group G). At 24, 72, and 96 hpi, IFN expression was reduced compared to SHRV-infected controls (group G).

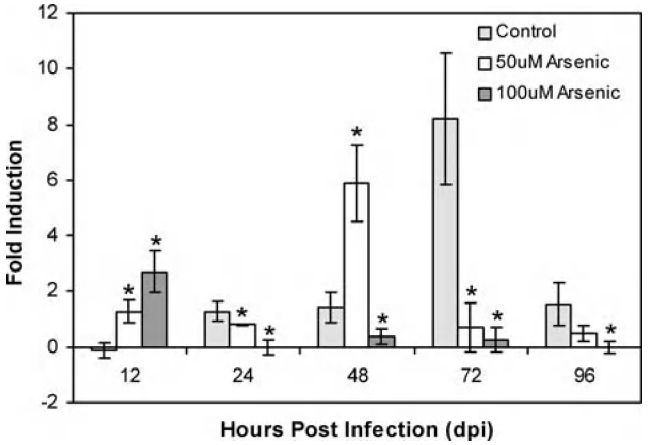

Fig. 5. IFN expression in arsenic-exposed larvae after exposure to SHRV, measured by quantitative real-time PCR. Larvae were exposed to 50 or 100 μM arsenic from the one-cell stage, and exposed to 10^6 $TCID_{50}$/ml SHRV. Treatment groups: Control (infected with SHRV) is group G; 50 μM arsenic (infected with SHRV), is group H; 100 μM arsenic (infected with SHRV), is group I. Total RNA was harvested at designated time points postinfection and reverse transcribed into cDNA. The resultant cDNA was used in a quantitative real-time PCR reaction using primers specific for zebrafish IFN. Expression values were normalized to Zebrafish β-actin. Fold induction was calculated by dividing copy numbers determined for arsenic-exposed larvae by those for unexposed controls. Unit induction was set equal to zero and induction or suppression was displayed as positive or negative values, respectively. IFN expression at each time point was assayed in triplicate. Error bars indicate the square root of the variance. (*Significant differences compared with SHRV-infected controls.) These results are representative of 3 independent experiments.

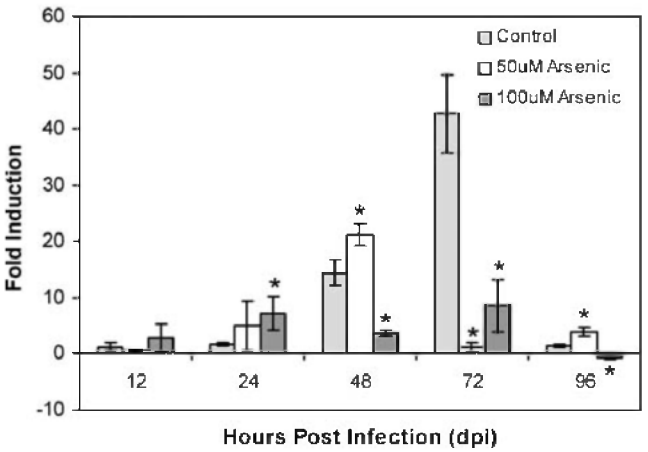

Fig. 6. Mx expression in arsenic-exposed larvae after exposure to SHRV, measured by quantitative real-time PCR. Larvae were exposed to 50 or 100 μM arsenic from the one-cell stage, and exposed to 10^6 $TCID_{50}$/ml SHRV. Treatment groups: Control (infected with SHRV) is group G; 50 μM arsenic (infected with SHRV), group H; 100 μM arsenic (infected with SHRV), group I. Total RNA was harvested at the designated time points postinfection and reverse transcribed into cDNA. The resultant cDNA was used in a quantitative real-time PCR reaction using primers specific for zebrafish Mx. Expression values were normalized to zebrafish β-actin. Fold induction was calculated by dividing copy numbers determined for arsenic-exposed larvae by those for unexposed controls. Unit induction was set equal to zero and induction or suppression was displayed as positive and negative values, respectively. Mx expression at each time point was assayed in triplicate. Error bars indicate the square root of the variance. (*Significant differences compared with SHRV-infected controls.) These results are representative of 3 independent experiments.

