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and Leigh Ann Ferren

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SUMMARY

The diffusion chamber system was used to monitor the toxicity of trivalent arsenic for human target cells in vivo. Cell-impermeable diffusion chambers containing human embryonic lung cells were implanted into the peritoneal cavity of hamsters that one day later were injected i.v. or i.p. with arsenic trioxide (As_2O_3) as aqueous solution (AsO_2^-). Control animals received an equivalent volume of water. $^{74}AsO_2^-$ was employed to quantitate the concentration of arsenic reaching the chambered target cells and to describe the toxicokinetics of arsenic in the hamster. Trivalent arsenic induced a growth inhibition in the target cells with the dosage level determining the extent of inhibition at 24 h and the recovery of cell proliferation at 48 h. The maximum concentration of arsenic in the chamber fluid was detected 2 h post i.p. injection, after which the elimination of ^{74}As from the chamber fluid paralleled the decline of ^{74}As activity in the plasma of the host animal. Limited tissue distribution studies showed that that arsenic was retained primarily in the pelt.

INTRODUCTION

Arsenic has been identified as a potentially hazardous trace element pollutant of the energy technologies associated with coal as well as a pollutant of the smelting and processing of non-ferrous metals [1-3]. The toxicity of this element is historically significant and more recently the mutagenic and carcinogenic potential of arsenic has been recognized [4,5]. The trivalent form of arsenic is more toxic than the pentavalent species due primarily to the reaction of trivalent arsenicals with sulfhydryl groups of enzymes [6]. Whereas both valence forms of inorganic arsenic are methylated in vivo, there are differences in the degree of methylation and in the whole body retention of the two species [7].

A number of studies have described the toxicity of trivalent arsenic at either the organismal level (the reaction of the host) or at the cellular level with tissue culture target cells [8-10]. The diffusion chamber system [11,12] offers a means of describing the arsenic-target cell interaction within an in vivo environment. Two aspects of this in vivo system are important: 1) the arsenic can undergo modifications (e.g., methylation) before reaching the target cells, and 2) the dynamic concentration of the arsenic within the chamber fluid reflects the changing levels in the plasma, thus simulating the arsenic interaction at the tissue level. There are additional benefits to the diffusion chamber assay in that target cells foreign to the host can be used for study and the cells can be easily retrieved and enumerated for toxicity determinations.

The purpose of this study is to describe the dosage and temporal effects of arsenic trioxide (As_2O_3) on the proliferation of human target cells within diffusion chambers implanted in hamsters. In addition, $^{74}AsO_2^-$ was used in order to determine the concentration of arsenic reaching the target cells as well as other selected tissues of the diffusion chamber host.

MATERIALS AND METHODS

Male Syrian golden hamsters (strain LVK/LAK) were obtained from Charles River Lakeview and were between 5-13 weeks of age for the studies. Drinking water and Purina Lab Chow were provided ad libitum.

Arsenic-74 was prepared by the $^{74}Ge(p,n)^{74}As$ nuclear reaction on natural germanium. The germanium was dissolved in aqua regia (HNO_3 + HCl), and then the excess HNO_3 was removed by boiling with 12 M HCl followed by adjustment to 6 M HCl. After addition of 50 mg of As_2O_3 carrier, Cl_2 gas was bubbled through the solution to form the nonvolatile As^{5+} oxidation state, while $GeCl_4$ was removed by distillation. Arsenic was reduced to the volatile 3+ oxidation state by addition of HBr and distilled into an ice-cooled water trap. Arsenic 5+ was reformed with Cl_2 gas prior to evaporation of the solution to near dryness. The residue was dissolved in distilled water, converted to As^{3+} with SO_2 gas, and the concentration adjusted to 1.46 mg As/ml. The solution was assayed by Spark Source Mass Spectrometry which indicated < 0.5 μg/ml of Ge. The only significant contaminant was sulfur at ~500 μg/ml which is consistent with the 625 μg/ml expected from the stoichiometric reduction of the arsenic 5+ to 3+ oxidation state by SO_2. Since dilute solutions of As^{3+}

are completely hydrolyzed to arsenous acid, the solution was considered equivalent to aqueous solutions of As_2O_3 (AsO_2^-).

