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Erionite Induces Production of Autoantibodies and IL-17 in C57BL/6 Mice

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Abstract

Background—Erionite has similar chemical and physical properties to amphibole asbestos, which induces autoantibodies in mice. Current exposures are occurring in North Dakota due to the use of erionite-contaminated gravel. While erionite is known to cause mesothelioma and other diseases associated with asbestos, there is little known about its effects on the immune system.

Objectives—We performed this study to determine whether erionite evokes autoimmune reactions in mice.

Methods—Bone marrow derived macrophages (BMDM) were used to measure toxicity induced by erionite. Cytokine production by BMDM and splenocytes of C57BL/6 mice was examined by bead arrays and ELISA following exposure to erionite, amphiboles and chrysotile. Wild type C57BL/6 mice were exposed to saline, erionite, amphibole asbestos (Libby 6-Mix) or chrysotile through intratracheal instillations at equal mass (60 µg/mouse). Seven months after exposure, sera were examined for anti-nuclear antibodies (ANA) and IL-17. Immunohistochemistry was used to detect immune complex deposition in kidneys.

Results—Erionite and tremolite caused increased cytokine production belonging to the T_H17 profile including IL-17, IL-6, TGFβ, and TNF-α. The frequency of ANA was increased in mice treated with erionite or amphibole compared to saline-treated mice. IL-17 and TNF-α were elevated in the sera of mice treated with erionite. The frequency of immune complex deposition in kidneys increased from 33% in saline-treated mice to 90% with erionite.

Conclusions—These data demonstrate that both erionite and amphibole asbestos induce autoimmune responses in mice, suggesting a potential for adverse effects in exposed communities.

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Conflict of Interest Statement

The authors confirm that there are no conflicts of interest.

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Keywords

Asbestos; Autoimmunity; Erionite; Th17; Immunotoxicology

¹INTRODUCTION

Erionite is a fibrous material classified in a group of minerals called zeolites. Erionite occurs naturally when volcanic ash reacts with ground water and forms fibrous masses inside the hollow spaces of rock. Erionite fibers share some morphologic and chemical similarities with amphibole asbestos. They are both mixtures of long and straight mineral fibers composed of long chain silicon oxides (averaging $\text{Si}_{14}\text{O}_{36}$ and Si_8O_{22} , respectively) with various cations depending on the specific type of fiber (Carbone et al. 2012). Both asbestos and erionite exposure can cause pleural fibrosis and malignant mesothelioma, and there have been extraordinarily high rates of mesothelioma in villages located in central Turkey where the residents have been exposed to erionite (Carbone et al. 2011).

Many geologic formations contaminated with erionite have been found throughout the western United States (Carbone et al. 2011; Van Gosen et al. 2013). Several gravel pits that are contaminated with erionite have been excavated in western North Dakota (Dunn County Erionite Workgroup, 2010). Since the 1980s, this gravel has been used to construct more than 300 miles of local roads, parking lots, baseball fields, playgrounds and other areas (Carbone et al. 2011). Activity based sampling demonstrated that exposures in Dunn County (0.175 fibers/cc) were higher or similar to those found in Turkish villages (0.0431–0.221 fibers/cc) experiencing mesothelioma rates as high as 1 in 1000. This is compared to rates of 1–15 per 10^6 in most areas of the United States even where asbestos exposure is high (Carbone et al., 2011). Fortunately, a rise in mesothelioma cases among exposed populations in North Dakota has not yet been observed. Of note, in Karain, a Turkish village with over 50% of all deaths (1990–2006) due to mesothelioma, it was reported that the average latency (time between exposure and the development of disease) for mesothelioma was 53.8 years (Metintas et al., 2010). Therefore, given the long latency between the development of mesothelioma and the relatively recent use of erionite-contaminated gravels in North Dakota (ND), it may still be too early to observe this health outcome. A recent study of Dunn County, ND, road workers, without a history of asbestos exposure, did report evidence of fibrotic pleural disease consistent with erionite exposures (Ryan et al. 2011). Thus, given the demonstrated toxicity and health effects associated with erionite exposures, it is important to implement preventive public health actions to reduce exposures and to follow exposed workers and communities for adverse effects and any opportunities for early medical interventions.

