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Decreased Immune Response in Zebra Finches Exposed to Sublethal Doses of Mercury

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Abstract Mercury (Hg) is a ubiquitous contaminant with deleterious effects on many wildlife species. Most studies to date have focused on fish-eating birds and mammals because much historical Hg pollution is aquatic. Recently, however, comparable blood-Hg levels have been found in terrestrial insectivorous songbirds. As a result, research is needed to clarify the effects of Hg exposure on songbirds. One fundamental end point that is still poorly understood is the effect of Hg on the songbird immune system. If Hg affects the functioning of the immune system, exposed songbirds may be less able to mount an appropriate immune response against invading pathogens. To gain insight into how Hg affects songbird immune function on a cellular level, a flow cytometric assay was developed to measure lipopolysaccharide-induced B-lymphocyte proliferation in zebra finches (*Taeniopygia guttata*). This is the first experimental (dosing) study of the potential effect of Hg on songbird immune system functioning. Decreased B cell proliferation was observed after lipopolysaccharide exposure in individuals with greater concentrations of Hg in their blood and tissues. In addition, these individuals had decreased ratios of proliferating-to-resting B cells. This decrease in lymphocyte proliferation in response to an effective mitogen suggests that environmental exposure to sublethal levels of Hg may inhibit or delay B cell proliferation in songbirds, potentially increasing susceptibility to disease and decreasing survivorship.

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Mercury (Hg) is a global contaminant that is toxic to wildlife and humans (Boening 2000; Holmes et al. 2009). Hg pollution usually enters waterways in an inorganic form and then can be bioconverted into methylmercury (MeHg), which has a greater bioavailability to organisms such as fish and birds (Boening 2000). In fish-eating birds, high accumulation of MeHg through biomagnification has been associated with endocrine disruption and decreased reproduction (Frederick and Jayasena 2011; Scheulhammer et al. 2007). In addition, recent studies have showed that terrestrial insectivorous songbirds (Passeriformes) can accumulate Hg concentrations in blood comparable with those found in fish-eating and aquatic species (Cristol et al. 2008; Rimmer et al. 2010). The apparent consequences of environmental exposure to MeHg on insectivorous songbirds include altered singing behavior (Gorissen et al. 2005; Hallinger et al. 2010), suppressed adrenocortical response (Wada et al. 2009), decreased reproductive success (Brasso and Cristol 2008; Hallinger and Cristol 2011), and altered immune function (Hawley et al. 2009). Although these field studies offer correlational evidence of the effects of Hg on avian fitness and condition, there is little or no evidence from experimental studies of songbirds pointing to Hg exposure as a cause of immune suppression.

Studies of the immunomodulatory effects of MeHg on songbirds have been particularly limited, in part due to a lack of songbird-specific reagents. However, there is evidence in songbirds that high Hg exposure is associated with immune suppression using the phytohemmaglutinin (PHA) skin test, and the same pattern, albeit not statistically significant, was observed for decreased antibody production

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using the sheep red blood cell (SRBC) assay (Hawley et al. 2009). The PHA skin test is generally used to measure T-lymphocyte function by injecting PHA, a T-lymphocyte mitogen (i.e., a substance used to trigger cell division), intradermally into the bird's wing and measuring subsequent swelling. However, PHA also stimulates a broad range of immune cells and is therefore not a simple measure of cell-mediated immunity alone (Martin et al. 2006; Vinkler et al. 2010). The SRBC assay measures antibody titer in response to injection of novel antigen as a proxy for humoral or B-lymphocyte immunity. A disadvantage of both techniques is that they do not establish what is occurring on a cellular level and do not allow ready extrapolation to disease susceptibility (Kennedy and Nager 2006).

B and T lymphocytes, the main players of acquired immunity, have essential roles in immune responses in all vertebrates, including birds (Warr 1981; Zwollo 2011). On antigen exposure, B lymphocytes differentiate into antibody-producing plasma cells, whereas activated T lymphocytes drive cell-mediated immune responses. Both B and T lymphocytes initially respond to pathogen-derived antigens through cell proliferation as a means of expanding the population of antigen-specific responder cells, which are necessary for efficient removal of the pathogen. When lymphocyte proliferation is inhibited, such as may be the case during exposure to environmental toxins, this typically correlates with decreased immune responses and lowered resistance to infection (Carlson et al. 2002; Christin et al. 2003). As such, lymphocyte proliferation is considered a key indicator of immune strength (Grasman 2002).

One commonly used metric of immunocompetence is the ability of cultured B lymphocytes to proliferate in response to mitogens, such as lipopolysaccharide (LPS) (Courrèges et al. 2003; Day et al. 2007), a large, highly immunogenic molecule present on the surface of gram-negative bacteria. Similarly, T cell proliferation can be measured by adding T cell mitogens in culture (Courrèges et al. 2003; Finkelstein et al. 2007; Das et al. 2008). Several vertebrate studies have measured the effects of MeHg on lymphocyte proliferation using such techniques. Increased levels of MeHg chloride (0–40 μM) added in vitro to cell cultures of peripheral blood leukocytes (PBLs) from harbor seals (*Phoca vitulina*) resulted in decreased mitogen-induced T-lymphocyte proliferation (Das et al. 2008). Similarly, B-lymphocyte proliferation was decreased in loggerhead sea turtles (*Caretta caretta*) in response to both environmental Hg exposure and Hg added to PBLs in culture (Day et al. 2007).

