In Vivo Tungsten Exposure Alters B-Cell Development and Increases DNA Damage in Murine Bone Marrow

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High environmental tungsten levels were identified near the site of a childhood pre-B acute lymphoblastic leukemia cluster; however, a causal link between tungsten and leukemogenesis has not been established. The major site of tungsten deposition is bone, the site of B-cell development. In addition, our in vitro data suggest that developing B lymphocytes are susceptible to tungsten-induced DNA damage and growth inhibition. To extend these results, we assessed whether tungsten exposure altered B-cell development and induced DNA damage in vivo. Wild-type mice were exposed to tungsten in their drinking water for up to 16 weeks. Tungsten concentration in bone was analyzed by inductively coupled plasma mass spectrometry and correlated with B-cell development and DNA damage within the bone marrow. Tungsten exposure resulted in a rapid deposition within the bone following 1 week, and tungsten continued to accumulate thereafter albeit at a decreased rate. Flow cytometric analyses revealed a transient increase in mature IgD+ B cells in the first 8 weeks of treatment, in animals of the highest and intermediate exposure groups. Following 16 weeks of exposure, all tungsten groups had a significantly greater percentage of cells in the late pro-/large pre-B developmental stages. DNA damage was increased in both whole marrow and isolated B cells, most notably at the lowest tungsten concentration tested. These findings confirm an immunological effect of tungsten exposure and suggest that tungsten could act as a tumor promoter, providing leukemic “hits” in multiple forms to developing B lymphocytes within the bone marrow.

Key Words: tungsten; leukemia; DNA damage; B-cell development.

Tungsten is a naturally occurring element within the earth’s crust; the use of tungsten is increasing, either alone or in alloys, with the advancement of technologies. Despite its widespread use, little is known of the health consequences of tungsten exposure (Kazantzis and Leffler, 2007). Human exposure to tungsten is correlated with occupations such as tungsten mining and processing although exposure to environmental tungsten can occur via ingestion of contaminated ground water or consumption of fruits and vegetables grown in high-tungsten areas.

The average urinary tungsten level in the United States is 0.093 μg/l (CDC, 2012), however, some regions with higher tungsten levels have been identified. In Churchill County, Nevada, tungsten levels from residential drinking water wells range from 0.27 to 742 μg/l (Koutsospyros et al., 2006), resulting in an elevated average community urinary level of 1.19 μg/l (CDC, 2003). This is well above the 95th percentile (0.50 μg/l) of average urinary tungsten levels as outlined in Centers for Disease and Prevention (CDC)’s National Report on Human Exposure to Environmental Chemicals (CDC, 2012). Interestingly, within the same county, the CDC validated a pediatric leukemia cluster, with the majority of cases being pre-B cell acute lymphoblastic leukemia (Rubin et al., 2007). Despite identifying increased levels of tungsten, as well as arsenic, JP-8 jet fuel, and pesticides, within the community, the CDC found no relationship between leukemia and tungsten exposure and discovered no environmental exposure that could explain the cluster of childhood leukemia (CDC, 2003). However, based on a lack of experimental data on which to base regulatory decisions, the National Institutes of Health have classified tungsten as a priority chemical for toxicological research (CDC, 2003).

Despite not having a clear link to the development of leukemia, tungsten exposure has been linked to carcinogenesis in animal models. Oral administration of sodium tungstate in drinking water significantly increased carcinoma incidence following a single dose of N-nitroso-N-methylurea compared with control groups (79.2 vs. 50.0%) (Wei et al., 1985). Furthermore, rats implanted with high-concentration tungsten alloy pellets (91.1% W, 6.0% Ni, 2.9% Co) developed high-grade, pleomorphic rhabdomyosarcoma significantly sooner than rats implanted with nickel pellets used as a positive control (Kalichnich et al., 2005).
Tungsten is distributed throughout the body via the blood, and experiments on rats indicate that excretion reduces the body’s burden by approximately 97% within 72 h (Kaye, 1968). The remaining tungsten preferentially deposits within bone but is also present in other organs in lower concentrations (spleen, kidneys, and liver) (Guandalini et al., 2011; McInturf et al., 2011). The pharmacokinetics of tungsten excretion and retention are based mostly on mathematical models extrapolated from several animal studies, but predicts that accumulation within the bone would increase gradually throughout the life of chronically exposed individuals (Leggett, 1997). An accumulation of tungsten within the bone, therefore, could result in higher exposure levels within the bone marrow, the site of the developing immune system, and the likely site of leukemogenesis.

