

Inorganic arsenite inhibits IgE receptor-mediated degranulation of mast cells

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ABSTRACT: Millions of people worldwide are exposed to arsenic (As), a toxicant which increases the risk of various cancers, cardiovascular disease and several other health problems. Arsenic is a potent endocrine disruptor, including of the estrogen receptor. It was recently shown that environmental estrogen-receptor disruptors can affect the signaling of mast cells, which are important players in parasite defense, asthma and allergy. Antigen (Ag) or allergen crosslinking of IgE-bound receptors on mast cells leads to signaling, culminating in degranulation, the release of histamine and other mediators. Because As is an endocrine disruptor and because endocrine disruptors have been found to affect degranulation, here we have tested whether sodium arsenite affects degranulation. Using the rat basophilic leukemia (RBL) mast cell model, we have measured degranulation in a fluorescence assay. Arsenic alone had no effect on basal levels of degranulation. However, As strongly inhibited Ag-stimulated degranulation at environmentally relevant concentrations, in a manner that is very dependent on concentrations of both As and Ag. The concentrations of As effective at inhibiting degranulation were not cytotoxic. This inhibition may be a mechanism underlying the traditional Chinese medicinal use of As to treat asthma. These data indicate that As may inhibit the ability of humans to fight off parasitic disease. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: arsenic; arsenite; mast cells; basophils; degranulation; RBL-2H3; endocrine disruptor; parasite; allergy; asthma

INTRODUCTION

The World Health Organization and Agency for Toxic Substances and Disease Registry consider arsenic (As) exposure to be a major human health concern (ATSDR, 1999; NRC, 1999). Worldwide, many millions of people around the world are exposed to elevated levels of As – in particular, inorganic arsenite – via drinking water, various foods (such as seafoods, Dabeka *et al.*, 1993; Tao and Bolger, 1999; Velez *et al.*, 1995; and poultry, Lasky *et al.*, 2004), pesticides, industry, toxic waste sites and mining sites. For example, millions of people are consuming excess As via their drinking water, particularly from unregulated wells contaminated from certain natural underground geological sources containing As (ATSDR, 1999; NRC, 1999). In recognition of emerging epidemiological and molecular/mechanistic data about the toxicity of As, the US EPA recently (January 2006) reduced the maximum allowable levels of As in public drinking water from 50 to 10 ppb. However, many people on private wells in the US and many others around the world continue to be exposed to elevated levels of As. For example, many regions of countries including Mexico, Bangladesh, India, Chile, Argentina and Vietnam have drinking water containing high levels of As, often above 50 ppb and sometimes ranging up to 2000 or 3000 ppb (ATSDR, 2007).

Many acute and chronic toxic health effects resulting from As exposure have long been well documented. Arsenic is classified as a human carcinogen by the US Environmental Protection Agency (EPA) and the World Health Organization (ATSDR, 1999; NRC, 1999). Chronic ingestion has been associated with increased risk of a number of serious diseases including various cancers (e.g. skin, lung, liver, kidney, bladder, and other internal cancers), type

II diabetes, cardiovascular disease and reproductive and developmental abnormalities (Abernathy *et al.*, 1999, 2003; ATSDR, 2005; NRC, 1999, 2001; Smith *et al.*, 1992; Tapio and Grosche, 2006; Watanabe *et al.*, 2003).

Arsenic also has been well described as a potent endocrine disruptor, an effect that may underlie many of its toxicities. Arsenic has been shown to disrupt transcription mediated by several steroid receptors: the receptors for glucocorticoids (Bodwell *et al.*, 2004; Hamilton *et al.*, 1998; Kaltreider *et al.*, 2001), progesterone (Bodwell *et al.*, 2006), androgens (Bodwell *et al.*, 2006; Rosenblatt and Burnstein, 2009), mineralocorticoids (Bodwell *et al.*, 2006) and estrogen (Davey *et al.*, 2007) as well as other related nuclear hormone receptors (i.e. receptors for thyroid hormone and retinoic acid; Davey *et al.*, 2008). For example, arsenic disrupted estrogen receptor-dependent gene transcription of the estradiol-inducible vitellogenin II gene in chick embryo livers *in vivo*, and similar results were found in human breast cancer MCF-7 cells (Davey *et al.*, 2007).

It has recently been shown that endocrine disruptors, in particular estrogen disruptors, can affect signaling of mast cells (Zaitzu *et al.*, 2007; Narita *et al.*, 2007), which are a key line of defense against parasites and are major asthma and allergy effector cells. These cells are found in the vast majority of human

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tissues, including heart (Dvorak, 1986), liver (Farrell *et al.*, 1995), kidney (Blank *et al.*, 2007), bladder (Theoharides and Sant, 1991), aorta (Pouchlev *et al.*, 1966), skin, lungs, connective tissues of various organs and mucosal epithelial tissues of respiratory, genitourinary and digestive tracts (Kuby, 1997). Specific antigen (Ag) or allergen crosslinking of IgE-bound FcεRI receptors on mast cells leads to signaling (Beaven and Metzger, 1993), beginning with a tyrosine phosphorylation cascade (Benhamou *et al.*, 1990; Paolini *et al.*, 1991) and proceeding to an influx of calcium into the cell (Ferris *et al.*, 1989; Putney *et al.*, 2001; Millard *et al.*, 1989). Degranulation follows and involves the release of cytoplasmic granules that contain histamine, leukotrienes, cytokines and enzymes which trigger the symptoms of allergy and asthma.

Allergy and asthma are more common in women than in men during early adulthood (after puberty), and many clinical and epidemiological studies have linked female reproductive hormones with increased incidence of asthma and allergy in humans (Debley *et al.*, 2004; Frye *et al.*, 2003; Lange *et al.*, 2001; Nicolai *et al.*, 2003). These epidemiological studies prompted Zaitzu and others to hypothesize that low, physiologically relevant levels (0.1 nM) of estradiol can increase the degranulation response of mast cells (Zaitzu *et al.*, 2007). These authors used rat basophilic leukemia (RBL-2H3) and other mast cell/basophil cell cultures, all of which naturally express the estrogen receptor alpha. They showed that estradiol alone causes degranulation, and estradiol also enhances Ag-stimulated degranulation in an estrogen receptor alpha-dependent manner (Zaitzu *et al.*, 2007). Subsequently, Narita and others showed that several environmental estrogenic endocrine disruptors, which affect estrogen receptor signaling, also enhance mast cell degranulation (Narita *et al.*, 2007). Thus, estrogen receptor-disrupting pollutants may affect allergy, asthma and parasite defense by altering mast cell degranulation.

In addition to its endocrine-disrupting activity, As can affect tyrosine phosphorylation cascades (Qian *et al.*, 2003) and other components of signaling pathways similar to those comprising the pathway leading to mast cell degranulation. For example, As has been shown to interfere with T cell signal transduction by affecting phosphorylation of the kinases Lck and Fyn (Soto-Pena and Vega, 2008). These findings motivated us to investigate As's effects on mast cell degranulation.

In addition, the recent correlation of estrogen receptor signaling to mast cell signal transduction (Narita *et al.*, 2007; Zaitzu *et al.*, 2007) has raised the question of whether or not As, as an estrogen receptor disruptor (Davey *et al.*, 2007), could also affect allergy signaling. Our laboratory has addressed this question by utilizing RBL cells, a well-accepted model system of allergic signaling that has been used extensively. RBL cells are homologous to mucosal mast cells with regard to histology and secretory granule biochemistry (Seldin *et al.*, 1985). We have sensitized RBL cells with anti-DNP (dinitrophenyl) IgE antibody (which binds to the high-affinity rat IgE receptor, FcεRI), stimulated them with DNP-BSA Ag in the presence of inorganic arsenite, and measured degranulation. We have measured degranulation in a fluorescence microplate assay based on release of the enzyme β-hexosaminidase from granules during degranulation (Naal *et al.*, 2004). β-Hexosaminidase is a good marker for degranulation because its release closely follows and is linearly related to release of the major allergic mediator histamine from granules (Schwartz *et al.*, 1979).