Larvae infected with SHRV and exposed to 100 μM arsenic (group I) showed a 2.7-fold induction of IFN expression at 12 hpi. At 24 hpi, IFN expression was abrogated and did not differ from mock-infected, 100 μM arsenic exposed larvae (group E). At 48 hpi, IFN expression was 0.38-fold induced, followed by a 0.26-fold induction at 72 hpi. At 96 hpi, IFN expression did not differ from control larvae (group E). IFN expression was significant at 12 and 48 hpi, but did not differ from mock-infected, 100 μM arsenic-exposed larvae (group E) at 24, 72, and 96 hpi. Overall, SHRV-infected, 100 μM arsenic-exposed larvae (group I), compared with infected controls (group G), showed an initial increase in IFN expression at 12 hpi, followed by a decrease in IFN expression at the remaining time points. Differences in IFN induction between SHRV-infected treatment groups were significant ($P < 0.05$). Variations in fold induction of IFN could be observed between different experiments, but overall trends were conserved.

Mx Expression After SHRV Infection. Expression of Mx mRNA was examined from 12 to 96 hpi after SHRV exposure (Figure 6). Mx expression in control larvae infected with SHRV (group G) was 1.3-fold induced at 12 hpi. At 24 hpi, a 1.8-fold induction of Mx expression was observed. At 48 hpi, Mx expression increased to 14.4-fold. This increase in Mx induction reached a peak at 72 hpi, when a 42.7-fold induction of Mx could be observed in SHRV-infected controls (group G). At 96 hpi, Mx mRNA expression in infected controls (group G) returned to baseline levels (group F) of 1.5-fold induction. Mx expression was significant for all time points.

When infected with SHRV, larvae exposed to 50 μM arsenic (group H) showed a 0.66-fold induction at 12 hpi. At 24 hpi, Mx expression increased to 5.1-fold. This increase continued at 48 hpi, when SHRV infection resulted in a peak of 21.2-fold induction of Mx expression. At 72 hpi, however, Mx expression had not increased further, and only a 1.3-fold induction of Mx expression could be observed. At 96

hpi, Mx expression in infected, 50 μM arsenic-exposed larvae (group H) was again increased to 4.0-fold induction. Mx expression was significant ($P < 0.05$) for all time points. During the observed time frame, SHRV infection of 50 μM arsenic-exposed larvae (Group H) resulted in an initial decrease in MX expression at 12 hpi, followed by a marked increase at 24 hpi compared with controls (group G). At 48 hpi, a peak in Mx expression was reached. At 72 hpi, Mx expression was reduced, followed by an increase in Mx expression at 96 hpi compared with SHRV-infected control larvae (group G). Mx induction was significantly different ($P < 0.05$) from induction in SHRV-infected controls.

Larvae infected with SHRV, and exposed to 100 μM arsenic (group I), showed a 2.9-fold induction in Mx expression at 12 hpi. At 24 hpi, a 7.2-fold increase in Mx expression was observed, however, at 48 hpi, Mx expression decreased to 3.7-fold. SHRV-infected, 100 μM arsenic-exposed larvae (group I) displayed a second peak of 8.6-fold induction in Mx expression at 72 hpi. At 96 hpi, larvae showed a 0.79-fold reduction compared with mock-infected, 100 μM arsenic-exposed larvae (group E). This reduction, however, was not significant compared with Mx expression levels of mock-infected, 100 μM arsenic-exposed larvae (group E). Mx expression was significant at all other time points ($P < 0.05$). SHRV-infected larvae exposed to 100 μM arsenic (group I) showed an initial induction of Mx expression at 12 and 24 hpi. At 48 through 96 hpi, Mx expression was decreased, compared with controls (group G). Variations in induction of IFN could be observed between different experiments, but overall trends were conserved.

