

The effect of iron on the biological activities of erionite and mordenite

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Abstract

Epidemiological data has demonstrated that environmental and/or occupational exposure to mineral particulates may result in the development of pulmonary fibrosis, bronchogenic carcinoma and malignant mesothelioma many years following exposure. It has been suggested that the genotoxic effects of fibrous particulates, such as asbestos, is due in part to the generation of reactive oxygen species (ROS) from iron associated with the particulates. However, the molecular mechanisms by which mineral particulates induce ROS that results in genotoxic damage remains unclear. The naturally occurring zeolites, erionite and mordenite share several physicochemical properties but they elicit very different biological responses, with erionite, a fibrous particulate, being highly toxic, and mordenite, a nonfibrous particulate, being relatively benign. We are using these natural zeolites as a model system to determine what physicochemical properties of these zeolites are responsible for their biological response(s) and to evaluate the parameters that influence these responses. The purpose of the present study was to determine the mutagenic potential of erionite and mordenite and to determine whether this mutagenic potential was modulated by iron. The results of this study using the Chinese hamster ovary cell line AS52 demonstrated that erionite was more cytotoxic than mordenite. However, the cytotoxicity of both zeolites was increased in the presence of physiological concentrations of ferrous chloride. Ferrous ions (5–20 μM) significantly ($p < 0.001$) increased the cytotoxicity of mordenite, but only at the highest concentration (16 $\mu\text{g}/\text{cm}^2$) of mordenite tested. Conversely, only the highest concentration (20 μM) of ferrous ion significantly ($p < 0.001$) increased the cytotoxicity of erionite, but this enhanced cytotoxicity occurred over a wider concentration range (6–16 $\mu\text{g}/\text{cm}^2$) of erionite. Mordenite was not mutagenic at any of the concentrations tested, and the mutagenic potential of mordenite was not enhanced by the addition of ferrous ion. Conversely, erionite was mutagenic in a dose-response manner at concentrations greater than 6 $\mu\text{g}/\text{cm}^2$ and the mutagenic potential of erionite was significantly enhanced by the addition of ferrous ions. These results suggest that while the cytotoxicity of mordenite and erionite may be related to the ability of these fibers to transport iron into a cell, the different coordination state of iron associated with the two fiber surfaces is critical for inducing genotoxic damage.

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1. Introduction

Epidemiological and experimental data have demonstrated that environmental and/or occupational exposure to mineral particulates may result in the development of pulmonary fibrosis, bronchogenic carcinoma and malignant mesothelioma (Mossman et al., 1990; Rom et al., 1991;

Heppleston, 1991). Asbestos, which is a family of fibrous silicates, is the most extensively studied mineral fiber. Over the past 30 years, numerous studies have been performed to determine the mechanisms by which asbestos causes disease. Various physicochemical properties such as fiber dimensions, fiber structure surface charge, ability to generate reactive oxygen species and biopersistence have been implicated in inducing disease (Mossman et al., 1990; Barrett, 1994; Kane, 1996; Vallyathan and Shi, 1997). However, the molecular mechanisms by which fibrous particulates induce cellular damage, as well as the cellular

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response to these particulates as it relates to carcinogenesis, remains unclear.

While there is considerable data on the cytotoxic effects of asbestos fibers, there is less information concerning the genotoxicity of asbestos fibers. The genotoxicity data varies greatly, ranging from no effect to inducing chromosomal aberrations (Hei et al., 1992; Both et al., 1994; Takeuchi and Morimoto, 1994; Dong et al., 1994; Both et al., 1995; Ault et al., 1995; Park and Aust, 1998; Okayasu et al., 1999a,b; Keane et al., 1999). This variation is probably the result of several factors such as the purity, source and type of the asbestos, the in vitro model employed and the endpoint examined (Jaurand, 1996, 1997). While several mechanisms have been proposed to explain the genotoxic effects of asbestos (Jaurand, 1996, 1997; Kane, 1996) several studies have focused on the potential role of reactive oxygen species in this process. Since asbestos fibers contain ferrous/ferric ions, it has been proposed that the cytotoxic and genotoxic effects of asbestos fibers may be related to the generation of reactive oxygen species and/or reactive nitrogen species mediated by the iron (Hansen and Mossman, 1987; Mossman and Marsh, 1989; Korkina et al., 1992; Kamp et al., 1992; Maples and Johnson, 1992; Chao et al., 1994; Kinnula et al., 1994; Hei et al., 1995; Xu et al., 1999; Unfried et al., 2002). However, the mechanism(s) by which asbestos associated iron induces genotoxic damage remains unclear.