We first tested for As cytotoxicity in RBL cells and found that high-dose (1500 ppb) As is not cytotoxic to RBL cells during a

short-term (1 h) exposure that simulates the exposure during the degranulation experiments. Clonal survival (clonogenic) 24 h cytotoxicity tests of As in RBL cells showed that the 24 h LC₅₀ of As on RBLs is greater than 2000 ppb. In contrast to the stimulatory effects found to be elicited by estradiol and xenoestrogens (Narita *et al.*, 2007; Zaitzu *et al.*, 2007), we have found that As alone has no effect on degranulation. Instead, interestingly, As inhibits crosslinker-stimulated degranulation. Moreover, this effect is dependent on the concentrations of both As and Ag. Our data show significant inhibition of degranulation by As, suggesting that As may suppress the body's defense against parasites. Also, this inhibition may be a mechanism underlying the traditional Chinese medicinal use of As to treat asthma.

METHODS

RBL-2H3 Cell Culture

We utilized RBL clone 2H3, which had been selected for its robust degranulation (Barsumian *et al.*, 1981); these cells express plentiful IgE receptors ($2\text{--}3 \times 10^5$ per cell) at the plasma membrane. RBL-2H3 (RBL) cells (Barsumian *et al.*, 1981) were a generous gift from D. Holowka (Cornell University, Ithaca, NY, USA). Monolayer cultures of RBL cells were maintained with Eagle's minimal essential medium (EMEM, with EBSS and L-glutamine; BioWhittaker Lonza, Walkersville, MD, USA) containing 20% fetal bovine serum (Premium; Atlanta Biologicals, Lawrenceville, GA, USA) and $10 \mu\text{g mL}^{-1}$ gentamicin sulfate (BioWhittaker Lonza) at 37 °C and 5% CO₂ for a maximum of 3 months per vial thawed. Cells were split once per week, using trypsin (0.05% porcine trypsin with EDTA in HBSS with no calcium and no magnesium; HyClone Laboratories Inc., Logan, UT, USA) for 5 min at 37 °C. All tissue culture plates and flasks used were made from polystyrene. No plastics containing known endocrine disruptors (e.g. bisphenol A) were used in experiments. All media and buffers were sterile filtered using ZapCap filters (0.2 μm filter; Whatman, Florham Park, NJ, USA). Tests for mycoplasma were regularly performed on supernatant media from RBL cell culture flasks with the MycoAlert mycoplasma detection kit (Lonza, Rockland, ME, USA). The manufacturer's recommended protocol and a microplate luminometer (Synergy 2; Biotek, Winooski, VT, USA) were used. No mycoplasma contamination was detected in these approximately monthly tests.

Arsenic Stock Preparation

Arsenic (As) for all experiments was prepared under sterile conditions as 10 mM stocks of inorganic sodium metaarsenite (+3 oxidation state; Baker Analyzed, 98.0% minimum, NaAsO₂, CAS no. 7784-46-5; JT Baker, Phillipsburg, NJ, USA), and dissolved in cell culture water (sterile, reverse osmosis, deionized, distilled water for Cell Culture Applications; BioWhittaker Lonza). It was next filtered with a 0.2 μm syringe filter for sterility, aliquoted into sterile polypropylene microfuge tubes, and frozen at −20 °C until the day of use.

Trypan Blue Cytotoxicity Assay

A trypan blue cytotoxicity assay was used to test whether As concentrations up to 1500 ppb were cytotoxic during a 1 h expo-

sure, analogous to the exposure during the degranulation assay (see below). RBL cells were plated at 1.5×10^6 cells per well in six-well, sterile, tissue culture-treated plates and were incubated overnight at 37 °C and 5% CO₂. On day two, the As dilutions were made with Tyrodes–BSA buffer. Tyrodes–BSA contains 135 mM NaCl (extra pure; EMD, Darmstadt, Germany), 5 mM KCl (JT Baker), 1.8 mM CaCl₂ (dihydrate; Acros organics, Morris Plains, NJ, USA), 1 mM MgCl₂ (hexahydrate; BDH VWR, West Chester, PA, USA), 5.6 mM D-(+)-glucose (anhydrous ACS reagent grade; MP Biomedicals, Solon, OH, USA), 20 mM HEPES sodium salt (ultra-pure bioreagent; JT Baker), and 1 mg mL⁻¹ bovine serum albumin (BSA; low heavy metals, fraction V; Calbiochem, La Jolla, CA, USA), at pH 7.4. All ingredients for all experiments were selected for at the highest possible purity to minimize the risk of contaminants causing toxicological effects.

Media from the incubated six-well plates was discarded, and 2 ml of each As sample in Tyrodes–BSA was added to the cells for a 1 h, 37 °C incubation. After the incubation, the buffer containing As was removed, and the wells were washed with 2 ml per well of fresh Tyrodes–BSA. Following the removal of this wash, the cells were next washed with 1 ml per well trypsin, which was then, likewise, removed and discarded. After a further addition of 1 ml per well of trypsin, the plate was left to incubate for 10 minutes at 37 °C for complete cell dislodgment. Harvesting of the cells was completed by repeated pipetting, up and down, dislodging the cells, and trypsin was quenched by addition of RBL media to each cell sample. Viable cells were identified using trypan blue stain (0.4%; BioWhittaker Lonza) exclusion and counted with a hemocytometer.

Each treatment group was repeated in three separate experiments, with three total wells of cells per treatment group per experiment. Statistical significance was determined with Prism (Graphpad, San Diego, CA, USA) as determined by one-way ANOVA with a Tukey's post-test. Error bars represent means $\pm$ SEM.

Lactate Dehydrogenase Cytotoxicity Assay

Lactate dehydrogenase (LDH) activity release was measured in the culture medium of As-exposed RBL cells by using an LDH assay kit (Cytotoxicity detection kit^{PLUS}; Roche Diagnostics, Indianapolis, IN, USA). This colorimetric assay, used as an indicator of cell membrane damage or cell death, is based on the reduction of a tetrazolium salt, an event that generates a red product only when LDH activity is present. To assay any cytotoxic effect As might have had on the RBL cells, cells were harvested and reconstituted to 0.1×10^6 cells mL⁻¹ in fresh RBL culture media. Aliquots of 100 μ L (10 000 cells) of this mixture were plated in each well of optically clear, flat-bottom 96-well plates (CoStar; Corning Inc., Corning, NY, USA); this cell concentration was found to be optimal (refer to kit instructions). The plate was then incubated at 37 °C, 5% CO₂ overnight.