Discussion

This report describes for the first time the effects of arsenic exposure on the innate immune response of zebrafish. It is shown that respiratory burst activity, cytokine expression, and induction of cytokines after viral infection are affected in arsenic-exposed larvae. The 96-hour LC_{50} value for adult zebrafish is 28.1 mg/ml (375 μM) (Tisler and Zagorc-Koncan, 2002). Zebrafish larvae were exposed to 50 and 100 μM arsenic, concentrations that did not cause mortality or result in visible toxic effects. These concentrations are within the range of arsenic concentrations used in both fish (Kothary and Candido, 1982; Hermesz et al., 2002; Wang et al., 2004) and mammals (Cavigelli et al., 1996; Y.C. Chen et al., 1998).

Both 50 and 100 μM arsenic lowered respiratory burst activity of zebrafish larvae. Respiratory burst activity of control larvae increased from 4 to 6 dpf, when it reached a maximum. Respiratory burst activity between 7 and 11 dpf gradually decreased until 11 dpf. In this time frame, variations in control induction were observed from experiment to experiment, but the overall trend of a decrease in ROS production in arsenic-exposed larvae was conserved. As described by Hermann et al. (2004), ROS production is only measured in phagocytes located at the surface of the embryo. Increased pigmentation in older larvae blocks the fluorescence of the indicator of ROS production.

The respiratory burst response, a measure of the immune health of an organism, is often used to assess the immunotoxic effects of heavy metals and environmental toxicants. Both stimulatory and inhibitory effects of these substances on respiratory burst activity have been described. The pesticide malathion, when administered to Japanese medaka, decreased respiratory burst activity (Beaman et al., 1999); however, malathion-exposed mice showed an increase in ROS production (Rodgers and Xiong, 1997). Cadmium, mercury, and zinc inhibited the ability of human monocytes to undergo a respiratory burst after LPS activation (Koropatnick and Zalups, 1997). However, cadmium was found to enhance respiratory burst activity in head-kidney macrophages of sea bass (Lemaire-Gony and Mayer-Gostan, 1994). Copper inhibited respiratory burst activity in rainbow trout leukocytes (Elsasser et al., 1986), but enhanced respiratory burst in goldfish (Jacobson et al., 1999).

Arsenic exerts seemingly contradictory effects on ROS production in connection with the respiratory burst response as well. Rat alveolar macrophages exposed to arsenic displayed a decrease in superoxide production and LPS-induced TNF-α expression (Lantz et al., 1995). Arsenic-containing waste incinerator dust also decreased superoxide and hydrogen peroxide production in rabbit alveolar macrophages, but increased production of ROS in bovine alveolar macrophages (Gulyas et al., 1990; Berg et al., 1993). Our results show that arsenic reduces ROS production in whole zebrafish larvae.

Cytokines are important mediators of immune function. Arsenic can modulate the expression of cytokines by affecting components of upstream signal transduction pathways in a dose-dependent manner. Kinases activated or inhibited by arsenic, such as protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and IκB kinase (IκK), modulate activation of the transcription factor NFκB, which in turn controls cytokine expression (Cavigelli et al., 1996; N.Y. Chen et al., 2000; Roussel and Barchowsky, 2000); (Wijeweera et al.,

2001). In acute promyelocytic leukemia cells, arsenic brought about an increase in IL-1β and G-CSF, but caused a decrease in IL-6 and IL-8 (Jiang et al., 2003). Arsenic resulted in overexpression of TNF-α, IL-8, IL-1α, GM-CSF, and TGF-α in human and murine keratinocytes (Yen et al., 1996; Germolec et al., 1996; Corsini et al., 1999). Expression of IL-1α, IL-1β, IL-8, and TNF-α, however, was down-regulated in arsenic-transformed human osteoblast-like cells (Yang and Frenkel, 2002).