Zeolites are a group of aluminosilicates that are characterized by a framework of interlocking tetrahedra of SiO_4 and AlO_4 (Breck, 1974). Naturally occurring zeolites include erionite and mordenite. Erionite, which is fibrous in nature, is a known carcinogen with a carcinogenic potential that is equal to or greater than crocidolite (Rom et al., 1983; Wagner et al., 1985; Eborn and Aust, 1995). Conversely, while mordenite, which is nonfibrous, has a chemical composition similar to that of erionite, it is not carcinogenic and has been reported to have “slight biological activity” (Guthrie, 1992). Erionite and mordenite contain little if any iron under natural conditions (Eborn and Aust, 1995; Fubini and Mollo, 1995). However, because of their ion-exchange capabilities, they can accumulate iron on their surface (Eborn and Aust, 1995; Fubini and Mollo, 1995). It has been suggested that the biological activity of erionite is due to the presence of iron on its surface (Eborn and Aust, 1995; Fubini and Mollo, 1995). We have been using these natural zeolites as a model system to determine what physicochemical properties of these zeolites are responsible for their biological response(s) and to evaluate the parameters that influence these responses (Hogg et al., 1996; Long et al., 1997; Fach et al., 2002). The purpose of the present study was to determine the mutagenic potential of erionite and mordenite and to determine whether this mutagenic potential was modulated by iron. For these studies, we used the Chinese hamster ovary cell line, AS52. AS52 cells are a transgenic cell line that lack the normal X-linked mammalian hypoxanthine guanine phos-

phoribosyltransferase (*hprt*) gene, but that contain a single functional copy of the *Escherichia coli* xanthine-guanine phosphoribosyltransferase (*gpt*) gene stably integrated into the CHO genome (Tindall et al., 1984, 1986, 1987; Tindall and Stankowski, 1989; Hart and Tindall, 1997). Since the *gpt* gene is not essential for growth or survival of AS52 cells, it is possible to isolate mutants containing a variety of mutations, including: point, interchromosomal deletions, mitotic recombinations, gene–chromosomal conversions and multilocus deletions. We, as well as others, have demonstrated that AS52 cells can be used to determine molecular and mechanistic features associated with mutagenesis in mammalian cells (Tindall et al., 1984, 1986, 1987; Tindall and Stankowski, 1989; Hsie et al., 1990; Ariza and Williams, 1996; Ariza et al., 1998).

2. Materials and methods

2.1. Materials

Ham's F-12 medium, Hank's balanced salt solution (HBSS) and dialyzed fetal bovine serum (DFBS) were purchased from GIBCO Life Technologies (Buffalo, NY). Mordenite and erionite were purchased from Minerals Research (Clarkson, NY). All chemicals and enzymes were of the highest purity available.

2.2. Methods

2.2.1. Size fractionation of zeolites

Mordenite and erionite were fractionated as we have described (Fach et al., 2002). Briefly, the zeolite was suspended in water and allowed to settle. The supernatant was removed, vacuum filtered (0.80 μm) and dried at 70 °C. The particle size of the fractionated material was determined by scanning electron microscopy. The size of the fractionated erionite ranged from 0.7 to 13 μm in length with a median size of 3 μm , while the median size of the mordenite used in this study was 3.1 μm . The zeolites were suspended in deionized distilled water and autoclaved. Prior to use, the zeolites were suspended in HBSS at a concentration of 1.5 mg/ml.