Normal B lymphocyte development occurs within the bone marrow after birth, in a linear process that can be fractionated into several stages of development (Fractions A–F; Fig. 1) based upon differential expression of surface protein markers (Hardy et al., 1991). During B-cell maturation, the genes encoding the antibody variable regions are assembled from the gene segments V (variable), D (diversity), and J (joining) through the process of double-strand breaks and subsequent DNA repair (Rajewsky, 1996). Developing lymphocytes then ensure proper immunoglobulin rearrangement, which when altered can lead to leukemogenic events (Nussenzweig and Nussenzweig, 2010). Thus, this process of maturation may leave developing B cells at risk for increased DNA damage. Our previous in vitro data show the developing pro-/pre-B CD43+ BU-11 cell line is sensitive to tungsten-induced growth inhibition and apoptosis, as well as increased DNA damage (Guilbert et al., 2011). Importantly, we have shown that tungsten-induced DNA damage occurs at lower concentrations than those required to induce cell death. We hypothesized, therefore, that tungsten accumulation within the bone after an oral exposure would increase the exposure of the developing B cells and consequently result in DNA damage. Interestingly, we observed that tungsten exposure increased DNA damage within the bone marrow resident B lymphocytes and that the percentages of these developing cells were altered.

MATERIALS AND METHODS

In vivo tungsten exposure. Animal experiments were performed under a McGill University Animal Care Committee approved protocol. Four-week-old male C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME) and were given food and water ad libitum. After 1 week of acclimation, mice were divided into four treatment groups (n = 5 per treatment group): control tap water or 15, 200, or 1000 mg/l tungsten. No other concentrations were tested. An additional study compared removal of tungsten for 4 and 8 weeks, following a 4-week exposure to 15 mg/l tungsten. The concentrations used in our study represent elemental tungsten, as opposed to sodium tungstate (1.795 g Na2WO4·2H2O = 1 g W). Sodium tungstate dihydrate (Na2WO4·2H2O; Sigma-Aldrich) was dissolved in tap water and was replaced every 2 or 3 days to limit conversion to polytungstates, with remaining water used to calculate average daily consumption. Exposure was conducted over a period of 16 weeks, with no animals being lost as a result of exposure.

Hematology, liver function, and tungsten analyses. Peripheral blood was drawn by cardiac puncture following CO₂ asphyxiation and used immediately for peripheral blood smears or collected in EDTA-containing Microvette tubes (Sarstedt, Nümbrecht, Germany) for automated complete blood count and differential analysis using a Scil Vet abc Hematology Analyzer (Vet Novations, USA).
**TABLE 1**

Antibody-Fluorochrome Staining Panel Used to Discriminate Cell Populations via Flow Cytometry

<table>
<thead>
<tr>
<th>Clone</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardy stain (for B-Cell precursors)</td>
<td>eB121-15F9</td>
</tr>
<tr>
<td>IgM—e450</td>
<td>M1/69</td>
</tr>
<tr>
<td>IgD—PerCP-Cy5.5</td>
<td>B220/CD45R—APC-Cy7</td>
</tr>
<tr>
<td>CD24—FITC</td>
<td>BP1—PE</td>
</tr>
<tr>
<td>CD43—APC</td>
<td>CD43—APC</td>
</tr>
<tr>
<td>CD20/CD45R—APC-Cy7</td>
<td>CD20/CD45R—APC-Cy7</td>
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</table>

**CLP Staining**

<table>
<thead>
<tr>
<th>Clones</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin Mouse Lineage Panel—SA-APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD127—PE</td>
<td>A7R34</td>
</tr>
<tr>
<td>CD117—PE-Cy7</td>
<td>2B8</td>
</tr>
<tr>
<td>Sca1—V450</td>
<td>D7</td>
</tr>
</tbody>
</table>

**Flow cytometry of B-cell development.** Bone marrow cells were isolated from both tibiae and femora for flow cytometric analyses, colony forming unit (CFU) assays, and assessment of DNA damage, with the flushed bone stored at ~80°C. Elemental tungsten concentration in bone was assessed by inductively coupled plasma mass spectrometry (ICP-MS) analysis by Chemical Solutions Ltd (Mechanicsburg, PA), with an elemental detection limit of 0.5 ppm for weeks 1 and 0.2 ppm for all other weeks.

