In Vitro Bioavailability of Heavy Metals in Pressure-Treated Wood Dust

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Pressure treatment with chromium, copper, and arsenic (CCA) is the most prevalent method for protecting wood used in outdoor construction projects. Although these metals are tightly bound to the wood fibers and are not released under most conditions of use, we examined the bioavailability of metals in CCA pressure-treated wood dust in vitro. Cytotoxicity and metallothionein (MT) mRNA expression were examined in V79 Chinese hamster lung fibroblast cells incubated with respirable-size wood dust generated by sanding CCA-treated and untreated (control) Southern yellow pine. In colony survival studies, increased cytotoxicity (p < 0.05) occurred in V79 cells treated with CCA wood dust (351 ± 77 μg/ml, mean ± SE) compared with control wood dust (883 ± 91 μg/ml). Increased cytotoxicity with CCA wood dust also occurred in an arsenic resistant subline of V79 cells, thus suggesting that arsenic was not responsible for the increased cytotoxicity. Metallothionein mRNA was significantly increased after 48 h of treatment with CCA wood dust compared with control wood dust. Incubation of CCA wood dust in cell culture media resulted in the transfer of copper, but not chromium or arsenic, into the media. Moreover, the treatment of cells with this filtered extract resulted in significantly increased metallothionein mRNA, suggesting that bioavailable copper is responsible for inducing metallothionein mRNA in V79 cells. Thus, these bioassays suggest that metals become bioavailable during in vitro culture of phagocytic V79 cells with CCA wood dust.

Key Words: CCA-treated wood; metals; gene expression; chromium; copper; arsenic; bioavailability; metallothionein.

Approximately 470 million cubic feet of lumber is treated with waterborne preservatives each year in the United States, and the majority of the waterborne treatments utilize the impregnation of softwood with chromated copper arsenate (commonly referred to as CCA) (American Wood Preservers Institute, 1997). A considerable degree of controversy has surrounded the safety of CCA wood. This controversy has centered primarily on the toxicity of chromium and arsenic and the potential for these metals to be released from the wood. Although these metals possess a wide range of toxic properties, they are tightly bound to the wood fibers (Dahlgren and Hartford, 1972) and are not extracted and available as contaminants under most conditions of use. The U.S. Environmental Protection Agency (U.S. EPA) ruled in 1986 that CCA pressure-treated wood is safe for interior use, play structures, garden edging, and vegetable stakes (U.S. EPA, 1986). This ruling was based on the tight, “irreversible” binding of the metals to the wood fibers (Dahlgren and Hartford, 1972). The metals can be made bioavailable, however, under certain conditions. Peters and colleagues (1983) reported that a family was stricken with a variety of illnesses, including bronchitis, pneumonia, gastrointestinal disorders, and severe alopecia, after extended burning of CCA-treated wood for heating purposes. Peters and colleagues later reported that two workers developed pulmonary, hematologic, and gastrointestinal symptoms after several days of construction of picnic tables using freshly treated CCA lumber in which the metals were inadequately fixed (Peters et al., 1986). Thus, in these exceptional circumstances, CCA-treated wood has produced adverse health effects.

Data regarding the actual kinetics of release of metals from CCA wood are limited in the peer-reviewed literature, but it appears that several factors, including time, temperature, and humidity, contribute to fixation and thus the bioavailability of metals in CCA-treated wood. In freshwater environments, minimal leaching of metals from the CCA wood occurs, but is increased under acidic conditions (Warner and Solomon, 1990). In marine water environments, Weis and colleagues have demonstrated that metals are present in the fine fraction of sediments near CCA-treated bulkheads in estuaries (Weis and Weis, 1992; Weis et al., 1998). These metals are generally confined to within 1 m of the bulkheads and resulted in a reduction in the nearby biotic community. McNamara observed that in Southern pine wood treated with CCA, the maximum concentration of chromium that could be released from wood occurred immediately after treatment (0.27%) and diminished rapidly over a 336-h period (McNamara, 1989). Thus, proper fixation of metals to the wood fibers after pressure treatment is essential to minimizing potential exposure of wood handlers to copper, chromium, and arsenic.