A population in Libby, Montana has been exposed amphibole asbestos due to the mining of contaminated vermiculite. Not only has this population been suffering from increased incidence of mesothelioma and other pulmonary diseases, but screening done by the Agency for Toxic Substances and Disease Registry (ATSDR) in 2000–2001 found that 6.7% of the

¹Abbreviations: ANA, antinuclear autoantibodies; BMDM, Bone marrow derived macrophages; SLE, Systemic Lupus Erythematosus; TGF, Transforming Growth Factor; TNF, Tumor Necrosis Factor; CBA; Cytometric Bead Array;

people screened were diagnosed with a systemic autoimmune disease including systemic lupus erythematosus (SLE), scleroderma, and rheumatoid arthritis, where the expected prevalence for these three diseases is less than 1% (Pfau et al. 2005). The Libby population was also found to have higher levels of autoantibodies when compared to a population with no known exposure to amphibole asbestos (Pfau et al. 2005). Further study of the Libby population showed that the risk for being diagnosed with a systemic autoimmune disease (SAID) increases with increased exposure to amphibole asbestos, supporting the hypothesis that asbestos exposure is linked to autoimmunity (Noonan et al. 2006).

Since erionite and amphibole asbestos share some physical properties and can cause similar diseases, the purpose of this study was to determine whether erionite is able to stimulate autoimmune responses, also similar to amphibole asbestos. Immune cells, specifically cultured macrophages and mixed splenocytes, were exposed to erionite *in vitro* and examined for various cytokines that have been implicated in autoimmune disorders: in particular, interleukin-17 (IL-17), which is produced by T Helper 17 (T_H17) cells. T_H17 cells form in the presence of TNF α , IL-6 and TGF- β , which are produced by innate immune cells including macrophages (Furuzawa-Carballeda et al. 2007). Some studies have shown IL-17 plays a part in the pathogenesis of rheumatoid arthritis by demonstrating elevated levels of IL-17 in synovial fluids of diseased joints and activation of osteoclasts (Kotake et al. 1999). Elevated serum IL-17 has been demonstrated in individuals with SLE, but the role of IL-17 in SLE is still unclear (Afzali et al. 2007).

Given the potential role of cytokines of the T_H17 lineage in autoimmune diseases, it was the hypothesis of this study that immune cells *in vitro* and *in vivo* would express T_H17 cytokines after exposure to amphibole asbestos, which has been associated with autoimmunity in the Libby, MT population. Also, since erionite and amphibole asbestos share similar physical characteristics, it is also hypothesized that erionite will evoke a similar response by immune cells to produce T_H17 cytokines.

Autoantibodies against ubiquitous antigens are hallmarks of systemic autoimmune diseases (Darrach and Andrade 2013). The presence of these antibodies was examined using C57BL/6 mice exposed to erionite through intratracheal instillations. Mice were also exposed to saline only, amphibole asbestos, and to chrysotile asbestos, which has not been associated with autoimmunity. A study done by Pfau et al. in 2008 demonstrated increased autoantibodies in C57BL/6 mice exposed to an amphibole asbestos, tremolite (Pfau et al. 2008). However, to our knowledge, this type of study has not been done using erionite. Therefore, this study went on to assess how erionite affects certain immune parameters that are associated with autoimmunity *in vivo*.

METHODS

Fibers

Erionite collected at Rome, Oregon, was provided by the U.S. Environmental Protection Agency (EPA) as a characterized sample of purified mineral fibers. Transmission Electron Microscopy (TEM) characterization of the EPA erionite was provided by Dr. Jed Januch, EPA (Table 1). Pure Korean tremolite asbestos (amphibole) was provided by Dr. Ann

Wylie, University of Maryland. Libby 6-Mix asbestos containing tremolite, other amphibole asbestos forms, and closely related asbestiform fibers of winchite and richterite, was provided by U.S. Geological Survey (Meeker et al. 2003). Chrysotile was provided by the National Toxicology Program by Dr. Dori Germolec (NTP 1990). Wollastonite was kindly provided by Dr. Andrij Holian (University of Montana). All fibers were suspended in sterile phosphate buffered saline (PBS, pH 7.4), and sonicated (Branson Ultrasonics, Danbury, CT) for five minutes prior to use to minimize aggregation of the fibers. Concentrations used in cell cultures are given in $\mu\text{g}/\text{cm}^2$ with the observation that the fibers precipitated to the bottom of the well, with densities being 2.5 g/cm^2 for erionite (Dogan et al. 2008), 2.6 g/cm^2 for intermediate chrysotile (NTP 1990), and 3.0 g/cm^2 for tremolite (Webber et al. 2008).

Mice

All experiments on mice were approved by the Idaho State University Institutional Animal Care and Use Committee (IACUC, protocol #692-0213). The mice used were female wild type C57BL/6 (Idaho State University Animal Care Facility, breeders from Jackson Labs, Bar Harbor, Maine). This strain is not genetically predisposed to systemic autoimmune responses, and was chosen in order to a) test the autoimmune responses in a genetic background that did not already produce disease, and b) to be able to compare to other published studies of ANA in mice (Pfau et al. 2008; Ferro et al. 2013). These mice were housed under specific pathogen free (SPF) conditions in ventilated cages (Tecniplast, West Chester, PA) with 12 hour light-dark cycle, constant temperature (22°C) and humidity (45%), and *ad libitum* food and water.