**CLP Staining**

Bone marrow cells were isolated from both tibiae and femora. Single cell suspensions were made and cryopreserved for batch analysis. After thawing, lymphocytes were counted using a Z2 Coulter Counter (Beckman Coulter, CA). Two million bone marrow cells were stained with a fixable viability dye (Invitrogen, OR) in PBS, incubated with rat anti-mouse CD16/CD32 Fc receptor block (BD technologies), and finally stained with specific antibodies or appropriate isotype controls in PBS supplemented with 5% fetal bovine serum and 10mM sodium azide. Specific antibody-fluorochrome staining panel and an example of the general gating strategy followed by Newman-Keuls post hoc test was used to determine differences between data sets, with significance determined and denoted based on the following hierarchy: * = p < 0.05, ** = p < 0.01, *** = p < 0.001. Statistical analyses were performed using GraphPad Prism 4 (San Diego, CA).

**Results**

Based on our in vitro results, we hypothesized that mice exposed to sodium tungstate would exhibit alterations in B lymphocyte development that would correlate with levels of tungsten within the bone. To test our hypothesis, we exposed C57BL/6J wild-type mice over 16 weeks to varying concentrations of tungsten in the drinking water. Tungsten concentrations were chosen based on literature review and reported environmental tungsten levels from Churchill County, Nevada, and included (1) a low dose of 15 mg/l tungsten, which has previously been shown to enhance respiratory syncytial virus (RSV)–associated pathology (Fastje et al., 2012), (2) a high dose of 1000 mg/l tungsten, representing the “hot-spot” concentration found in surface dust (Sheppard et al., 2007), and (3) an intermediate dose of 200 mg/l tungsten. No other concentrations other than those listed here were tested. The lowest concentration of 15 mg/l tungsten used in this study is approximately 20× higher than the highest private residential well water sampling reported within Churchill County (Koutsospyros et al., 2006). Concentrations used are much lower than the 2000 mg/l being evaluated for antidiabetic (Muñoz et al., 2001) and antiobesity (Claret et al., 2005).
properties of tungstate. Daily water consumption between groups did not differ (± 0.16 ml/g).

We did not observe liver toxicity as assessed by peripheral blood AST and ALT enzyme levels (Supplementary table S1), confirming previously published data (Barberà et al., 1997; Pawa and Ali, 2004; Uskoković-Marković et al., 2007). Although animal weight was significantly lower in animals given the highest concentration of tungsten compared with all other groups,
Tungsten Exposure Results in Increased Percentage of pre-B Cells Within the Bone Marrow

Previous data suggest that tissue accumulation of tungsten following chronic exposure is not uniform, with the highest concentration occurring in the skeleton (Guandalini et al., 2011). The amount of tungsten in the tibia bones of exposed animals was quantitated using ICP-MS over the 16-week experiment. Tungsten concentration increased rapidly following the first week of exposure (Fig. 3A). Differential analysis of the blood showed that the percentage of lymphocytes, monocytes, and granulocytes within the leukocyte compartment remained the same, indicating a dose-dependent panleukopenia as a result of exposure (Fig. 3B). In later weeks, the decreased WBCs were only significant at 12 weeks of exposure at the highest concentration tested although the observed trend was present following 8 weeks of exposure.

Chronic Tungsten Exposure Results in a Dose-Dependent Concentration of Tungsten Within the Bone

Previous data suggest that tissue accumulation of tungsten following chronic exposure is not uniform, with the highest concentration occurring in the skeleton (Guandalini et al., 2011). The amount of tungsten in the tibia bones of exposed animals was quantitated using ICP-MS over the 16-week experiment. Tungsten concentration increased rapidly following the first week of exposure, after which the accumulation rate decreased significantly (p < 0.001), for the remainder of the experiment (Fig. 4). Tungsten content of tibia was dependent upon the concentration of tungsten to which the individual was exposed. Interestingly, lower exposures suggest an incomplete saturation of the tibia compartment, as tungsten content here peaked at values below the bone concentration achieved with our highest exposure.