Flow 2000 cells (human embryonic lung cells) obtained from Flow Labs (Rockville, MD) were maintained in monolayer culture on Earle's Minimal Essential Media (EMEM) containing 10% heat-inactivated fetal calf serum. These cells display density-dependent growth inhibition with a doubling time of 24 h.

Diffusion chambers were prepared by fixing 0.2 μm porosity polycarbonate filters (Nucleopore Corp., Pleasanton, CA) to each side of 14 mm Lucite rings (Millipore Corp., Bedford, MA) with the Millipore adhesive. The assembled diffusion chambers were sterilized by 80° heating for 24 h prior to use. Target cells were harvested from monolayer culture by trypsinization and standardized to $5x10^5$ cells/ml in EMEM without serum. A 0.1 ml volume of the cell suspension ($5x10^4$ cells) was dispensed into each chamber through the radial hole in the Lucite ring, and the chamber hole sealed with paraffin. The seeded diffusion chambers were implanted surgically in the peritoneal cavity of anesthesized hamsters (3 chambers per animal). One day following chamber implantation the hosts received an i.v. (lateral marginal vein) or i.p. injection of aqueous solution of As_2O_3 (AsO_2^-) or an equivalent volume of water. For i.v. injections, the AsO_2^- or water was mixed 1:1 with hamster plasma. In all cases, the concentration of As_2O_3 was 1.8 mg/ml solution, and the volume administered was calculated for each animal.

At 24 h intervals after injection the hamsters were killed and the chambers removed. The exterior of the chamber was wiped with gauze to

remove adhering cells. After removal of the chamber fluid, the chamber interior was treated with a 0.5% pronase solution (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C to remove attached target cells and to obtain a single cell suspension for counting. The pronase solution was combined with the original chamber fluid and diluted to 1.0 ml with serum-free EMEM. Cells were counted either by hemocytometer or in a Royco cell counter with periodic microscopic evaluation of the cell population using trypan blue dye exclusion test as a check for cell viability. Six to nine chambers comprised a test or control group. The percentage of inhibition of cell growth was calculated as:

$$1 - \frac{\text{mean no. cells in test group}}{\text{mean no. cells in control group}} \times 100.$$

The differences in the mean cell counts were evaluated for statistical significance with Wilcoxon two sample test [13].

In studies where $^{74}AsO_2$ was injected, the animals were killed at various times prior to the 24 and 48 h growth-inhibition check. The target cells removed from the chambers were initially counted under the microscope using hemocytometers and then separated from the chamber fluid by filtration through a 0.45 μm Swinnex filter unit (Millipore Corp., Bedford, MA) followed by a wash with 2 ml of serum-free media. In some studies, selected hamster tissues were also removed, washed with saline, blotted dry, put in scintillation vials and weighed. The ^{74}As activity in the samples was measured in a Packard Autogamma counter. The ^{74}As activity in the cellular fraction of blood was calculated as:

(^{74}As in 1 ml blood) - (^{74}As in 1 ml plasma) (Vol. plasma in 1 ml blood).

RESULTS

Dosage effect of AsO_2^- on growth of target cells.

When hamsters bearing diffusion chambers were injected intraperitoneally with AsO_2^- at dosage levels of 2.5, 5.0, 7.5, or 10.0 mg/kg As_2O_3, the mean target cell numbers in the chambers of all arsenic-exposed groups were significantly lower ($0.04 > p > 0.001$) than the mean cell count in chambers of control hamsters 24 h after injections. In Fig. 1 the average cell count of each group is plotted against the dosage