The Roche Cytotoxicity detection kit^{PLUS} was used at room temperature (<25 °C) to assay cytotoxicity on the second day, according to the manufacturer's instructions. Briefly, As was diluted into Tyrodes–BSA buffer (to make 10–1500 ppb As samples). Plated cells were washed twice with Tyrodes–BSA. Aliquots of 100 μ L As samples were added to cell wells. Aliquots of 100 μ L plain Tyrodes–BSA buffer were added to cell wells for high controls and to wells containing no cells for background controls. The reaction mixtures were incubated for 1 h at 37 °C. Following this, 5 μ L of Lysis solution was added to high

control samples only, and the plate was incubated for another 15 min at 37 °C. Manufacturer's instructions were followed for the remainder of the procedure, and the plate was read using a spectrophotometric microplate reader (Synergy 2; Biotek) at an absorbance of 490 nm. Experimental conditions were optimized to result in non-saturating absorbance values. The average background control absorbance value was subtracted from all raw data values, and 'percentage cytotoxicity' was calculated per kit instructions. Each treatment group was repeated in five separate experiments, each of which contained three total wells of cells per treatment group. Statistical significance was determined with Prism as determined by one-way ANOVA with a Tukey's post-test. Error bars represent means $\pm$ SEM.

To eliminate the possibility that data were the result of As's having a direct effect on LDH enzyme activity, we directly tested the effects of As on activity of purified LDH enzyme. A 50 μ L aliquot of 0.000078 U mL⁻¹ purified LDH enzyme standard (Cayman chemical company, Ann Arbor, MI, USA) was incubated for 1 h at 37 °C with 50 μ L of each 2 $\times$ As stock in Tyrodes–BSA in each well of a clear, flat-bottom 96-well plate (CoStar; Corning Inc.; three replicates per treatment group per experiment and two separate experiments were done). The remainder of the experiment followed the manufacturer's recommended protocol (Cytotoxicity detection kit^{PLUS}, Roche Diagnostics) and the procedure described above. 'Relative absorbance units of LDH standard' were calculated by dividing the average absorbance value for each sample by that of the 0 ppb As-LDH standard control, and statistical significance was determined by one-way ANOVA with a Tukey's post-test (in Prism). LDH activity was not affected by As up to 1500 ppb (data not shown), indicating that As has no effect on the LDH enzyme itself.

Clonogenic Cell Survival Cytotoxicity Assays

RBL cells were grown to confluence in complete RBL media (see above) until clonogenic assays were started. All steps of clonogenic assays were performed using standard RBL media. Approximately 300 cells per well were plated into six-well plates and allowed to grow overnight at 37 °C and 5% CO₂. They were then exposed to 5–15 000 ppb As in 2 ml cell culture media per well for 24 h at 37 °C and 5% CO₂. At the end of this 24 h incubation, the media were changed to wash out the As. After 4–5 days of cell growth at 37 °C and 5% CO₂, colonies of 25 cells or more had formed in control wells, so the media were removed, cells were washed with phosphate buffered saline (with no calcium or magnesium; BioWhittaker Lonza) buffer (PBS), and the cells were fixed with 100% methanol (ACS grade; BDH) for 5 min at room temperature. Following the removal of the methanol, the cells were stained with 0.4% (w/v) Giemsa stain (MP Biomedicals) in 70% methanol for 90 min at room temperature. Excess stain was washed away with PBS, and colonies were counted. Results are given as a percentage of the 0 ppb As control, and each condition was repeated in triplicate within each experiment. Five independent experiments were performed, with each individual As dose repeated in two to five separate experiments. Error bars represent average $\pm$ SEM. The sigmoidal dose–response curve was produced with Prism's dose–response curve analysis, using the mean value for each dose. The 24 h LC₁ and LC₅₀ values were determined using EPA's Probit analysis program.

Degranulation Assay

Degranulation of RBL cells releases the enzyme β -hexosaminidase into the cells' supernatant. β -Hexosaminidase levels can be quantified with the synthetic substrate 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide. The enzyme cleaves this substrate and releases the fluorescent product methylumbelliferone. Degranulation was determined using the standard β -hexosaminidase microplate assay as described previously in detail (Naal *et al.*, 2004). A description of the assay used in this current study, with alterations from this published assay (Naal *et al.*, 2004), is included here.

RBL cells were harvested after 2–4 days of growth via trypsinization (5 min, 37 °C), were centrifuged at 500 *g* for 8 min, and were resuspended at 0.5×10^6 cells mL^{-1} in fresh RBL media. Cell aliquots (100 μL) were plated in black flat-bottom, tissue-culture treated, sterile 96-well plates (Greiner Bio-One) in a randomized fashion so as to minimize systematic error. All subsequent washes and additions to the plates in this assay were randomized in order to eliminate systematic error. Plates were incubated overnight in a 5% CO_2 incubator at 37 °C.

Each experimental plate was designed to contain the following: (1) Antigen (Ag) stimulation, IgE sensitization followed by Ag stimulation of cells to elicit the degranulation response; (2) spontaneous release, to measure basal release of β -hexosaminidase in cells exposed to neither IgE nor Ag, only Tyrodes–BSA buffer; and (3) Triton-X 100 detergent cell lysis, to measure total release of β -hexosaminidase from cellular granules upon Triton-X 100 (Surfact-Amps X-100, 10%, low carbonyl and peroxide; Thermo scientific, Rockford, IL, USA) detergent lysis of RBL cells (exposed to neither IgE nor Ag).

On the second day, cells in the Ag grouping received 0.1 $\mu\text{g mL}^{-1}$ of anti-dinitrophenyl (DNP) mouse IgE (monoclonal, clone SPE-7; Sigma, St Louis, MO, USA) in RBL cell media (100 μL per well) for 1 h at 37 °C and 5% CO_2 for sensitization of the IgE receptors. After the 1 h IgE incubation, the media was removed from the plate, and each well was twice washed with Tyrodes–BSA buffer to remove excess IgE.

Cell samples then received 200 μL aliquots of either Ag (for Ag stimulation), plain Tyrodes–BSA (for Spontaneous Release) or Triton-X 100 (for Triton-X 100 detergent cell lysis), as planned, with simultaneous As additions (to Ag, spontaneous or Triton-X 100 samples) as indicated in the Results. Antigen samples were solutions of Tyrodes–BSA containing various concentrations of the multivalent Ag DNP–BSA, which is BSA conjugated with an average of 15 DNP groups. The DNP–BSA was prepared as described previously (Hardy, 1986) and was a generous gift from D. Holowka and B. Baird (Cornell University). (Multiple DNP haptens on the BSA carrier create a multimeric Ag which is capable of crosslinking many anti-DNP IgEs together.) The Triton-X 100 was diluted to 0.2% Triton-X 100 detergent in Tyrodes–BSA. Tyrodes–BSA was added to three cell-free wells on each plate to measure background fluorescence. The plate was then incubated at 37 °C with 5% CO_2 for 1 h of Ag stimulation/As treatment.

Following this incubation, degranulation was stopped by placement of the cells on ice. To determine quantitatively the amount of β -hexosaminidase released by the cells, 25 μL of each well-mixed cell supernatant and 100 μL of 1.2 mM β -hexosaminidase-substrate (4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide; Calbiochem), in acetate buffer, were mixed together in another 96-well plate. (The 100 μL substrate samples

had been aliquoted into the new plate in advance.) Acetate buffer contained 0.12 M acetic acid (glacial, ACS and USP grade; BDH Aristar/VWR, West Chester, PA, USA) titrated to a pH of 4.4 with sodium hydroxide (10N, Baker Analyzed; JT Baker). This plate containing the enzyme–substrate mixtures was then incubated for 30 min at 37 °C. The reaction was quenched by addition of 200 μL per well of glycine-carbonate buffer at room temperature. The glycine-carbonate buffer contained 0.356 M glycine (Omnipur; EMD, Gibbstown, NJ, USA) and 0.444 M sodium carbonate (anhydrous, granular, ACS grade; Mallinckrodt, Phillipsburg, NJ, USA), titrated to pH 10.