2.2.2. Mutagenesis

AS52 cells were obtained from Dr. Kenneth Tindall (present address: North Carolina Biotechnology Center, Research Triangle Park, NC). AS52 cells were maintained, as described previously (Ariza and Williams, 1996), at 37 °C in a humidified 5% CO_2 environment in F-12 medium, which contains 3 μM $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, supplemented with 5% (v/v) heat-inactivated DFBS and MPA additives (10 $\mu\text{g}/\text{ml}$ mycophenolic acid, 25 $\mu\text{g}/\text{ml}$ adenine, 50 μM thymidine, 250 $\mu\text{g}/\text{ml}$ xanthine and 3 μM aminopterin).

Mutagenesis was performed with minor modifications of the procedures that we have described previously (Ariza and

Williams, 1996). Briefly, AS52 cells (10^6) were exposed to various concentrations of zeolites (expressed as $\mu\text{g}/\text{cm}^2$ of culture dish) with and without added soluble iron (ferrous chloride 1–20 μM) for 24 h in Ham's F-12 medium containing 5% DFBS. Following treatment, cells were washed three times with HBSS and subcultured to allow for phenotypic expression. Selection was performed 6–8 days following treatment, by culturing 10^6 AS52 cells at a density of 2×10^5 cells per 100 mm dish in F-12 medium containing 5% DFCS and 10 μM 6-thioguanine (TG). Cells were incubated for 10–14 days and examined for the development of TG-resistant (TG^r) clones. Only those clones containing greater than 50 cells were counted.

Cytotoxicity and relative cloning efficiency (RCE) was determined, as we have described (Ariza and Williams, 1996). Briefly, cytotoxicity is determined 24 h after initiation of treatment, by determining the RCE of the treated population when compared to nontreated controls. RCE is also determined for each population simultaneously with TG selection. The cloning efficiency of nontreated AS52 cells is 76–96%. The historical spontaneous mutation frequency of AS52 cells in our laboratory is 10.5 ± 2.7 TG^r mutants/ 10^6 clonable cells. As a positive control, AS52 cells were treated with ethylmethanesulfonate (EMS) as described previously (Tindall et al., 1984; Ariza and Williams, 1996). The mutation frequency under these conditions was 346.7 ± 22.4 TG^r mutants/ 10^6 clonable cells.

2.2.3. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as we have described previously (Long et al., 1997). Briefly, the cells were fixed in 3% glutaraldehyde. Following overnight fixation, the cells were rinsed with cacodylate (0.1 M) using two 15-min rinses. The cells were then placed in osmium tetroxide for 1 h. The cells were then dehydrated using alcohols, progressively from 50% to 100% for 30 min each. They were then embedded in Medcast (Ted Pella, Redding, CA). The cells were then examined using a Philips 300 microscope (Philips Electronics Inst., Mahwah, NJ) to evaluate their ultrastructural morphology.

2.3. Statistical analyses

Statistical analyses of the data were performed using Student's *t*-test. Statistical analysis was applied to obtain 95% confidence intervals. *p*-values of 0.05 or less were considered significant.

3. Results

Our previous study demonstrated that in cell free systems, erionite, a known carcinogen, induces the generation of hydroxyl radicals to a greater degree than mordenite, a zeolite fiber that is not carcinogenic (Fach et al., 2002). To further examine the role of reactive oxygen species induc-

tion as it relates to the cytotoxic and genotoxic properties of these fibers, we utilized the AS52 cell line as a model system to determine the role iron may have in modulating the biological activity of these fibers.