Although these results suggest that Hg might suppress B-lymphocyte proliferation in other taxa, studies in birds have thus far been limited. In songbirds, the paucity of studies is due mainly to a lack of available antibody tools to identify proliferating B lymphocytes. The objectives of this study were to develop an assay of mitogen-induced B cell proliferation for zebra finches (*T. guttata)* and to measure effects of sublethal MeHg exposure on B cell proliferation in this model system.

Materials and Methods

We developed a method for measuring mitogen-induced B cell proliferation in zebra finches based on a technique that is well established in other organisms, including humans, mice, and teleost fish (Barr et al. 2011; Chehrehasa et al. 2009; Yu et al. 2009). We harvested cells from fresh spleens of birds exposed to control or MeHg diets in captivity and then cultured these cells and stimulated proliferation with the B cell mitogen, LPS. We then added the nucleoside analog, 5-ethynyl-2′-deoxyuridine (EdU), which incorporates into the DNA of replicating cells, to distinguish proliferating from resting B cells (Salic and Mitchison 2008). Proliferation as measured by EdU incorporation positively correlates with both [^{3}H]thymidine incorporation (Yu et al. 2009) and 5-bromo-2-deoxyuridine (BrdU) labeling (Capella et al. 2008). However, we used EdU as an alternative because it allows for additional antibody labeling, does not require handling the radioactivity of [^{3}H]thymidine assays (Yu et al. 2009), and does not involve the harsh denaturation steps of BrdU labeling (Salic and Mitchison 2008).

After permeabilization and paraformaldehyde fixation, we used an antibody against the B cell specific transcription factor, Paired box protein 5 (Pax5), to distinguish B cells from other immune cells. Flow cytometry allowed us to quantify populations of proliferating and nonproliferating B cells in exposed and control populations.

Experimental Dosing

Zebra finches were housed in an indoor aviary with one breeding pair per cage ($n = 38$ pairs). We established pairs randomly from a large (>300 individuals) outbred colony. All zebra finches lived in cages (45 × 45 × 75 cm) with ad libitum drinking water, a nest box with ample nesting material, and adequate sources of calcium, grit and vitamins. The temperature was 20–23 °C, and photoperiod was 14:10 h light to dark.

Pairs were randomly assigned to the following treatment groups, which varied in how much MeHg they received in their diet and what form of Hg was added: (1) the control group (group 1; $n = 10$ pairs) received ad libitum reconstituted seed diet (ZuPreem Fruitblend) with 0 μg/g Hg; (2) the low Hg group (group 2) received the same food with 0.5 μg/g Hg (fresh weight) mixed into their seed ($n = 14$ pairs); and (3) the high Hg group (group 3) received

1.0 μg/g Hg mixed into their seed ($n = 14$ pairs). Within each of the latter groups (groups 2 and 3), Hg was added in one of two forms: MeHg cysteine ($n = 7$ of the 14 pairs in group 2 and $n = 7$ of the 14 pairs in group 3) or MeHg chloride ($n = 7$ of the 14 pairs in group 2 and $n = 7$ of the 14 pairs in group 3). All Hg was added in an aqueous solution representing 10 % of the weight of the food. MeHg cysteine was made with commercial MeHg chloride diluted into a 2-fold molar excess of cysteine in a degassed aqueous solution. Control diet was augmented with 10 % water and cysteine alone. All diets were mixed in a rock tumbler to homogenize, and each batch was tested to ensure it was within 10 % of the expected total Hg concentration. The diet was tested and consumed on a wet-weight basis. In fact, the finch diet as provided contained 13.9 % moisture and thus doses were equivalent to 0.58 and 1.16 ppm Hg on a dry-weight basis.

This rendered five treatment groups with seven pairs of birds in each of the MeHg-exposure groups and 10 pairs of birds in the control group. Because no differences were found in the total blood or other tissue Hg concentrations of birds given MeHg chloride versus MeHg cysteine (Varian-Ramos [unpublished data]), results obtained from the two groups were combined for analysis, effectively resulting in three treatments groups (0, 0.5, and 1.0 μg/g MeHg). Birds were humanely killed by rapid decapitation 60 days after hatching of their first successful clutch, which occurred 24–32 weeks after initial Hg exposure. Birds that failed to successfully reproduce ($n = 2$ pairs in control group, $n = 1$ pair in 0.5 μg/g group, and $n = 1$ pair in the 1.0 μg/g) were killed after 32 weeks of dietary Hg exposure and included in this study.

Tissue Collection

To obtain sera for Western blots, we obtained jugular vein blood samples with a 30-gauge insulin syringe immediately before killing one undosed zebra finch and one undosed European starling (*Sturnus vulgaris*) from the large outbred colony. These samples were transferred to a microcentrofuge tube containing 5 μl heparin and spun at 250 g for 10 min at 4 °C. The serum was removed and stored at −20 °C. For Hg analysis, blood samples were taken from each zebra finch immediately before killing in the same manner and stored in a 75-μl heparinized capillary tube.