β -Hexosaminidase activity in the cell supernatants was determined by measuring the fluorescence intensity of the hydrolyzed substrate in a fluorescence microplate reader (Synergy 2; Biotek) using 360/40 nm excitation and 460/40 nm emission filters, normal speed, sensitivity setting 35, top 50% optics position and 7 mm top optical offset. The described experimental conditions resulted in non-saturating fluorescence values. Raw data were processed by subtracting background fluorescence (substrate in Tyrodes–BSA buffer alone, with no cell supernatant) from all other readings. Each sample's background-corrected value was then divided by the plate's average Triton-X 100 value in order to give a 'percentage degranulation' value. For each experiment performed, the percentage degranulation response that was elicited in the presence of each dose of As was normalized to that of the 0 ppb As control on the same experimental day; this was done before averaging data from multiple days. Appropriate controls were performed to ensure that the plate position of the samples to be tested did not affect the degranulation values obtained through fluorescence.

For Fig. 2(A), five separate experiments were performed, and each dose was repeated in four or five separate experiments, with three replicates per dose per experiment. Statistical significance was determined with Prism as determined by one-way ANOVA with a Tukey's post-test (as compared to the 1 ppb sample is the source of the significance levels indicated by the asterisks). For Fig. 2(B), 12 separate experiments were performed, and each dose was repeated in 3–12 separate experiments, with three replicates per dose per experiment. Statistical significance was determined with Prism as determined by one-way ANOVA with a Tukey's post-test (as compared to the 5 ppb sample). For Fig. 2(C), four separate experiments were performed, and each dose was repeated in three or four separate experiments, with three replicates per dose per experiment. Statistical significance was determined with Prism as determined by one-way ANOVA with a Tukey's post-test (as compared with the 1 ppb sample). Error bars represent means $\pm$ SEM in Fig. 2(A–C). In Fig. 2(D), error bars represent the standard deviation of triplicate samples for each Ag dose (except for only two replicates for the 0.0002 $\mu\text{g mL}^{-1}$ sample) from a single representative data set. For the Fig. 4 data, five separate experiments were performed, and each dose was repeated in three to five separate experiments, with three replicates per dose per experiment. Statistical significance was determined with Prism as determined using a one-way ANOVA with a Tukey's post-test. Error bars represent means $\pm$ SEM.

Degranulation Assay with Anti-IgE Antibody Stimulation

The degranulation protocol was followed exactly as above, except that 10 $\mu\text{g mL}^{-1}$ mouse anti-IgE IgG antibody (clone M0784; Sigma) was used in place of DNP–BSA Ag to crosslink the IgE receptors so as to stimulate the RBL cells. For Fig. 3, four

separate experiments were performed, and each dose was repeated in three or four separate experiments, with three replicates per dose per experiment. Statistical significance was determined with Prism as determined by one-way ANOVA with a Tukey's post-test (as compared with the 10 ppb sample, which is the source of the significance levels indicated by asterisks). Error bars represent means $\pm$ SEM.

β -Hexosaminidase activity control assay

The accuracy of this assay for degranulation depends on a productive β -hexosaminidase–substrate interaction. Therefore, we tested whether or not As had a direct effect on β -hexosaminidase's enzyme activity. Cells were *not* exposed to As. Instead, IgE-sensitized cells were exposed to 0.0004 $\mu\text{g ml}^{-1}$ DNP–BSA alone, and cell supernatants – essentially crude preparations of β -hexosaminidase enzyme – were collected. These cell-free supernatants were combined and incubated for 1 h at 37 °C with arsenic plus 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide substrate in acetate buffer, so As was present *only* during the enzyme–substrate reaction step. All other experimental steps and data analyses were identical to those explained above for the degranulation assay (four separate experiments, with three replicates per treatment group per experiment). We found that As did not affect β -hexosaminidase's activity and ability to form the fluorescent product (data not shown).

RESULTS

High-dose (1500 ppb) As is not Cytotoxic to RBL Cells During a Short-term (1 h) Exposure

We first tested whether As, up to a rather high dose (1500 ppb), is cytotoxic to RBL cells during a short 1 h exposure, keeping with the conditions of the subsequent degranulation experiments. The effects of As (in Tyrodes–BSA buffer) on RBL cell survival were assessed by a trypan blue-exclusion cytotoxicity assay (Fig. 1A). Viable cells excluded trypan blue dye and were counted. The data in Fig. 1(A) show no statistical significance to suggest toxic effects up to 1500 ppb.

The effects of As (in Tyrodes–BSA buffer) on RBL cell survival were also assessed by an alternative cytotoxicity assay, the LDH release assay (Fig. 1B; Roche Diagnostics). LDH is a cytoplasmic enzyme found in all cells and released upon cell death or plasma membrane damage. The data in Fig. 1(B) show that, over a wide range of As doses, there was no significant LDH release from the cells, signifying that there is no plasma membrane damage or death up to 1500 ppb As during a 1 h exposure. Together, the data in Fig. 1(A, B) strongly suggest that there is no cytotoxic effect of As, up to 1500 ppb, on RBL cells during a 1 h exposure in Tyrodes–BSA.

Cytotoxicity (24 h) of As in RBL Cells

The clonal survival (clonogenic) assay is considered the gold standard of cytotoxicity tests (Komissarova *et al.*, 2005), and it detects cell death via both necrosis and apoptosis. Thus, we used this colony-forming method to determine the longer-term (24 h) cytotoxic doses of As in RBL cells (Fig. 1C). After a 24 h exposure to As, cells were changed into fresh media and allowed to grow for

several days to form colonies. Counting was done to determine colony-forming ability after the As exposure. We found that As has a 24 h LC_{50} value of 2100–2600 ppb (95% confidence interval), which is far higher than the doses showing effects on degranulation (see below). Also, the 24 h LC_1 value was found to be 350–760 ppb (95% confidence interval), near the high range of the doses we use in our 1 h degranulation assay.

As Inhibits the Degranulation Response of RBL Cells to Various Antigen Doses

The effects of As on antigen-stimulated RBL cell degranulation were assessed by a fluorescence-based assay (Naal *et al.*, 2004) for the release of the granular enzyme β -hexosaminidase into cell supernatant. In this 96-well plate assay, attached cells were treated with anti-dinitrophenyl (DNP) mouse IgE, to sensitize cell surface IgE receptors. Next, cells were stimulated for 1 h with various doses of DNP–BSA antigen. Various non-cytotoxic (see Fig. 1) As doses were mixed with the DNP–BSA, in Tyrodes–BSA, in order to assay the effects of As on degranulation. Additionally, a group of cell samples received Triton-X 100 detergent during this period, in order to measure the total amount of β -hexosaminidase in the cells, for the purpose of normalization (to generate 'percentage degranulation' values). After the As/antigen incubation period, the assay for released β -hexosaminidase was performed as described in 'Methods,' and the relative degranulation response, normalized to the 0 ppb As control average value, is plotted against the doses of As that the cells received (Fig. 2A–C).

In these experiments, a low to maximal range of antigen doses were used. These antigen doses were chosen based on the relative degranulation responses that they elicited when no As was present. A representative curve for DNP–BSA antigen dose response in the RBL cells is shown in Fig. 2(D). First, a low antigen dose, 0.00016 $\mu\text{g ml}^{-1}$, which elicited an average absolute degranulation response of $12 \pm 2\%$ (SEM; ~23% of the maximal response; see Fig. 2D) in the absence of As, was chosen for As dose–response experiments (Fig. 2A). Statistically significant inhibition of degranulation function began at 100 ppb As, where degranulation levels were 0.86-fold $\pm$ 0.03 (SEM) of the 0 ppb control levels (Fig. 2A). Increasing levels of As exposure further inhibited degranulation, with a clear As dose–response. At 250 ppb As, degranulation levels were 0.74-fold $\pm$ 0.02 (SEM) of the 0 ppb control levels. At 500 ppb As, degranulation levels were 0.59-fold $\pm$ 0.04 (SEM) of the 0 ppb control levels. At 750 ppb As, degranulation levels were reduced to 0.49-fold $\pm$ 0.04 (SEM) of the 0 ppb control levels. These data indicate a strong inhibition of the multivalent antigen-stimulated degranulation response in RBL mast cells due to As co-exposure.

