2004

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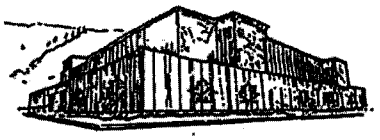

MECHANISMS OF SILICA EXACERBATED SYSTEMIC AUTOIMMUNE DISEASE IN NEW ZEALAND MIXED MICE

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Presented in partial fulfillment of the requirements for the
Doctor of Philosophy in Toxicology Degree

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May 2004

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Jared Michael Brown Ph.D. 2004 Toxicology

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ABSTRACT

Silica exposure has been associated with formation of autoantibodies and development of several autoimmune diseases, however, mechanisms leading to these events are unknown. Therefore, the effect of silica on autoimmune disease progression in autoimmune-prone New Zealand mixed mice was studied. Following silica exposure, NZM mice developed high levels of proteinuria and died prematurely due to immune complex and complement deposition within the glomerulus of the kidney as compared to saline and TiO_2 exposed mice. Further, silica exposure resulted in higher levels of autoantibodies to nuclear antigen, specifically histones, than saline or TiO_2 exposed mice. These effects were mediated in part by the migration of high numbers of CD4+ T cells and B1a B cells to local lymph nodes as well as the decrease in the number of CD4+CD25+ regulatory T cells. Silica exposure induced a shift in the Th1/Th2 balance in favor of a Th1 response as measured by alterations in immunoglobulin isotypes. The increase in autoantibodies following silica exposure in NZM mice involved autoantibodies which preferentially recognized apoptotic cells. Following silica exposure, alveolar macrophages from NZM mice were shown to have increased gene expression levels of several apoptosis genes. Most prominent was an increase in pro-apoptotic protein kinase Cδ in alveolar macrophages from silica exposed NZM mice. Using an *in vitro* model, PKCδ was found to mediate apoptosis in silica treated bone marrow derived macrophages. Further, TNF-α was found to be chronically elevated in lung lavage fluid possibly adding to the induction of apoptosis. Inhibiting the apoptotic response induced by silica in alveolar macrophages using an inhibitor of PKCδ *in vivo* significantly reduced silica exacerbated systemic autoimmune disease. Inhibition of PKCδ *in vivo* resulted in decreased proteinuria, kidney disease and anti-histone autoantibodies. These results suggest that silica exposure resulted in apoptosis of alveolar macrophages and by blocking apoptosis through PKCδ the exacerbation of systemic autoimmune disease in NZM mice was inhibited. These data taken together may provide insights into mechanisms by which xenobiotics induce apoptosis and the role apoptosis may play in the progression of systemic autoimmune disease.

ACKNOWLEDGMENTS

First and foremost, I would like to thank the Center for Environmental Health Sciences and the Department of Biomedical and Pharmaceutical Sciences for providing me with the educational opportunity and resources to complete this dissertation.

I extend my heartfelt appreciation to the members of my dissertation committee: Drs. Beall, Holian, Pfau, Putnam, Minnick and Thompson.

In particular, I want to express my gratitude to my mentor, Andrij Holian for his guidance throughout the past years and development of an exciting project.

I would also like to acknowledge the American Foundation for Pharmaceutical Education (AFPE) for providing financial assistance with an AFPE Pre-doctoral Fellowship from 2002-2004.

Lastly, to my wife, son and family, I am grateful for all that you have done to support me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Relatively little is known regarding mechanisms associating environmental exposures with the development of autoimmune disease. However, several environmental factors have been implicated in initiating or accelerating systemic autoimmune disease including mercury, iodine, vinyl chloride, certain pharmaceuticals and crystalline silica. In addition, there is increasing epidemiological evidence supporting the hypothesis that occupational silica exposure is associated with a variety of systemic autoimmune diseases, including scleroderma (SSc), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), glomerulonephritis (GN) and small vessel vasculitis (SVV) (Steenland and Brown 1995; Steenland and Goldsmith 1995; 1997; Parks 1999; Parks 2002; Parks 2003). However, there have been few mechanistic studies examining silica exposure and autoimmune disease initiation and progression. This introduction will cover background information on systemic autoimmune diseases and human epidemiology data linking crystalline silica exposure with autoimmune disease. Further, this introduction will cover mechanisms of apoptosis and how silica induced apoptosis may be involved in the progression of autoimmune disease. Finally, background data on the effects of silica on lymphocytes will be presented followed by specific aims of this dissertation.

Systemic Autoimmune Disease

Systemic autoimmune diseases are characterized by immune dysregulation resulting in the production of autoantibodies, circulating immune complexes and activation of the complement system. These collections of diseases, which includes systemic lupus erythematosus, rheumatoid arthritis and scleroderma, can involve the

joints, skin, kidney, brain, lung, heart and gastrointestinal tract. The pathological hallmark of these diseases is recurrent, widespread and diverse vascular lesions due to immune complex and complement deposition. Women appear to have higher incidences of systemic autoimmune diseases, often accounting for greater than 75% of the cases.

The etiology of many autoimmune diseases remains unknown. A genetic predisposition, sex hormones and environmental triggers likely contribute to the development and progression of autoimmunity. A role for genetics is suggested by increased frequency of several MHC haplotypes in patients with autoimmune disease (NIH 2003). The role for heredity is further supported by a high concordance for autoimmune disease among monozygotic twins. However, the concordance rate of autoimmune disease among polyzygotic twins is only between 25 and 40%, thereby suggesting a role for environmental factors (NIH 2003).

Crystalline Silica

Commonly known as quartz, crystalline silica is an abundant mineral found in rock, sand and soil. The highest exposures to silica occur in the dusty trades, such as mining, farming, foundry work, ceramic and pottery making, glass making, drilling and sandblasting. Silica is widely used in materials, such as, absorbents, dessicants, catalysts, fillers, lubricants, thickening agents, paints, etc. Prolonged or acute high exposures of silica can cause pulmonary inflammation and fibrosis, which is termed silicosis (1997). The potential to develop silicosis depends on the concentration of silica in the ambient air, the content of crystalline silica in inhaled dust, the size of the particles (under 1 μm being most fibrogenic) and the duration and dose of exposure (Hughes 1982).

Silica occurs in crystalline and amorphous forms with the crystalline form being more biologically active. Cutting, grinding and milling of silica probably results in the formation of fractured crystal resulting in the generation of Si and Si-O radicals on its surface. Thus, fractured crystalline silica may have surface properties that make it more reactive in the lung. Crystalline silica has been shown to have a negative charge on its surface which provides an increased potential to adsorb and interact with cellular components (Castranova 1996).

Silica particles of respirable size, generally less than 1 μm are deposited in the alveolar spaces of the lung. The particles are phagocytosed by alveolar macrophages which are removed by the mucociliary movement of macrophages up the respiratory tract or drained to the lymphatics (Brody 1982). However, at high concentrations it is reported that the mucociliary clearance of silica particles is impaired resulting in an accumulation of silica laden macrophages within the lymphatics and interstitial spaces of the lung (Brody 1982). Consequently, these silica-laden macrophages are able to interact with other immune cells, possibly mediating many of the immune effects of silica, including systemic autoimmune disease.

Silica Exposure and Autoimmune Disease (Human Data)

There have long been case reports linking silica exposure to several autoimmune diseases, however recently there have been several population based studies examining silica exposure as a risk for the development of autoimmune disease (Parks 1999; Parks 2002; Parks 2003). Parks *et al* (2002) reported a higher prevalence of systemic lupus erythematosus (SLE) in patients following silica exposure than a control population. Further, they reported an association between the level of silica exposure and the

development of SLE. These data suggest that silica exposure may promote the development of SLE in some individuals and that the development of disease may be more prevalent in highly exposed individuals. They further examined several common types of autoantibodies and found a strong association between silica exposure and anti-DNA and anti-Sm autoantibody levels. An association between silica exposure and anti-neutrophil cytoplasmic autoantibody formation has also been reported to be involved in small vesicle vasculitis (Hogan 2001). Therefore, it appears that silica exposure results in the production of several specific types of autoantibodies.

Lymphocytes have been examined in regards to activation and abnormalities in patients with silicosis and these same abnormalities may play a role in the progression of autoimmune disease. The number of T helper cells was reported to be increased in the lymph nodes of the lung hilus in silicosis patients, however, the overall number of all classes of T lymphocytes was decreased (Watanabe 1987). Further, silicosis patients who were reported to have a higher prevalence of autoimmune disease also had a significant decrease in B, T and NK cells as compared to healthy volunteers (Subra 2001). Although a significant increase in the percentage of activated CD3+ T cells was reported (Subra 2001). However, it is not known whether the increase of activated T cells represents chronic stimulation by silica and if this is occurring directly or indirectly through other immune cells or cytokines. Further, it is not known if the overall decrease in B, T and NK cells is due to apoptosis of these cells. Decreased numbers of lymphocytes (lymphopenia) have been reported to induce autoimmune diseases experimentally through reduction of regulatory T cells that help control T helper cell responses.

Therefore, this may provide an additional mechanism for the effects of silica on the immune system (Shevach 2000).

As mentioned above, there is strong evidence linking silica exposure with several autoimmune diseases. While these data may provide clues to underlying mechanisms involved in silica-induced autoimmunity, there are relatively little data examining detailed mechanisms in animal models that may then be applied to therapeutic intervention.