The present study is based upon the premise that the use of CCA pressure-treated wood at outdoor construction sites and in fabrication industries may result in the exposure of workers to potentially hazardous wood dust. The extent of metal exposure associated with the inspirable fraction of wood dust encoun-
tered by workers cutting, sanding, and routing CCA pressure-treated wood has been previously examined (Decker et al., 2001; Nygren et al., 1992) and, in certain job assignments, found to exceed recommended occupational exposure levels for arsenic (Decker et al., 2001). In regards to the bioavailability of metals bound to inhalable CCA wood dust, we speculate that the greater surface area of inhalable particles (relative to CCA-treated lumber), the acidic environment of phagolysosomes in phagocytic cells, and the retention time of wood particles in the respiratory tract may each contribute to an increase in bioavailability of heavy metals from CCA wood dust. Therefore, we examined the bioavailability of chromium, copper, and arsenic in vitro by examining cytotoxicity and metallothionein (MT) mRNA induction in cells treated with respirable-size particles generated by the sanding of CCA pressure-treated wood.

MATERIALS AND METHODS

Cytotoxicity experiments. Chinese hamster lung fibroblast (V79) cells were obtained from American Type Culture Collection (Manassas, VA). The arsenite-resistant cell line AsR27 was established previously in this laboratory (Wang and Rossman, 1993). All cells were maintained as a monolayer culture in F12 medium containing 5% fetal calf serum, 5 mM glutamine, 100 units penicillin/ml, and 100 μg streptomycin/ml (GibcoBRL, Grand Island, NY). Cytotoxicity was determined by colony survival as previously described (Wang and Rossman, 1993). Briefly, exponentially growing cells were trypsinized, counted, and plated at 300 cells per 60-mm dish (Becton Dickin-son, Oxnard, CA). At 24 h after plating, wood dusts were diluted to 62.5, 125, 250, 500, 1000, and 1250 μg/ml in F12 culture media (without fetal calf serum) just prior to the start of incubation with cells. All experiments were performed in duplicate or triplicate. These treatment concentrations were based upon preliminary work demonstrating effects ranging from no effect to complete killing. The dust remained in culture with the cells throughout a 24-h incubation period. The wood dust was removed by washing three times with PBS and replaced with fresh medium with serum. The cells were then incubated for 6 days to allow colonies to grow to visible size (greater than approximately 1 mm), fixed with methanol, and stained with Giemsa (Harleco, Gibbstown, NJ). The number of colonies was scored using a semi-automated image analysis system using a Macintosh Ilcx computer, NIH Image software (freeware, National Institute of Mental Health), a data acquisition board (Scion Corp., Frederick, MD), and a Dage CCD camera. To examine whether phagoctosis was required for metals to become bioavailable, CCA-treated wood dust was placed in flasks containing F12 culture media (without fetal calf serum) and no cells for 24 h at 37°C in a 5% carbon dioxide incubator at a concentration of 750 μg/ml. The wood dust sample was removed, the media was filtered with a 0.22-μm sterile filter (Costar, Cambridge, MA), and the filtered media incubated with V79 cells for 24 h as described above.

Metal analysis. Samples of wood dust and cell culture media were analyzed for metal content using nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES) as described previously (Decker et al., 2001). Briefly, samples were leached/digested in concentrated (70%) nitric acid for 5 min and then diluted and leached in a 7% nitric acid matrix for at least 24 h. The samples were then filtered with a 0.45-μm pore size filter and analyzed by IPC-AES (Thermal Jarrell Ash Polyscan 61E). The ICP was calibrated prior to sample analysis using a 7% nitric acid solution with no metals as the blank and a 7% nitric acid solution containing 5 ppm each of chromium, copper, and arsenic. Low (0.25 ppm) and high (0.5 ppm) concentration quality control samples were analyzed along with blanks during the wood dust analysis. In addition, a 5.0-ppm calibration standard was reseed periodically.

