Development of Biomarkers for Haloacetonitrile-Induced Cell Injury

Project Scope

Treated drinking water commonly contains a mixture of halogenated hydrocarbons (disinfection byproducts, or DBPs) that form when residual chlorine reacts with natural organic substances. Among these disinfection by-products are the halogenated acetonitriles (HANs), which have been shown to be toxic and mutagenic *in vitro* and *in vivo* assays, and carcinogenic in exposed laboratory animals.

The overall goal of this research project was to develop unique biomarkers in a readily accessible compartment such as peripheral (i.e., circulating) blood, for HAN exposure and HAN-induced cellular injury. The development and validation of biomarkers for use in human risk assessment can significantly improve the quality and reduce uncertainty in risk estimates associated with exposure to HANs in drinking water. The specific research objectives were to:

1. Evaluate some of the responses to HANs in peripheral blood cells *in vitro*, and develop an animal model for exposure to, and effects of, HANs; and
2. Identify target tissues of HAN-induced toxicity, cellular injury, macromolecular damage, and impaired cellular functions.

To address these objectives, an extensive series of *in vitro* experiments and supportive *in vivo* experiments were conducted using dichloroacetonitrile, chloroacetonitrile, and dibromoacetonitrile. The results and implications of some of the major experiments are summarized below.

Grant Title and Principal Investigator

Development of Biomarkers for Haloacetonitriles-Induced Cell Injury in Peripheral Blood (EPA Grant #R825955)

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Key Findings and Implications

Analytical Accomplishments:

- Demonstrated that *in vitro* and *in vivo* exposure to halogenated acetonitriles (HANs; disinfection by-products) produce toxic effects through interactions with DNA and other cellular macromolecules.
- Demonstrated HAN-induced *in vitro* toxicity in gastric and intestinal tissues under conditions analogous to human exposures through drinking water.
- Demonstrated that oxidative stress and damage to key macromolecules were the most important mechanisms by which HANs induce cellular injury and DNA damage.

Implications of Research and Impacts of Results:

- Supports the development of mechanism-based biomarkers of adverse human health effects resulting from HAN exposure in drinking water.
- Strengthens the basis for development of regulations and guidance concerning acceptable levels of chronic human exposure to HANs in drinking water.

Publications include 6 peer reviewed journal articles and 14 conference/symposium presentations.

Project Period: October 1997 to September 2000

Project Results and Implications

Molecular Interaction of Halogenated Acetonitriles with DNA: To evaluate HAN-induced DNA damage of peripheral blood, the investigator conducted a series of *in vitro* experiments was conducted on the effect of dichloroacetonitrile on the degradation of supercoiled plasmid DNA to circular or linear forms. The concentration- and time-dependence of the reaction of dichloroacetonitrile with DNA were also evaluated. Hydrogen peroxide was added to the incubation system containing dichloroacetonitrile and plasmid DNA to generate reactive oxygen species (ROS) that are necessary to conduct the experiments.

Relevance to ORD's *Drinking Water Research Multi-Year Plan* (2003 Edition)

This project contributes directly to the first of three Long-term Goals for drinking water research: (1) by 2010, develop scientifically sound data and approaches to assess and manage risks to human health posed by exposure to regulated waterborne pathogens and chemicals, including those addressed by Arsenic, M/DBP, and Six-Year Review Basis.

The M/DBP rules are an interrelated set of national regulations designed to provide public health protection against waterborne pathogens while minimizing the risks posed by exposure to DBPs. This research furthers understanding of the mechanisms of adverse effects of halogenated acetonitrile (HAN) exposure. Building on these results, mechanism-based biomarkers of HAN-exposure in humans can be developed. Furthermore, this research strengthens the basis for development of regulatory guidelines and policies governing acceptable levels of chronic human exposure to HANs in drinking water.

The results showed that dichloroacetonitrile oxidizes to reactive intermediates by ROS-generating systems *in vitro*. Furthermore, dichloroacetonitrile induces oxidative DNA damage and hydrogen peroxide mediation of dichloroacetonitrile-induced DNA damage is a possible mechanism of its genotoxicity.

The investigator conducted further *in vitro* experiments to examine the effect of the addition of iron (Fe II) on dichloroacetonitrile-induced DNA oxidative damage, since iron is typically present in mammalian tissues. Iron complexes are thought to catalyze the production of ROS such as hydroxyl (OH) radicals. Dichloroacetonitrile-induced damage to supercoiled plasmid DNA was evaluated in the presence of hydrogen peroxide with and without iron in the form of ferrous ammonium sulfate. Combined exposures to iron and hydrogen peroxide resulted in a 32 percent increase in DNA damage compared to hydrogen peroxide exposure alone. These results demonstrate the role and importance of iron in the mechanism of HAN-induced genotoxicity. They also support a hypothesis that metal-catalyzed oxidation of dichloroacetonitrile to reactive intermediates by ROS is a possible mechanism for HAN-induced DNA damage and genotoxicity.

Melatonin is secreted by the pineal gland and is important in the regulation of many hormones in the human body. It is a highly effective antioxidant and a known endogenous OH radical scavenger. In light of this, the researcher investigated the ability of melatonin to reduce dibromoacetonitrile-induced oxidative plasmid DNA damage in another series of *in vitro* experiments. The protective effect of vitamins E and C, two well-known endogenous cellular antioxidants, and ethanol, an exogenous antioxidant, were also tested. All tested concentrations of melatonin were found to protect the DNA from dibromoacetonitrile-induced oxidative damage. Vitamins C and E and ethanol were also found to demonstrate a dose-related protective effect against the degradation of plasmid DNA. These results confirm that oxidative stress-mediated activation of dibromoacetonitrile to a reactive intermediate is capable of inducing DNA strand breaks *in vitro*.