Muscle, brain, liver, and kidney samples were collected immediately after killing and frozen at 20 °C for later Hg analysis. All Hg analyses were performed using a direct Hg analyzer (DMA-80; Milestone, Shelton, CT). Tissues were freeze-dried before Hg analysis, whereas blood samples were analyzed fresh. Quality assurance included analyses of at least 20 % duplicate samples. In addition, two samples of each of two solid standard reference materials were run (DORM3 and DOLT4, National Institute of Standards, Canada) with each batch of 20 samples, and samples of blood were spiked with standard reference material to assess recovery. Relative percent difference of duplicates was <10 %, and recovery of standard reference material was close to 100 % for spiked samples.

The spleen from each killed bird was immediately placed in 5 ml cold RPMI-1640 (Invitrogen Life Technologies, Grand Island, NY) and macerated with a syringe (BD Biosciences, Franklin Lakes, NJ). The cell suspension was drawn up and released from the syringe 10–15 times before being forced through a 40-μm cell strainer (BD Biosciences) into a 50-ml conical tube (BD Biosciences) on ice. Initial testing using Histopaque-1077 to exclude red blood cells (RBCs) showed that the presence of RBCs did not affect lymphocyte proliferation. Therefore, to maximize splenocyte yield, we did not use the Histopaque lymphocyte enrichment procedure on our samples. Cells were spun at 250 g for 10 min, and the supernatant was removed. Next, cells were washed twice in sterile Hank's balanced salt solution (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, and 20 mM Hepes [pH 7.05]) and counted using a hemacytometer. Pellets were either frozen at −80 °C in aliquots of 1×10^6 cells for Western blots or washed in medium (RPMI-1640, 10 % FBS, 50 μM 2-ME, and 100 U/ml each penicillin and streptomycin) at a concentration of 1×10^7 cells/ml for culturing.

B Cell Antibodies

Due to a lack of songbird-specific antibodies against B cell surface proteins, we took an alternative approach of using the transcription factor Pax5 as a B cell marker. Because of their highly conserved nature, use of transcription factors, including Pax5, has proven fruitful in previous studies of both mammalian and teleost fish B cell development (Zwollo 2011). Furthermore, a Pax5-specific antibody, ED-1 (Zwollo et al. 1998), was already available (Zwollo et al. 2005, 2008). This polyclonal rabbit antimouse antibody recognizes the highly conserved complete paired domain (DNA binding domain, aas 16-142) of human, mouse, and rainbow trout Pax5 (Adams et al. 1992), thus making it a good candidate for use as a B cell marker in songbirds. For flow cytometric analyses, the anti-Pax5 antibody was conjugated to Alexa Fluor 555 using protein-labeling kits according to the manufacturer's instructions (Molecular Probes, Grand Island, NY). Isotype-control antibodies included rabbit immunoglobulin (Ig)G (molecular probes) conjugated to Alexa 555 (C555) or Alexa 647 (C647). Antibody aliquots were stored in 1 % bovine serum albumin (BSA) at −20 °C.

Western Blots: Testing Potential B Cell Markers

To verify the specificity of the ED-1 antibody, we performed a Western blot using songbird (European starling and zebra finch) spleen cell lysate and serum. Cell lysates were prepared by adding a sample buffer containing 5 % 2-ME to serum (5 µl) or cell pellets containing 1×10^6 cells, collected as described previously, and proteins were separated by size using denaturing 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels as described previously (Zwollo et al. 2005). Proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Membranes were incubated in blocking solution of 5 % dry milk in 1X phosphate-buffered saline (PBS) for 1 h on a rotomixer, followed by incubation in blocking solution containing primary antibody (1 µg/ml) for 1 h. Four 5-minute washes in PBS (1.9 mM $NaH_2P04.H_20$, 8.1 mM $Na_2HP04.7H_201$ 37 mM NaCl, and 2.6 mM KCl, pH 7.4; 1X PBS) were then followed by a 1-hour incubation with secondary Ab goat antirabbit IgG-horse radish peroxidase (0.1 µg/ml) conjugate (Zymed Laboratories, San Francisco, CA) in blocking solution. Membranes were then washed four more times in 1X PBS and developed using a chemiluminescence-PLUS kit (ECL-Plus; GE Health Care Life Sciences, Pittsburgh, PA).

Spleen Cell Culture for Flow Cytometry

Spleen cells were cultured at a concentration of 1×10^7 cells/ml culture medium (RPMI-1640, 10 % FBS, 50 µM 2-ME, and 100 U/ml each penicillin and streptomycin) and plated in 96-well plates at 200 µl/well. Lipopolysaccharide (055:B5 from *Escherichia coli*; Sigma, St Louis, MO) was added at a final concentration of 50 µg/ml. Cells were incubated for a total of 24 h (37 °C, 5 % CO_2).