Bone Marrow Derived Macrophages

To examine innate immune system cells, we used bone marrow derived macrophages (BMDM) as a model for alveolar, pleural or peritoneal macrophages. The bone marrow used was from C57BL/6 mice and collected and differentiated as previously described (Overocker and Pfau 2012). The media used for these cells was RPMI 1640 1X with L-glutamine and 25 mM HEPES (Mediatech, Manassas, VA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and penicillin-streptomycin solution (Sigma, St. Louis, MO). All cultures were maintained in a humidified 5% CO_2 incubator at 37°C.

Cell Viability

The CyQUANT Proliferation Assay (Invitrogen, Eugene, OR) quantifies cell proliferation or death in culture based on the amount of DNA, using a green fluorescent dye, CyQUANT GR, that binds to nucleic acids. A cell suspension of BMDM macrophages was obtained in media at a concentration of 10^6 cells ml^{-1} . One hundred microliters of this cell suspension were added to each well in a 96 well white opaque plate (Fisher Scientific, Santa Clara, CA). The plate was incubated at 37°C in 5% CO_2 for 60 minutes to allow the macrophages to adhere to the plate. The sonicated fiber suspensions were added to cultures to give final concentrations (0 μg to 105 $\mu\text{g}/\text{cm}^2$) in equal volume in all wells. The macrophages were exposed to the fibers for 48 hours in the same incubating conditions as before, and then the media was carefully removed from each well and the plate was frozen at -80°C overnight to

lyse the cells. The plate was then thawed and brought up to room temperature. Two hundred microliters of the 1× cell lysis buffer with CyQuant dye was added to each well and was incubated for five minutes at room temperature. The plate was read using the BioTek Synergy HT plate reader (Winooska, VT) and Gen 5.0 software at a fluorescence setting where excitation was 480 nm and emission at 520 nm. Data are presented as percent viable, calculated from values established by untreated cells compared to cells treated with 1% Triton-X100 to induce cell death.

TNF α expression by BMDM treated with fibers

Expression of TNF α by BMDM was assayed using an ELISA set from BD Biosciences according to the manufacturer's instructions. Kit standards were prepared in order to create a standard curve, and culture supernatants were diluted 1:5 in PBS containing 10% FBS for the assay. Wells of a high-binding ELISA plate were coated with capture antibody overnight, washed with PBS/Tween-20, and then blocked for an hour with the PBS/FBS solution. After another wash step, the samples and standards were added and kept at room temperature for 2hr. The plate was then thoroughly washed followed by addition of the detection antibody for 1 hr. The detection antibody solution was washed out and then the enzyme reagent was added and incubated in the plate for 30 min. After a final wash step, the TMB substrate was added and then the reaction was stopped with 2N H₂SO₄. The plates were read at 450 nm using the BioTek Synergy HT plate reader and Gen 5.0 software.

Mixed Cell Culture (Splenocytes)

Mixed splenocytes were collected from the spleens of untreated C57BL/6 mice. To collect the spleens, the mice were euthanized in a CO₂ chamber and the spleens were quickly collected and minced using the two frosted ends of microscope slides (VWR Scientific, West Chester, PA). To lyse the red blood cells, the cells were incubated with 5 ml of red blood cell lysis buffer (eBioscience, San Diego, CA) for five minutes at room temperature. The lysis buffer was then diluted with PBS. The white blood cells were collected through centrifugation and resuspended in 1.0 ml of supplemented RPMI media as described above. The cells were counted using a Z-series Coulter counter (Beckman-Coulter, Brea, CA) and diluted to 10⁶ cells per 1.0 ml of RPMI media. One milliliter of this cell suspension was placed in each well of 24-well culturing plates (Becton Dickinson, Franklin Lakes, NJ) to give 10⁶ cells in each well.

The cultures were exposed to fibers at a dose of 35 $\mu\text{g}/\text{cm}^2$ for 48 hours, incubated at 37° C at a 5% CO₂ concentration. The supernatants in each well were collected and kept at 4° C until analyzed.