Silica and Apoptosis

Apoptosis, or programmed cell death, has several possible roles in the pathogenesis of autoimmune disease; including generation of apoptotic material as a source of antigen, altered immune cell apoptosis as a cause of immune dysregulation and tissue specific apoptosis as a mechanism of organ specific autoimmune disease. Possible mechanisms of silica induced autoimmune disease may involve apoptosis of the alveolar macrophage, an immune cell of the lung that is the first line of defense and a cell involved in the clearance of apoptotic cells. It has previously been reported that uptake of silica leads to apoptosis of alveolar macrophages and appears to be in part mediated through scavenger receptor class A (SR-A) (Iyer 1996; Thibodeau 2003). SR-A is characterized by broad ligand binding specificity and is referred to as a pattern recognition receptor. SR-A has been mainly studied for its role in atherosclerosis due to the binding of modified low density lipoproteins (Krieger 1997). However, silica has also been reported to bind SR-A (Kobzik 1995). Silica induced apoptosis of alveolar macrophages leads to release and uptake of silica by other alveolar macrophages, producing a cyclical process of inflammation and cell death (Cooper 2002). This may

provide an environment of excess antigen being presented to the immune system, thereby breaking immune tolerance. In support of this hypothesis, the presence of concentrated autoantigens has been reported within the blebs of apoptotic cells (Rosen and Casciola-Rosen 1999). Further, lupus autoantigens are represented by structures that are chemically cleaved or modified during apoptosis and if this material is not removed by non-inflammatory processes, apoptotic material could be presented by specialized antigen presenting cells to induce immune responses (Utz and Anderson 1998; Ronchetti 1999). In an *in vitro* system, macrophages were found to prevent immunity to apoptotic material by competing with dendritic cells for uptake of apoptotic blebs, demonstrating the importance of the cell population silica is targeting for injury (Albert 1998). Consistent with this hypothesis, animals intravenously exposed to apoptotic cellular material have been reported to develop autoantibodies (Mevorach 1998). Therefore, it appears apoptosis may play a role in the development of autoimmune disease following silica exposure so mechanisms of silica-induced apoptosis, including caspase activation, Fas/Fas ligand and TNF-α will be reviewed.

Silica and Caspase Activation

The central effectors in apoptotic cell death involve the activity of a group of cysteine proteases known as caspases. They are termed caspases because they cleave their target protein at specific aspartic acid residues. Caspases are synthesized in the cell as inactive precursors, or procaspases, which are typically activated by cleavage at aspartic acid residues by other caspases. Once activated, caspases cleave and thereby activate other procaspases resulting in an amplifying proteolytic cascade. The activated caspases can then cleave key cellular proteins, such as structural proteins, cell cycle

proteins, signaling proteins, or they can activate DNA degrading enzymes leading to apoptosis of the cell. Thirteen caspases have been identified and are named caspase-1 through –13. Two main pathways of caspase activation have been studied, the intrinsic and extrinsic pathways.

The intrinsic pathway involves mitochondrial damage resulting in redistribution of cytochrome c from the mitochondria to the cytoplasm of the cell. The activation of caspases depends on the release of cytochrome c and the presence of Apaf-1 (apoptotic protease activating factor). When cytochrome c is released from the mitochondria, Apaf-1 binds caspase-9 in a cytochrome c and ATP dependent manner. The activation of caspase-9 by Apaf-1 in turn leads to the activation of caspase-3. Caspase-3 is termed an effector caspase because it mediates many of the apoptotic processes.

In addition to activation of caspases through mitochondrial release of cytochrome c and Apaf-1, caspases can be activated through cell surface receptors. The extrinsic pathway of caspase activation involves several tumor necrosis factor receptor (TNFR) family members, such as TNFR-1 and CD95 (Fas). These receptors have an intracellular signaling domain referred to as the death domain (DD). Upon receptor engagement by TNF or Fas ligand, the DD is bound by another class of proteins referred to as adaptor proteins which can activate caspase-8 and –10. Through downstream events, caspase-8 and –10 lead to the cleavage and activation of caspase-3 which results in apoptosis of the cell.

Silica has been reported to alter mitochondrial membrane permeability inducing release of procaspase-9 (Thibodeau 2003). Silica has been reported to specifically lead to the production of the active p39 subunit of caspase-9 and not the p37 subunit, which in

turn leads to cleavage and activation of caspase-3 thereby initiating apoptosis in MH-S cells (Thibodeau 2003). Using a general caspase inhibitor (z-VAD-fmk) or a caspase-9 specific inhibitor (z-LEHD-fmk), Thibodeau *et al* (2003) were able to inhibit silica induced activation of caspase-9 and –3 in MH-S cells. They further reported that inhibition of the mitochondrial permeability pore by cyclosporin A partially decreased caspase-9 and –3 activation in silica exposed MH-S cells. Therefore, activation of caspases by silica appears to be partially dependent on loss of mitochondrial integrity.

Borges *et al* (2002) has reported a role for apoptosis in silicosis by using caspase inhibitors in silica exposed BALB/c mice. Treatment with pan-caspase inhibitors reduced inflammation and collagen deposition in the lungs of BALB/c mice that received 20 mg of silica (Borges 2002). Silica-induced apoptotic cells were also found in the draining lymph nodes of these silica-instilled BALB/c mice where they were able to interact with lymphocytes (Borges 2002). They further reported that within the draining lymph nodes of these silica treated mice, electron microscopy showed lymphocytes undergoing activation induced cell death. Therefore, it appears apoptosis plays a role in silicosis and that silica-induced apoptotic cells are found outside of the lung, suggesting a systemic immune response.

Silica and Fas/FasL

Fas is a cell surface receptor protein involved in apoptosis which belongs to the TNF receptor family and Fas abnormalities have been reported in human autoimmune diseases such as SLE and RA. Fas ligand (FasL) induces apoptosis by binding its membrane receptor Fas. Outside of the thymus, most of the T cell receptor mediated apoptosis of T cells, referred to as activation induced cell death (AICD) is induced

through the Fas pathway. Cytotoxic T lymphocytes also have the ability to use FasL to trigger apoptosis in Fas bearing cells, such as virus infected cells or cancer cells. The importance of Fas in AICD is demonstrated by the *lpr* and *gld* strains of mice which have alterations in the Fas gene. *Lpr* and *gld* mice develop a lymphoproliferative disorder that resembles systemic lupus erythematosus. The MRL/*lpr* and *gld* mice have been studied with regards to silicosis, however, no studies have examined autoimmune disease following silica exposure in these models (Davis 1998; Borges 2002). In MRL/*lpr* mice, silica has been reported to result in increased prominent cellular infiltrates and fibrosis as compared to BALB/c or C3H mice which have normal levels of Fas (Davis 1998). Borges *et al* (2002) reported that silica exposed T cells from BALB.*gld* mice were resistant to AICD as compared to wild-type BALB/c mice suggesting that self-reactive T cells may be able to escape apoptosis in this silicosis model. Further, silicosis patients with autoimmune disease have been reported to have elevated serum soluble Fas levels as compared to healthy volunteers (Otsuki 1998; Tomokuni 1999; Hamzaoui 2003). Soluble Fas is produced as an alternatively spliced product of the Fas gene which protects the cell from apoptosis by antagonization of the binding between membrane bound Fas and FasL. Therefore, it is possible that self-reactive immune cells may survive by escaping Fas mediated apoptosis or silica may allow for excess lymphocyte activation induced cell death leading to immune abnormalities.

Silica and TNF-α

Tumor necrosis factor-α (TNF-α) is a potent cytokine mediator of inflammatory and immune functions. TNF-α mediates a broad range of biological activities, but was originally characterized by its ability to induce apoptosis of tumor cells. TNF-α is

predominately produced by macrophages and signals through TNF receptors which are found on nearly all cell types. There are two types of TNFR, type I (p55) and type II (p75). The type I receptor is found on most cells, while the type II receptor is found mainly on hematopoietic cells. The TNFR has a death domain in the cytoplasmic tail which signals an adaptor protein named TRADD (TNF receptor associated death domain). For apoptosis signaling, TRADD recruits FADD (Fas associated death domain) which in turn recruits and activates caspase-8 and –10. As discussed previously, caspase-8 and –10 in turn can activate caspase-3 through downstream signaling events leading to apoptosis.

TNF-α has been studied extensively in silica-induced fibrosis, however, the same role TNF-α plays in fibrosis may be relevant to silica-induced autoimmune disease. When macrophages are exposed to silica *in vitro*, enhanced TNF-α production occurs (Savici 1994; Ortiz 1999). Increases in TNF-α production activate an inflammatory cascade which induces release of cytokines, including TNF-α, and activation of several cell signaling cascades (Pfeffer 2003). Inhibition of TNF-α significantly reduces fibrosis and addition of recombinant TNF-α increases collagen deposition in the lungs of mice (Piguet 1990; Ortiz 1999). Ortiz *et al* reported that silica-exposed C57Bl/6 and BALB/c mice upregulated mRNA levels of TNF-α and the p75 TNFR and that these mice developed fibrosis. They also reported that silica-exposed TNFR knockout mice did not develop fibrosis as compared to wild-type mice. Thus, silica induced lung injury involves TNF-α production and signaling by macrophages. TNF-α has been extensively studied as a pro-apoptotic cytokine, therefore, it possible that silica-induced production of

TNF-α by macrophages may lead to apoptosis and that apoptosis may be playing a role in lung injury.

Abnormalities at many points in the process of apoptosis may be related to autoimmune disease pathogenesis. Given the amount of evidence and the many pathways for the induction of apoptosis due to silica exposure, it is likely that apoptosis is playing a role in the progression of autoimmunity. Figure 1 shows an overview of possible silica-induced signaling pathways involved in apoptosis. Overall these data suggest that silica-induced apoptosis may provide excess antigen, altered antigen and/or impaired clearance of apoptotic material leading to systemic autoimmunity.

