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Arsenite interacts with DBC at low levels to suppress bone marrow lymphoid progenitors in mice

Peace C. Ezech, Fredine T. Lauer, Ke Jian Liu, Laurie G. Hudson, and Scott W. Burchiel*

Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, New Mexico, USA

Abstract

Arsenite and Dibenzo[*d e f, p*]chrysene (DBC), a polycyclic aromatic hydrocarbon (PAH), are found in nature as environmental contaminants. Both are known to individually suppress the immune system of humans and mice. In order to determine their potential interactive and combined immunosuppressive effects, we examined murine bone marrow (BM) immune progenitor cells' responses following combined oral exposures at very low levels of exposure to As^{+3} and DBC. Oral 5-day exposure to DBC at 1 mg/kg (cumulative dose) was found to suppress mouse BM lymphoid progenitor cells, but not the myeloid progenitors. Previously established no-effect doses of As^{+3} in drinking water (19 and 75 ppb for 30 days) produced more lymphoid suppression in the bone marrow when mice were concomitantly fed a low dose of DBC during the last 5 days. The lower dose (19 ppb) As^{+3} had a stronger suppressive effect with DBC than the higher dose (75 ppb). Thus the interactive toxicity of As^{+3} and DBC *in vivo* could be As^{+3} -dose dependent. *In vitro*, the suppressive interaction of As^{+3} and DBC was also evident at low concentrations (0.5 nM), but not at higher concentrations (5 nM) of As^{+3} . These studies show potentially important interactions between As^{+3} and DBC on mouse BM at extremely low levels of exposure *in vivo* and *in vitro*.

Keywords

Arsenite; Dibenzo[*def,p*]chrysene; Bone marrow; Immunosuppression; Lymphoid; PAHs

Introduction

Arsenic and polycyclic aromatic hydrocarbons (PAHs) are environmental toxicants that are individually known to produce immunotoxicity in humans and mice [1–5]. Our previous studies showed that As^{+3} given orally at low levels selectively suppressed bone marrow (BM) lymphoid progenitor cells responses *in vivo* and *in vitro*, and spleen T-dependent antibody responses (TDAR) *in vitro* [6]. Immunosuppression is one of the major adverse manifestations of As^{+3} and PAH exposures. Dibenzo[*def,p*]chrysene (DBC), like the highly toxic 7,12-dimethylbenz(a)anthracene (DMBA), is known to suppress T-dependent antibody responses (TDAR) of murine spleen cells following *in vivo* and *in vitro* exposures [7,8,9,10]

*Corresponding author sburchiel@salud.unm.edu, Phone: 505-272-0920, Fax: 505-272-6749.

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and, in combination with As^{+3} , these PAHs were found to produce synergistic immunosuppression of the TDAR *in vitro* [4].

Our studies were motivated by the knowledge that PAHs produce immunosuppression in lymphoid organs, such as spleen and BM, through the formation of reactive metabolites that form DNA adducts with induction of p53 [7–9, 11, 12,]. Since PAH-adducts can be repaired by both DNA Base Excision Repair (BER) and Nucleotide Excision Repair (NER) pathways that are sensitive to inhibition by As^{+3} [13–14], we postulated that low concentrations of PAHs that are not cytotoxic to BM may produce increased immunotoxicity in the presence of As^{+3} .

We sought to investigate the possible influence of co-exposure to both pollutants and their possible interactions in the BM in mice at very low levels through physiologically relevant routes of exposure. Concentrations of 19 and 75 ppb As^{+3} in 30 day drinking water were previously established to be non-toxic to the BM [6], and 0.1 and 1 mg/kg cumulative doses of DBC in pill forms [10] were chosen based on previous dose-range studies.