T_H 1/T_H2/T_H17 Cytometric Bead Array (CBA)

The cytometric bead array (CBA) mouse T_H1/T_H2/T_H17 cytokine kit (BD Biosciences) examines seven cytokines, including interleukin-2 (IL-2), IL-4, IL-6, IL-17, IL-10, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α). The CBA procedure was carried out according to the manufacturer's instructions. Cell culture supernatants were assayed without dilution, and mouse sera were analyzed after dilution in assay buffer at 1:2. All the samples were analyzed on the FACS Calibur flow cytometer (BD Biosciences) at the

Flow Cytometry Core Facility at Idaho State University, using CellQuest software (BD Biosciences) for acquisition and analysis.

Mouse Immunoglobulin Isotyping CBA

The mouse immunoglobulin isotyping CBA kit (BD Biosciences, San Diego, CA) measures the relative concentration of antibody subtypes including IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, and IgE. This kit examined supernatants from the exposed mixed cell culture and also serum diluted 1:2 in PBS from mice exposed to erionite or saline. The CBA procedure was carried out according to the kit instructions, and all the reagents accompanied by the kit were used. All the samples were analyzed on the FACS Calibur flow cytometer using CellQuest software (BD Biosciences) for acquisition and analysis.

ELISA for Measurement of Transforming Growth Factor- β

Transforming growth factor- β (TGF- β) was examined using enzyme-linked immunosorbent assays (ELISA)(eBioscience). The procedure was carried out according to the manufacturer's instructions for measuring total TGF- β . The samples used for this procedure were culture supernatants from BMDM exposed to erionite, tremolite and wollastonite at 35 $\mu\text{g}/\text{cm}^2$. The ELISA plates were read at 450 nm using the BioTek plate reader and Gen 5.0 software.

Intratracheal Instillations

Mice were exposed to fibers using an injection of the fiber suspension directly into the trachea. Mice were sedated by intraperitoneal injection of a combination of 50 μl of Xylazine, 40 μl of ketamine and 40 μl of Torbugesic solution. An incision down the center of the neck exposed the trachea and 30 μl of 1mg/ml suspensions of the fibers were injected into the trachea. This dose was used because it has been shown to induce autoantibodies in these mice when treated with amphibole asbestos (Pfau et al. 2008). The incision was closed using an adhesive and cleaned using betadine. The mice recovered from the procedure in a warmed cage. All the mice received two instillations of 30 μg one week apart and lived for seven months afterward, at which time they were euthanized by CO_2 asphyxiation. The serum, kidneys, and spleen were collected from each mouse for analysis.

Anti-Nuclear Antibody (ANA) Assay

The serum for each mouse was analyzed for anti-nuclear antibodies (ANA). The serum was collected by cardiac puncture, aspirating the blood from the heart using a 1.0 ml syringe (Becton, Dickinson and Company). The blood was collected in microtainer tubes that contained a serum separator (Becton, Dickinson and Company). The ANA test (ImmunoConcepts, Sacramento, CA) is a semi-quantitative analysis that uses indirect immunofluorescence to detect any auto-reactive antibodies in the serum. It has recently been validated by the American College of Rheumatology as the test of choice for screening (Meroni et al. 2010; Agmon-Levin et al. 2014). The assay was carried out according to the manufacturer's instructions (www.immunoconcepts.com/products/ifa-slides/), modified for analysis of mouse serum instead of human. Each sample was diluted 1:40 for screening by adding 5.0 μl of serum in 195 μl of PBS. Twenty microliters of each diluted sample was

added to their corresponding wells on the substrate slide and incubated at room temperature for 30 minutes and washed with PBS. The fluorescent antibody reagent was prepared by adding 2.0 μ l of goat anti-mouse IgG antibody conjugated to AlexaFluor 488 (Invitrogen, Eugene, OR) to 1.0 ml of PBS (1:500). Twenty microliters of the antibody solution were added to each well and incubated in the dark at room temperature for 30 minutes. The slide was washed with PBS and coverslipped with Fluorosave (Calbiochem, La Jolla, CA). The slides were viewed by two independent blinded readers using the FITC (488nm) filter on the Leica DMRB fluorescence microscope in the Advanced Imaging Core Facility at Idaho State University.

Immune Complex Staining

Kidneys were collected from mice exposed to saline and erionite and submerged in histochoice fixative for 24 hours at 4° C. The tissues were sunk in 30% sucrose solution and then placed in cryomolds and frozen in O.C.T at -80° C. The kidneys were cut using a Leica 3050 cryostat in 10 μ m sections and placed on pre-coated superfrost slides (Fisher).

The O.C.T. was removed from the sections with PBS, and then the slides were boiled in 0.01M sodium citrate buffer followed by washes with distilled water and PBS. The sections were blocked with 4% fetal bovine serum in PBS and stained for IgG using goat anti-mouse IgG antibody conjugated to AlexaFluor 488 (Invitrogen, Eugene, OR). The slides were viewed using the FITC (488nm) filter on the Leica DMRB fluorescence microscope.