FIGURE 1

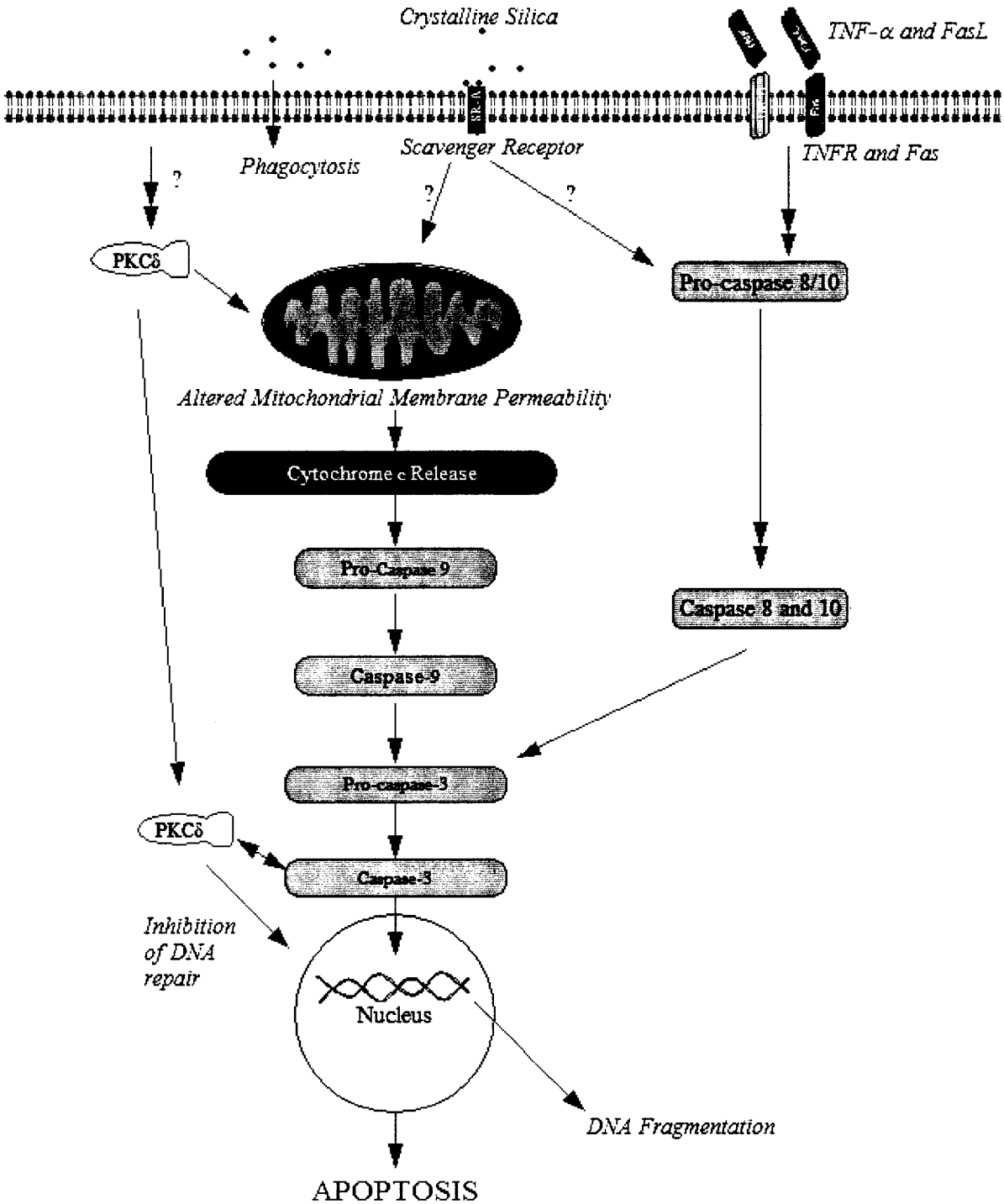

Figure 1.

Possible signaling pathways involved in apoptosis of alveolar macrophages induced by silica exposure. Possible signaling pathways involve Fas/FasL, TNF-α, PKCδ, activation of caspases through the mitochondria and signaling through the scavenger receptor.

Protein Kinase Cδ

An additional pro-apoptotic protein which was examined in this study following silica exposure is protein kinase Cδ (PKCδ) (Fig. 1). PKCδ is a novel PKC family member that has been reported to play a role in the initiation of apoptosis in many cell types (Pongracz 1999; Webb 2000; Bertho 2002; Lounsbury 2002; Brodie and Blumberg 2003; Shukla 2003). PKCδ is activated by diacylglycerol (DAG)/phorbol esters in a calcium-independent manner. PKCδ activation and translocation are induced by a variety of apoptotic stimuli in many different cellular systems. PKCδ is activated in neutrophils undergoing spontaneous apoptosis, in H_2O_2-induced apoptosis, TNF-α induced apoptosis, Fas mediated apoptosis and asbestos-induced apoptosis of alveolar epithelial cells (Pongracz 1999; Webb 2000; Bertho 2002; Lounsbury 2002; Brodie and Blumberg 2003; Shukla 2003).

As shown in Figure 2, PKCδ is regulated not only by the binding of DAG or phorbol ester but also by molecular mechanisms such as phosphorylation and proteolytic reactions. PKCδ has several conserved serine and threonine phosphorylation motif sites as well as three different tyrosine phosphorylation sites which have been reported to be important in H_2O_2 induced activation (Konishi 2001; Kikkawa 2002). In addition to phosphorylation, a catalytically active fragment of PKCδ is generated by proteolysis in cells induced to undergo apoptosis in response to anti-Fas, DNA damaging drugs and ionizing radiation (Emoto 1995; Mizuno 1997; Takahashi 1998). The catalytically active fragment of PKCδ is reported to be cleaved by caspase-3 at Asp-327 and is involved in inhibiting the function of DNA-dependent kinase thereby contributing to DNA damage induced apoptosis (Bharti 1998). All three forms of PKCδ activation have been reported

to lead to apoptosis, however, the targets and cellular localization of the various activated PKCδ isoforms may vary (Kikkawa 2002; Basu 2003).

Cell lines have been established that overexpress PKCδ providing a mechanism to elucidate the role of PKCδ. In general, growth inhibition is observed in cells overexpressing PKCδ (Kikkawa 2002). H_2O_2-induced apoptosis is enhanced in CHO cells overexpressing PKCδ (Kikkawa 2002). Further, transgenic mice have been generated that overexpress PKCδ in basal epidermal cells resulting in mice that are resistant to skin tumor formation induced by phorbol ester (Reddig 1999). In addition to overexpression of PKCδ, knockout mice have been generated. The PKCδ-null mice were reported to have increased proliferation of B cells and were prone to autoimmune disease (Miyamoto 2002). This was attributed to the deficiency of PKCδ preventing the establishment of B cell tolerance through deletion of self-reactive B cells (Miyamoto 2002). In addition to these immune effects, decreased apoptosis of smooth muscle cells was reported to have led to arteriosclerotic lesions in PKCδ deficient mice (Leitges 2001). Even though PKCδ is ubiquitously expressed, it is unclear why PKCδ null mice only show a clear phenotype in certain cell types.

A study by Shukla *et al* (2003) reported a role for PKCδ in apoptosis of alveolar type II cells induced by exposure to asbestos. They reported that asbestos induced PKCδ leading to its translocation to the mitochondria inducing release of cytochrome c and activation of caspase-9. Pre-treatment of these cells with rottlerin, a selective inhibitor of PKCδ, prevented activation of caspase-9 and apoptosis of asbestos exposed type II cells (Shukla 2003). These results suggest that apoptosis of alveolar type II cells is dependent on PKCδ and similar mechanisms may be involved in silica-induced apoptosis.

FIGURE 2

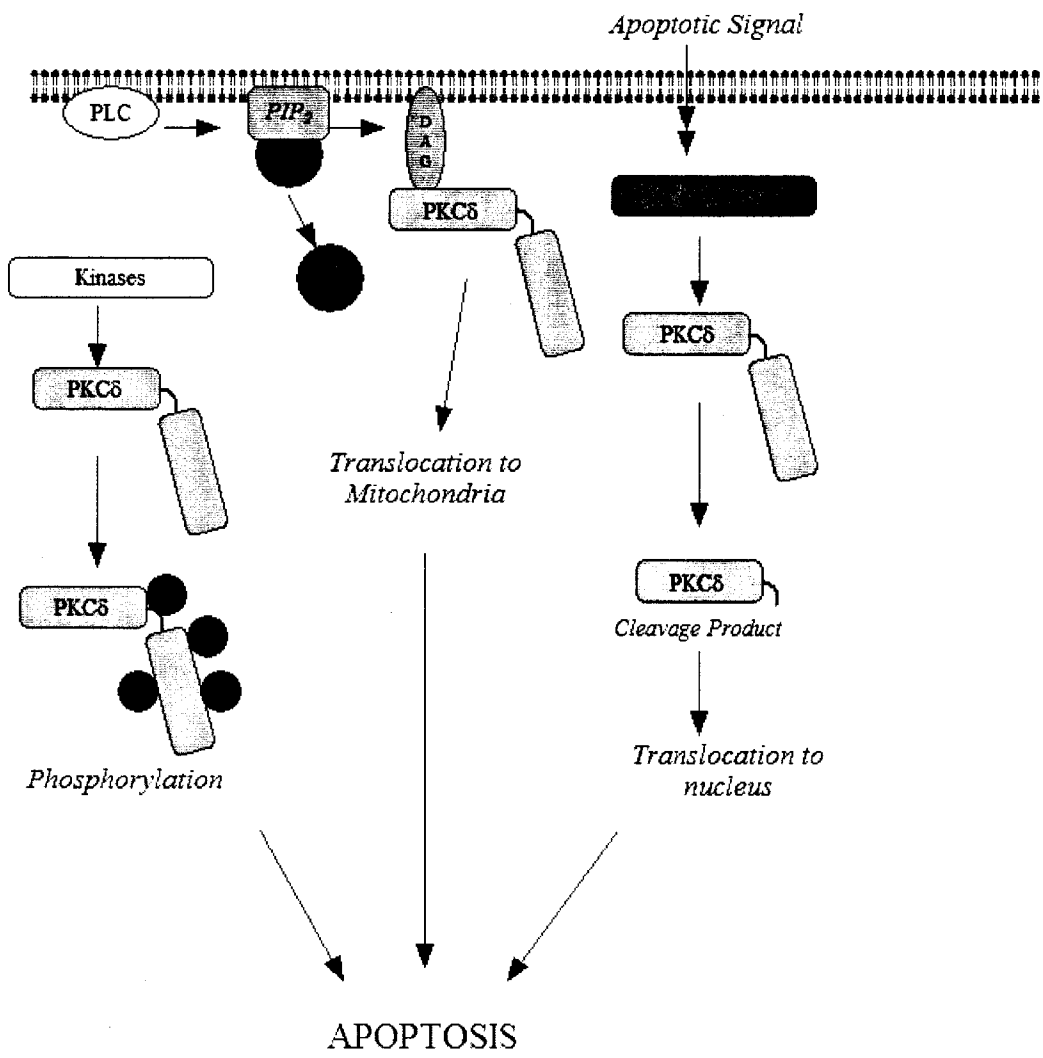

Figure 2

Diagram of the activation of PKCδ by three distinct mechanisms: Diacylglycerol (DAG), caspase-3 and phosphorylation. In addition to activation by DAG, PKCδ is activated by phosphorylation at several serine/threonine and tyrosine residues. PKCδ is also cleaved by caspase-3 to form a catalytically active fragment that is able to translocate to the nucleus to inhibit DNA protein kinase thereby preventing DNA repair.