Next, a mid-range antigen dose, 0.0004 $\mu\text{g ml}^{-1}$, which elicited an average absolute degranulation response of $25 \pm 1\%$ (SEM; ~47% of the maximal response; see Fig. 2D) in the absence of As, was selected for As dose–response experiments (Fig. 2B). The 100 ppb dose of As was not statistically significantly effective with this level of antigen stimulation, unlike in the low-dose antigen exposure, but a downtrend trend was seen at 100 ppb. Statistically significant inhibition of degranulation function began at 250 ppb As, where degranulation levels were 0.84-fold $\pm$ 0.02 (SEM) of the 0 ppb control levels (Fig. 2B). Increasing levels of As exposure further inhibited degranulation, with a clear As dose–response. At 500 ppb As, degranulation levels were 0.74-fold $\pm$ 0.02 (SEM) of the 0 ppb control levels. At 750 ppb As,

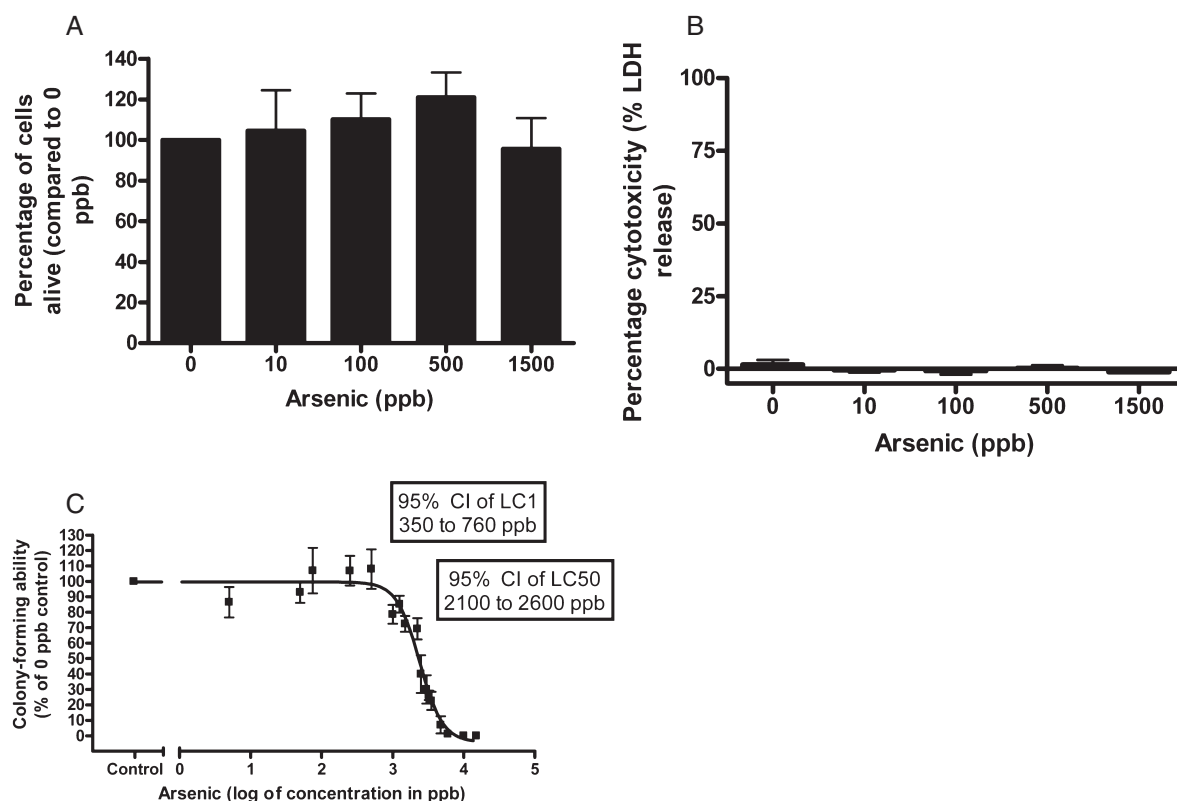


Figure 1. High-dose (1500 ppb) As is not cytotoxic to RBL cells during a short-term (1 h) exposure and 24 h cytotoxicity of As in RBL cells. (A) Effects of As on RBL cell survival were assessed by a trypan blue-exclusion cytotoxicity assay. RBL cells were cultured and the assay was performed as described in the 'Methods' section. Briefly, the cells were treated with As, at the concentrations shown, in Tyrodes-BSA buffer. After a 1 h As exposure at 37 °C, cells were harvested, mixed with trypan blue, and counted on a hemocytometer. The percentage of viable cells (normalized to the 0 ppb control samples) is plotted against the doses of As that the cells received. Error bars represent the standard error of the mean of the data from three repeat experiments; three replicates per dose were done on each experimental day. (B) Effects of As on RBL cell survival were assessed by a LDH release cytotoxicity assay. RBL cells were cultured and the assay was performed as described in the 'Methods' section. Briefly, RBL cells in clear 96-well plates were treated with As, at the concentrations shown, in Tyrodes-BSA buffer. After a 1 h As exposure at 37 °C, cytotoxicity was measured according to the manufacturer's protocol for the Cytotoxicity Detection Kit (LDH) from Roche. The percentage of cytotoxicity is plotted against the doses of As that the cells received. Error bars represent the standard error of the mean of the data from five repeat experiments; three replicates per dose were done on each experimental day. (C) Effects of As on RBL cell survival were assessed by a colony-forming assay. Cells were cultured and the assay was performed as described in the 'Methods' section. Briefly, the cells were treated with As, at the concentrations shown, in complete cell culture medium for 24 h at 37 °C. After the exposure, the media containing As was removed, and the cells received fresh media, in which they were cultured until control samples had colonies of approximately 25 cells. Colonies were fixed, stained and counted as described in the 'Methods' section. The colony-forming ability, as a percentage of the number of colonies in the 0 ppb control samples, is plotted against the log of micromolar As concentrations that the cells received. Error bars represent the standard error of the mean of the data from five repeat experiments (each individual dose was tested in a total of two to five experiments); three replicates per dose were done on each experimental day. The 95% confidence intervals of the LC₅₀ and LC₁ for cell survival were determined using Probit software.

degranulation levels were 0.63-fold $\pm$ 0.02 (SEM) of the 0 ppb control levels. These data indicate inhibition of the multivalent antigen-stimulated degranulation response in RBL mast cells due to As co-exposure, but the effect is somewhat weaker than when the lower antigen concentration is used. For example, 100 ppb As with low antigen (Fig. 2A) is similarly effective to 250 ppb As with maximal antigen (Fig. 2C). Figure 2(A–C) shows clearly that As has an inhibitory effect on degranulation of RBL mast cells; however, the antigen concentration plays a strong role in the severity of the effect.