Methods

Animals and Treatment Exposures

C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, Maine, USA, at 8–10 weeks old and housed in our AAALAC-approved facility, for use in this study at 12–16 weeks. These animals were handled according to procedures and protocols that have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico Health Sciences Center. About 2–3 animals were housed per cage and exposed to different concentrations of arsenite (As^{+3}) in parts per billion (ppb) for 30 days via drinking water and/or given Dibenzo[*def*, *p*]chrysene (DBC) by voluntary ingestion of a pill containing the chemical. Double processed tissue culture water (Sigma Aldrich), was used to prepare arsenite stock solutions. The cookie pill containing the DBC was prepared using Transgenic Dough Diet, DMSO, bromophenol blue and DBC according to the procedure previously described [10].

Water samples were collected periodically and sent to University of Arizona Laboratory for Emerging Contaminants (ALEC, Tucson AZ) to determine the As^{+3} concentrations in the control (no As^{+3}) drinking water and to confirm As^{+3} levels in prepared solutions used for these studies. The concentration of As^{+3} in the control drinking water and that used to make solutions was found to be less than 5 ppb. For the water consumption of the mice in each cage, the water bags containing water were weighed prior to placement in the cage and again after water consumption by the mice. Mice were fed a Mouse 2020X Teklad Global Soy Protein-Free Extruded Rodent Diet purchased from Harlan Laboratories Inc, Madison Wisconsin, USA, www.harlan.com. The total arsenic content of this diet was found to be 0.16 mg/kg (160 ppb), with approximately 10–15% estimated to be inorganic arsenic, As^{+3} and As^{+5} , as discussed previously [2].

For *in vivo* studies, 45 male C57BL/6J mice were exposed to As^{+3} via drinking water for 30 days and/or exposed to DBC via pill ingestion. The mice were treated in groups of five for

each treatment and bone marrow cells (BM) from each exposed mouse was isolated and analyzed in triplicate. For *in vitro* studies, bone marrow cells isolated from both femurs of three untreated C57BL/6J mice were pooled together, exposed to each treatment and analyzed in triplicate. All animals were handled and disposed of in accordance with the University of New Mexico's Department of Safety and Risk Services.

Chemicals and Reagents

Sodium arsenite (CAS 774-46-5, NaAsO₂) was purchased from Sigma-Aldrich (St. Louis, MO). The PAHs were purchased or obtained at greater than 95% purity from the following sources: dibenzo[*def,p*]chrysene (DBC, Accustandard), DBC-11,12 dihydrodiol was provided by Dr. David Williams at Oregon State University. MethoCult GF Methylcellulose Medium (Cat. No. M3534) with recombinant cytokines (without EPO) for mouse cells was purchased from Stem Cell Technologies (Vancouver, BC, Canada). Mouse Methylcellulose Complete Medium for pre-B cells (Cat. No. HSC009) was purchased from R&D Systems (Minneapolis, MN).

Arsenic and PAH handling

Arsenic, DBC and DBC-diol and all animals exposed to these chemicals were handled and disposed according to the University of New Mexico's Department of Safety and Risk Services.

Isolation of Mouse Bone Marrow Cells

Bone marrow cells were isolated according to the procedure outlined in the Stem Cell Technologies Technical Manual version 3.1.1 (<http://www.stemcell.com/>) and as described in previous work [6]. Briefly, each mouse was euthanized and the femurs were sterilely harvested, then placed and held on ice in Hanks' Balanced Salt Solution (HBSS) from Lonza Walkersville, MD, and transported to the laboratory. Bone marrow cells were extracted from the femurs using a 25 gauge needle with a 1 cc syringe, filled with approximately 1 ml cold sterile medium which was used to flush through the femur several times to release cells into a cell culture dish containing RPMI medium supplemented with FBS. The cells solution was immediately transferred to a 15 ml culture tube and placed on ice until needed. Cells were washed by centrifugation at 4°C, 300 × g for 10 min and were resuspended in RPMI Medium for culturing. Cell recovery and viability was determined by manual counting or automated counting using the Nexcellom Cellometer 2000.