Metallothionein mRNA experiments. After treatment of V79 cells with Southern yellow pine or CCA-treated wood dust for 24 to 48 h, total cellular RNA was isolated by a rapid guanidinium-phenol extraction method using Trizol (GibcoBRL). RNA quantity and purity was determined by A260/A280 spectrophotometric absorbances. Integrity of RNA was confirmed by ethidium bromide staining of ribosomal RNA following gel electrophoresis. Standard slot-blot RNA analyses were carried out by direct application of 5 μg glyoxylated RNA samples onto nylon membrane filters (Nittran, Schleicher & Schuell, Keene, NH) in a vacuum manifold (Schleicher & Schuell). Membrane-bound RNA was hybridized to nick-translated, 3P-labeled cDNA probes in the presence of dextran sulfate by a modification of the procedure of Wahl et al. (1979). A rat MT-1 plasmid (Andersen et al, 1983) was provided by Dr. R. H. Herschman, UCLA, and the β-actin probe was purchased from ATCC (Be-thesda, MD). Following hybridization, filters were washed to a final stringency of 0.4× standard saline citrate + 0.1% sodium dodecyl sulfate at 65°C for 30 min. Specifically hybridized mRNA was visualized by film autoradiography at −70°C using Kodak XAR-5 film plus Cronex intensifying screens (DuPont, DE). Autoradiogram signal strengths of hybridized mRNA were quantitated by the measurement of optical densities using BioImager (Millipore, Ann Arbor, MI). All MT gene expression results were normalized to actin expression which served as an internal control to ensure that artifacts such as unequal loading of RNA onto filters were not responsible for any observed differences in autoradiographic signal strengths.

Collection of wood dust. The CCA pressure-treated wood was a gift of Hickson Corporation (Conley, GA). Initially, Southern yellow pine boards (5/4 × 4 × 8”) were cut in half. One half was treated with a CCA treating solution for one full treatment cycle and the remaining half was saved as the control wood. The CCA-treated wood was wrapped in plastic, wet fixed for 7 days, and then air dried at ambient temperature. Each board was tested with chromatographic acid to ensure complete fixation. X-ray fluorescence analysis of cross sections of the treated wood demonstrated that the American Wood-Preservers’ Association fixation standard of 0.4 pounds/ft2 was achieved. Upon delivery at NYU School of Medicine, the boards of CCA-treated wood and control wood were singly wrapped in autoclavable paper and autoclaved to sterilize the samples. A special sanding chamber was constructed to collect respirable size wood dust under as sterile as possible conditions for use in the in vitro experiments. A disc sander was placed in a 2 × 3 × 3’ acrylic chamber supplied with HEPA-filtered air. A control board was sanded first by feeding the wood through a cut in the end of the wrapping paper into a slot in the side wall of the acrylic chamber directly opposite the sanding disc. As the wood dust particles became airborne, a cascade centripetal impactor (BGI, Inc., Waltham, MA) sampled the chamber atmosphere at 30 l/min. After appropriate sampling periods (20–30 min), the centripetal sampler was opened within the sterile atmosphere of a bio-safety hood, and dust collected on the stages with effective cutoff diameters of 1.4–3.4 μm and less than 1.4 μm was pooled and stored in sterile test tubes at 4°C. The generation system was cleaned and the identical sanding and sampling procedure was repeated with a board of CCA-treated wood.

Statistics. The lethal concentration of the test material which killed 50% of the colonies (LC50) was calculated manually using log-probit graph paper. Statistical comparisons of LC50 values between treatment groups were done with a Fisher’s exact test. Evaluation of MT differences among treatment groups were done with a one-factor analysis of variance followed by a Student-Neumann-Keuls post hoc test at each time point. All data are presented as the mean ± SE; p values ≤ .05 were considered statistically significant.