Molecular Interaction of Haloacetonitriles with Cellular Functions: In this phase of the grant, the investigator evaluated the relationships between dichloroacetonitrile exposure and apoptosis (programmed cell death) and necrosis in cultured mouse peritoneal macrophages. The macrophages were exposed to increasing concentrations of dichloroacetonitrile and resulting phagocytic activation was characterized by measuring ROS production and secretion of TNF-α (tumor necrosis factor precursor). The ratio of intracellular antioxidants GSH/GSSG (glutathione/oxidized glutathione) was also evaluated as an indicator of oxidative stress. Following dichloroacetonitrile treatment, increased GSSG levels and activation of macrophages were observed. A dose-dependent increase in apoptic cell death and degradation of genomic DNA was also observed in treated macrophages. Cellular necrosis was also

increased following dichloroacetonitrile exposure along with decreased viability. Together, these results suggest that the dose-dependent dichloroacetonitrile-induced apoptosis or necrosis in mouse peritoneal macrophages results from a disturbance in GSH/GSSG ratio and the subsequent oxidative damage.

Additional *in vitro* experiments on the cellular toxicity of dibromoacetonitrile in mouse fibroblasts resulted in apoptosis at low exposure levels and severe membrane damage at higher concentrations. "Comet" assays were used in a series of experiments to further characterize biomarkers for HAN-induced DNA damage and apoptosis in peripheral blood and target organs. In the comet assay, damaged cellular DNA unwinds and migrates faster than intact DNA under electrophoresis, giving an appearance of a comet, and serves as a sensitive indicator of apoptosis. In human leukemia cells, the investigator observed increases in the frequency of "comet cells" with increasing dibromoacetonitrile exposure. The treated cells were also examined using differential staining and light microscopy to help relate comet assay results with dibromoacetonitrile-induced apoptosis. The results show that dibromoacetonitrile has a damaging effect on cellular DNA and that it triggers programmed cell death signals, which in turn leads to apoptosis.

Target Organ Specificity of Halogenated Acetonitrile Toxicity: Gastrointestinal (GI) tissues are potential target sites of acute and chronic toxicity resulting from exposure to HANs. To examine the mechanism of HAN toxicity in GI tissues, the investigator dosed rats orally with chloroacetonitrile. *In vitro* effects on GSH homeostasis and its impact on oxidative DNA damage in rat gastric mucosal cells were also examined. Following a single low or high dose of chloroacetonitrile, all rats were sacrificed at different times up to 24 hours and gastric mucosal samples were collected. Gastric GSH contents and the integrity of genomic gastric DNA were assessed as was oxidative damage to gastric DNA. These experiments suggested that chloroacetonitrile-induced acute toxicity may be partially mediated by depletion of GSH, interruption of the energy metabolism, and induction of oxidative stress, leading to oxidative damage to gastric DNA. Cytotoxic effects and oxidative stress induction by chloroacetonitrile were next assessed in cultured rat gastric epithelial cells. Cytotoxicity was measured by assessing cell viability, lactate dehydrogenase release (an indication of tissue damage), changes in GSH levels, and lipid peroxidation. The results suggested that chloroacetonitrile has a potential cytotoxic effect in rat gastric epithelial cells, and that thiol group donors, antioxidants, and iron chelators can play a critical role in mediating CAN-induced cellular damage.

Summary: Research under this grant demonstrated that HAN exposure results in oxidative stress and oxidative damage to macromolecules, cells, and a range of tissues. HAN exposure was observed to increase both apoptosis and necrosis, apparently mediated by oxidative mechanisms. Collectively, these studies provide an improved understanding of the toxic mechanisms of HANs, and support the use of oxidative stress and DNA damage as biomarkers for adverse human health effects.

Investigator

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For More Information

NCER Project Abstract and Reports:

http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/195/report/0

Peer Reviewed Publications

Jacob, S., Abdel-Aziz, A.H., Shouman, S.A., and Ahmed, A.E. 1998. Effect of glutathione modulation on the distribution and transplacental uptake of 2-[14C] chloroacetonitrole (CAN) quantitative whole-body autoradiographic study in pregnant mice. Toxicology and Industrial Health 14(4):533-546.

Ahmed, A.E., and Jacob, S. 1999. Dichloroacetonitrile induces oxidative stress as a mediator of apoptosis or necrosis in mouse peritoneal macrophages (MPM). The Toxicologist 48(1):155.

Ahmed, A.E., Jacob, S., and Nouraldeen, A.M. 1999. Chloroacetonitrile (CAN) induces glutathione depletion and 8-hydroxylation of guanine bases in rat gastric mucosa. Journal of Biochemical and Molecular Toxicology 113:119-126.

Ahmed, A.E., Aronson, J., and Jacob, S. 2000. Induction of oxidative stress and TNF-alpha secretion by dichloroacetonitrile, a water disinfection byproduct, as mediators of apoptosis or necrosis in a murine macrophage cell line (RAW). Toxicology In Vitro 2000;14(3):199-210.

Ahmed, A.E., Johnson, B., and Jacob, S. 2005. Apoptic potency and cytotoxic effects of dibromomoacetonitrile in human leukemia cells. Journal of Toxicology and Environment Health (submitted).

Chiryil, A., Jacob, S., and Ahmed, A.E. 2005. Hydrogen peroxide catalyzed dibromoacetonitrile-induced DNA strand breaks: effect of dietary modulators and antioxidants. Pharmacology and Toxicology (submitted).