To detect cell proliferation, the zebra finch cells that had been cultured for 16 h were incubated in the presence of 30 µM EdU (Click-iT, Invitrogen, Grand Island, NY). After 8 h of EdU incorporation, cells were collected and fixed immediately. Cells were spun at 110 g for 5 min at 4 °C, and pellets washed in 1X PBS with 0.02 % sodium azide and resuspended in 500 µl of a fixative containing 1 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 1X PBS. Cells were counted before fixation. Samples were incubated at 4 °C for 15 min, followed by one wash in 1 ml 1X PBS plus 0.02 % sodium azide. Cell pellets were vortexed for 20 s to create a cell suspension, and 1 ml 80 % ice cold methanol was added drop wise. Cells were stored at −20 °C for a minimum of 16 h and no more than 2 weeks. To save cells for later analysis, they were refixed in a 100-µl volume of 1 % paraformaldehyde, washed, and resuspended in 100 µl FBS followed by 100 µl FBS plus 20 % dimethylsulfoxide in a CryoTube (NUNC, Rochester, NY) for a final concentration on 10 % DMSO. Samples were then stored at −80 °C for later analysis.

Flow Cytometric Analysis to Quantify B-Lymphocyte Populations

Frozen cell samples were thawed partially and immediately resuspended in 1 ml 1X PBS plus 5 % FBS in a 1.5-ml Eppendorf tube. Cells were spun in a microcentrifuge at 450 g for 3 min at 4 °C. After removing the supernatant, cells were resuspended in 45 µl Perm/Wash (BD Biosciences, San Jose, CA) containing 5 % FBS and plated on a 96 well round-bottom enzyme-linked immunosorbent assay plate (Fisher Scientific, Waltham, MA). The plate was placed on a nutator for 15 min at 4 °C in the dark. Five microliters of 10 × antibody solution (0.15 mg/ml) were added for a final concentration of 0.015 mg/ml antibody/sample, and cells were incubated for 90 min on a nutator in the dark at 4 °C. Two hundred fifty microliters of Perm plus 2 % FBS was then added to each sample, and cells were spun at 250 g for 5 min at 4 °C. After removing supernatant, pellets were resuspended in 250 µl Perm plus 2 % FBS and incubated in the dark on nutator at room temperature for 10 min. Cells were spun and pellets were again washed with Perm and 2 % FBS and incubated for 10 min as before.

Click-iT EdU reaction cocktail (Invitrogen, Grand Island, NY) was prepared according to the manufacturer's instructions. Cells were spun as before and pellets resuspended in 250 µl Click-iT reaction cocktail, followed by a 30-minute incubation in the dark at room temperature. After this incubation, cells were washed twice in 280 µl Perm plus 2 % FBS and resuspended in 100 µl Perm plus 2 % FBS. Samples were immediately analyzed using flow cytometry, with 50,000 events acquired per sample, using BD FACSArray (BD-Biosciences, San Jose, CA). Flow cytometry graphs and plots were generated using WinMDI 2-8 (J. Trotter 1993–1998, West Lafayette, IN) software. Mean fluorescence intensities (MFIs) were determined by calculating the difference in MFI between Pax5− and Pax5+ populations using BDFACS Array software (San Jose, CA).

Statistical Analyses

Principal component analysis (PCA) was used to decrease five intercorrelated measures of Hg concentration in different tissues (blood, muscle, brain, kidney, and liver) to a single component (Hg PC1 [total body Hg]). We did this to generate a single index of tissue total Hg load for each individual. Hg PC1 accounted for 95.8 % of the tissue Hg variation. Loadings explain the weight that each variable (tissue) contributed to the PC score; all of the respective

Table 1 Loading factors of Hg PC1

Tissue	Zebra finch PC1
Muscle	0.4538
Brain	0.4514
Liver	0.4498
Kidney	0.4455
Blood	0.4353

tissue variables loaded in a highly positive manner with Hg PC1 (Table 1).

Although subjects within each treatment group received the same Hg concentration in their diet, Hg accumulation in tissues was highly variable among individuals (Fig. 1). Therefore, instead of comparing treatment groups, we analyzed the relationships between individual Hg level (Hg PC1) and our indices of immune system functioning using generalized linear models. For the analyses of the frequency of proliferating and resting B cells, we used a binomial distribution and a logit link where the response was the number of proliferating or resting B cells, and the binomial was the total number of events (cells) acquired per sample (50,000). For the analysis of the ratio of proliferating to resting B cells, we used a gamma distribution and a log link. The analyses of cell number and MFIs used a normal distribution and an identity link. PCA was performed with PAST Statistics (Hammer et al. 2001), and the generalized linear models were performed in SPSS 19 (IBM, Armonk, NY).