RESULTS

Cytotoxicity

Treatment with CCA-treated wood dust for 24 h significantly reduced colony survival in comparison with control wood dust (p ≤ 0.05). Over five experiments, the mean (± SE)
The LC$_{50}$ for the CCA-treated wood and control wood treatments was $351 \pm 77 \, \mu g/ml$ and $883 \pm 91 \, \mu g/ml$, respectively. A representative concentration-response curve is presented in Figure 1. The toxicity of the CCA-treated wood dust appeared to decrease during the course of the five experiments (Table 1). Therefore, fresh CCA-treated wood dust was generated from the same piece of CCA-treated wood and its cytotoxicity was compared with the older dust in one experiment. As seen in Figure 1, the newly generated CCA-treated wood dust shifted the concentration-response curve to the left, thus suggesting that aging of the wood dust reduced its cytotoxicity.

The filtered extract of the CCA-treated wood dust was less cytotoxic to V79 cells than was the treatment with CCA-treated wood dust. As seen in Figure 2, the filtered extract of 750 $\mu g/ml$ CCA-treated wood dust produced little toxicity. ICP analysis of the extract indicated that measurable amounts of only copper were transferred from the wood dust to the cell culture media during the 24-h incubation period of 750 $\mu g/ml$ CCA-treated wood dust. In comparison with 1.83 ppm copper

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*Note.* Expressed as the LC$_{50}$ in $\mu g/ml$ (mean ± SE) except for PTW on 1/2/97. On that date, the LC$_{50}$ was below the lowest concentration of PTW tested (250 $\mu g/ml$).
in the CCA-treated wood dust, 1.32 ppm copper was measured in the filtered extract. Thus, approximately 72% of the available copper in the CCA-treated wood dust particles was transferred to the pH 7.2 cell culture media after a 24-h incubation at 37°C.

Treatment of arsenic resistant R27 cells with CCA-treated wood dust produced cytotoxicity results that were similar to those observed with V79 cells. CCA-treated wood dust was significantly more cytotoxic than control wood dust in R27 cells (Fig. 3, performed in triplicate). In comparison to V79 cells, R27 cells were resistant to arsenic cytotoxicity, but equally susceptible to chromium and copper (Fig. 4).

Metallothionein mRNA

Treatment with the CCA-treated, but not control, wood dust induced a time- and concentration-dependent increase in steady-state metallothionein mRNA levels in V79 cells. As seen in Figure 5, significant increases in metallothionein mRNA levels were observed at 24 and 48 h for the 500 and 750 μg/ml doses. The expression level of metallothionein mRNA was significantly elevated in arsenic resistant R27 cells treated with CCA-treated wood dust (MT-1/actin ratio of 0.99 ± 0.08, mean ± SE) compared with R27 cells treated with control wood dust (0.12 ± 0.02). Treatment of V79 cells with the extract of soaked CCA-treated wood dust (750 μg/ml) also produced a significant increase in the steady-state levels of metallothionein mRNA at 24 and 48 h (Fig. 6).