Silica Exposure and Lymphocytes

As discussed previously, silica-induced apoptosis of alveolar macrophages may provide excess or modified antigen that is presented to lymphocytes thereby eliciting autoantibody production and T cell mediated responses. Further, the inflammatory response initiated by silica provides activation signals via cytokines to lymphocytes. Several studies have further examined silica's effects on lymphocytes using animal models.

Silica exposure has been reported to result in massive increases in lymph node size and sustained activation of distinct T cell populations suggesting silica is mitogenic for T cells (Garn 1997; Davis 2000; Garn 2000). Lymph nodes from silica exposed rats have been reported to be increased in weight up to 35-fold 52 weeks after silica exposure and that the increase was due to an early influx of CD4+ T cells and later influx of B cells (Friedetzky 1998). Six weeks following silica exposure, Friedetzky *et al* (1998) reported finding accumulations of macrophages with ingested silica scattered throughout the lymphoid tissue and these macrophages were interacting with lymphocytes undergoing apoptosis. Garn *et al* (1997) further reported that T cells from lymph nodes of silicotic rats had become activated with enhanced IFN-γ gene transcription, thereby resulting in macrophage activation. Garn *et al* (2000) further examined cytokine production from T cells in silicotic rats and reported a shift towards production of the Th1 cytokines: IL-12, IL-18 and IFN-γ. Therefore, T cells seem to be migrating into the lymph nodes of silicotic animals and are producing cytokines, thereby providing additional signals for immune activation. Studies using T cell deficient nude mice, have suggested a role for T lymphocytes in the migration of macrophages to the lung and in the termination of a

neutrophil response following silica exposure (Hubbard 1989). In this study, Hubbard (1989) reported that silica exposure in nude mice resulted in a massive influx of neutrophils that persisted over two months, whereas in the T cell sufficient mice, the neutrophil response was short lived and followed by a migration of macrophages into the lung. This suggests that T cells play a role in influencing the cell type within the lung of silica exposed mice, however, the silica exposed nude mice did not develop fibrosis (Hubbard 1989). These results suggest that T cells may play a role in the migration of macrophages to the lung and that macrophages are playing a major role in the induction of the fibrotic process. A similar role for T cells inducing macrophage migration to the lung may also be an important mechanism for the progression of autoimmune disease.

Lymphocytes appear to be migrating to regional lymph nodes of silica-exposed animals, however antigen-presenting cell and T cell interactions may be occurring within the lung itself. The accumulation of lymphocytes has been reported within the alveolar spaces of the lung and lymphoid tissue of silica treated C3H/HeN mice (Davis 2001). These lymphocytes were reported to consist primarily of CD4+ T cells, but also numerous CD8+ T cells, NK cells and γδ T cells (Davis 2001). Further, Davis *et al* (2001) reported enhanced IFN-γ production and mRNA transcripts for IL-12 and IL-18, thereby suggesting a Th1 response in silica-exposed mice. Using IFN-γ deficient C57Bl/6, Davis *et al* (2001) further reported less extensive silicosis than in wild-type mice. Lymphocytes are found within the lung of silica-exposed animals, however, very little data exist as to their role in the progression of autoimmune disease.

Taken together, these data suggest that silica exposure results in T and B cell activation primarily within the draining lymph nodes of the lung. It appears silica and apoptotic material-laden antigen presenting cells are migrating to these lymph nodes and presenting self-antigen to T cells, thereby inducing autoimmune responses. The activation of T cells can then lead to B cell activation and autoantibody production further inducing immune complex formation and complement activation.

New Zealand Mixed Mouse Model of Autoimmune Disease

Several mouse models of systemic autoimmune disease exist, however, many of the models have a rapid and severe onset of disease, thereby masking any potential to observe environmental effects. The New Zealand mixed (NZM) mouse is a genetically mixed hybrid of the New Zealand white X New Zealand black F_1 (NZWxNZB F_1) crossed with a NZW father mouse. Several lines of autoimmune prone NZM mice were generated from this breeding, of which the NZM2410 is the most studied (Rudofsky and Lawrence 1999). NZM2410 mice were selected due to the presence of renal disease and development of autoantibodies. NZM2410 mice do not have a full complement of autoimmune genes as with other mouse models of autoimmunity, but do carry many susceptibility loci. Thus, an environmental effect may be more readily observed in this moderately autoimmune prone strain rather than strains of mice with high disease penetrance. Rudofsky *et al* (1999) reported that NZM mice develop proteinuria and 50% of the mice succumb to glomerulonephritis around 6 months of age. They further reported that these mice develop autoantibodies to dsDNA, ssDNA, IgG, histones, chromatin, nuclear antigens, nucleosomes and myeloperoxidase. Thirty percent of these mice also develop anti-erythrocyte autoantibodies leading to anemia (Rudofsky and

Lawrence 1999). All NZM strains are deficient in complement C4 and C5 thus Rudofsky *et al* (1999) have proposed these genes as susceptibility loci. Due to the lower disease penetrance in the NZM2410 mouse model, it provides an appropriate model to assess the effects of silica on the development of autoimmune disease.

Specific Aims

Using the autoimmune prone New Zealand mixed mouse model, this project examined the ability of silica exposure to exacerbate systemic autoimmune disease in a genetically susceptible population. It is possible that many people have varying levels of genetic susceptibility to autoimmune disease, but have not developed the disease, however an environmental exposure such as silica may push the immune system to a point at which the susceptibility genes may be involved and a disease results. By examining the following 3 specific aims, we will better understand mechanisms by which silica and possibly other relevant environmental agents can alter the immune system resulting in autoimmunity.

SPECIFIC AIMS

Specific Aim I. Characterize the exacerbation of systemic autoimmune disease by silica in autoimmune prone-New Zealand mixed mice (Chapters 1 and 2)

Specific Aim II. Characterize the role of apoptosis in the production of autoantibodies in silica exposed NZM mice (Chapter 3)

Specific Aim III. Characterize the role of protein kinase Cδ in silica-induced apoptosis and systemic autoimmune disease in NZM mice (Chapter 4)

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CHAPTER 1

Silica accelerated systemic autoimmune disease in lupus-prone New Zealand mixed mice

Abstract

The genetic backgrounds of lupus-prone murine models are a valuable resource for studying the influence of environmental exposure on autoimmune diseases in sensitive populations. Epidemiological studies have shown associations between silica exposure and several autoimmune diseases, including scleroderma and systemic lupus erythematosus. To determine whether silica exposure can exacerbate systemic autoimmunity in genetically predisposed animals, New Zealand mixed mice were intranasally instilled twice with saline or saline suspensions of 1 mg silica or 500 μg TiO_2, a dose equivalent in surface area, and were evaluated with respect to health and immune status. Survival in silica exposed NZM mice was decreased compared to saline and TiO_2 exposed mice. Proteinuria levels were elevated in silica exposed mice. Levels of circulating immune complexes, autoantibodies to nuclear antigen (ANA), histone, and double stranded DNA were measured every two weeks by ELISA. Circulating immune complexes showed a trend towards an increased acceleration in levels in the silica exposed mice compared to saline and TiO_2 exposed mice. ANA levels were significantly higher in silica exposed animals compared to saline and TiO_2 exposed animals (0.237 ± 0.03 vs. 0.140 ± 0.029 and 0.125 ± 0.03, $p < .05$) 16 weeks post-exposure. Autoantibodies to histone were also significantly elevated after 16 weeks in silica exposed animals compared to saline and TiO_2 exposed animals (0.227 ± 0.03 vs. 0.073 ± 0.015 and 0.05 ± 0.03, $p < .05$). In contrast, serum IgG levels were decreased in silica exposed NZM mice compared to the saline controls, however IgM levels were

unaffected. Lungs of the silica-exposed mice had increased inflammatory infiltrates as well as fibrotic lesions characterized by excess collagen deposition. Therefore, although NZM mice are susceptible to SLE, silica exposure significantly exacerbated the course of disease.

Introduction

Silica is ubiquitous in the environment as an abundant mineral found in rock, sand, and soil. Occupational silicosis results from acute or chronic exposures to high levels of silica dusts in many manufacturing and construction processes as well as mining operations. Silicosis leads to deceased pulmonary function and increased susceptibility to other diseases of the respiratory tract (1997). In addition, inhalation of silica has been associated with increased incidence of systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) and scleroderma (Parks, Conrad et al. 1999). Human silicosis patients have exhibited increased autoantibodies, immunoglobulins and immune complexes (Doll, Stankus et al. 1981). However, the mechanisms leading to autoantibody production and the relationship to subsequent pathology have not been elucidated and these autoimmune reactions do not occur in all silica exposed individuals, suggesting a genetic component. Although some of the enhanced humoral response could be attributed to a non-specific adjuvant effect, the tendency towards select autoimmune syndromes suggests a more specific effect.

A study by Steenland and Brown demonstrated that 3000 gold miners exposed to silica had increased risk for autoimmune diseases, including SLE, rheumatoid arthritis, and scleroderma (Steenland and Brown 1995). Haustein and Anderegg created a scleroderma registry from 1980-1997 and showed a 12-fold increase in scleroderma in males over the age of 40 with silicosis (Haustein and Anderegg 1998). Several other groups have reported an increased incidence of SLE associated with silicosis (Wilke, Salisbury et al. 1996; Brown, Gridley et al. 1997).