CFU-B Assay

The procedure for Mouse Colony-Forming Cell (CFC) assays described in the Stem Cell Technologies Technical manual version 3.1.1 (<http://www.stemcell.com/>) was used to determine the pre-B and granulocyte-monocyte colony forming units (CFU) counts per pair of mice femurs and per million bone marrow cells. Briefly, bone BM cells marrow cells isolated from femurs of each treated or untreated mouse (for *in vivo* studies) or pooled from femurs of three untreated mice (for *in vitro* studies), were suspended in RPMI 1640 Medium supplemented with 2% heat inactivated Hyclone Fetal Bovine Serum (Fisher Scientific, Pittsburgh, PA) at 1×10⁶ cells/ml. 400 µl (4×10⁵ cells) of the cell suspension was transferred

to a 16 ml (17×100 mm) sterile culture tube which containing 4 ml Mouse Methylcellulose Complete Media for pre-B Cells. The tube was vortexed and left to sit for 20 min to release air bubbles. 1 ml of the methylcellulose-cell suspension was placed in a 35 mm culture dish (Stem Cell Technologies) using a 3 cc syringe with a 16G×11/2" Monoject Aluminum Hub, blunt cannula needle Covidien Mansfield, MA. Samples were ran in triplicate. The mixture was evenly dispersed in the dish by rocking the dish. One sterile water dish and two sample dishes were placed into a 100 mm culture dish and incubated at 37°C, 5% CO₂, in a humidified incubator for 10 days. CFU-B colonies were counted and recorded for statistical analysis.

CFU-GM Assay

This assay was performed using the same procedure as in the CFU-B assay described above except that the isolated bone marrow cells were suspended in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with 2% heat inactivated Hyclone Fetal Bovine Serum at 2×10⁵ cells/ml, and 400 µl (8×10⁴ cells) of the cell suspension was transferred to a 16 ml (17×100 mm) sterile culture tube containing 4 ml MethoCult GF Methylcellulose Medium (Stem Cell Technologies). Like in the CFU-B assay described above, the mixture was dispersed evenly in the dish and sample dishes (35 mm) were placed in a 100 mm culture dish and put in the humidified incubator at 37°C and 5% CO₂ for 14 days. CFU-GM colonies were counted and recorded for statistical analysis.

Data Analysis and Statistics

Sigma Stat version 3.5 and Sigma Plot 12.0 software were used for data analysis. One-way analysis of variance (ANOVA) and Dunnett's t-test were applied to determine differences between control and treatment groups. In the *in vivo* studies, treatment groups consisted of four or five animals and each animal was analyzed in triplicate. Bone marrow cell recovery was expressed as the mean number of recovered bone marrow cells obtained from both femurs. Results were reported as CFU-B and CFU-GM per million cells and also per pair of femurs. For the *in vitro* experiments, a treatment group consisted of three replicates of one chemical treatment of pooled bone marrow cells or control. CFU-B and CFU-GM colony counts were reported as per million cells.

Results

C57BL/6J male mice, consisting of 4 mice per treatment dose, were exposed to 0, 0.01, 0.1, 1 or 10 mg/kg cumulative dose of DBC via voluntary ingestion of DBC pills daily for 5 days. As shown in Table 1, at these exposure concentrations, there was no effect on the recovery of viable cells from the BM. A dose-dependent decrease in CFU-B formation was detected when expressed on a per million cell basis (Fig. 1a) and on a per pooled femur basis (Fig. 1b). Greater than 90% inhibition of CFU-B colony formation was observed at the 10 mg/kg total cumulative dose of DBC given over 5 days.