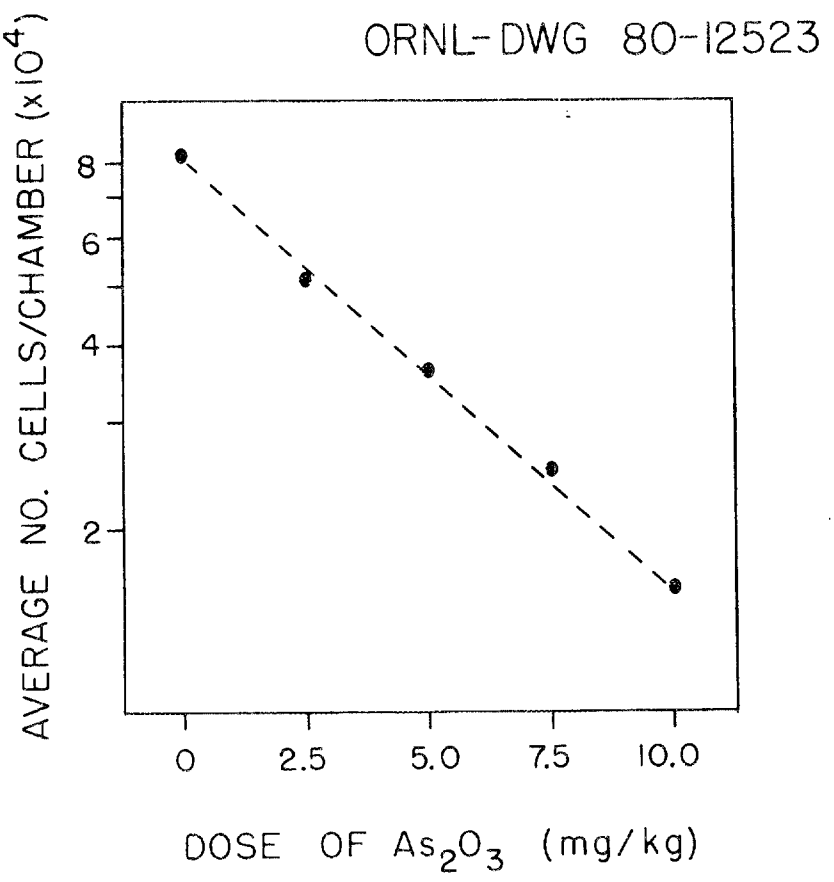

Fig. 1. Effect of AsO_2^- exposure on Flow 2000 cell number within diffusion chambers 24 h after i.p. injection.

level of AsO_2^- employed. For these selected doses a linear dose-response exists. The 5.0 mg/kg dose was used in several separate studies as a test of the reproducibility of the diffusion chamber assay for arsenic-induced inhibition of cell growth. With the age of host animals ranging from 6-13 weeks and the route of administration either i.p. or i.v., there was close agreement in the percentages of growth inhibition induced by the 5.0 mg/kg dosage level (Table 1).

Table 1. Inhibition of cell growth in chambers of hamsters injected with 5.0 mg As_2O_3/kg body weight.

Age of animals (wk)	Injection route	% Growth inhibition
6	i.p.	56[a]
8	i.p.	52,[b] 52[b]
11	i.p.	51[c]
13	i.v.	53[c]

[a] Difference from control, $p < 0.05$

[b] Difference from control, $p < 0.01$

[c] Difference from control, $p < 0.001$

Two dosages (5 and 10 mg/kg) were selected for a temporal study of arsenic effects on Flow 2000 cell growth in the chambers. In animals receiving the 5 mg/kg dosage, the growth inhibitory effect observed at 24 h was temporary; by 48 h post injection, the target cells had regained the doubling time of cells in control chambers (Fig. 2). In the group receiving the 10 mg/kg dosage, however, the inhibitory effect was more prolonged in that these target cells never achieved the control growth