The present study was designed to test the hypothesis that silica exposure could exacerbate the development of autoimmune disease in a genetically susceptible murine model. Studies by Pollard, *et al* have demonstrated that autoimmune prone mice are a valuable resource for studying the effects of environmental exposures on genetically susceptible populations (Pollard, Pearson et al. 1999). The New Zealand mixed (NZM) 2410 mouse model was selected to assess the effects of silica exposure and the exacerbation of predisposed autoimmune disease. NZM mice spontaneously develop features of SLE between 6 months and 1 year of age (Rudofsky and Lawrence 1999). This model characteristically develops anti-dsDNA, anti-histone, and anti-nuclear antigen (ANA) autoantibodies as well as increasing levels of circulating immune complexes (Rudofsky and Lawrence 1999). This strain also develops glomerulonephritis by one year of age in 80% of males and females (Rudofsky and Lawrence 1999). The New Zealand mixed mouse was incorporated into this study due to the lower number of autoimmune susceptibility loci compared with murine models that have a full complement of SLE genes, such as the MRL/*lpr* or NZBxNZW F1, in which the severe autoimmune phenotype could mask any environmental insult (Rudofsky and Lawrence 1999).

The overall objective of the study was to test the hypothesis that inhaled silica, and not saline or a control particle (TiO_2), could exacerbate the natural progression of systemic autoimmune disease in SLE prone NZM mice. The disease course was measured by following the development of autoantibodies, serum immunoglobulins, immune complexes, proteinuria, and pulmonary fibrosis.

Materials and Methods

Mice

Male and female New Zealand mixed (NZM 2410) mice were obtained from Taconic (Germantown, NY) and maintained in microisolation containers in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council. The animal room is set on 12-hour dark/light cycles with food and water provided *ad libitum.*

Treatment of mice

At six weeks of age, mice were instilled intranasally with either 30 μL saline (n=5) or 30 μL saline suspensions of 1 mg crystalline silica (n=14) or 500 μg TiO_2 (n=5) as a control particle equivalent to silica in surface area. All mice received 2 instillations 2 weeks apart in order to represent several exposures over a period of time. Control and experimental groups were matched for the number of male and female mice. Silica was obtained from Pennsylvania Glass Sand Corp. (Pittsburgh, PA). TiO_2 was obtained from Fisher Scientific (Denver, CO). Mice were bled for sera before the first instillation and at 2-week intervals following instillations to monitor autoantibody levels. A second cohort of NZM mice was instilled with 30 μL saline (n=8) or 30 μL of a saline suspension of 1 mg silica (n=8) to use for histological examinations at 14 weeks. After 14 weeks, blood was collected for sera by cardiac puncture. The lungs and kidneys were removed for histology and the superficial cervical lymph nodes and spleens were weighed.

Detection of serum autoantibodies

ANA was detected by indirect immunofluoresence using HEp-2 cell slide kits (Immunoconcepts, Sacramento, CA). Manufacturer's protocol was followed. ANA, anti-dsDNA, anti-histone antibodies and circulating immune complexes were detected by

ELISA kits (Alpha Diagnostics, San Antonio, TX). Sera were diluted 100-fold before assay and manufacturer's protocol was followed. Samples with a positive circulating immune complex level were determined by using a cut-off value as determined by the manufacturer. The reported values are mean optical density (OD) values from each treatment group.

Serum immunoglobulin quantitation

Serum IgG and IgM levels were quantitated by ELISA. 96 well Polysorp Nalge-Nunc ELISA plates (Fisher) were coated with 100 μL of 1 μg/mL goat anti-mouse kappa light chain antibody (Southern Biotechnology Associates, Birmingham, AL) diluted in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Plates were blocked with 200 μL PBS-1% bovine serum albumin (BSA) for 1 hour at room temperature followed by 3 washes with PBS-0.1% Tween 20. Sera were diluted in PBS-0.1% Tween 20/1% BSA. Standard curves were generated using unlabeled IgG (Southern Biotechnology Associates) starting at 0.5 μg/mL and unlabeled IgM (Sigma Chemical, St. Louis, MO) starting at 1 μg/mL. Diluted sera were added to wells and incubated in duplicate for 1 hour at room temperature followed by 3 washes with PBS-0.1% Tween 20. HRP-conjugated goat anti-mouse IgG (Fc-fragment specific) (Jackson ImmunoResearch Laboratories, West Grove, PA) and IgM (Jackson Laboratories) were diluted 2000-fold in PBS-0.1% Tween 20/1%BSA and incubated for 1 hour at room temperature followed by 3 washes with PBS-0.1% Tween 20. 100 μL One Step TMB substrate (Zymed, San Francisco, CA) was added and incubated for 15 minutes followed by the addition of 100 μL 2N H_2SO_4 to stop the reaction. The optical density was read at

450 nm and serum immunoglobulin concentration was calculated by extrapolation from the linear portion of the standard curve. All samples were tested in duplicate.

Urinary Protein

Proteinuria was measured by Chemstrip 2 GP test strips as described by the manufacturer (Boehringer Mannheim Diagnostics, Indianapolis, IN). Milligram protein per deciliter was measured between groups following the provided scale (0 = negative, trace, 1+ = 30 mg/dL, 2+ = 100 mg/dL, 3+ = 500 mg/dL).

Histology

Animals were given a lethal injection of sodium pentobarbital (5 mg i.p.) and the lungs were perfused with Histochoice fixative (Amresco Inc, Solon, IL). Lungs and kidney were removed and routinely processed using an automated processor (ThermoShandon, Pittsburgh, PA). The lungs and kidneys were embedded in paraffin wax and sectioned 5-7 μM thick, then collected on poly-L-lysine coated slides (Sigma Chemical, St. Louis, MO). Using an automated stainer (ThermoShandon, Pittsburgh, PA) lung samples were stained with hematoxylin and eosin for general cellular morphology and Gomori Trichome for collagen deposition. Samples were blinded and examined by light microscopy. Kidney sections were immunohistochemically stained using an antibody for IgG immune complex and complement deposition. The kidney sections were boiled in a 0.01 M sodium citrate buffer for 10 minutes followed by washes in distilled water and phosphate buffered saline. The kidney sections were then blocked with 4% fetal bovine serum in phosphate buffered saline. Goat anti-mouse IgG-FITC antibody (ICN Biomedicals, Irvine, CA) and a goat anti-mouse C3-FITC (ICN Biomedicals, Irvine, CA) were used for the detection of immune complexes and complement deposition. A goat

anti-rat IgG antibody (ICN Biomedicals, Irvine, CA) was used as an isotype control. Samples were blinded and examined using a fluorescent microscope.

Statistical Analysis

Differences between silica treated and saline or TiO_2 treated mice were assessed using analysis of variance (ANOVA) and unpaired *t*-test. All values are reported as mean ± SE; $p \leq 0.05$ was considered significant.

Results

Effects of silica and TiO_2 on mortality, proteinuria and circulating immune complexes in NZM mice

Mortality, proteinuria and immune complexes have been reported with silicosis (Doll, Stankus et al. 1981), therefore these biomarkers were examined in NZM mice following instillation of saline or saline suspensions of TiO_2 or silica. Mortality in silica instilled NZM mice was exacerbated compared to saline and TiO_2 instilled animals (Figure 3). Mortality in silica exposed NZM mice began around 10 weeks following instillation, while mortality in the saline and TiO_2 instilled mice didn't begin until 16 weeks following instillation. Within 22 weeks following instillation of silica, only 22% of the mice survived, while 60% of the mice instilled with saline or TiO_2 survived within the same time and continued to live until sacrificed at 9 months following exposure.

Although NZM mice have a rapid onset of glomerulonephritis with proteinuria levels greater than 500 mg/dL in both males and females (Rudofsky and Lawrence 1999), silica exposure exacerbated the development of proteinuria (Figure 4). NZM mice instilled with silica developed proteinuria levels of 500 mg/dL within 10 weeks following instillation, while the saline instilled mice did not develop the same levels until 16 weeks following exposure.

FIGURE 3

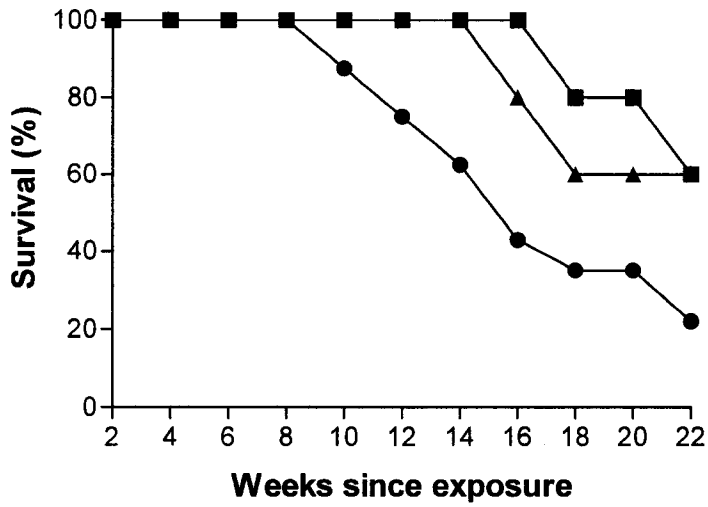


Figure 3.

Survival of saline (■) (n=5), TiO_2 (▲) (n=5) and silica (•) (n=14) instilled NZM mice. Silica exposed NZM survival decreased more rapidly and to a greater extent than saline and TiO_2 exposed mice.

The TiO_2 instilled mice developed 500 mg/dL proteinuria levels 14 weeks following exposure. Sixteen weeks following exposure, 87.5% of the silica instilled NZM mice had 500 mg/dL levels of proteinuria, while only 33% of saline and TiO_2 had high proteinuria levels.