AS52 cells are cultured in F-12 medium, which contains 3 μM ferrous sulfate. Under these conditions, the RCE ranges from 76% to 96%, and the spontaneous mutation frequency is 10.5 ± 2.7 TG^r mutants/ 10^6 clonable cells. To determine the effects of iron, erionite and mordenite on AS52 cells, cells were treated with either zeolite alone or in combination with iron and the effects of the treatment on cytotoxicity and the mutation frequency of the *gpt* recorder gene were determined and compared to values on nontreated controls. Treatment of cells in F-12 medium supplemented with ferrous chloride (1–20 μM) for 24 h resulted in a typical dose–response curve indicating there was a correlation between the concentration of ferrous ion and cell viability. There was a statistically significant ($p < 0.005$) increase in the cytotoxicity of cells treated with concentrations of ferrous chloride greater than 1 μM when compared to the nontreated control (Fig. 1a). Treatment of AS52 cells with a 20 μM concentration of ferrous chloride resulted in a $22.6 \pm 2.6\%$ decrease in cell viability. Exposure of AS52 cells to either erionite or mordenite also resulted in a dose-dependent cytotoxicity of the treated cells when compared to the nontreated control. There was a statistically significant ($p < 0.001$) increase in the cytotoxicity of cells treated with mordenite at concentrations of 6 $\mu\text{g}/\text{cm}^2$ or greater (Fig. 1b). Erionite, however, was consistently more cytotoxic than mordenite (Fig. 1c). There was a statistically significant ($p < 0.005$) increase in the cytotoxicity of erionite-treated cells at concentrations as low as 2 $\mu\text{g}/\text{cm}^2$. The addition of ferrous ion to the mordenite-containing medium enhanced the cytotoxicity of mordenite when compared to cells treated with fiber alone, but this enhancement was only significant ($p < 0.001$) in cells treated with 16 $\mu\text{g}/\text{cm}^2$ mordenite and ferrous ion concentrations from 5 to 20 μM . Likewise, co-treatment of AS52 cells with iron and erionite significantly ($p < 0.001$) enhanced the cytotoxicity as compared to cells treated with either iron or erionite, but only at an iron concentration of 20 μM and only at erionite concentrations greater than 6 $\mu\text{g}/\text{cm}^2$.

Treatment of cells with ferrous ion (1–20 μM) for 24 h also resulted in a dose-dependent increase in the mutation frequency, ranging from a 1.6-fold increase at 1 μM to a 4.6-fold increase at 20 μM when compared to the nontreated control, which had a spontaneous mutation frequency of 12.25 ± 3.11 TG^r mutants/ 10^6 clonable cells. (Fig. 2a). This increase in mutation frequency was statistically significant ($p < 0.001$) at iron concentrations of 5 μM and greater. There was no increase in the mutation frequency of cells treated with mordenite or with mordenite and ferrous ion when compared to the appropriate controls (nontreated and ferrous ion-treated cells) (Fig. 2b). There did appear to be a slight decrease in the relative mutation frequency in cells treated with mordenite and ferrous ion when compared to