Tungsten Exposure Results in Increased Percentage of pre-B Cells Within the Bone Marrow

Our previous in vitro data demonstrated that a murine bone marrow–derived pro-/pre-B cell line is sensitive to tungsten-induced apoptosis and DNA damage (Guilbert et al., 2011). We, therefore, hypothesized that tungsten exposure would result in a skewing of B lymphocyte populations, particularly in the early stages of development. To test this hypothesis, the percentages of live B lymphocytes from each developmental fraction in the bone marrow were determined using multiparameter flow cytometry. No significant changes were observed in fractions A or B, representing the early pre-pro and pro-B cells (Table 3). However, after 16 weeks of exposure, all tungsten-exposed groups had a significantly greater percentage of cells in fraction C/C′, which constitutes the late pro- and large pre-B cell stages (Fig. 5A). The greater percentage of fraction C/C′ cells resulted in a significant increase in the total number of C/C′ cells (15 and 200 mg/l concentrations) when normalized to bone marrow cellularity (Supplementary table S4). In addition, we identified an increase in fraction F (mature IgD+ B cells (Fig. 5B). This increase was observed only in animals exposed to the intermediate and high concentrations of tungsten and persisted only until 8 weeks of treatment (Table 3). No changes were observed in CD43−, IgD− B-cell populations (Fractions D and E). Importantly, total bone marrow cellularity only differed significantly after 16 weeks of treatment with 1000 mg/l tungsten (Supplementary table S5).

Tungsten Exposure Results in Clonogenic Capacity of Lymphoid Lineage Progenitors

We investigated whether tungsten exposure would induce changes in B lymphoid precursors, in either their cell number or activity. We first assessed the number of progenitor cells that had committed to the lymphoid lineage, the CLPs. CLPs can differentiate into either B or T lymphocytes and are postulated to be a target cell type in acute lymphoblastic leukemia (Hoebek et al., 2007). The number of CLP (defined as Lin−, Sca-1+CD43+ c-kit+, and IL-7R+) was assessed by flow cytometry in both control and tungsten-exposed animals, but no differences were observed (Fig. 6A). We next assessed changes in clonogenicity of bone marrow progenitor cells into lineage-specific lymphoid precursors ex vivo in response to IL-7 using...
the CFU-pre-B assay. In vivo tungsten treatment resulted in a dose-dependent increase in the number of colonies detected compared with age-matched controls following 16 weeks of exposure, and although not significant, this trend was observed in as early as 1 and 4 weeks of exposure (Fig. 6B). These data indicate that the clonogenicity, rather than the number, of CLPs was increased.

**FIG. 3.** Tungsten exposure decreases total WBC count in peripheral blood. (A) Total WBC count was assessed over 16 weeks. (B) White cell differential following 1-week exposure is shown. Data represent the mean value of treatment group (n = 5), with error bars showing standard error. *p < 0.05; **p < 0.01; ***p < 0.001.

Tungsten Exposure Results in Increased DNA Damage Within Developing B Cells

Our previous *in vitro* study demonstrated that tungsten, even at lower concentrations, can induce DNA damage in the BU-11 cell line. Therefore, DNA damage was assayed using the alkaline comet assay in the nonadherent bone marrow population, which includes the B lymphocytes. Tungsten exposure resulted
in a significant increase in comet tail moment. Intriguingly, we observed a significant increase at the lower concentration of tungsten but not at the highest concentration (Fig. 7A). Furthermore, we performed the comet assay on CD19+ B cells isolated from the marrow of animals exposed for 1 or 4 weeks to tap water with and without tungsten. Again, increased DNA damage was observed at lower tungsten concentrations of 15 and 200 mg/l but not the 1000 mg/l concentration (Fig. 7B). To confirm these results, we used another marker of DNA damage, γH2AX, the histone variant that is phosphorylated when DNA double-stranded breaks occur (Rogakou et al., 1998). Immunoblotting experiments of CD19+ B cells isolated from control and animals exposed to tungsten for 1 week showed an increase in γH2AX, particularly at the lower concentrations (Fig. 7C). This correlates with our data using the comet assay, confirms our in vitro data, and indicates that the developing B lymphocytes are targets for tungsten-induced DNA damage in vivo (Guilbert et al., 2011).

Continued Effects on B-Cell Development and DNA Damage Following Removal of Tungsten Exposure

We sought to determine whether removing tungsten from the drinking water of exposed mice would decrease bone concentrations and reverse the observed effects. Animals were exposed to 15 mg/l tungsten for 4 weeks followed by tap water for an additional 4 or 8 weeks. After 4 weeks without tungsten, tibia tungsten levels decreased significantly to approximately half the levels of those animals with continuous tungsten exposure. Surprisingly, we found no further decrease from this level after 8 weeks on tap water (Fig. 8A).