Levels of circulating immune complexes showed a trend towards accelerated elevation in silica instilled mice compared to saline and TiO_2 instilled mice (Figure 5). The silica instilled NZM mice developed detectable levels of circulating immune complexes within 6 weeks following instillation, while it required 10 weeks post-exposure for the TiO_2 exposed mice to develop detectable levels of circulating immune complexes. The saline exposed mice did not show detectable levels of circulating immune complexes until 12 weeks following instillation. However, within 14 weeks following exposure, all of the mice within the three groups developed circulating immune complexes. 16 weeks following exposure, fewer saline and TiO_2 exposed mice had detectable levels of circulating immune complexes, while all of the silica exposed mice continued to have detectable levels of circulating immune complexes. Taken together, these results demonstrate that silica exposure in NZM mice exacerbates mortality, possibly due to increases in proteinuria levels and circulating immune complexes.

FIGURE 4

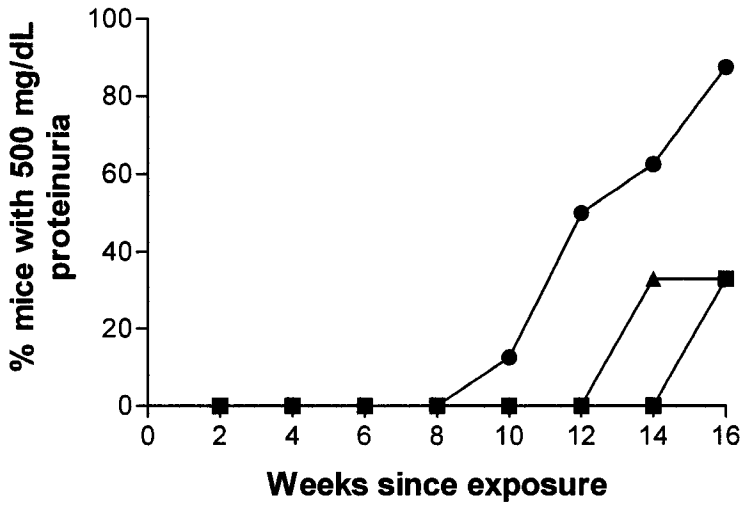


Figure 4.

Proteinuria levels greater than 500 mg/dL in saline (■) (n=5), TiO_2 (▲) (n=5) and silica (●) (n=14) instilled NZM mice measured by Chemstrip 2GP proteinuria/glucose strips every two weeks. Silica exposed NZM mice developed high proteinuria levels earlier and in greater numbers than the saline or TiO_2 exposed mice.

FIGURE 5

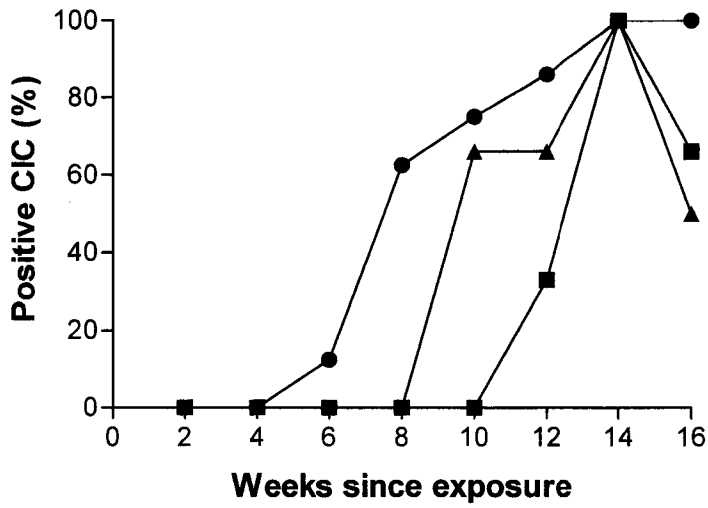


Figure 5.

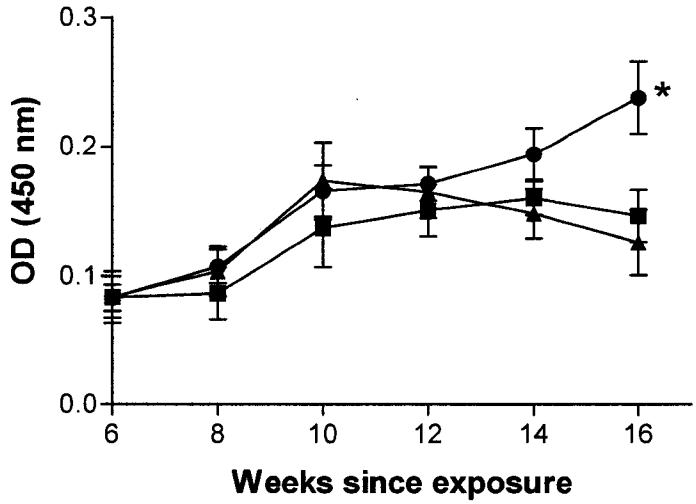
Levels of circulating immune complexes (CIC) in serum from silica exposed NZM mice. Shown is the percentage of saline (■) (n=5), TiO_2 (▲) (n=5) and silica (●) (n=14) exposed NZM mice that developed a positive level of CIC measured by ELISA every two weeks. Serum samples with an OD reading above a certain cut-off value that was set according to the manufacturer was considered to have positive levels of CIC.

Effects of silica and TiO_2 on autoantibody levels in NZM mice

Although NZM mice are susceptible to autoantibody formation, typically developing autoantibodies within six months of age (Rudofsky and Lawrence 1999), silica significantly elevated levels of serum ANA and anti-histone antibodies within 16 weeks following exposure compared to saline and TiO_2 (Figure 6). ANA levels measured by ELISA were exacerbated in the silica-exposed animals compared to the saline and TiO_2 mice (0.237 ± 0.03 vs. 0.140 ± 0.029 and 0.125 ± 0.03, $p < .05$ respectively) (Figure 6A). ANA was also confirmed by HEp-2 fluorescent staining that consisted of a homogenous nuclear pattern (Data not shown). Anti-histone antibody levels were also elevated in silica treated mice compared to saline and TiO_2 treated animals (0.227 ± 0.03 vs. 0.073 ± 0.015 and 0.05 ± 0.03, $p < .05$ respectively) (Figure 6B). Autoantibodies to dsDNA were also examined, however there were no differences between treatments (data not shown). These results demonstrate that silica exposure is able to elevate levels of autoantibody production in an autoimmune prone mouse model.

FIGURE 6

A.



B.

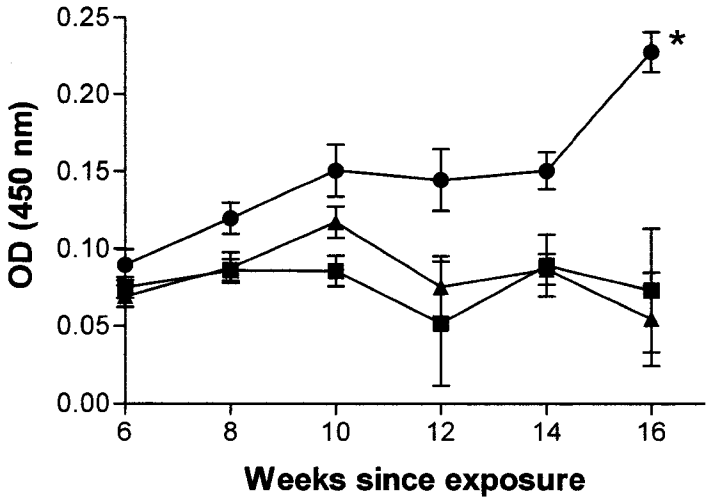


Figure 6.

Development of anti-nuclear antigen (ANA) and anti-histone autoantibodies in saline (■) (n=5), TiO_2 (▲) (n=5) and silica (●) (n=14) exposed NZM mice measured by ELISA every two weeks. Silica exposure significantly elevated ANA levels 16 weeks following exposure compared to saline and TiO_2 (A). Levels of anti-histone autoantibodies 16 weeks following exposure were also elevated compared to saline and TiO_2 exposed mice (B). The values reported are mean values for each treatment group. The levels of ANA and anti-histone autoantibodies before 6 weeks were not different between treatments and therefore not shown. *($p \leq 0.05$ using an unpaired t-test)

Effects of silica on serum immunoglobulin levels in NZM mice

Serum immunoglobulin levels have been reported to be increased in several models of silicosis (Huang, Hubbs et al. 2001; Weissman, Hubbs et al. 2001), therefore IgG and IgM levels were examined in NZM mice following instillations of saline and silica. IgM and IgG levels were examined by ELISA using serum from saline and silica 14 week exposed mice that were sacrificed for lung histology. Fourteen weeks following silica exposure, IgG levels were decreased from an average of 3024 μg/mL in the saline exposed mice to 1638 μg/mL in the silica exposed mice (Figure 7). However, the levels of IgG remained the same from 2-12 weeks (data not shown). There was no difference with IgM levels in the saline or silica exposed mice from 2 weeks to 14 weeks following exposure (data not shown). These results demonstrate that silica exposure specifically decreases IgG in the NZM mouse model contrary to increases in immunoglobulin levels in other models of silicosis.

Effects of silica on pathological changes in NZM mice

Saline and silica exposed NZM mice were sacrificed at 14 weeks following exposure to collect lung samples for histology and to examine lymph node and spleen weights. Lung sections were stained with H&E and Gomori's trichrome to examine the development of fibrosis. Histological examination of stained lung sections of silica exposed NZM mice revealed fibrotic lesions with excess collagen deposition, while the saline exposed mice had minimal collagen deposition (Figure 8). H&E staining showed increased inflammatory infiltrates in the silica exposed NZM mice compared to the saline exposed mice (data not shown).