Next, a maximal antigen dose, 0.005 μ g ml⁻¹, which elicited an average absolute degranulation response of 52 $\pm$ 1% (SEM; compared with the maximal response shown in Fig. 2D) in the absence of As, was used for As dose-response experiments (Fig. 2C). A slight downward trend of inhibition is indicated by ~250 ppb, but statistically significant inhibition of degranulation function did not begin until 750 ppb As. At 750 ppb As, the degranulation level was 0.89-fold $\pm$ 0.02 (SEM) of the 0 ppb

control level. These data indicate a modest inhibition of the multivalent antigen-stimulated degranulation in RBL mast cells due to As co-exposure, but the effect is much weaker than when the lower antigen concentrations are used. For example, 100 ppb As with low antigen (Fig. 2A) is similarly effective to 750 ppb As with maximal antigen (Fig. 2C). Figure 2(A–C) shows clearly that As has an inhibitory effect on degranulation of RBL mast cells; however, the antigen concentration plays a strong role in the severity of the effect.

As Inhibits Degranulation Stimulated by Different Types of IgE Receptor Crosslinkers

Above, we showed that As inhibits the degranulation response of RBL cells to the multivalent antigen DNP-BSA. Next, by using an anti-IgE antibody in place of DNP-BSA, we were able to test an

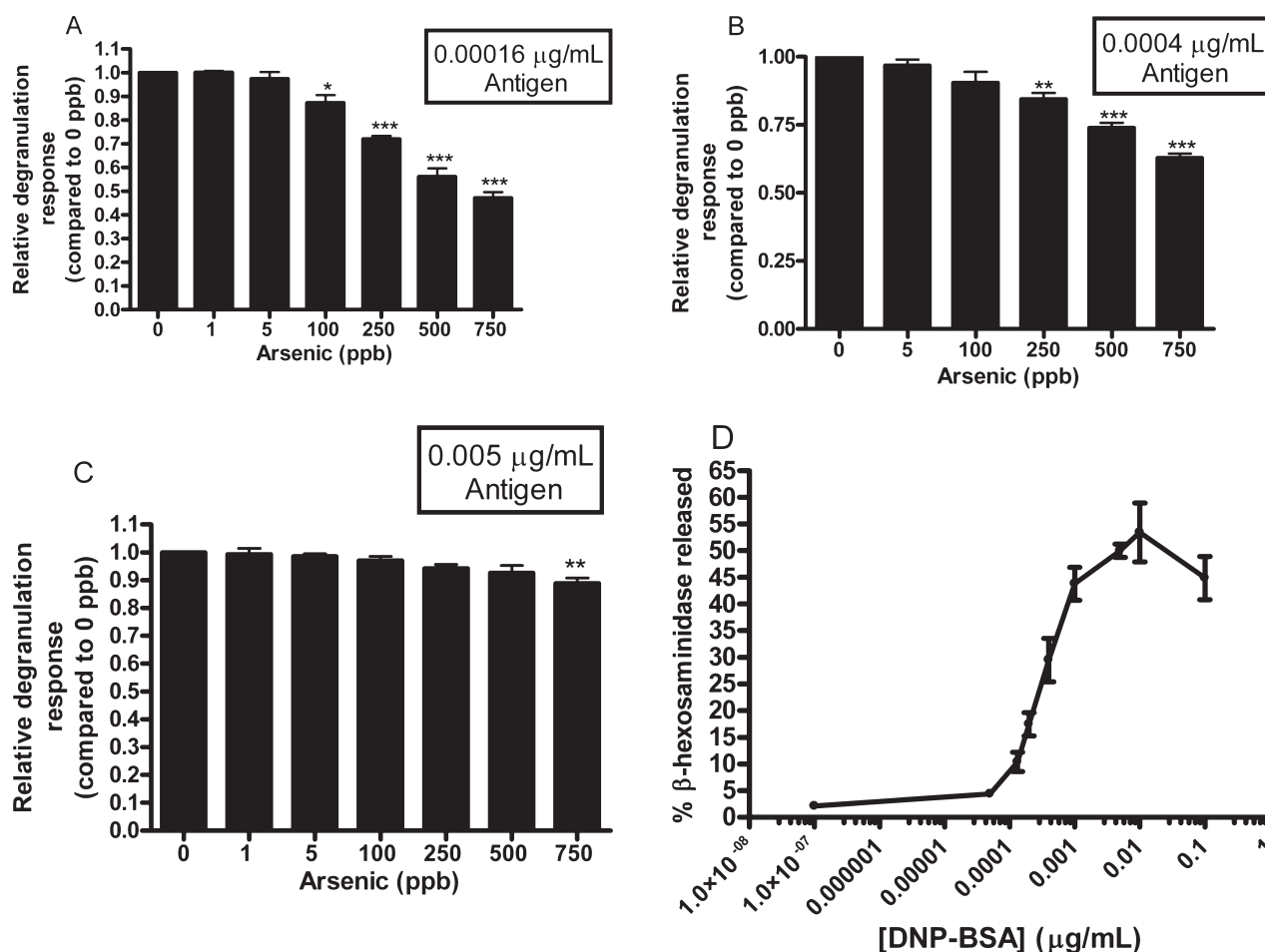


Figure 2. Inhibition of the degranulation response of RBL cells to various antigen doses by As. (A–C) Effects of As on antigen-stimulated RBL cell degranulation were assessed by a fluorescence-based assay. RBL cells were cultured and the assay was performed as described in the 'Methods' section. Briefly, RBL cells were incubated with $0.1 \mu\text{g mL}^{-1}$ of anti-DNP-BSA IgE to sensitize the IgE receptors. Next, cells were stimulated with (A) $0.00016 \mu\text{g mL}^{-1}$, (B) $0.0004 \mu\text{g mL}^{-1}$ or (C) $0.005 \mu\text{g mL}^{-1}$ DNP-BSA antigen along with the As concentrations shown for 1 h at 37°C . β -Hexosaminidase released into cell supernatants was measured as described in 'Methods'. The relative degranulation response (β -hexosaminidase released, normalized to the 0 ppb As, antigen-stimulated control) is plotted against the doses of As that the cells received. Error bars represent the standard error of the mean of the data from (A) five, (B) 12 and (C) four repeat experiments (each individual dose was tested in a total of three to thirteen experiments); three replicates per dose were done on each experimental day. One-way ANOVA tests, with Tukey's post-tests [comparing data points with 1 ppb (A and C) or to 5 ppb (B) samples], were performed using Prism software, and asterisks on the graphs indicate that a particular exposure produced a significant response to the following levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (D) Antigen dose–response effects on RBL cell degranulation were assessed (no As present). RBL cells were cultured and the assay was performed as described in the 'Methods' section. This experiment was done as described above for (A)–(C), except without As and with a wide range of DNP-BSA antigen doses. The percentage absolute degranulation response (% β -hexosaminidase released) is plotted against the doses of DNP-BSA antigen used (on a logarithmic scale). Error bars represent the standard deviation of triplicate samples for each antigen dose (except for only two replicates for the $0.0002 \mu\text{g mL}^{-1}$ sample) from a single representative data set.

alternative IgE receptor-crosslinker (Fig. 3). We performed this experiment to determine whether different modes of RBL stimulation can be inhibited by As. A mouse anti-IgE IgG antibody specific to the Fab portion of the IgE molecule was used to cross-link receptors and stimulate degranulation, and the effects of As on anti-IgE-stimulated degranulation were assessed using the same fluorescence-based assay and analysis described above.