DISCUSSION

Pesticide application of chromium and arsenic in wood is regulated by the U.S. EPA in the United States. Based upon the tight binding of the metals to the wood fibers and available environmental toxicity data, the U.S. EPA ruled in 1986 that the use of CCA-treated wood in the outdoor construction of decks, marine structures, garden edging, and vegetable stakes is permissible (U.S. EPA, 1986). It is clear that these metals can be released under certain conditions, such as incomplete fixing of the metals to the wood fibers, acidic environments, and wood combustion, and that these conditions are associated with significant adverse health effects (Peters et al., 1983). There is little available data, however, on the toxicity of CCA-treated wood in mammalian species, and thus there has
metallothionein mRNA, thus providing strong evidence that the copper-containing extract produced significant increases in or complexed to amino acids or secreted proteins. Regardless, cells, it is not known whether the copper was in the free state the three metals. During the incubation of this extract with the 1 ppm) and no detectable As or Cr. The limit of detection by resulted in the transfer of copper into the media (approximately 48 h produced significant increases in metallothionein mRNA. Although arsenic is capable of inducing metallothionein mRNA (Albores et al., 1992; Thor- nalley and Vasak, 1985), our data suggest that copper was responsible for the induction of metallothionein mRNA by the CCA-treated wood dust. In terms of potential adverse health effects, the increase in metallothionein mRNA in cells incubated with CCA-treated wood dust is unclear. An increase in metallothionein protein or mRNA can occur in response to a variety of toxic agents or stressors and is often considered to be an adaptive response to the toxic effects of metals or oxidative injury (Thornalley and Vasak, 1985; Wang et al., 1994). Regardless, our findings determined that significant amounts of copper can be transferred from CCA-treated wood dust into pH 7.2 cell culture media. Because this large degree of leaching is in sharp contrast to the relatively small amount of metals that are released from CCA-treated lumber into freshwater and marine environments, it suggests that the large surface area of respirable wood dust and mammalian body temperature may significantly increase the bioavailability of metals in fixed CCA-treated wood. These factors, as well as the phagocytic ability of pulmonary macrophages, make the inhalation of CCA-treated wood dust an occupational concern.

As determined in the colony survival bioassay, CCA-treated wood dust was more cytotoxic than the untreated Southern yellow pine wood dust generated from the same, but untreated, piece of lumber. This increase in cytotoxicity suggests that one or more of the metals becomes bioavailable during the 24-h incubation period by either release into the cell culture media or after intracellular compartmentalization of wood dust particles. Importantly, the CCA-treated wood dust maintained this greater cytotoxicity (than the control wood dust) in the arsenic-resistant subline of cells. This finding suggests that either chromium or copper was responsible for the cytotoxic effects of CCA-treated wood dust. We can further speculate that copper played little role in the cytotoxic effects of CCA-treated wood dust because the LC50 for copper chloride was approxi- mately 150 μM (or 9 ppm), which is considerably higher than the concentration of copper in the cell culture media extract even if all of the copper was theoretically released from the CCA-treated wood dust. Because neither Cr nor As was re- leased into the cell culture media, it is likely that the observed cytotoxicity was not due to metals that were leached from the wood dust directly into the cell culture media during the incubation period. It is more likely that the CCA-treated wood dust-induced cytotoxicity was a result of the phagocytosis of wood particles and the subsequent release of metals, presumably Cr, from lysosomal vacuoles within the cells. Because the preservative metal oxides are present in CCA-treated wood at 0.8% by weight, the equivalent EC50 of arsenate and chromate in CCA-treated wood would be approximately 5μg/ml (from Fig. 1, upper panel). Comparison of this value with the actual EC50s for chromium and arsenic as their inorganic aqueous solutions (Fig. 4) suggests that the chromium and arsenic in CCA-treated wood may be up to 7-fold less toxic to cultured V79 cells than the inorganic water-soluble forms of these metals. Thus, bioavailability of the preservative metals in treated wood appears to play a role in the cytotoxicity of...
CCA-wood dust. Of course, this speculation does not take into account any potential interaction(s) of the metals that are present in combination in the CCA-treated wood.

In conclusion, these studies have demonstrated that compared with control wood dust, CCA-treated wood dust is more cytotoxic in vitro and induces an increase in the steady-state expression level of metallothionein mRNA. Thus, under the conditions of our experiments, the metals bound to wood fibers appear to become bioavailable. This bioavailability occurs despite the complete (as defined by the pressure-treated wood industry) fixation of the metals to the wood fibers. Interestingly, we observed a general decrease in cytotoxicity of the CCA-treated wood dust over a 2-month study period. This observation suggests that the freshly generated CCA-treated wood dust encountered in the workplace may represent a greater hazard than the wood dust that was used over a period of time in our bioavailability studies. Inhalation toxicology studies using respirable dust particles freshly generated from CCA-treated wood would address this issue.

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