Next, we assessed the persistence of observed tungsten-induced changes in B-cell development. Tungsten exposure

### TABLE 3

<table>
<thead>
<tr>
<th>Exposure duration</th>
<th>Treatment group</th>
<th>Hardy fraction</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1 Week</td>
<td>Control</td>
<td>2.44±0.37</td>
</tr>
<tr>
<td></td>
<td>15 mg/l</td>
<td>2.39±0.16</td>
</tr>
<tr>
<td></td>
<td>200 mg/l</td>
<td>2.36±0.39</td>
</tr>
<tr>
<td></td>
<td>1000 mg/l</td>
<td>2.05±0.41</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>Control</td>
<td>2.67±0.46</td>
</tr>
<tr>
<td></td>
<td>15 mg/l</td>
<td>2.17±0.52</td>
</tr>
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<td></td>
<td>200 mg/l</td>
<td>3.42±0.53</td>
</tr>
<tr>
<td></td>
<td>1000 mg/l</td>
<td>2.89±0.51</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>Control</td>
<td>1.56±0.16</td>
</tr>
<tr>
<td></td>
<td>15 mg/l</td>
<td>1.74±0.32</td>
</tr>
<tr>
<td></td>
<td>200 mg/l</td>
<td>1.55±0.12</td>
</tr>
<tr>
<td></td>
<td>1000 mg/l</td>
<td>1.75±0.15</td>
</tr>
<tr>
<td>12 Weeks</td>
<td>Control</td>
<td>1.80±0.25</td>
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<tr>
<td></td>
<td>15 mg/l</td>
<td>1.86±0.18</td>
</tr>
<tr>
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<td>200 mg/l</td>
<td>1.41±0.27</td>
</tr>
<tr>
<td></td>
<td>1000 mg/l</td>
<td>1.58±0.26</td>
</tr>
<tr>
<td>16 Weeks</td>
<td>Control</td>
<td>1.20±0.14</td>
</tr>
<tr>
<td></td>
<td>15 mg/l</td>
<td>1.21±0.08</td>
</tr>
<tr>
<td></td>
<td>200 mg/l</td>
<td>1.27±0.11</td>
</tr>
<tr>
<td></td>
<td>1000 mg/l</td>
<td>1.39±0.16</td>
</tr>
</tbody>
</table>

Note. Cryopreserved bone marrow was stained for flow cytometric analysis in order to determine the percentage of B cells within each stage of development. Results shown represent the mean value of treatment groups (n = 5) ± SD.

*p < 0.05; **p < 0.01; ***p < 0.001.
induced an increase in Fraction F (IgM+; IgD+ B cells) after 8 weeks, but after removing tungsten for 4 or 8 weeks, this fraction was decreased below control values (Fig. 8B). In addition, we found that the percentage of cells in fraction D (small pre-B cells) was significantly decreased after tungsten was removed for 8 weeks compared with control animals. Interestingly, we found that removal of tungsten for 4 weeks increased the percentage of early pre-pro-B cells and pro-/large pre-B cells (fractions A and C/C′), where no changes had been observed upon continuous tungsten exposure at this time point. However, the changes observed in these early B-cell populations were not sustained after removal of tungsten for 8 weeks.

Finally, we compared the extent of DNA damage in whole marrow with and without removal of tungsten for 4 weeks by assessing γH2AX phosphorylation. The increase in γH2AX staining that was observed with our 15 mg/l exposure not only persisted following a 4-week removal period but was increased compared with tungsten-exposed and control animals (Fig. 8C). Together these data indicate that tungsten is not removed from the bone as fast as it accumulates and that some effects of tungsten, e.g., an increase in DNA damage and an ability to skew B-cell developmental fractions, can persist following removal of the exposure.

**DISCUSSION**

This study provides evidence that chronic tungsten exposure results in a dose-dependent accumulation of tungsten within the bone and that exposure results in an altered B-cell development profile, an increase in DNA damage within B lymphocytes, and an increased activity of lymphoid lineage progenitors.