Based on our previous work, we were interested in determining whether concomitant exposure of mice to low or no-effect doses of As⁺³ with DBC could produce significantly more suppression of the BM. In an *in vivo* experiment using 5 mice per dose group, mice

were exposed to 0, 19, or 75 ppb As⁺³ in drinking water for 30 days in the absence or presence of DBC at 0.1 or 1 mg/kg given during the last 5 days of As⁺³ exposure. As expected, results showed that neither As⁺³ nor DBC suppressed CFU-B levels on their own when expressed on a per million cell basis (Fig. 2a), but the combined exposures to 19 ppb As⁺³ and 1 mg/kg DBC did show significant suppression which was more than that seen in the 75 ppb As⁺³ and DBC individual exposures. DBC produced a small decrease in BM cell recovery at the 1 mg/kg exposure (Table 2) which resulted in a significant effect on CFU-B formation when expressed on an individual mouse pooled femur basis (Fig. 2b). An additional finding was that these combinations of As⁺³ and DBC did not suppress the formation of CFU-GM colonies (Fig. 2c, 2d). These findings are consistent with our previous studies showing the selective suppression of CFU-B in mouse BM following 30 day low level As⁺³ exposures [6].

To further investigate the possibility of As⁺³ interaction with DBC in the BM, mice BM cells were exposed to various concentrations of As⁺³, DBC, and DBC-diol (a DBC metabolite known to be the immediate precursor of DBC-diol, epoxide, which is thought to be important in DBC adduct formation in DNA) *in vitro*. Based on preliminary studies to determine no-effect doses of agents, we used 0.5 and 5 nM concentrations of As⁺³ and 0.1 and 1 nM concentrations of DBC (Fig 3a). We found that these levels of As⁺³ and DBC given alone did not suppress CFU-B formation *in vitro*. However, low concentrations of As⁺³ (0.5 nM) produced CFU-B suppression when combined with low level (0.1 nM) of DBC or DBC-diol (Fig 3a). The colony count in the As⁺³/DBC-diol combination was significantly different compared to both control and the individual parent compounds at the same doses, suggesting interaction. Increased As⁺³ concentration (5 nM) also produced suppression of CFU-B in the presence of 0.1 nM and 1 nM DBC, but not in the presence of DBC-diol. There were no interactions between As⁺³ and DBC at the higher concentrations of exposure.

Because we previously found that MMA⁺³ (an As⁺³ metabolite) may be responsible for the toxicity of As⁺³ on BM *in vivo* [6], we investigated possible interaction of MMA⁺³ with DBC or DBC-diol in *in vitro* cultures, using one concentration (5 nM) that was previously established to be minimally toxic to the BM and another at a ten times lower concentration (0.5 nM). Both concentrations of MMA⁺³ suppressed CFU-B formation in mouse BM (Fig 3b). However, we observed MMA⁺³/DBC interaction at the higher concentrations of both chemicals but not at the lower concentrations nor with DBC-diol.

Discussion

The present studies demonstrate for the first time that As⁺³ interacts with DBC when given orally at low doses *in vivo* and selectively suppresses pre-B formation in mouse BM. It was also shown that DBC alone is selectively toxic to BM CFU-B formation at a 1 mg/kg oral cumulative dose given over a five day period. In previous work, an acute intraperitoneal injection and oral administration of 50 mg/kg DMBA (a DBC-like PAH), suppressed BM CFU-B and CFU-GM in mice [3]. The effect of the DBC following oral administration on the BM has not been reported previously, nor has the toxicity of DBC at the concentrations we examined been studied. DBC toxicity is likely caused by the products of its

biotransformation [15]. DBC-dihydrodiol epoxide (DBCDE) is formed through the action of two enzymes, CYP1B1 and epoxide hydrolase [16]. DBC radical cation is formed through the action of peroxidases [17], and DBC-dione obtained through the breakdown of DBC-11, 12-dihydrodiol by aldoketoreductases (AKR), [18]. Thus, in addition to the formation of DNA adducts by DBCDE, there is also potential redox cycling leading to the production of reactive oxygen species (ROS) with resultant oxidative stress.