FIGURE 7

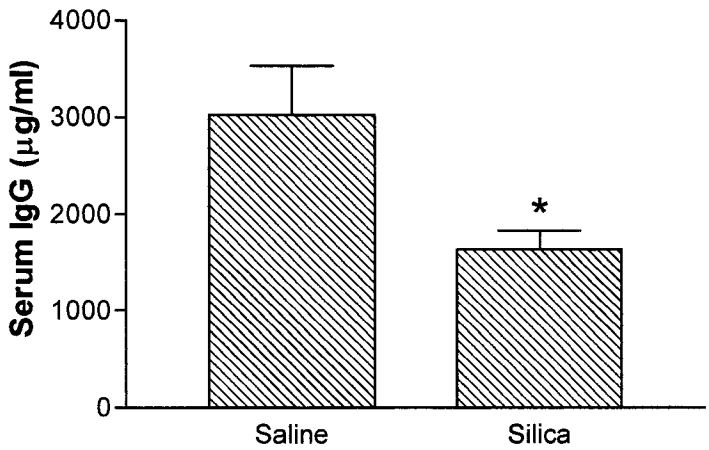


Figure 7.

Serum IgG levels in saline and silica exposed NZM mice 14 weeks following exposure measured by ELISA. IgG levels were significantly reduced in silica exposed mice (n=8) compared to saline control mice (n=8) *($p \leq 0.05$ using an unpaired t-test).

FIGURE 8

A.

B.

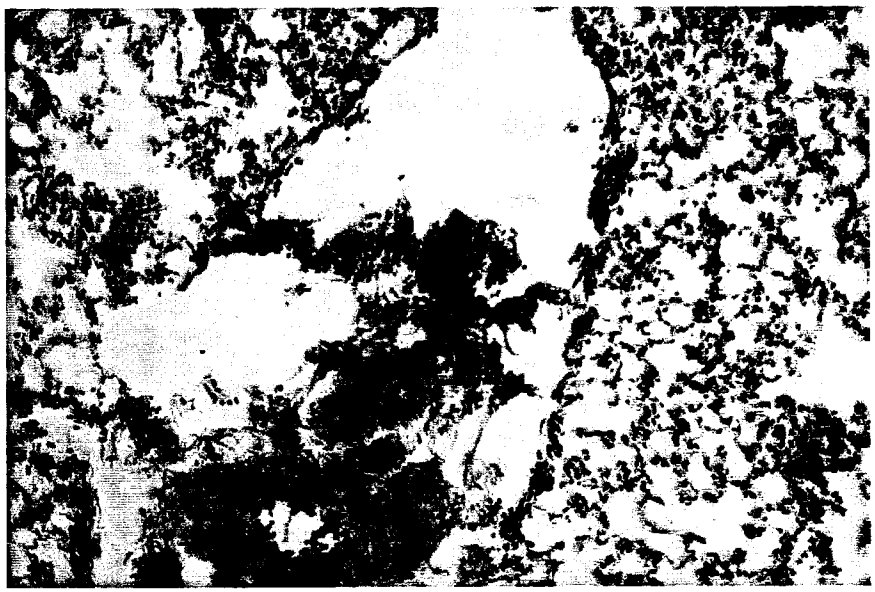


Figure 8.

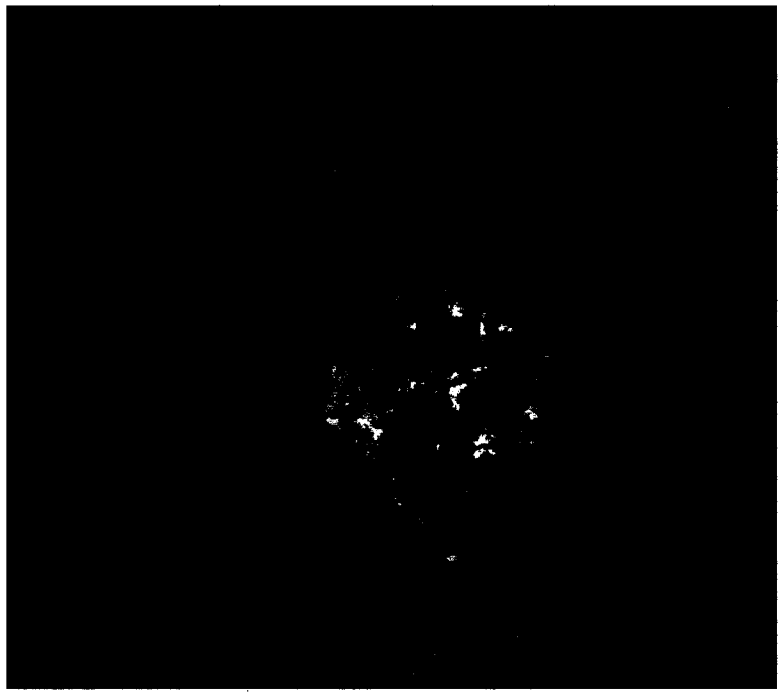
Representative examples of Gomori trichrome staining of a saline (A) and silica (B) exposed NZM mouse 14 weeks following exposure. The silica exposed mouse shows extensive fibrotic lesions and excess collagen deposition represented by the blue staining indicating development of silicosis. Saline exposed mice had very little collagen deposition.

The kidneys of saline and silica exposed NZM mice were examined 14 weeks following exposure for the presence of immune complex and complement deposition. Immunohistochemical staining of kidneys revealed extensive IgG immune complex deposition within the glomeruli of silica exposed mice (Figure 9). The saline exposed mice showed minimal staining for IgG immune complexes within the glomeruli. The kidneys were also stained for complement C3 deposition and showed similar patterns of staining within the glomerulus as the IgG immune complex staining (data not shown).

The superficial cervical lymph node was removed and weighed upon sacrifice of saline and silica treated NZM mice. A trend towards an increase in the average size of the lymph node in the silica treated mice (117.4 g ± 33.3) compared to the saline treated mice (69.04 g ± 20.7) was observed. However, there was no difference in spleen weight between the silica exposed (152 g ± 15.7) and the saline exposed mice (146 g ± 13.3). These results demonstrate that silica exposure in NZM mice is leading to development of silicosis with increased inflammation in the lung and increased immune complex and complement C3 deposition within the kidney.

FIGURE 9

A.



B.

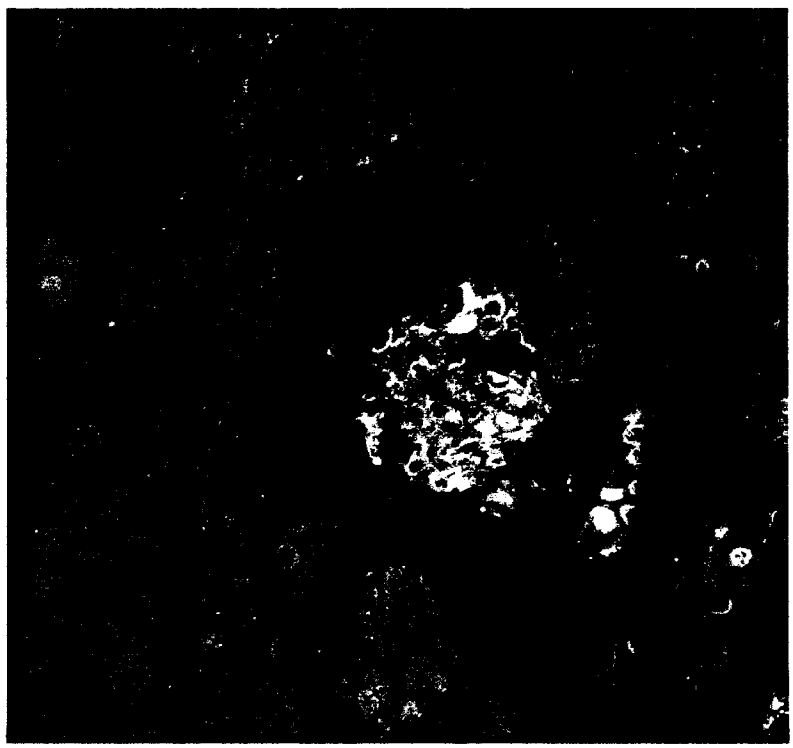


Figure 9.

Representative examples of immunohistochemical staining of kidney sections from saline (A) and silica (B) exposed NZM mice 14 weeks following exposure. A goat anti-mouse IgG-FITC antibody was used to stain immune complexes within the glomeruli of the kidney. The silica-exposed mice showed extensive immune complex deposition within the glomeruli of the kidney while the saline exposed mice had minimal staining for immune complexes. (*Magnified 60x)

Discussion

The exacerbation of systemic autoimmune disease by silica exposure in New Zealand mixed mice identifies a potential model for the study of xenobiotic effects on autoimmune susceptible populations. This study demonstrates that silica is able to exacerbate the development of autoimmune disease in a genetically susceptible mouse model. The disease acceleration involved increases in mortality, proteinuria, autoantibody levels, circulating immune complexes, pulmonary fibrosis, immune complex deposition and complement C3 deposition within the kidney. The disease acceleration appears to be specific to silica and is not seen with a control particle, TiO_2, which has been reported to cause mild inflammation (Yuen, Hartsky et al. 1996).

The most pronounced response to silica exposure in the NZM mouse was a decrease in survival. The silica exposed NZM mice died significantly earlier than the saline or TiO_2 exposed mice, which corresponded with the development of proteinuria levels greater than 500 mg/dL. This suggests that mortality may have been associated with development of glomerulonephritis in the silica-exposed mice. Silica particles have been reported to accumulate in the kidney leading to renal fibrosis and localized inflammation (Slavin, Swedo et al. 1985). However, our model indicates that circulating autoantibodies are deposited within the kidney resulting in an immune complex mediated glomerulonephritis. This is supported by the rapid development of high titers of circulating immune complexes, proteinuria and immune complex deposition within the kidneys of the silica-exposed mice.

Possible mechanisms of silica induced autoimmune disease may involve the alveolar macrophage, the primary immune cell of the lung that is the first line of defense

and the primary cell involved in the clearance of apoptotic cells within the lung. It has previously been reported that phagocytosis of silica leads to a caspase dependent apoptosis in human alveolar macrophages (Iyer, Hamilton et al. 1996). Silica induced apoptosis of alveolar macrophages leads to release and phagocytosis of silica by other alveolar macrophages, producing a cyclical process of inflammation and cell death (Cooper, Miller et al. 2002). This constant inflammation and cellular death may provide excess antigen that is being presented to the immune system, thereby breaking immune tolerance.