Statistics

Experiments using BMDM cultures were analyzed using a one-way analysis of variance (ANOVA). The cytokine data collected from the mixed splenocyte cultures is presented as fold change from the control and analyzed by comparing the treated groups to the control using Mann-Whitney U tests. The cytokine concentrations in mouse sera were analyzed using a two-way t-test with unequal variance. The mean values are reported in the figures with standard error of the mean (SEM) used to create the error bars. Percent positive antinuclear antibody and immune complex results were analyzed using Chi-squared tests to compare the treated groups to the saline control. Statistical significance for all experiments was determined by a p value < 0.05.

RESULTS

BMDM Cell Viability and TNF alpha Production

BMDM were treated with a range of fiber concentrations bracketing concentrations previously shown to induce cytokine production without overt toxicity when using asbestos (Overocker et al. 2012). Figure 1A shows a gradual decrease in viability as fiber concentration was increased in BMDM cultures. At concentrations above 35 μ g/cm², cell viability dropped below 90% for all fibers. In a separate experiment using 8 replicates for erionite compared with no treatment, the change in values for the assay reached statistical significance (p<0.05) for erionite at 50 μ g/cm². Therefore, to avoid effects attributed simply to cell death, all subsequent experiments were performed using a dose at 35 μ g/cm² of fibers, where most of the cells were still viable and the macrophages were still adherent and

functionally responsive in terms of cytokine release (Figure 1B). Erionite and all asbestos fibers induced increases in TNF α production above control, with erionite $\gg$ amphibole (tremolite or Libby 6-Mix) > chrysotile. Wollastonite, a control fiber, did not induce TNF α production.

T_H1/T_H2/T_H17 Cytokines

The cell culture model used consisted of splenocytes collected from the spleens of C57BL/6 mice, directly exposed to fibers *in vitro* for 48 hours. The culture media was examined for cytokines from T_H1, T_H2 and T_H17 lineages using cytometric bead arrays (CBA). Tremolite, an amphibole asbestos, was used to compare with erionite to determine if erionite evokes similar responses by the immune cells. Chrysotile, a different type of asbestos that does not seem to promote autoimmunity (Birch et al. 2012; Ferro et al. 2013), was also used in this study. The cytokine data are presented as a ratio of the concentration values obtained from the treated cultures to the concentrations values of the untreated control showing the relative changes from the control values. The control values were normalized at a value of 1.0 and indicated by the dashed line on the figures.

Three cytokines were examined that are associated with the T_H1 lineage, and it was found that the production of IFN- γ and TNF- α was significantly increased after exposure to erionite almost 4 and 9 times higher than the control, respectively. In contrast, IL-2 production was significantly decreased by almost half after erionite exposure. After exposing these cells to tremolite, TNF- α increased almost 4 times higher than control, while IFN- γ significantly decreased more than half from the control. IL-2 showed no change from the untreated samples. Treatment of cells with chrysotile decreased the production of all three of the T_H1 cytokines (Figure 2).

Two cytokines associated with the T_H2 lineage, IL-4 and IL-10, were also examined. The only cytokine to increase significantly was IL-10 after exposure to erionite. The increase was almost 9 times higher than the untreated samples. Chrysotile treatment decreased IL-10 production by almost half. Values for IL-4 were very close to the untreated values resulting in no physiologically significant changes (Figure 3).

Figure 4 shows the production of T_H17 cytokines, IL-6, IL-17 and TNF- α , after each fiber treatment. TNF- α has roles in both the T_H1 and the T_H17 lineages, apparently being essential for maintaining T_H17 cells (Furuzawa-Carballeda et al. 2007). Therefore, this cytokine was also included in this figure. All the cytokines were significantly increased after erionite and tremolite exposure by two times or more, yet decreased after exposure to chrysotile.

TGF- β is an important cytokine that plays a role in initiating the development of the T_H17 lineage in combination with IL-6. TGF- β was not included in the CBA, but its production by BMDM was examined using ELISA. Figure 5 shows the production increased after erionite and tremolite exposure. For this assay, another fiber that has not been associated with autoimmunity, wollastonite (Pfau et al. 2008), was used as a comparison. This fiber caused a significant increase in TGF- β compared to control, but was significantly lower than the erionite-induced levels.

The same cytokines were also examined in the serum of mice exposed to erionite or saline. Figure 6 shows that TNF- α and IL-17 were elevated in the serum of erionite-exposed mice compared to those exposed to saline.