Previous reports have shown that although most tungsten is rapidly excreted (Kaye, 1968), the remaining tungsten preferentially deposits within the skeleton and to a lesser extent in the spleen and kidneys (Guandalini et al., 2011). We also observed an initial rapid accumulation of tungsten within the bone, which was followed by a significant decrease in the accumulation rate after 1 week of exposure. From 4 weeks onward, we observed a trend where accumulation rate continuously slows, suggesting a stable concentration may be achieved within the tibia bone following chronic exposure. Furthermore, our data indicate that this skeletal accumulation is proportional to ingested levels of tungsten. These data contradict a previous model, which predicts that bone accumulation would continue gradually throughout the lifetime of chronically exposed individuals at a rate that would remain relatively constant following approximately 30 years of chronic exposure (Leggett, 1997). We hypothesize that, even if exposure is discontinued, an endogenous exposure would result as tungsten is released from the bone through continuous turnover of the skeleton. This mechanism of continued exposure as a result of bone remodeling exists for other exposures, such as fluoride (Gibson, 1998). In fact, removing tungsten from the drinking water after a 4-week exposure resulted in

**FIG. 5.** Effect of sodium tungstate exposure on the percentage of live B-cell populations according to developmental stage. Bone marrow was stained for flow cytometric analysis in order to determine the percentage of B cells within each stage of development. (A) Fraction C/C′ staining following 16 weeks of exposure. (B) Fraction F B cells following 8 weeks of exposure. *p < 0.05; **p < 0.01; ***p < 0.001.
a significant decrease in bone tungsten concentration. However, the removal of tungsten from bone was much slower than the accumulation. This is analogous to lead concentrations in the bone (Bergdahl et al., 1998; Rabinowitz, 1991). In former lead workers, increased lead levels in bone were still present long after exposure, which resulted in increased lead levels in blood, confirming an endogenous exposure as stored lead is released from the bone (Glenn et al., 2003).

We observed a significant initial decrease in peripheral WBC counts and a trend toward leukocytopenia after 8 weeks of exposure. These data correlate with data from human peripheral blood mononuclear cells. Osterburg et al. (2010) found that treating human peripheral blood lymphocytes in vitro with 1mM and 10mM (183.84 and 1838.4 mg/l elemental tungsten, respectively) sodium tungstate resulted in a dose-dependent increase in the number of cells undergoing early apoptosis within 24 h. A decrease in WBC count could decrease immune surveillance and increase susceptibility to pathogens or malignant transformation in exposed individuals. In fact, exposure to tungsten increased RSV-associated morbidity and mortality in comparison to mice exposed to RSV alone (Fastje et al., 2012).

We hypothesized that tungsten would alter B-cell development, particularly early fractions. Indeed, we observed a significant increase in the percentage of cells in the late pro- and early pre-B cell fractions after 16 weeks of exposure to tungsten. Importantly, these fractions are undergoing or have completed rearrangement of the heavy immunoglobulin chain, a stage we hypothesize will have increased susceptibility to DNA damaging agents (Nussenzweig and Nussenzweig, 2010). Notably, tungsten did not increase the C/C′ Hardy fraction to levels present in leukemia, but any increase in this population would provide a larger pool of cells wherein a second oncogenic hit could occur. Furthermore, tungsten exposure correlated with an increase in the percentage of mature IgD+ B cells (Fraction F) following 1 week of treatment—a trend that was sustained until 8 weeks of treatment (Table 3, Fig. 5B). This only occurred at the intermediate and highest concentrations of tungsten. The increase observed in mature IgD+ B cells may be a result of an inability to properly control the release of cells from the marrow, or a block within a specific stage of B-cell development, but at present, the exact cause for this phenomenon remains unclear.

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The percentage of developing B cells varies with age (Miller and Allman, 2003), and therefore, the observed effects may be specific to the relatively young mice tested in our experiment. The timing of the exposure may significantly alter the effects of tungsten on developing B cells. Tungsten exposure may have a greater effect very early in the developmental process, e.g., in utero, when these cells are expected to be at their highest concentrations. Alternatively, if bone remodeling alters tungsten disposition, older mice with greater changes in bone biology may be more susceptible to the effects of tungsten. These different exposure scenarios are currently under investigation.