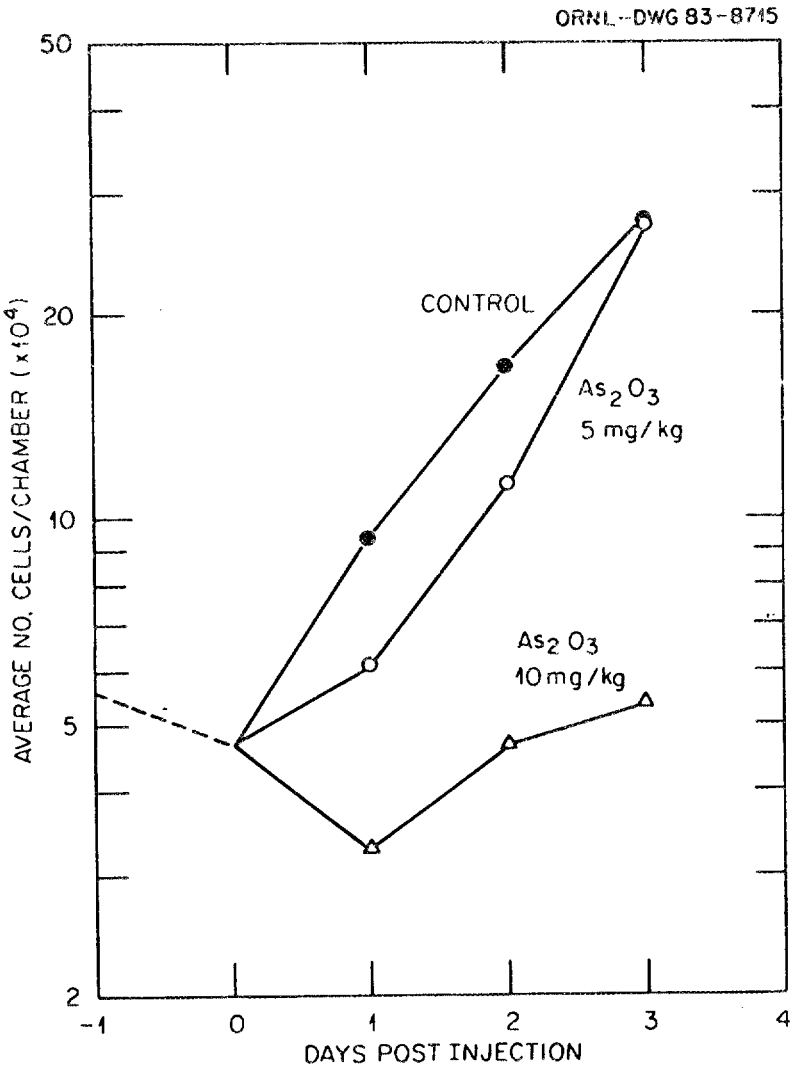

Fig. 2. Effect of As_2O_3 dosage (as AsO_2^-) on the proliferation of Flow 2000 cells in diffusion chambers.

rate. In this study the percentage growth inhibition at 24 h post-injection was lower than that observed in earlier studies, but this may have been due to the change in methods of enumerating the target cells (i.e. earlier studies were done with hemocytometers).

Concentration of ^{74}As in tissues and fluid.

Table 2 shows the early distribution of ^{74}As in the blood, plasma and chamber fluid of hamsters injected i.p. with $^{74}AsO_2^-$ (10 mg/kg).

Table 2. Distribution of ^{74}As in blood, plasma, and chamber fluid of hamsters injected i.p. with 10 mg $^{74}As_2O_3$/kg body weight (% dose/ml fluid: $\bar{x} \pm$ S.D.).

	Whole blood	Plasma	Chamber fluid
2 h	0.44 ± 0.06	0.30 ± 0.05	2.26 ± 0.62
4 h	0.34 ± 0.05	0.17 ± 0.08	0.27 ± 0.01
	0.30 ± 0.05	0.14 ± 0.05	0.18 ± 0.05
	0.27 ± 0.01	-	0.23 ± 0.005
8 h	0.19 ± 0.06	0.05 ± 0.007[a]	0.05 ± 0.03
24 h	0.11 ± 0.05	0.05[a]	0.04 ± 0.01
	0.10 ± 0.03	-	0.07 ± 0.02

[a] These values are the average of 2 animals. All other values are means of 3 animals (blood and plasma) or 6-9 chambers.

At all assay periods, the majority of ^{74}As activity found in whole blood was determined to be associated with the cellular component. At 2 and 4 h post injection, the level of ^{74}As was higher in chamber fluid than in circulating plasma. By 8 h, however, the ^{74}As activities in plasma and chamber fluid were comparable, verifying the fluid permeability of the diffusion chamber. Within the selected assay times, the maximum arsenic concentration contacting the target cells in the chambers was calculated to be 110 μM AsO_2^-. Levels of ^{74}As activity associated with the human target cells recovered from the chambers were quite low. The maximal concentrations (4 h post-injection) was calculated to be 2 ng As/chamber cell population or 0.7 ng As/10^4 viable cells.