Antibody Production from Splenocyte Cultures

Media from the mixed cell cultured was also examined for antibodies using a cytometric bead array (CBA) mouse isotyping kit to determine if B lymphocytes were activated. Both erionite and tremolite caused an increase in production of IgG1 and IgG2a. These two treatments also caused an increase in IgM by at least two times more than the control. Chrysotile did not seem to evoke any changes in antibody production in this model (Figure 7).

Anti-Nuclear Antibodies (ANA) in C57BL/6 Mice

Wild type C57BL/6 mice were exposed intratracheally to saline, erionite, Libby 6-Mix asbestos or chrysotile and seven months later, the serum was tested for the presence of anti-nuclear antibodies (ANA) in the blood serum. Figure 8 shows the percentage of mice having positive ANAs seven months after the exposures. Only four of eighteen mice treated with saline developed serum ANAs (22%). Of the ten mice exposed to erionite, eight of them tested ANA positive (80%). Three out of seven mice treated with chrysotile were ANA positive (42%). Of the eleven mice exposed to Libby 6-Mix, eight developed ANA (73%). Only erionite ($p=0.005$) and Libby 6-Mix ($p=0.018$) treatment groups produced significantly different values from the saline treated mice.

Immune Complex Deposition in Glomeruli

The kidneys from mice exposed to erionite and saline were stained for the presence of IgG in the glomeruli indicating the deposition of immune complexes. Nine out of ten mice exposed to erionite showed evidence of immune complex deposition, and only three out of nine mice exposed to saline presented positive for these complexes. Figure 9 shows the frequency of mice that had immune complexes in their kidneys for each treatment group. Figure 10a shows a representative image of glomeruli without any staining (a saline-exposed mouse) and Figure 10b shows an image of a positive stain for IgG from an erionite-exposed mouse.

DISCUSSION AND CONCLUSION

There is increasing concern for public health consequences of environmental exposures to erionite in the western U.S. Erionite shows many similar characteristics to amphibole asbestos. They are both made of silicon and oxygen and both are long and straight fibers (Dogan et al. 2008). Like amphibole and chrysotile asbestos, erionite also causes respiratory diseases in addition to mesothelioma, such as fibrosis and pleural plaques (Emri et al., 2002). Recent investigations in Libby, Montana (Pfau et al. 2005) indicate an increased frequency of systemic autoimmune diseases in the residential population. It was the purpose of this study to investigate whether erionite disrupts the immune system in a similar manner, thus promoting autoimmunity.

This study first examined the toxicity of erionite in macrophages, which are an important part of the innate immune system by having the ability to phagocytize invading pathogens and fibers. When macrophages internalize erionite and asbestos fibers, they tend to produce reactive oxygen species (ROS) (Carbone et al. 2012; Hogg et al. 1996), which contribute to carcinogenicity and may also play a role in the pathogenesis of autoimmune diseases. The oxygen radicals may alter self-antigens in a way that causes the immune system to lose self-tolerance and mount an attack on those antigens (Cooper et al. 2008). Erionite treatment for 48 hr caused a decrease in BMDM survival in cell culture similar to other fibers, but only a slight decrease was seen at the experimental concentration of 35 $\mu\text{g}/\text{cm}^2$. Alveolar macrophages have also been shown to undergo apoptosis as a result of asbestos exposure (Holian et al. 1997), and it is thought that apoptotic blebs provide a potential source of self-antigens that will be presented to the immune system and leading to autoimmunity (Casciola-Rosen et al. 1994; Pfau et al. 2004). It is possible that this mechanism is also taking place over time with erionite exposures, but further study is needed to explore that possibility.

Using the mixed cell cultures of splenocytes, we were able to investigate the cross talk between innate and adaptive immune cells in the form of cytokines. This model was used to determine if cytokine signals from macrophages caused T Helper (T_H) cells to become activated and differentiate into T_H1 , T_H2 or T_H17 subsets.

IL-2 and IFN- γ are cytokines produced by T_H1 cells. The data demonstrate that tremolite and chrysotile did not cause an increase in any of the T_H1 cytokines examined in mixed splenocyte cultures, suggesting that these fibers do not activate the T_H1 response. Erionite caused an increase in IFN- γ but a decrease in IL-2. Further, neither of these cytokines was significantly increased in the serum of the erionite-exposed mice, suggesting that this pathway was not completely activated in these mice. Further interpretation of these results will be much more meaningful once the responses to erionite are examined in other mouse strains and in humans. To our knowledge, cytokine levels have not yet been measured in the serum of individuals exposed to erionite.