The proinflammatory cytokine TNF-α primes phagocytic cells for potentiated PKC-dependent processes, such as the respiratory burst, by inducing the synthesis of myristylated alanine-rich C kinase substrate (MARCKS). MARCKS, a substrate for PKC, is phosphorylated when cells are stimulated with PMA (Thelen et al., 1990). Other studies have shown that TNF-α increases the expression of PKC (Phillips et al., 1992) and the cytosolic components of the respiratory-burst-producing NADPH oxidase, p47 and p67 (Green et al., 1994). An increase in expression of the membrane-bound complex of NADPH oxidase, flavocytochrome b558 (Ward et al., 2000), is mediated by TNF through exocytosis of intracellular granules in a process regulated by p38 MAPK. TNF-α, via TNF receptor 1 (TNFR), primes a respiratory burst by inducing protein tyrosine-kinase–dependent phosphorylation of p47 (Dewas et al., 2003). A lack of TNF-α, as demonstrated by antibody inhibition, resulted in decreased PMA-stimulated superoxide production (Niwa et al., 1996). TNF-α expression of arsenic-exposed larvae was examined here to elucidate a possible mechanism for arsenic-mediated reduction of respiratory burst activity. The current study demonstrates that both TNF-α and respiratory burst activity are reduced in arsenic-exposed larvae. Exact values of TNF-α suppression varied between experiments, but the overall trend of reduced TNF-α expression was conserved. The results of this study support the idea that arsenic, by decreasing TNF-α levels in the zebrafish, inhibits priming of the respiratory burst, resulting in a decrease in ROS production upon PMA stimulation of arsenic-exposed larvae.

Upon infection with a virus, infected cells produce IFN, which binds to IFN receptors on neighboring cells. This leads to expression of IFN-inducible genes, like *Mx*, which inhibit protein and mRNA synthesis, preventing propagation of the virus. The initial IFNα/β expression after viral infection is mediated through the TLR-signaling pathway (Matsumoto et al., 2002). TLR3 recognition of double-stranded viral RNA results in binding of TLR-receptor interacting factor (TRIF), which ultimately leads to activation of IFN response factor 3 and to IFN mRNA expression (Yamamoto et al., 2002). A second pathway involves binding of tumor necrosis factor receptor associated factor 6 (TRAF6) to TLR-3-bound TRIP and subsequent activation of NFκB and mRNA expression of IFN (Sato et al., 2003).

Baseline IFN mRNA expression in arsenic-exposed zebrafish larvae showed a gradual increase over the observed time period. IFN expression in arsenic-exposed larvae was first less than and then greater than that of the controls. Exact values for IFN expression differed between experiments, but the overall trend of IFN increase over time was conserved. Arsenic may increase IFN production over a longer incubation period by inducing the activation of MAPKs and subsequently NFκB within the IFN-inducing TLR pathway.

Mx expression was decreased at all times in arsenic-exposed larvae and, unlike IFN, did not increase in expression at later time points. Arsenic may, therefore, be interfering with the induction of Mx by IFN. Again, values of Mx reduction differed between experiments, but the overall trend of Mx reduction was conserved. The IFN receptor mediates signals through phosphorylations of Janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins. Arsenic has been found to inhibit this pathway by interfering with JAK1 phosphorylation (Cheng et al., 2004), leading to an inhibition of IFN-inducible genes such as the Mx and IFN, genes. The reduction of Mx seen in arsenic-exposed larvae could be due to inhibition of the JAK/STAT pathway.