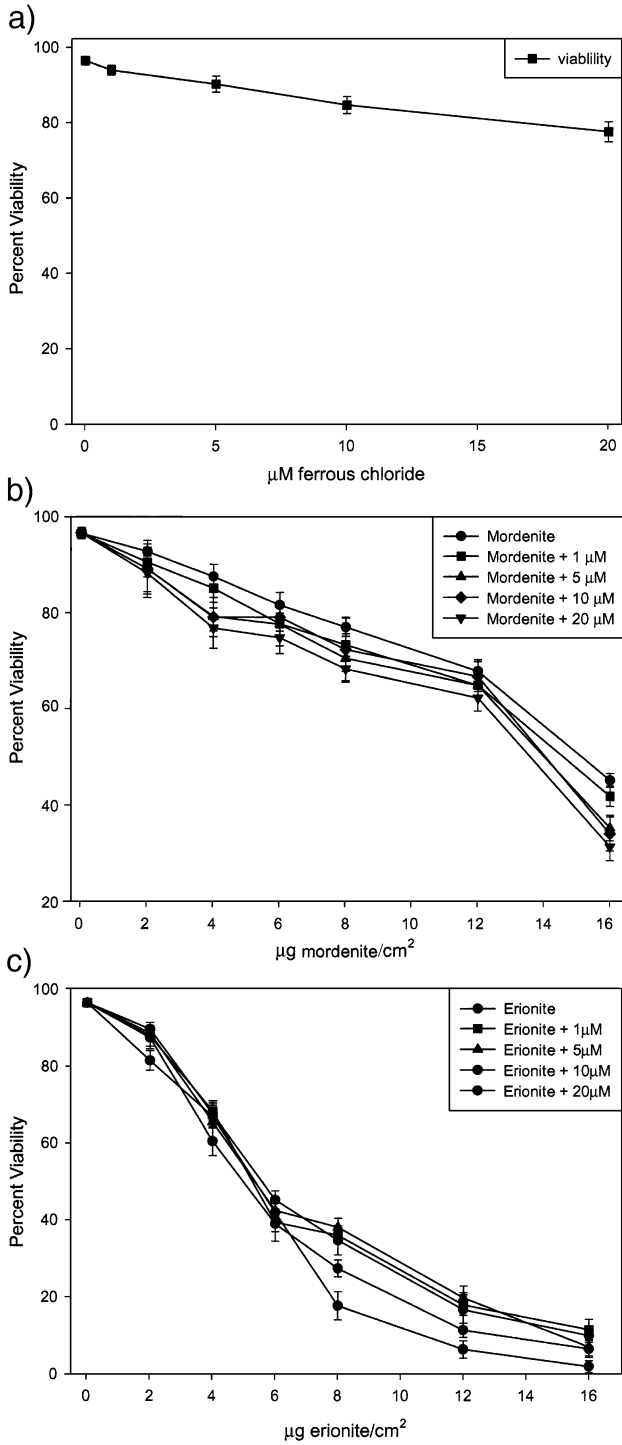


Fig. 1. Concentration–inhibition curves of ferrous chloride, mordenite and erionite on viability of AS52 cells. Cells were treated and viability determined as described in Section 2. Values shown are the means \pm S.D. of a minimum of three independent experiments. (a) Ferrous chloride-treated cells. (b) Mordenite-treated cells. (c) Erionite-treated cells.

ferrous ion treated controls, but this was not statistically significant except for cells treated with 5 μ M ferrous ion and mordenite (12 and 16 μ g/cm²). The relevance of this decrease is not known.