IL-4 is a cytokine important in initiating the T_H2 lineage; it was not increased by any treatment *in vitro*, and was not increased in serum of erionite-exposed mice. Erionite caused an increase production of IL-10 from the splenocytes, primarily made by T_H2 cells, while the other fibers seemed to have no effect on this cytokine. IL-10 was also increased in serum of the erionite-treated mice, although the change did not reach statistical significance. This cytokine is often considered anti-inflammatory, so its increase here requires further study to determine whether this also occurs in humans and what the significance of this might be. Tremolite and chrysotile failed to cause an increase in either of the two T_H2 cytokines tested.

IL-6 is important in the development of the T_H17 lineage, which has been shown to have a potential role in autoimmune disorders (Furuzawa-Carballeda et al., 2007). While chrysotile caused a decrease in both TNF- α and IL-6 production, these cytokines were increased in splenocyte cultures exposed to erionite and amphibole. IL-17 levels more than doubled after treating cultures with erionite and tremolite, and chrysotile had little effect on IL-17 levels.

Thus, erionite and tremolite led to increases in all of the T_H17 pathway cytokines tested, suggesting activation the T_H17 lineage. Further, TGF- β was also shown to increase in BMDM cultures after erionite and tremolite exposure. Along with IL-6, these two cytokines are vital in initiating the differentiation of progenitor T cells into T_H17 cells (Weaver et al. 2006). In support of these *in vitro* findings, TNF- α and IL-17 were significantly increased in the serum of the erionite-exposed mice. Similarly, TNF- α and IL-17 have also been found to be elevated in humans who have SLE (Afzali et al., 2007). Such findings support the possible association between human autoimmunity and both amphibole asbestos and erionite exposure.

In previous studies, chrysotile has not been shown to produce elevated autoantibodies in humans (Birch et al., 2012) or mice (Ferro et al. 2013). Chrysotile also did not induce increased serum cytokines other than IL-6, while Libby 6-Mix induced both IL-17 and IL-6 (Ferro et al. 2013). It has been proposed that chrysotile has an inhibitory effect on the immune system, which may increase the potential for cancers to become established (Kumagai-Takei et al., 2011; Kumagai-Takei et al., 2013). In our study, we did note a decrease in the production of all of the T_H1 cytokines (IL-2, IFN- γ , TNF α) and T_H17 cytokines (IL-6 and IL-17) after chrysotile exposure in the splenocyte cultures, consistent with immunosuppressive effects.

B cells, which are antibody-producing lymphocytes, secreted more antibodies after being treated with erionite or tremolite in this cell culture model. These two fibers greatly increased IgG1 and IgM production in particular. This is another example of how erionite evokes a similar response to tremolite asbestos and how it may lead to autoimmunity since over activated B cells may be more likely to produce autoantibodies *in vivo*.

A hallmark of systemic autoimmunity is the production of autoantibodies against nuclear antigens. Seven months after being exposed to erionite, the serum samples from these mice were collected to detect the presence of these antibodies. Only erionite or amphibole asbestos increased the frequency of positive ANA tests in the mice: chrysotile did not. There was also evidence of immune complex deposition in the kidneys of erionite-exposed mice. These mouse model data suggest that erionite may behave similarly to amphibole asbestos and may potentially promote the production of autoantibodies commonly seen in people with systemic autoimmune diseases.

Most studies involving erionite have focused on evaluating its ability to cause cancer or lung fibrosis in humans and various animal models. To our knowledge, this is the first study to investigate the effects of erionite on the adaptive immune system and its association with autoimmunity. Our work suggests that erionite exposures are potentially associated with other health endpoints, in this case adverse autoimmune effects, in addition to pulmonary cancers and fibrosis. Further studies screening for autoantibodies in erionite exposed human populations will be important in establishing the relationship between erionite exposure and autoimmune health effects in humans.

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Highlights

- Erionite is a fibrous mineral that is a current public health concern in the Northern Rocky Mountain states
- Erionite exposure induces antinuclear autoantibodies in mice exposed intratracheally
- Erionite induces a clear Th17 cytokine response in vitro (splenocyte culture) and in vivo (serum)
- These responses were distinct from responses caused by amphibole or chrysotile asbestos
- These data in mice raise the possibility that exposed humans may be at risk for autoimmune responses.

Figure 1. Viability and cytokine production by BMDM exposed to fibers

A. Cell viability was determined by using a CyQUANT cell proliferation assay (Life Technologies) to detect DNA content in BMDM cultures following treatment for 48 hr at concentrations shown. Erionite was compared to three forms of asbestos that were subsequently used in either *in vitro* or *in vivo* experiments: chrysotile, tremolite, and Libby Amphibole (6-Mix). The mean percent viable $\pm$ range are shown for comparison of four different fiber types; $n=2$ at each time period. B. Tumor Necrosis Factor alpha (TNF α) in culture supernatants was measured by ELISA (BD Biosciences), and data are shown as the mean with error bars = SEM. * = $p < 0.05$, ** = $p < 0.005$.