Phelan et al. (2004) have shown previously that SHRV infection of zebrafish larvae results in a maximum of IFN expression at 72 hpi, and this induction was also observed in the present study. When 50 μM arsenic-exposed larvae were infected with SHRV, IFN expression reached a maximum at 48 hpi. This maximum of IFN expression in 50 μM arsenic-exposed larvae at 48 hpi was not significantly reduced compared with the maximum at 72 hpi in controls, but no significant induction of IFN could be observed at 72 hpi in 50 μM arsenic-exposed larvae. Larvae exposed to 100 μM arsenic completely lacked IFN induction at either 48 or 72 hpi, but showed a maximum of IFN expression at 12 hpi. One possible explanation is that the shift of maximal IFN induction upon viral infection to earlier time points in both 50 and 100 μM arsenic-exposed larvae indicates a faster immune response to viral infection compared with controls. Arsenic may weaken immune defense mechanisms of zebrafish larvae, resulting in more rapid infection with SHRV and earlier induction of IFN. In order to confirm this theory, future experiments will measure viral gene

expression kinetics or viral yields. Arsenic-exposed larvae infected with SHRV showed similar cumulative percentage of mortality as SHRV infected controls (data not shown), indicating that arsenic does not kill the virus or prevent infection of the larvae. Other than when IFN was maximalls induced, expression of IFN in arsenic-exposed larvae was lower overall than in the controls. Arsenic may interfere with the JAK/STAT pathway and the TLR pathway and thus reduce the virus-mediated induction of IFN. Values of IFN induction differed between experiments, but overall trends were conserved.

Mx expression in 50 μM arsenic-exposed larvae after viral infection was increased at 24 hpi compared with controls and reached a maximum at 48 hpi, but failed to increase further at 72 hpi, when Mx expression was abrogated. The maximum extent of Mx expression at 48 hpi in 50 μM arsenic-exposed larvae corresponded with the maximum in IFN expression seen at the same time point in 50 μM arsenic-exposed embryos. Unlike IFN induction, induction of Mx was significantly decreased compared with controls. This indicates that arsenic interfered with the IFN-dependent Mx expression and may have affected the JAK/STAT pathway. Infection of 100 μM arsenic-exposed larvae with SHRV led to an increase in Mx induction at 12 and 24 hpi. An increase at 12 hpi was also observed in IFN expression. The IFN-inducible gene Mx was expected to be induced after induction of IFN. The maximum of IFN expression could subsequently induce Mx at both 12 and 24 hpi. Low amounts of IFN were present at 48 and 72 hpi in 100 μM arsenic-exposed larvae, which would account for Mx induction at these times. Further studies are needed to elucidate the specific mechanism of arsenic interference with Mx and IFN induction.

In conclusion, arsenic reduced respiratory burst activity and in general reduced levels of immune factors of the innate immune response in whole zebrafish larvae. The timing of reduction in TNF-α expression correlated with reduction in respiratory burst, lending support to the idea that arsenic reduces ROS production by inhibiting priming of the respiratory burst response with TNF-α. Mx and IFN mRNA expression were affected differently by arsenic. While arsenic inhibited Mx expression at all time points, it first suppressed, and then induced IFN expression increasingly over time. In virus-infected larvae, arsenic exhibited dose-dependent effects on Mx and IFN expression. IFN and Mx mRNA expression were reduced overall compared with controls at both arsenic concentrations. Differences between IFN and Mx induction point toward interference of arsenic with Mx induction by IFN. Our data suggest that arsenic reduces the innate immune response of zebrafish with potentially adverse effects on their resistance to viral infection. Arsenic exposure of zebrafish larvae results in developmental immunotoxic effects, which could also alter immune functions of adult zebrafish. Future work will address the exact mechanisms and signal transduction pathways involved in arsenic-inhibited immune response to infection. The observed immunotoxic effects of arsenic on the innate immune system of zebrafish most likely apply to other fish species living in arsenic contaminated waters. In addition to promoting cancer and other diseases, arsenic consumed via contaminated fish or well water might also affect the immune response of humans to pathogen infection. This study is the first step in examining the immunotoxic effects of arsenic on zebrafish. Future experiments will evaluate immunologic parameters after chronic arsenic exposure and look at a wide spectrum of arsenic concentrations.

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