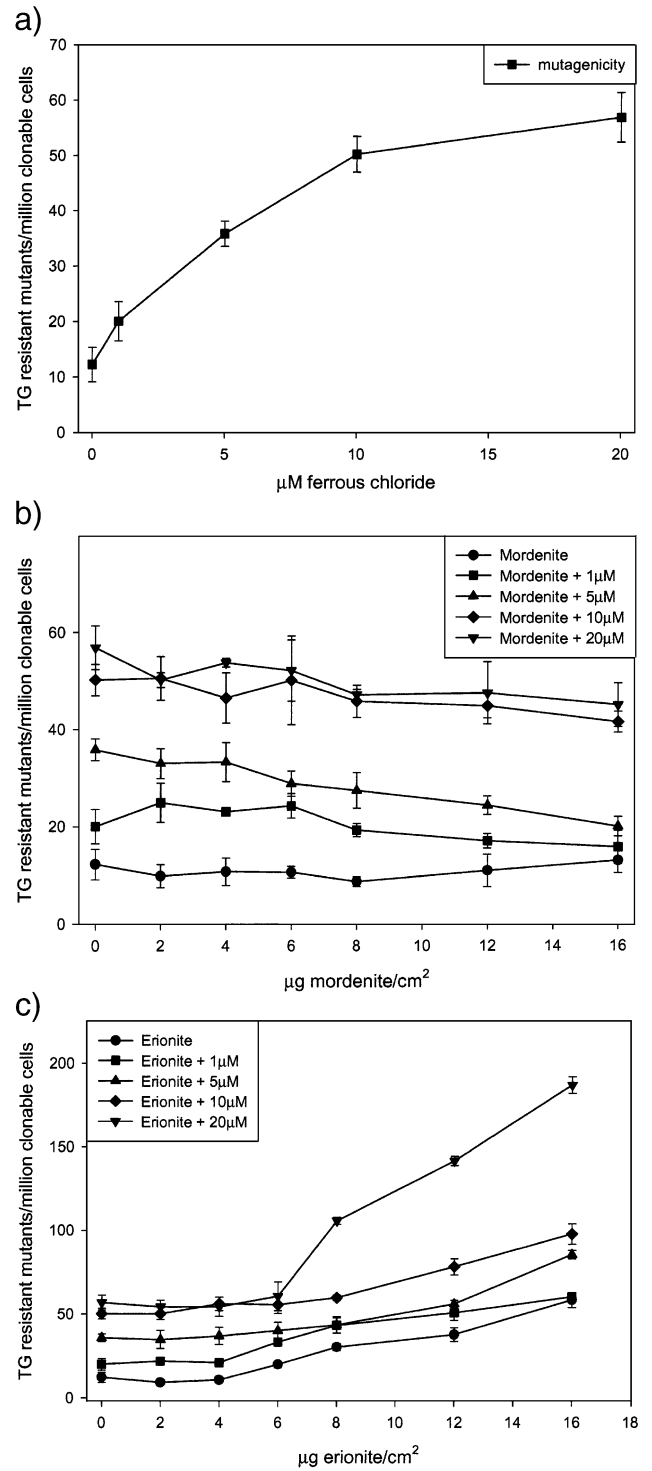


Fig. 2. Iron- and zeolite-induced mutagenesis of AS52 cells. Mutagenesis was performed as described in Section 2. Values shown are the means \pm S.D. of a minimum of three independent experiments. The positive control, treatment of AS52 cells with EMS (300 μ g/ml), resulted in a mutation frequency of 421.49 ± 63.58 TG^r mutants/10⁶ clonable cells. (a) Ferrous chloride-treated cells. (b) Mordenite-treated cells. (c) Erionite-treated cells.

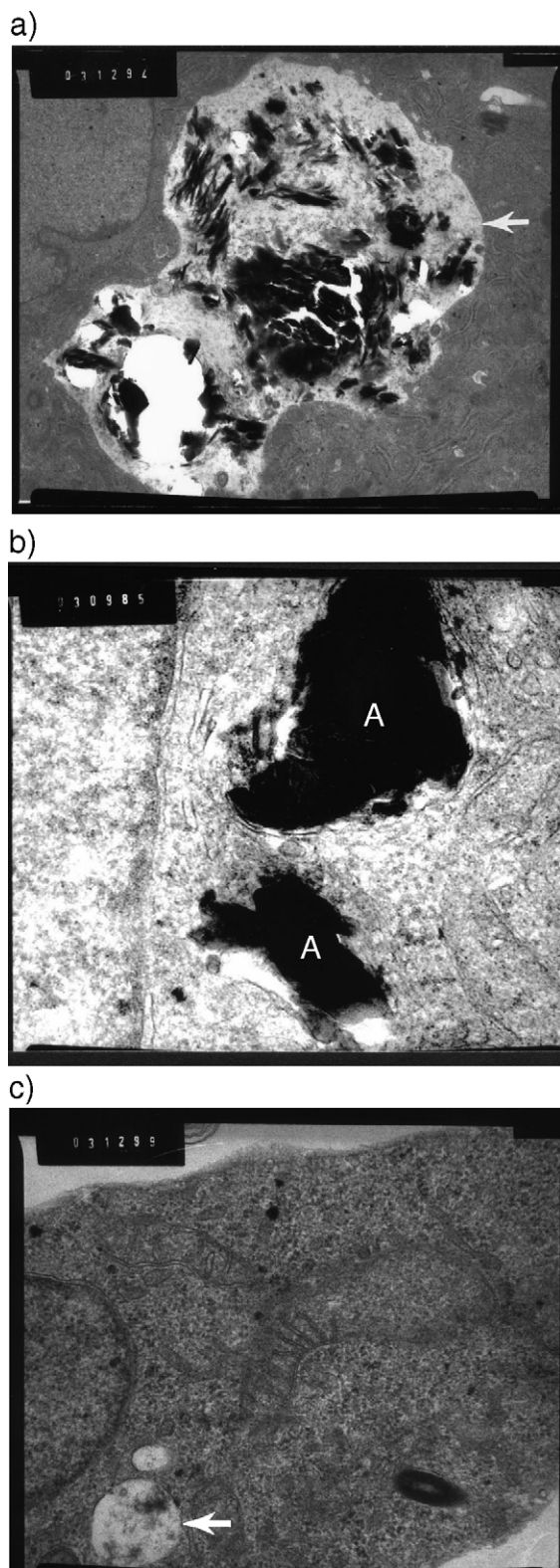


Fig. 3. TEM of AS52 cells treated with mordenite or erionite. (a) AS52 cell exposed to mordenite at 24-h posttreatment. Lysosome contains numerous particulates. Magnification 11,000 \times . (b) AS52 cell exposed to erionite at 24-h posttreatment. Dense clusters of particulates (A) are present in the cytoplasm. Magnification 53,000 \times . (c) AS52 cell (control) at 24 h. Arrow points to apparent autophagy material. Magnification 28,000 \times .