Figure 2. T_H1 Cytokine production in splenocyte cultures exposed to fibers

Cytokines in the cultures exposed to fibers at 35 $\mu\text{g}/\text{cm}^2$ were measured using the Mouse T_H1 Cytokine Bead Array (CBA), and data are reported as the mean fold change compared to no treatment (dotted line). n=5; error bars = SEM; (*) indicates statistical significance (p<0.05) compared to No Treatment.

Figure 3. T_H2 Cytokine production in splenocyte cultures exposed to fibers

Cytokines in the cultures exposed to fibers at 35 $\mu\text{g}/\text{cm}^2$ were measured using the Mouse T_H2 CBA, and data are reported as the mean fold change compared to no treatment (dotted line). n=5; error bars = SEM; (*) indicates statistical significance ($p < 0.05$) compared to No Treatment.

Figure 4. T_H17 Cytokine production in splenocyte cultures exposed to fibers

Cytokines in the cultures exposed to fibers at 35 $\mu\text{g}/\text{cm}^2$ were measured using the Mouse T_H17 CBA, and data are reported as the mean fold change compared to no treatment (dotted line). n=5; error bars = SEM; (*) indicates statistical significance ($p < 0.05$) compared to No Treatment.

Figure 5. TGF- β production from BMDM cells exposed to erionite, tremolite or wollastonite fibers at 35 $\mu\text{g}/\text{cm}^2$
TGF- β (total) was measured in macrophage cultures exposed to fibers at at 35 $\mu\text{g}/\text{cm}^2$ using ELISA. n=6; error bars = SEM; (a) indicates statistical significance ($p<0.05$) compared to No Treatment; (b) indicates statistical significance compared to erionite treatment.

Figure 6. Serum Cytokine analysis of sera collected from erionite and saline instilled mice

Serum cytokines in mice exposed for 7 months to erionite (60 $\mu\text{g}/\text{mouse}$) were measured using the T_H1, 2, 17 CBA. Erionite n=5; Saline n=4; (*) indicates statistical significance ($p < 0.05$) comparing erionite to saline-exposed mice.

Figure 7. Altered serum antibody profiles after fiber exposure

Serum immunoglobulin isotypes were measured in serum of mice exposed for 7 months to fibers (60 $\mu\text{g}/\text{mouse}$) using a mouse isotyping bead array. Data are shown as fold change from saline-exposed mice. $n=5$; error bars = SEM; (*) indicates statistical significance ($p<0.05$) compared to saline only treatment.

Figure 8. The frequency of C57BL/6 mice that tested positive for serum antinuclear antibodies (ANA)
Serum ANA were measured in serum of mice exposed for 7 months to fibers (60 $\mu\text{g}/\text{mouse}$) using indirect immunofluorescence. Saline n=18; Erionite n=10; Libby 6-Mix n=11; Chrysotile n=7; (*) indicates statistical significance ($p<0.05$).

Figure 9. The frequency of mice showing IgG deposition in kidney glomeruli

Ninety percent of mice exposed to erionite showed positive for IgG in the glomeruli. Only 33% of mice treated with saline had a similar stain for IgG. This difference was found to be statistically significant after a chi-squared test. Saline n=9; Erionite n=10;

(*) indicates statistical significance ($p < 0.05$).

Figure 10. Immune complex deposition of IgG

These sections were sliced at 10 μm thick and stained for IgG and images taken at a total magnification of 100X. A: a representative image of the kidney of a saline exposed mouse, with little or no staining in the glomeruli (arrow). B: a representative image of the kidney from an erionite exposed mouse with bright glomerular staining indicating immune complex deposition.

Table 1

Summary Characteristics for Fibers used in this study.

Fiber	Length (um)	Diameter (um)	Aspect ratio (L/D)	Surface Area (m ² /g)	Reference	Source
Libby 6-Mix	7.21 ^a	0.61 ^a	11.8	5	Hillegass, JM., 2010	USGS
Korean Tremolite	5.49 ^a	0.32 ^a	17.16		Bernstein, DM., 2003	Dr. AG Wylie, Univ. of Maryland
Intermediate Chrysotile	0.82 ^b	0.089 ^b	8.435	20.2 ± 0.1	NTP TR 246, 1990	NIEHS
Oregon Ertomite	6.21 ^a 4.15 ^b	0.83 ^a 0.53 ^b	13.37 ^a 9.23 ^b		Januch, J. TEM Data	EPA

^a Mean

^b Median