Results

Optimization of a Zebra Finch B Cell Marker

Our first goal was to find a B cell marker that would identify songbird B cells. A fluorescence-conjugated anti-chicken IgM (μ heavy chain) antibody (Rockland Immunochemicals) failed to react with passerine Ig using either Western blot or flow cytometry (data not shown). The only antibody that has been developed specifically for use in a passerine species, a rabbit anti-red-winged blackbird IgG antibody (Hasselquist et al. 1999), failed to specifically recognize heavy-chain Ig mu by Western blot analysis (data not shown). As an alternative approach, we used an anti-Pax5 antibody that has been used to identify B lymphocytes in other vertebrates (Zwollo et al. 2005). The antibody was tested using Western blot analysis with spleen cellular extracts and blood serum of two different songbirds (European starling and zebra finch). An approximately 53-kDa band, corresponding to the reported size for Pax5 protein (Zwollo et al. 1998), was detected in samples containing cellular extract but not in serum (Fig. 2). Therefore, Pax5 is an effective antibody with which to detect Pax5 protein in passerine B cells and was subsequently used in flow cytometric analysis.

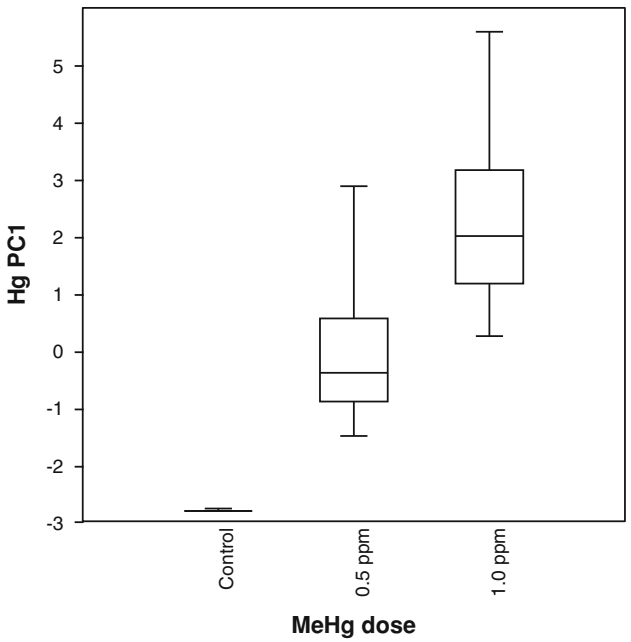

Fig. 1 Index of body total Hg (Hg PC1) for each treatment group of zebra finches. The *center line* of the box represents the median value. The *upper* and *lower limits* of the box are 75th and 25th percentile intervals, respectively. *Error bars* indicate 95th percentile ranges. Note that there is substantial among-individual variation in Hg PC1 within each treatment group

Cytometric Analyses of Proliferating Lymphocytes from Cultured Spleens

Zebra finch splenic cell cultures were analyzed 24 h after LPS-induction (day 1). The total number of zebra finch spleen cells isolated was typically between 1 and 2 × 10^6

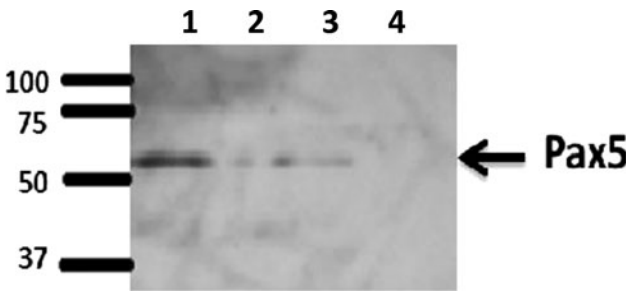

Fig. 2 Western blot analysis to determine the ability of anti-Pax5 antibody to detect Pax5 protein in zebra finch or European starling immune tissues. Molecular weight markers (in kD) are shown on the left. *Lane 1*: zebra finch whole spleen cell lysate (from 10^6 cells); *lane 2*: zebra finch serum (5 μl undiluted); *lane 3*: starling whole spleen whole cell lysate (from 10^6 cells); and *lane 4*: starling serum (5 μl, undiluted); $N = 4$

cells/spleen, which was sufficient for only one time point sample. Therefore, proliferation could not be measured during multiple days. We chose to collect cells at 24 h after culture because we wished to determine not only whether Hg suppresses but also if it *delays* B cell proliferation. Therefore, measuring B cell proliferation at an early time point was essential. Previous work from our laboratory (Zwollo 2011) had suggested that 24 h was a valid time point because approximately 20 % of splenic mouse B cells had already incorporated EdU at that time. In addition, an early study reported that 26 % of splenic mouse B cells already incorporated [^{3}H]thymidine within 12 h of LPS exposure in culture (DeFranco et al. 1982).

All cells were exposed to proliferation marker EdU 8 h before collection, and cells were fixed and permeabilized before anti-Pax5 antibody staining and flow cytometric analysis. A lymphoid population with characteristic low side scatter (SSC [indicates cell complexity]) and varying in forward scatter (FSC [a measure of cell size]) was present in zebra finch spleen samples as shown in the dot plot graph (Fig. 3a). This lymphocyte population was similar to that observed in other species, including chickens (Dalgaard et al. 2010). We gated this population, which ranged from 43 to 78 % of the entire splenic fraction (Fig. 3a), so that only lymphocytes would be included in our analysis of different B cell populations (Fig. 3b–e). The same gating was used for all further analyses.