A dose of anti-IgE antibody, $10 \mu\text{g mL}^{-1}$, which elicited an average absolute degranulation response of $34 \pm 1\%$ (SEM; ~64% of the maximal response found with DNP-BSA stimulation and shown in Fig. 2D) in the absence of As, was selected for As dose–response experiments (Fig. 3). Increasing levels of As exposure inhibited degranulation, with a clear As dose–response. At

500 ppb As, degranulation levels were $0.75\text{-fold} \pm 0.02$ (SEM) of the 0 ppb control levels. At 750 ppb As, degranulation levels were $0.70\text{-fold} \pm 0.03$ (SEM) of the 0 ppb control levels. These data indicate inhibition of the anti-IgE-stimulated degranulation response in RBL mast cells due to As co-exposure. Interestingly, the fractional inhibition values at 250, 500 and 750 ppb (Fig. 3) are similar to those values found with the mid-range DNP-BSA antigen dose (data in Fig. 2B), suggesting again that the level of stimulation of degranulation (relative to the maximal possible stimulation) determines the effect that As can have. Overall, Fig. 3 indicates that As's inhibition of RBL mast cell degranulation is not specific to a single type of antigen crosslinker used on the mast cell surface.

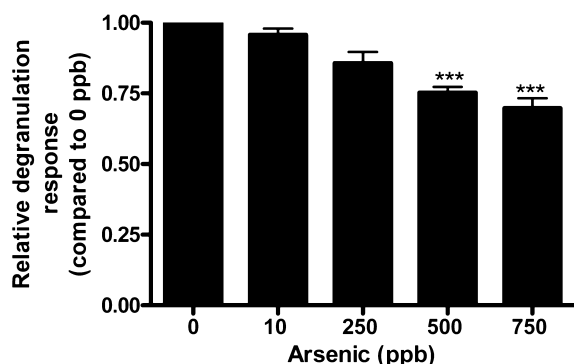


Figure 3. Arsenic inhibition of the RBL cell degranulation response to stimulation via anti-IgE antibody crosslinking of IgE receptors. Effects of As on anti-IgE antibody-stimulated (rather than DNP-BSA antigen-stimulated) RBL cell degranulation were assessed by a fluorescence-based assay. The assay was performed as described in the 'Methods' section and as summarized in the legend to Fig. 2, with the exception that $10 \mu\text{g mL}^{-1}$ anti-IgE IgG antibody was used as the cell-stimulating crosslinker instead of DNP-BSA. The relative degranulation response (β -hexosaminidase released, normalized to the 0 ppb As, antigen-stimulated control) is plotted against the doses of As that the cells received. Error bars represent the standard error of the mean of the data from four repeat experiments (each individual dose was tested in a total of three or four experiments); three replicates per dose were done on each experimental day. One-way ANOVA with a Tukey's post-test (compared to the 10 ppb sample) was performed using Prism software, and asterisks on the graphs indicate that a particular exposure produced a significant response to the following levels: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

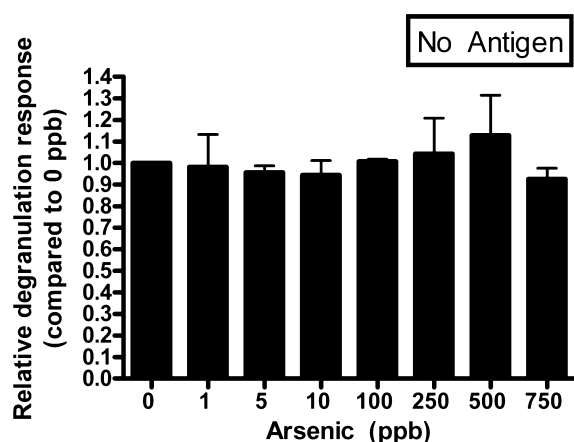


Figure 4. Arsenic has no effect on spontaneous degranulation. Effects of As on unstimulated RBL cell degranulation were assessed by a fluorescence-based assay. RBL cells were cultured and the assay was performed as described in the 'Methods' section. Briefly, RBL cells (which received neither IgE nor DNP-BSA or other stimulator) were incubated with the As concentrations shown for 1 h at 37°C . β -Hexosaminidase released spontaneously into cell supernatants was measured as described in 'Methods'. The relative degranulation response (β -hexosaminidase released, normalized to the 0 ppb As, unstimulated control) is plotted against the doses of As that the cells received. Error bars represent the standard error of the mean of the data from five repeat experiments (each individual dose was tested in a total of three to five experiments); three replicates per dose were done on each experimental day.

As has no Effect on Spontaneous Degranulation of Mast Cells

Other endocrine disruptors have a demonstrated ability to cause spontaneous degranulation in mast cells that are not simultaneously stimulated via antigen or other receptor crosslinkers (Narita *et al.*, 2007). To determine whether As could cause spontaneous degranulation of RBL mast cells, we measured the effects of As on unstimulated RBL cell degranulation (Fig. 4). Here, neither IgE nor antigen was used. Cells were incubated with As diluted into Tyrodes-BSA for 1 h. β -Hexosaminidase released spontaneously into cell supernatants was measured as described in 'Methods'. The average spontaneous degranulation of the 0 ppb As controls was $1.1 \pm 0.1\%$ (SEM; $\sim 2\%$ of the maximal response found with DNP-BSA stimulation and shown in Fig. 2D). There was no significant change in the levels of spontaneous degranulation due to As up to 750 ppb (Fig. 4). There is some apparent (but not statistically significant) variation, which is due to the fact that these values reflect low levels of fluorescence which are fairly close to background levels. This lack of an As effect on spontaneous β -hexosaminidase release indicates that As's effect on mast cell degranulation only occurs when antigen, a second environmental agent, is present.

DISCUSSION

The main finding of this study is that inorganic As inhibits RBL mast cell degranulation, which is an important physiological process in parasite defense, asthma and allergy. RBL cells exposed to environmentally-relevant doses of As exhibited reduced responses to Ag stimulation, in a As- and Ag-dose-responsive fashion.

RBL cell viability was not reduced, even with high As doses, up to 1500 ppb during a short-term (1 h) exposure that simulated the exposure (timing, dosing, buffer, etc.) conditions during the degranulation experiments. We tested this short-term As cytotoxicity to RBL cells via two different experimental methods, trypan blue exclusion and LDH release, in order to avoid the possibility of experimental artifacts affecting our interpretation of the data. While LDH release tests directly for plasma membrane damage, trypan blue exclusion tests for either plasma membrane damage or other metabolic effects that would cause the cells to not be able to pump out the trypan blue dye. Clonal survival (clonogenic) 24 h cytotoxicity tests of As in RBL cells additionally showed that the 24 h LC₅₀ of arsenite on RBLs is greater than 2000 ppb. While the 24 h LC₁ value is somewhere between 350 and 760 ppb, the high end of our dosage range, we are only exposing the cells for 1 h during the degranulation. Because we have found As effects on mast cell degranulation at concentrations as low as 100 ppb As and because all doses used in the degranulation experiments were less than or equal to 750 ppb, a non-cytotoxic dose at 1 h, the degranulation effects we have seen are not related to cell death.

We have found that, unlike estradiol and xenoestrogens (Narita *et al.*, 2007; Zaitzu *et al.*, 2007), As alone has no effect on degranulation. However, interestingly, As inhibits crosslinker-stimulated degranulation, and this effect is dependent on the concentrations of both As and Ag. It is interesting that a second environmental agent (antigen crosslinker, analogous to allergens such as pollens, dander, etc.) is needed in addition to the As in order to see a cellular degranulation effect due to As. Similar conditions were also found in studies of As's effects as an endocrine disruptor.

tor, in which both hormone and As were typically needed in order for As to exhibit an effect on steroid receptor-mediated transcription (Bodwell *et al.*, 2004, 2006; Davey *et al.*, 2007).