As⁺³ and its metabolites are known to be toxic. The biotransformation of As⁺³ by arsenite methyltransferase (AS3MT) produces methylated species with varying toxicities, including monomethylated forms such as monomethylarsonous acid (MMA⁺³) and monomethylarsonic acid (MMA⁺⁵), also dimethylated forms such as dimethylarsonous acid (DMA⁺³) and dimethylarsonic acid (DMA⁺⁵) [19, 20]. MMA⁺³ is considered the most toxic arsenic species [19] and has been previously implicated by our lab in BM toxicity [6]. Thus, an increase in the formation of MMA⁺³ from *in vivo* As⁺³ is expected to produce more BM suppression than the parent compound.

The combination of As⁺³ and DBC *in vivo* showed that there may be some interactions at low doses with slightly increased and selective suppression of CFU-B when 19 ppb, 30-day drinking water As⁺³ was given in combination with 1 mg/kg, 5-day oral cumulative dose of DBC. These doses did not significantly impact bone marrow cell recovery and viability in mice, nor did they suppress BM cell differentiation when given individually. The observed lack of significant effect on BM cell differentiation by individual exposures to these compounds may be due to a number of reasons, including: insufficient bioactive metabolites from the low doses administered, lack of induction of the enzymes responsible for their bioactivation or a redirection of metabolites to other pathways not associated with toxicity. It is however, noteworthy that 1 mg/kg DBC showed a statistically significant suppression of pre-B cells, but not CFU-GM when results were averaged for mice and expressed on a pooled femur basis. The selective and significant suppression of CFU-B over CFU-GM colony formation observed with 19 ppb As⁺³ and 1 mg/kg DBC combination, suggest that there may be some interaction at these doses. Although we could not further define the precise parameters responsible pertaining to the dose and time responses for interactions, we know that As⁺³ and/or DBC metabolites could be involved in the toxicity observed. It is possible that the presence of both compounds in the system could influence their individual metabolism and resultant toxicity or lack of toxicity, such that ratio may also be important. It is known that in the liver, As⁺³ alters the regulation of enzymes that are responsible for PAH bioactivation (CYP1s), thereby decreasing PAH metabolite formation [21, 22]. Other studies [23, 24] also indicate that As⁺³ diminishes the induction of the CYP1 enzymes. However, these studies were conducted at higher concentrations of As⁺³ (micromolar ranges) than used in our present work. As with DMBA [7], DBC is mostly metabolized by CYP1B1 in mouse lymphoid tissues [16]. Therefore, if high doses of As⁺³ interfere with the induction or activity of CYP1B1, this may explain the lack of interaction between As⁺³ and DBC at the higher concentration of As⁺³.

For our *in vitro* studies, the combination of no-effect, low levels (0.5 and 5 nM) of As⁺³ with no-effect, low levels (0.1 and 1 nM) of DBC and DBC-diol, produced significant

suppression of pre-B colonies that was greater than the individual parent compounds, but the suppression did not always increase with higher concentration of chemicals as expected.

We show evidence of complicated dose responses in As⁺³/DBC combination that may be explained by the different direct and indirect effects of the individual compounds as well as their metabolites and associated enzymes. As expected, 0.5 and 5 nM MMA⁺³ were more potent than the same concentrations of As⁺³ *in vitro*. However, our studies demonstrate significant MMA⁺³ interactions with DBC at the higher MMA⁺³ and DBC doses *in vitro*. This suggests possible differences in the mechanisms of action between As⁺³ and MMA⁺³ in their interactions with DBC *in vitro*. Therefore further studies are needed to understand the reason for the lack of interaction between DBC and the higher doses/concentrations of As⁺³ and MMA⁺³ used in the present studies in murine BM. The mechanisms responsible for interactions between DBC and As⁺³/MMA⁺³ are currently under investigation. We speculate that the interactions occur at multiple levels and involve both DNA damage and repair pathways (genotoxicity) as well as non-genotoxic pathways associated with cell signaling, and perhaps oxidative stress.