Limited tissue distribution studies were performed with hamsters receiving the 10 mg/kg dose of $^{74}AsO_2^-$ using both animals with and without implanted diffusion chambers. Table 3 shows the ^{74}As activity in the

Table 3. Distribution of ^{74}As in tissues of hamsters injected i.p. with 10 mg $^{74}As_2O_3$/kg body weight (% dose/gm tissue: $\bar{x} \pm$ S.D.; 6 animals).

	4 h	24 h
liver	2.22 ± 0.19	0.71 ± 0.26
kidney	1.17 ± 0.27	0.41 ± 0.07
spleen	0.79 ± 0.12	0.26 ± 0.09
heart	0.65 ± 0.11	0.23 ± 0.04
skeletal muscle	0.47 ± 0.16	0.32 ± 0.06
pelt	0.44 ± 0.06	0.53 ± 0.15

selected tissues following i.p. injection. The decline in activity between 4 and 24 h was 65-68% for liver, kidney, spleen and heart. Skeletal muscle showed a smaller loss (24%); and, as expected, arsenic was retained in the hamster pelt.

DISCUSSION

The diffusion chamber assay provided a means of examing the interaction of the arsenic with tissue culture grown cells in an in vivo setting. Exposure of hamsters to trivalent arsenic in the form of AsO_2^- resulted in an inhibition of human cell proliferation in implanted diffusion chambers, and the dosage administered determined both the degree of inhibition observed at 1 d and the reversibility of the inhibition over a three-day period. A similar effect was observed by Gurley et al [10] who described the in vitro response of Chinese hamster ovary (CHO) cells to arsenite exposure. In their studies concentrations of 25 and 50 μM arsenite caused irreversible inhibition of CHO growth, whereas at lower concentrations such as 10 μM, the cell division delay was followed by a spontaneous recovery even in the continued presence of

the arsenic. In our studies a 10 mg/kg dosage of As_2O_3 to hamsters resulted in a 110 μM concentration of arsenic in the chamber fluid 2 h post-injection and a significant inhibition of target cell growth in the chambers for a three day period.

It is difficult to compare the arsenic concentration and associated biological effects observed in the chamber with the results reported from in vitro studies due to the differences between the dynamic nature of the diffusion chamber environment where concentrations are changing and the static character of tissue culture systems. Another complexity in the comparison is the fact that arsenic administered in vivo undergoes biotransformation to mono- and dimethylated forms [7, 14-17]. Although the metabolism of trivalent arsenic in the hamster has yet to be described, the metabolism of pentavalent arsenic has been examined. In hamsters 24 h after i.v. dosing with ^{74}As-arsenic acid, urinary analysis showed dimethylarsenic acid to be the predominant metabolite (53% of total activity) with inorganic arsenic comprising 43% of the ^{74}As activity in the urine [18]. In the rabbit there is a rapid increase in the plasma of dimethylated arsenic within 6 h after i.v. injection of $^{74}AsO_2^-$ [14]. It is likely, therefore, that the arsenic detected in the chamber fluid relects a mixture of organic and inorganic arsenic species in a constantly changing ratio, a very different situation from the in vivo condition.

Because of the marked differences in the metabolism of inorganic arsenic by various animal species, selection of an animal model for man is difficult. The rat has been shown to be a poor choice because of an unusually prolonged retention of arsenic due to the accumulation of

arsenic in red blood cells [19] and a decreased ability to methylate inorganic arsenic [7]. It has been demonstrated that hamster erythrocytes do not share with the rat the high binding capacity for arsenic [20], but thus far the studies on arsenic metabolism and tissue distribution have been limited to the less toxic pentavalent species [18, 21]. Analysis of the biotransformation patterns for trivalent arsenic in hamsters must be done before a final decision is made on the suitability of the hamster as an experimental animal model.

In this study the diffusion chamber system provided a means of examining the in vivo toxicity of arsenic for human cells. The same system has been combined with the sister chromatid exchange assay in studies on the in vivo mutagenicity of cyclophosphamide [22]. With this application of the diffusion chamber system, studies on the health effects of arsenic can now be expanded to include the mutagenic potential of arsenic for human fibroblasts and lymphocytes.

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