In contrast to mordenite, there was a significant ($p < 0.01$ – 0.001) increase in the relative mutation frequency of cells treated with erionite when compared to nontreated cells (Fig. 2C) at concentrations of erionite equal to or greater than greater than $6 \mu\text{g}/\text{cm}^2$. The fold increase ranged from 1.6 to 4.8 for cells treated with 6 and $16 \mu\text{g}$ of erionite/ cm^2 respectively. At concentrations of erionite below $8 \mu\text{g}/\text{cm}^2$, the mutation frequency of cells treated with erionite and ferrous ion were not significantly different from the mutation frequency obtained for cells treated with ferrous ion. However, at erionite concentrations equal to or greater than $8 \mu\text{g}/\text{cm}^2$, there was a statistically significant ($p < 0.001$) increase in the relative mutation rate of cells treated with erionite and ferrous ion when compared to cells treated with only ferrous ion. This ranged from a 1.2-fold increase in cells treated with $8 \mu\text{g}$ of erionite/ cm^2 and $20 \mu\text{M}$ ferrous chloride to a 3.3-fold increase in cells treated with $16 \mu\text{g}$ of erionite/ cm^2 and $20 \mu\text{M}$ ferrous chloride.

TEM was performed to determine the cellular location of mordenite (Fig. 3a) and erionite (Fig. 3b) in treated cells. Cells cultured without zeolites were employed as controls (Fig. 3c). In zeolite-treated cells, small masses of fine particulates could often be seen external to the cells and lightly adherent to the cell periphery. Small clusters of particulate material could also be observed within the cytoplasm of cells, exposed to either mordenite (Fig. 3a) or erionite (Fig. 3b), but not in the control cells (Fig. 3c). These clusters were sharply delineated from surrounding cytoplasm and were characterized by an irregularly angular contour. Often there was evidence of their having been enclosed in a membrane-bound rounded structure such as a lysosome (Fig. 3a). Frequently, particulates were free in the cytoplasm with no evidence at this time (24-h postexposure) of being in a membrane-bound structure. In these cells, adjacent organelles showed nearly normal morphology (such as in Fig. 3b) aside from a diffusion of finely granular material. This fine granularity had the effect of “outlining” the contours of immediately adjacent organelles such as endoplasmic reticulum (Fig. 3b). The nature of this was not evident but could be an example of membrane-facilitated calcification in which calcium ions bind to the phospholipids present in the membranes.

4. Discussion

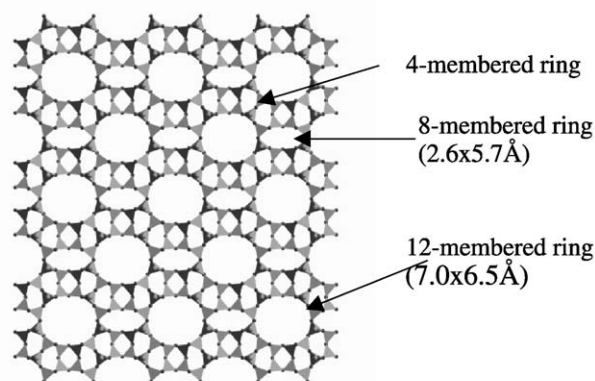
There are over 70 varieties of synthetic inorganic fibers with different physiochemical and morphological characteristics that are used in a variety of applications. With the increased use of natural and synthetic fibers in anthropogenic activities, there is increased risk of human exposure to these fibers. However, with the exception of asbestos there have been few studies to address possible genotoxic risks associated with exposure to these fibers. Furthermore, the molecular mechanisms by which mineral particulates are genotoxic remain unclear.