Removal of tungsten reversed the increase in the IgD+ B-cell populations such that the IgM/D+ cell percentage seen at 8 and 12 weeks was significantly decreased. Intriguingly, although 8 weeks of tungsten exposure did not significantly increase fraction C/C′ staining, removal of tungsten for 4 weeks significantly enhanced the percentage of this fraction. This effect,
however, was transient and not observed after 8 weeks of tungsten removal. Finally, several developmental fractions (Fig. 8B, Fractions A and D) that were not affected by continuous exposure were altered after removal of tungsten. Fraction A, representing pre-pro-B cells, was increased after 4 weeks of tungsten removal, whereas fraction D (small pre-B cells) was decreased after 8 weeks. Although no pattern is discernible in these data, considerable tungsten levels are still within the bone at this time, and perhaps the effects on these specific fractions are the result of an endogenous exposure of tungsten as it is removed from the skeleton. In fact, the changes observed within B-cell fractions A and C′ at 4 weeks posttungsten treatment may be the result of a rapid initial release of tungsten from the bone in the first 4 weeks following tungsten removal.

Sodium tungstate significantly increased the number of colonies formed by B-cell progenitors following 16 weeks of exposure. An increase in the number of colonies detected suggests either an increase in the number of progenitor cells relative to whole marrow or a change in clonogenicity. Flow cytometric data revealed no difference in the numbers of CLP populations (defined as: Lin−, Sca-1−, c-kit−, IL-7R+) of treated compared with nontreated mice, suggesting that the clonogenic capacity
FIG. 8. Effect of tungsten removal from the drinking water of exposed mice. C57BL/6J mice were exposed to 15 mg/l tungsten via drinking water for a period of 4 weeks, followed by a period of tap water for either 4 weeks (n = 4) or 8 weeks (n = 4). (A) After removal of bone marrow, tibia bones were analyzed for their elemental tungsten content by ICP-MS. The lower limit of detection for week 4 was 0.5 ppm, whereas the lower detection limit for weeks 8 and 12 had a value of 0.2 ppm. This limit is based upon element detected, instrument, and sample weight. (B) B lymphocyte developmental profile following removal of tungsten exposure. Gating was performed to exclude dead cells and debris from analysis. (C) Immunoblot for γH2AX and lamin B expression in whole marrow, with corresponding densitometry. Lanes correspond to different animal treatments as follows: 1–3, control; lanes 4–8, 15 mg/l tungsten for 8 weeks; and lanes 9–12, 15 mg/l tungsten for 4 weeks followed by tap water for 4 weeks. Each lane represents a separate mouse. *p < 0.05; **p < 0.01; ***p < 0.001.
of this progenitor population increased when exposed to tungsten in vivo.

Our study demonstrates that tungsten exposure, particularly at 15 mg/l and 200 mg/l, results in increased DNA damage in bone marrow resident B lymphocytes. The highest, 1000 mg/l, tungsten concentration often showed less DNA damage than the lower, 15 mg/l, concentration. It is possible that this highest tungsten concentration increases DNA repair efficiency or causes significant apoptosis of damaged cells so that our assays do not detect damage. These results warrant similar studies to be conducted with lower tungsten concentrations, e.g., 0.75 mg/l, to better assess the effects that environmental tungsten levels detected in the area of Churchill County, Nevada, may have on developing B lymphocytes. Additionally, we showed that DNA damage, as detected by γH2AX expression, not only persists following removal of tungsten from the drinking water but is enhanced. Again, this finding demonstrates tungsten’s continued cellular effects despite termination of oral administration as was shown in our B-cell developmental profile results. We did not show that tungsten is directly genotoxic although it has been reported that metallic tungsten particles can cause breaks in plasmid DNA (Mazuš et al., 2000). In this regard, tungsten would be similar to other members of the Group 6 periodic family—chromium (Zhitkovich et al., 2001) and molybdenum (Titenko-Holland et al., 1998), which have been shown to be genotoxic. We are actively investigating the potential for tungsten to interact with nuclear DNA.

We have shown that tungsten exposure results in several changes that could promote leukemogenesis. An increase in DNA damage, along with altered B-cell development and increased activity of progenitor cells, could possibly promote and expand a malignantly transformed or immature cell population. Changes within the immune system could further exacerbate/facilitate this process. Several other groups have linked tungsten exposure to tumorigenesis (Kalinich et al., 2005; Wei et al., 1985). We propose that tungsten could act as a tumor promoter by providing leukemic “hits” in multiple forms to developing B lymphocytes within the bone marrow. Tungsten exposure warrants continued investigation in genetically modified “pre-leukemic” mouse models.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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