Pax5 is expressed at high levels in nonproliferating, mature B cells in the spleen and is also expressed in proliferating plasmablasts. However, it is absent in terminally differentiated plasma cells (Nera and Lassila 2006). Pax5 staining in conjunction with the proliferation marker EdU resulted in three cell populations (Fig. 3c–e): Pax5+/EdU− (nonproliferating B cells [quadrant I]), Pax5+/EdU + (proliferating B cells [plasmablasts, quadrant II]), and Pax5−/EdU− (nonproliferating spleen cells, [quadrant III]). No Pax5−/EdU+ proliferating cells (non-B cells, quadrant IV) were detected in any of our samples. Plasma cells would be present in quadrant III together with a variety of other immune cells. Hence, their frequency could not be determined.

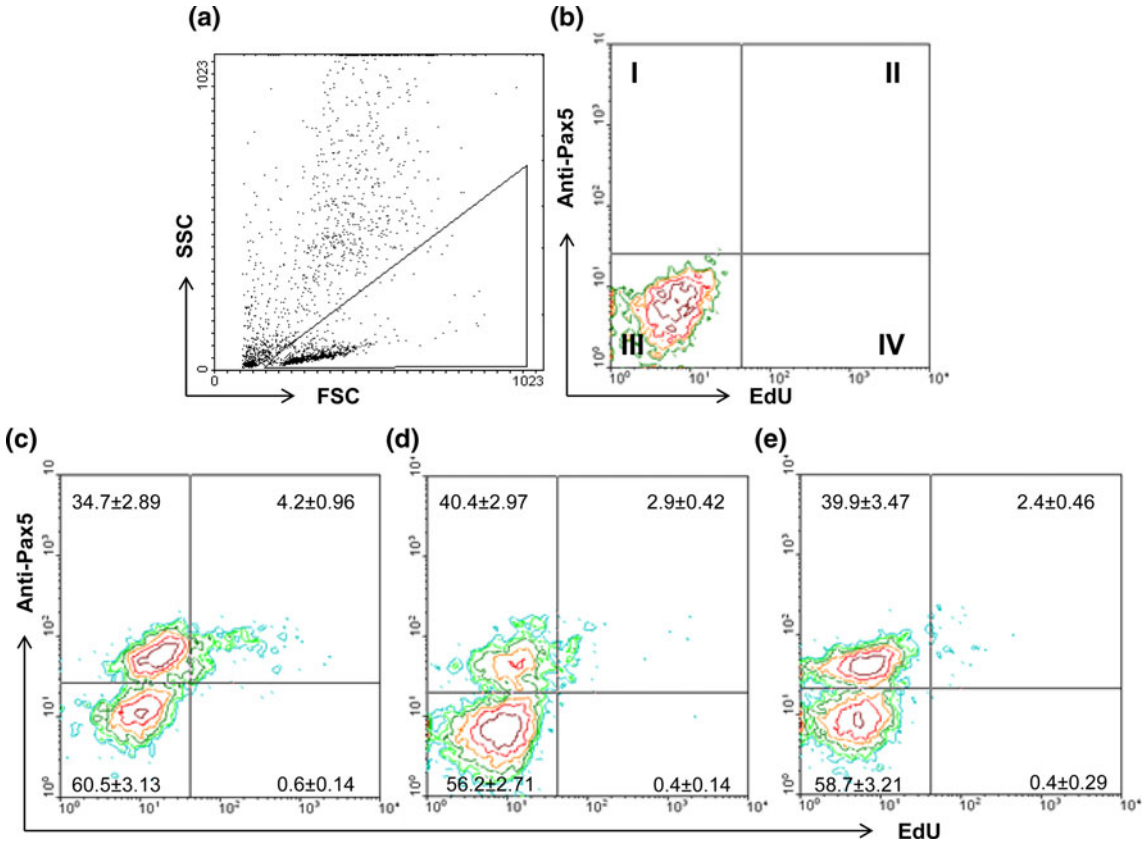

Fig. 3 Flow cytometric analysis of splenic B cells isolated from a representative zebra finch. **a** Dot plot of FSC versus SSC with gated lymphocyte population. **b** Isotype control staining with rabbit control IgG antibody C555, cells cultured in the absence of EdU but stained with Click-iT. Quadrants are labeled numerically for reference. **c–e** Staining with Pax5 antibody and EdU (with Click-iT). Four quadrants (as labeled in **b**) are defined as follows: quadrant I = Pax5+/EdU− (nonproliferating B cells); quadrant II = Pax5+/EdU + (proliferating B cells); quadrant III = Pax5−/EdU− (nonproliferating non-B cells or plasma cells); and quadrant IV = Pax5−/EdU+ (proliferating non-B cells). Overall mean and SE of percent cells in a quadrant are included on representative contour plots for **c** the control, undosed group, **d** the 0.5 ppm Hg group, and **e** the 1.0 Hg group

Hg and B Cell Proliferation

Those samples that lacked sufficient cell numbers to form a clear population of lymphocytes in a dot plot (Fig. 3a) were not included in analyses, thus decreasing our sample sizes to the following: controls ($n = 17$), 0.5 μg/g Hg ($n = 25$), and 1.0 μg/g Hg ($n = 22$). B cell proliferation decreased as Hg PC1 increased ($\beta = -0.10 \pm 0.01$, Wald $\chi^2 = 4448.3$, $df = 1$, $p < 0.001$; Fig. 4a). The frequency of resting B cells was weakly but significantly positively related to Hg PC1 ($\beta = 0.04 \pm 0.01$, Wald $\chi^2 = 6804.4$, $df = 1$, $p < 0.001$; Fig. 4b).