We are using the natural zeolites, erionite and mordenite as model mineral particulates for determining which physicochemical properties of these molecules are responsible for eliciting biological responses as it relates to lung disease. While erionite and mordenite share several physicochemical properties, they elicit very different biological responses (Wagner et al., 1985; Guthrie, 1992; Both et al., 1994; Eborn and Aust, 1995). Our studies have demonstrated that erionite is more efficient than mordenite at generating hydroxyl radicals in cell free systems (Fach et al., 2002). To further examine the relationship between zeolite-induced ROS production and the biological response to zeolite exposure, we used the AS52 cells as a model to quantitate the cytotoxicity and mutagenic potential of these zeolites.

In this study, we demonstrate that erionite and mordenite differ in their cytotoxic and mutagenic capabilities, thus, supporting conclusions from earlier studies that erionite and mordenite elicit different biological responses (Wagner et al., 1985; Guthrie, 1992; Both et al., 1994; Eborn and Aust, 1995). The data also demonstrate that the biological effects of both erionite and mordenite are increased in the presence of iron. The cytotoxicities of the zeolites were enhanced by ferrous ions, but the increase in cytotoxicity was only significant at higher iron and/or zeolites concentrations. Mordenite was not mutagenic and ferrous ion did not alter mordenite's mutagenic potential. Conversely, erionite ($2\text{--}16\ \mu\text{g}/\text{cm}^2$) was mutagenic and the mutagenic potential was significantly increased in the presence iron ($20\ \mu\text{M}$) when compared to nontreated cells or cell treated with only iron.

Naturally occurring erionite and mordenite contain little or no iron. However, their ion exchange capabilities allow them to accumulate iron from their environment (Guthrie, 1992). We demonstrated that erionite and mordenite accumulate iron in cell free systems, and that while erionite contains more iron/gm, the amount of iron accumulated on the surface is similar in both zeolites (Fach et al., 2002). Since the concentration of iron in serum ranges from 4 to $30\ \mu\text{M}$, some of the biological effects of these zeolites may be the result of their ability to adsorb iron on their surface and transport the iron intracellularly. However, if the biological effects of these zeolites were merely due to their ability to transport iron into a cell, then the toxicity and mutagenic potential of mordenite should be similar to erionite, but this is not the case. While erionite and mordenite are aluminosilicates, their structures are very different (Fig. 4). Erionite surface has a network of eight rings ($3.6 \times 5.1\ \text{\AA}$), whereas mordenite has 12-ring ($7.0 \times 6.5\ \text{\AA}$) openings, as well as eight and four rings. The iron on the surfaces of these zeolites is coordinated to different aluminosilicate rings, suggesting that their coordination environments are very distinct. The coordination environment around the iron can influence the Fenton reaction by limiting access of iron to hydrogen peroxide and by modifying the iron redox potential. The coordina-

a) Mordenite



b) Erionite

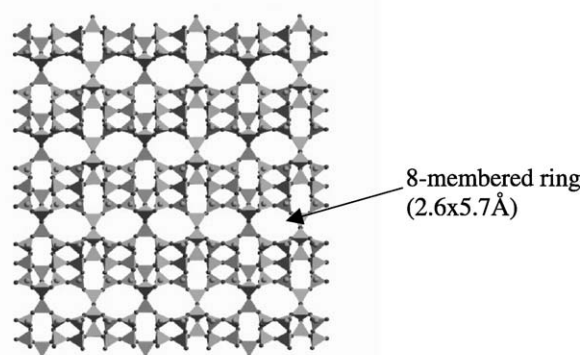


Fig. 4. The structure of mordenite and erionite.

tion chemistry of ferrous/ferric ions is important for the generation of hydroxyl radical (Graf et al., 1984) and the iron redox potential can be modified by the coordination state of the iron associated with the zeolite (Senaratne et al., 1996). Results of our study in a cell-free system suggests that not all iron species on a zeolite surface are Fenton-active (Fach et al., 2002), and this supports the premise of Fubini et al. (1995), who suggested that only a few iron species on minerals with the correct redox potential and coordination state are active in hydroxyl radical generation. Furthermore, while the role of serum proteins was not addressed in this study, they can be adsorbed to the surface of the zeolite particle and further modify surface chemistry of the particle. While additional studies are necessary, these studies support the hypothesis that the coordination state of iron and thus the structure of the zeolite is a critical factor in the biological response to the particulate.

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