In conclusion, we have observed that low doses of DBC selectively target the lymphoid progenitors in mice, and that *in vivo* and *in vitro* there is a complex non-linear immunosuppressive interaction between As⁺³ and DBC. These findings are important because these interactions occur at environmentally relevant doses and via normal physiological routes of exposure.

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Fig. 1.

Pre-B colony formation in mice post 5 day *in vivo* cumulative exposure to DBC Number of CFU-B colonies per million BM cells [a] or per set of femurs [b] in mice following 5 day *in vivo* exposure to DBC via oral voluntary pill ingestion, 10 days post plating in mouse methylcellulose media for pre-B cell selection. Data shown are Means $\pm$ SD. *Significantly different compared to control ($p < 0.05$).

Fig. 2.

CFU-B and CFU-GM colony formation in mice post *in vivo* exposure to low level of As⁺³ ± DBC

Number of CFU-B colonies per million BM cells [a] or per set of femurs [b]; Number of CFU-GM colonies per million BM cells [c] or per set of femurs [d] in mice following 30 day *in vivo* exposure to As⁺³ via drinking water ± 5 day DBC exposure via oral ingestion. BM cells were plated in mouse methylcellulose media for pre-B cell selection for 10 days or in methocult media for GM selection for 14 days. Data shown are Means ± SD. *Significantly different compared to control (p<0.05). #Significantly different compared to effect of DBC or As⁺³ given as individual treatments at the selected dose.

Fig. 3.

In vitro exposure of mice BM cells to As⁺³ ± DBC or DBC-diol. Number of CFU-B colonies per million cells in untreated mice BM cells post 10 day plating in pre-B mouse methylcellulose media containing As⁺³ ± DBC or DBC-diol [a], or containing MMA⁺³ ± DBC or DBC-diol [b]. Data shown are Means ± SD. #Significantly different compared to effect of DBC or As⁺³ given as individual treatments at the selected dose. *Significantly different compared to control (p<0.05).

Table 1Effect of cumulative 5 day *in vivo* exposure to DBC on mice BM cell recovery and viability^a

DBC (mg/kg)	BM Cell Count ($\times 10^7$)	Viability (%)
0	5.9 $\pm$ 1.6	98.8 $\pm$ 2.4
0.01	6.8 $\pm$ 1.9	100 $\pm$ 0.0
0.1	7.4 $\pm$ 1.7	99.3 $\pm$ 1.5
1	4.8 $\pm$ 0.9	100 $\pm$ 0.0
10	5.8 $\pm$ 3.0	93.0 $\pm$ 8.6

^aResults shown are the Means $\pm$ SD

Table 2

In vivo low dose As⁺³ ± DBC has no effect on the cell recovery and viability of mice BM cells^a

Treatment	Cell count ($\times 10^7$)	Viability (%)
Control	3.2 $\pm$ 1.2	93.1 $\pm$ 3.6
19 ppb As ⁺³	3.0 $\pm$ 3.2	92.4 $\pm$ 1.6
75 ppb As ⁺³	3.0 $\pm$ 7.9	93.1 $\pm$ 2.8
0.1 mg/kg DBC	3.0 $\pm$ 6.8	94.0 $\pm$ 2.2
1 mg/kg DBC	2.3 $\pm$ 4.9	93.0 $\pm$ 1.1
19 ppb As ⁺³ + 0.1 mg/kg DBC	3.3 $\pm$ 3.7	92.7 $\pm$ 2.8
19 ppb As ⁺³ + 1 mg/kg DBC	3.2 $\pm$ 7.3	94.4 $\pm$ 1.4
75 ppb As ⁺³ + 0.1 mg/kg DBC	3.4 $\pm$ 4.4	92.8 $\pm$ 2.2
75 ppb As ⁺³ + 1 mg/kg DBC	3.2 $\pm$ 6.7	93.1 $\pm$ 2.5

^aResults shown are the Means $\pm$ SD