The frequencies of the two B cell populations (proliferating and resting) are relative to the total number of cells and may be driven by variability in the frequency of non-B cell populations. Thus, we calculated a ratio of proliferating to resting B cells as an index of proliferation that is independent of non-B cell populations. This ratio of proliferating B cells decreased significantly with increasing Hg PC1 ($\beta = -0.14 \pm 0.04$, Wald $\chi^2 = 10.69$, $df = 1$, $p = 0.001$; Fig. 4c), indicating that there was less B cell proliferation in birds with greater Hg body burdens.

Two additional end points were analyzed for a potential relationship with Hg PC 1. Although we did not measure apoptosis directly, we determined the total number of splenic cells before and after proliferation to assess whether Hg may affect viability. We found no relationship between cell counts and Hg PC1 ($F = 0.02$, $df = 1.57$, $p = 0.89$). Therefore, there was no detectable difference between Hg-exposed and control samples in cell viability within the 24-hour culture period. Second, we asked whether Hg affects Pax5 expression in B cells. Analysis of the MFIs for Pax5 of each sample suggested that Hg did not affect Pax5 levels in our study ($F = 0.11$, $df = 1.63$, $p = 0.75$).

Discussion

We optimized a flow cytometric assay for measuring in vitro B cell proliferation of individual cells, which can be used for analysis of a songbird humoral immune system. This assay was used to measure potential effects of in vivo MeHg exposure on zebra finch B cell proliferation using splenic cell cultures stimulated by the B cell mitogen, LPS. We found that splenic B cells from individuals with greater Hg accumulation have decreased proliferation in response to LPS-induction (Fig. 4c). This result was strengthened by the observation that splenic cultures from MeHg-exposed birds had more resting B cells on LPS-induction than did cultures from control animals. These patterns are consistent with a separate experiment that was conducted in another species, European starling, in which there was also a tendency for birds with greater Hg accumulation to show decreased B cell proliferation (Lewis 2012).

The decrease in B cell proliferation associated with increasing Hg accumulation in the blood and organs suggests that Hg suppresses or delays the onset of B cell proliferation in response to antigen. This observation is consistent with earlier findings from avian studies in which the SRBC assay, which measures antibody secretion, indicated that exposure to Hg was associated with decreased primary immune response in one species dosed in captivity (Kenow et al. 2007; but see Fallacara et al. 2011), and a similar, although nonsignificant, pattern was found for another species exposed environmentally (Hawley et al. 2009).

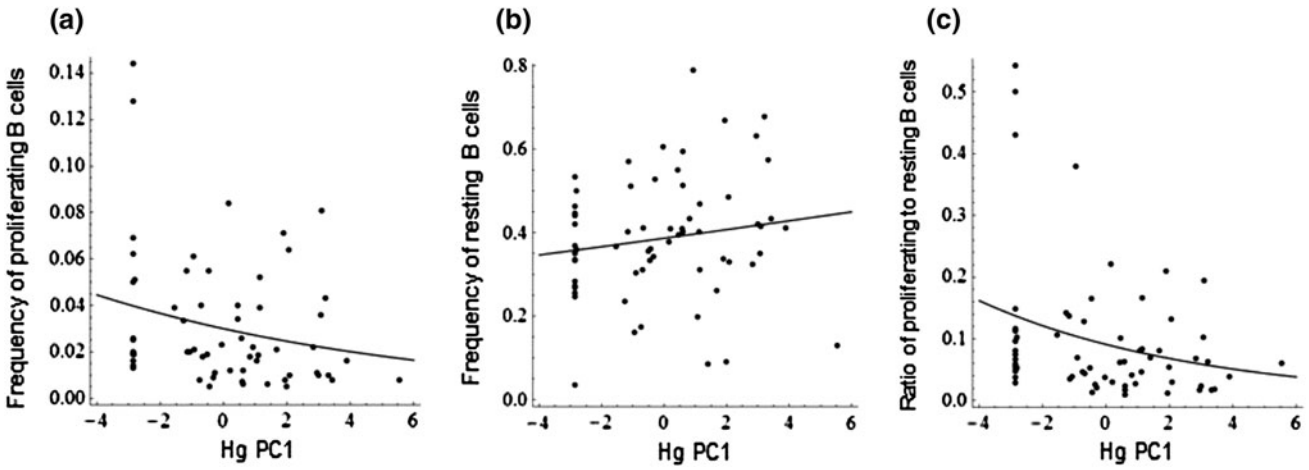

Fig. 4 Generalized linear models of the frequency of resting and proliferating B cell populations and body total Hg (Hg PC1). **a** Frequency of proliferating B cells versus Hg PC1. **b** Frequency of resting B cells versus Hg PC1. **c** Ratio of proliferating to resting B cells per sample versus Hg PC1. Each point represents the data from a single bird, and the *lines* shown are the generalized linear models back-transformed to the original scale

There is little consensus on the mechanisms by which Hg affects the immune response. It is well known that Hg readily binds sulfhydryl groups, including tubulin, thus blocking microtubule formation (Brown et al. 1988; Miura et al. 1984). Microtubules are critical for many aspects of basic cell functioning, including vesicular transport and cell structure. If microtubule disassembly leads to inhibition of these basic cellular processes, it is likely that entry into mitosis would be inhibited or slowed. This could explain the increase in resting B cells and the decrease in proliferating plasmablasts in Hg-exposed samples.