As had its greatest effects when a low DNP-BSA antigen concentration ($0.00016 \mu\text{g ml}^{-1}$) was used, leading to moderate (~23% of maximal) degranulation responses in the no-As controls and to strong, dose-dependent As inhibition of this degranulation. This inhibition started at 100 ppb As, with ~50% inhibition by 500 or 750 ppb As. As the Ag concentration was increased, As's effectiveness as an inhibitor was reduced in a dose-responsive manner, to the point that maximally-stimulated cells could only be marginally affected by As, with just an 11% inhibition at 750 ppb As. At this point, we do not know why there are different As dose-response effects when different Ag concentrations are employed. However, the data would suggest that the As-sensitive elements in the signal transduction cascade are themselves less important to the degranulation response when antigen concentration is maximal or that these same elements are inaccessible to As binding when maximal degranulation is occurring.

We performed control tests of As's effect on β -hexosaminidase's activity in isolation of cellular effects. We found that As does not interfere with β -hexosaminidase's activity and, therefore, does not interfere with the degranulation assay itself. Thus, the As effects on degranulation must be due to an As effect on cellular processes.

The levels of degranulation that we have observed due to As exposure (~500–750 ppb, in the low-antigen samples) in this study are approximately 50%, relative to the 0 ppb As controls. This strong level of inhibition is similar in magnitude to the inhibitory effect of having just one, rather than two, Fc ϵ R1 γ chains (single chain vs dimeric receptors), in studies done with variable transmembrane domain chimeric IgE receptors (Gosse *et al.*, 2005). Thus, 500–750 ppb As is approximately as effective as an inhibitor as removing half of the major signaling component (removing one of the two γ subunits) of the IgE receptor.

By using an anti-IgE antibody in place of DNP-BSA, we tested whether different modes of RBL stimulation can be inhibited by As. Because the As dose-response effects on degranulation were very similar when either anti-IgE or DNP-BSA crosslinking was utilized, we showed that As's inhibition of RBL mast cell degranulation is occurring somewhere in the degranulation process in the cell and is not specific to the type of antigen crosslinker used on the mast cell surface.

At this time, the mechanism of inorganic As's effects on mast cell degranulation is not known. Arsenic has previously been shown to affect tyrosine phosphorylation in T cell signal transduction (Soto-Pena and Vega, 2008). Tyrosine phosphorylation is also a key component of the signal transduction cascade connecting Ag stimulation to degranulation in RBL mast cells and, therefore, could be an As target. Additionally, other workers have found that As affects endocrine signaling (Bodwell *et al.*, 2004, 2006; Davey *et al.*, 2007; Hamilton *et al.*, 1998; Kaltreider *et al.*, 2001) and that other endocrine disruptors can affect mast cell degranulation, so As may affect degranulation via its endocrine-disrupting effects. We hypothesize that As's mechanism of action is either via its endocrine-disrupting effects or via an inhibitory effect of some step in the signal transduction cascade (tyrosine phosphorylation, calcium signaling, or other steps), and we will pursue these questions in future studies.

A different form of As from the inorganic arsenite used in this study, the organic phenylarsine oxide form of As, has been used

as an inhibitor of protein phosphotyrosine phosphatases in many investigations of cellular signal transduction (Kumada *et al.*, 1994; Massol *et al.*, 1998; Santini and Beaven, 1993). In fact, phenylarsine oxide has previously been shown to inhibit RBL cell degranulation (Adamczewski *et al.*, 1992). However, the effect of the more environmentally relevant form of As, inorganic arsenite (the form of As used in our current study), could not be assumed since different species of As often have very different biological reactivities. In 2007, Schmidt and co-workers incubated phenylarsine oxide and, separately, inorganic arsenite with sulfur-containing amino acid (cysteine), peptide (glutathione) and protein solutions and then obtained mass spectra of the mixtures (Schmidt *et al.*, 2007). Mass spectra of solutions in which phenylarsine oxide was incubated with cysteine, glutathione, or sulfur-containing protein indicated a pH-stable covalent bond between the compound's As atom and sulfur (Schmidt *et al.*, 2007). However, under these same conditions, no interactions of inorganic arsenite with the cysteine, glutathione or sulfur-containing protein could be detected with sensitive mass spectrometry (Schmidt *et al.*, 2007). It is quite likely that the mechanisms underlying inorganic arsenite's and phenylarsine oxide's inhibition of degranulation are different from one another. Because inorganic arsenite is much more prevalent in environmental and occupational exposures of people to As, we have used this compound for our studies. It is likely that the mechanism underlying inorganic arsenite's inhibition of degranulation is neither due to direct thiol reactivity nor to phosphatase inhibition.

As it is the case that mast cells perform their functions within tissues, it is most relevant to consider concentrations of As within mast cell-containing tissues rather than in blood. This consideration is particularly necessary because blood concentrations of As in As-exposed individuals tend to be very low compared with both the As concentration in their drinking water (Hughes, 2006) and the As concentrations in target tissues. Blood As concentrations are a poor proxy for As concentrations in the tissues where mast cells act: for instance, in a study of human organs immediately following a fatal arsenic trioxide poisoning, the concentrations of total As in all organ tissues were ~7- to 350-fold higher than in blood (Benramdane *et al.*, 1999). A study of the levels of As in tissues of 20 autopsied human cadavers in Japan found 551 ppb (wet weight) of total As in the aorta, 327 ppb As in the adrenal gland, 153 ppb As in the skin, 129 ppb As in the liver, 129 ppb As in the kidney and 104 ppb As in the lung (Yamauchi and Yamamura, 1983). These Japanese individuals had not been environmentally or occupationally exposed to particularly high levels of As, but their organs still contained levels of arsenic similar to the concentrations used in our experiments. Another human study found liver (dry weight) concentrations of As ranging between 500 and 6000 ppb As in biopsied human patients who had been exposed chronically to high (220–2000 ppb) drinking water As concentrations in West Bengal. Additionally, 690 ppb (wet weight) total As was found in the lungs of an autopsied 9-month-old human male and 590 ppb As was found in the lungs of an autopsied 69-year-old human female (Schroeder *et al.*, 1968). Moreover, a study of dogs chronically fed low-level dietary sodium arsenite found that the animals had liver and kidney As concentrations between 540 and 1240 ppb (wet weight) after several weeks of exposure (Neiger and Osweiler, 1992). Overall, there is evidence that mast cell-containing tissues may realistically contain 100–750 ppb As (the

As concentration range found to inhibit degranulation in this study) or higher As concentrations in individuals exposed to As.

Inorganic As has long been used in traditional Chinese herbal medicine for treatment of bronchial asthma (ATSDR, 2007), and cases of As poisoning have been found in people using these anti-asthmatic herbal preparations (Tay and Seah, 1975). The inhibition of mast cell degranulation that we have found in this study may be a mechanism underlying the traditional Chinese medicinal use of As to treat asthma.

This inhibition of degranulation by As suggests that As may suppress the body's defense against parasites by inhibiting the ability of mast cells to fight parasites. Children living in households with As-contaminated well water in Bangladesh were found to be significantly more wasted (to have lower body mass indices) than children unexposed to drinking-water As at home (Minamoto *et al.*, 2005). A reason for this effect was not elucidated in this manuscript, but many children in this study were infected with parasites, and it is possible that inhibition by As of the body's immune defense against parasites could lead to more or more severe parasitic infections, leading to greater wasting. Overall, our data indicate that As suppresses the immune function of mast cell degranulation, which is needed to defend humans against parasites.

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