Microtubule polymerization is also a central process in mitosis and cell proliferation. Correct microtubule formation and alignment is the critical mitotic checkpoint; if a cell cannot pass this checkpoint, it will be suspended in mitosis until microtubule alignment is corrected or, in the case of missegregated chromosomes, apoptosis will eventually be induced (Gorbsky 2001). In our study, such proliferating B cells would have incorporated EdU, would also be positive for Pax5, and would appear as plasmablasts in two-color flow cytometry (Fig. 3c–e [quadrant II]). If Hg inhibits proliferation by suspending cells at the metaphase checkpoint, we would have observed higher levels of proliferating cells in the Hg-exposed populations; however, this was not the case. However, if apoptosis is induced in cells suspended at this checkpoint, then this could lead to the observed decrease in the Pax5+/EdU + population in Hg-exposed samples.

There is strong evidence to suggest that Hg directly induces apoptosis in immune cells, but the mechanism and susceptible cell types are unclear. Studies suggest that low levels of MeHg lead to apoptosis primarily in resting T lymphocytes (Shenker et al. 1997), whereas somewhat greater levels lead to the oft-reported decrease in the population of mitogen-stimulated proliferating lymphocytes (Brown et al. 1988; Day et al. 2007; Das et al. 2008) accompanied by inhibition of microtubule polymerization (Brown et al. 1988). Induction of cell death during mitosis on greater Hg exposure is one reasonable explanation for the decreased population of proliferating B cells. Although our data did not show a correlation between cell numbers and Hg accumulation, the possible effects of Hg on lymphocyte apoptosis using direct measures will be an important area for future study.

It is important to note that whereas the current study isolated spleen cells from zebra finches exposed to dietary Hg throughout their adult lifetimes, the majority of previous studies that explored potential mechanisms have added Hg to peripheral blood lymphocyte cultures in vitro during the span of a few days (Haase et al. 2011; Pheng et al. 2000; Shenker et al. 1997; Ziemba et al. 2006). Several studies that tested the effects of environmental and in vitro Hg exposure on T and B lymphocytes found a dose-dependent decrease in lymphocyte proliferation in both cases, implying that both types of Hg exposure have the same effect (Das et al. 2008; Day et al. 2007).

Although the mechanism remains unknown, the immunomodulation observed here is likely also observed in some free-living songbirds with environmental Hg exposure. The high dose (1.0 μg/g) resulted in zebra finches accumulating blood Hg concentrations (approximately12 μg/g) comparable with the highest individual birds at a site of industrial contamination (Cristol et al. 2008), whereas the intermediate dose (0.5 μg/g) resulted in levels (approximately 5 μg/g) comparable with many songbirds at the contaminated site. At sites without direct point sources of Hg pollution, few individual songbirds would have blood Hg levels as high as those investigated here: Saltmarsh sparrows (*Ammodramus caudacutus*) across the east coast of North America averaged <2 μg/g Hg (Cristol et al. 2011), whereas rusty blackbirds (*Euphagus carolinus*) averaged 0.3 and 1 μg/g Hg on breeding grounds in Alaska and Nova Scotia, respectively (Edmonds et al. 2010).

Our study was limited by a relatively low number of zebra finch spleen cells and by monitoring changes soon after LPS-induction of the B cells. It is therefore unknown how well Hg-exposed B cells would proliferate at later time points. Using a similar experimental design, mouse splenic B cells reach their peak proliferation on days 2–4, whereas rainbow trout B cells have the highest proliferation between days 4 and 6 (Zwollo 2011). Measuring proliferation at later time points in zebra finches would be particularly interesting because it would test whether B cells from MeHg-exposed birds would still have the capacity to proliferate, albeit in a delayed manner, to the same extent as B cells from nonexposed birds.

In summary, based on our data, we conclude that sublethal levels of MeHg inhibit or delay B cell proliferation in zebra finch immune tissues, and we hypothesize that this makes it more difficult for MeHg-exposed birds to mount an effective adaptive immune response. We further hypothesize that this would lead to a greater susceptibility to parasites, which could pose a major threat to birds in the wild. Future studies exploring both T- and B cell proliferation, apoptosis, and the underlying signal transduction pathways are needed to help provide a more complete understanding of the effects of Hg on songbird health. Nevertheless, it is clear from this study that dietary Hg exposure can suppress B cell proliferation in a model songbird system.

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