In this study, autoantibodies to nuclear antigen, including dsDNA and histone were examined as biomarkers of disease acceleration because NZM mice have been reported to develop high titers of these within 6 months to 1 year of age (Rudofsky and Lawrence 1999). Silica exposure significantly elevated ANA levels and autoantibodies to histone, but had little effect on dsDNA autoantibody levels. The increase in titers of these autoantibodies may be the result of silica induced apoptosis leading to excess apoptotic material being presented to the immune system.

Several studies have examined immunoglobulin responses to experimental silicosis. These studies have reported that silicosis is associated with elevated IgG and IgM levels in the blood of Fisher rats (Huang, Hubbs et al. 2001). Another study examined IgG subclass responses in experimental silicosis, also in Fisher rats, and reported increased IgG-secreting spot-forming cells of all IgG subclasses in lung associated lymph nodes (Weissman, Hubbs et al. 2001). However, our results with NZM mice show a decrease in IgG levels 14 weeks following silica exposure and no change in IgM levels from 2 to 14 weeks post-exposure. In spite of the decrease in total IgG levels

there was an increase in autoantibodies of the IgG class. This suggests silica may be decreasing total IgG, but a higher percentage of the immunoglobulin appears to be autoreactive. The decrease in IgG levels may be in part explained by the overall health of the mice. The majority of the silica exposed mice died within 16 weeks of exposure and the IgG levels were measured 14 weeks following silica exposure. Severe weight loss occurred one to two weeks before the silica exposed mice died. It is possible the immune system was compromised at this point and unable to produce normal levels of IgG. However, a trend towards an increase in the size of the superficial cervical lymph nodes in the silica-exposed mice was observed, suggesting increased immune activity.

The silica exposed NZM mice developed pulmonary fibrosis, visualized with Gomori trichrome stain for collagen deposition, while the saline exposed mice did not develop fibrosis. However, it is unknown whether the fibrosis is due to direct action of the silica or could be exacerbated by the increase in autoantibodies and immune complexes. Future studies will examine if deposition of immune complexes occurs in these silicotic lungs. H&E staining further showed significant infiltration of inflammatory cells into the lungs of the silica treated NZM mice. Future studies will examine which inflammatory cells are being recruited to the lung and the role they may play in the development of autoimmune disease. Staining of kidney sections from silica exposed NZM mice for immune complex and complement C3 deposition revealed extensive immune complex mediated glomerulonephritis.

In summary, silica exposure in the NZM 2410 mouse model of SLE appears to accelerate the development of autoimmune disease. This exacerbation of disease involved increased mortality, proteinuria, autoantibodies, immune complexes, pulmonary fibrosis and

immune complex mediated glomerulonephritis. The NZM mouse represents a good model to further study the mechanisms of xenobiotic induced autoimmune diseases in a genetically susceptible population.

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CHAPTER 2

Immunoglobulin and lymphocyte responses following silica exposure in New Zealand mixed mice

Abstract

Epidemiological studies have shown strong associations between silica exposure and several autoimmune diseases, including scleroderma and systemic lupus erythematosus. We previously reported that the New Zealand mixed mouse develops silicosis and exacerbated autoimmunity following crystalline silica exposure, including increased levels of autoantibodies, proteinuria, circulating immune complexes, pulmonary fibrosis and glomerulonephritis. In this study, the NZM mouse was used to examine changes in immune activation following silica exposure by measuring levels of immunoglobulin, cytokines and lymphocyte populations. Levels of IgG1 were significantly decreased from 1124 ± 244 μg/ml in saline exposed mice to 614 ± 204 μg/ml in silica exposed mice suggesting a decrease in the Th2 response. The levels of TNF-α were significantly increased (1.5 fold) in the bronchoalveolar lavage fluid of the silica-exposed mice as compared to the saline exposed mice. The number of B1a B cells were significantly increased 6-fold within the superficial cervical lymph nodes of silica-exposed mice as compared with saline exposed mice. Following silica exposure, CD4+ T cells significantly increased 3-fold within the superficial cervical lymph nodes. During this increase in the number of CD4+ T cells, the number of CD4+CD25+ regulatory T cells were not significantly changed therefore altering the ratio of regulatory T cells to T helper cells from 1:5 to 1:8 following silica exposure. Therefore, the silica induced alterations in immunoglobulin levels, increased TNF-α, increased B1a B cells and CD4+ T cells with decreased regulatory T cells may provide an environment that allows for

increased autoreactivity. These studies begin to provide possible mechanisms for environmentally induced autoimmune diseases that have been reported in many epidemiological studies.

Introduction

Silica is ubiquitous in the environment as an abundant mineral found in rock, sand and soil. Silicosis is an occupational disease resulting from acute or chronic high levels of silica exposure that leads to decreased pulmonary function and increased susceptibility to other diseases of the respiratory tract (1997). Silicosis has also been associated with increased incidence of systemic autoimmune disease, such as systemic lupus erythematosus (SLE) and scleroderma (Wilke et al. 1996; Parks et al. 1999; Parks et al. 2002). Human silicosis patients have exhibited increased autoantibodies, immunoglobulins and immune complexes (Doll et al. 1981). Although some of the enhanced humoral response could be attributed to a nonspecific adjuvant effect, the tendency towards select autoimmune diseases suggests a more specific effect.

Although genetics have been shown to be partly responsible for autoimmune disease, identical twin studies have consistently found only a 20-30% chance that both twins will develop autoimmune disease suggesting an environmental component. A recent study by Parks, *et al* reported an association between silica exposure and the development of SLE in a case control study in the Southeastern United States (Parks et al. 2002). Earlier studies by Steenland and Brown demonstrated that 3000 gold miners exposed to silica had increased risk for autoimmune diseases, including SLE, rheumatoid arthritis and scleroderma (Steenland et al. 1995). Haustein and Anderegg created a scleroderma registry from 1980 to 1997 and reported a 12-fold increase in scleroderma in males over the age of 40 with silicosis (Haustein et al. 1998).

Previously, a good animal model of silicosis with development of autoimmune disease has not been available to study these effects, however we recently reported that

the New Zealand mixed (NZM) mouse develops silicosis and increased autoimmune disease upon exposure to crystalline silica (Brown et al. 2003). However, the mechanisms leading to autoantibody production and the relationship to subsequent pathology have not been elucidated. The silica-exposed mice develop higher levels of autoantibodies, proteinuria, circulating immune complexes, pulmonary fibrosis and glomerulonephritis than saline or titanium dioxide exposed control mice (Brown et al. 2003).

In the present study, the NZM mouse model to examine changes in immunoglobulin, cytokine and lymphocyte numbers following the exacerbation of autoimmunity due to silica exposure. The hypothesis was that NZM mice will exhibit increased immune activity as measured by increases in immunoglobulin and cytokine levels as well as increases in lymphocyte numbers following silica exposure that may play a role in the exacerbation of autoimmune disease. We conducted studies that examined alterations in the ratio of Th1 and Th2 immunoglobulin profiles as well as increases of cytokines within the lungs of silica exposed NZM mice. We further investigated alterations in B and T cell numbers within the spleen and superficial cervical lymph node of silica treated NZM mice. We examined both the B220+ and B1a B cell populations as well as CD4+ T helper cells and CD4+CD25+ T regulatory cell populations. These studies may provide possible mechanisms for not only silica-induced autoimmune disease, but also other environmentally relevant particles.

Materials and Methods

Mice

Male and female New Zealand Mixed (NZM2410) mice were obtained from Taconic (Germantown, NY) and maintained in microisolator containers in accordance with the *Guide for the care and use of laboratory animals* prepared by the Institute of Laboratory Animal Resources, National Resource Council. The animal room is set on 12 hour light/dark cycles with food and water provided *ad libitum.*

Treatment of mice

At six weeks of age, equal numbers of male and female NZM mice were intranasally instilled with 30 μL saline (n=8) or 30 μL saline suspensions of 1 mg crystalline silica (n=8). All mice received 2 instillations 2 weeks apart in order to represent several exposures over a period of time. Silica was obtained from Pennsylvania Glass Sand Corp. (Pittsburgh, PA). Mice were bled for sera every two weeks until sacrificed at 14 weeks post-exposure. At 14 weeks blood was collected for sera by cardiac puncture.

Serum immunoglobulin quantitation

Serum IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 levels were quantitated by ELISA. Polysorp Nalge-Nunc 96 well ELISA plates (Fisher) were coated with 100 μL of 1 μg/mL goat anti-mouse kappa light chain antibody (Southern Biotechnology Associates, Birmingham, AL) diluted in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Plates were blocked with 200 μL PBS-1% bovine serum albumin (BSA) for 1 hour at room temperature followed by 3 washes with PBS-0.1% Tween 20. Sera were diluted in PBS-0.1% Tween 20/1% BSA. Standard curves were generated using unlabeled IgG (Southern Biotechnology Associates) starting at 0.5 μg/mL, unlabeled IgM (Sigma

Chemical, St. Louis, MO) starting at 1 μg/mL, unlabeled IgG1 (Southern Biotechnology Associates) starting at 0.1 μg/mL, unlabeled IgG2a (Sigma) starting at 0.25 μg/mL, unlabeled IgG2b (Southern Biotechnology Associates) starting at 0.05 μg/mL, unlabeled IgG3 (Sigma) starting at 0.5 μg/mL. Diluted sera were added to wells and incubated in duplicate for 1 h at RT followed by 3 washes with PBS-0.1% Tween 20. HRP-conjugated goat anti-mouse IgG (Fc-fragment specific) (Jackson ImmunoResearch Laboratories, West Grove, PA), IgM, IgG1, IgG2a, IgG2b and IgG3 (Caltag) were diluted 2000-fold in PBS-0.1% Tween 20/1%BSA and incubated for 1 hour at room temperature followed by 3 washes with PBS-0.1% Tween 20. 100 μL One Step TMB substrate (Zymed, San Francisco, CA) was added and incubated for 15 minutes followed by the addition of 100 μL 2N H_2SO_4 to stop the reaction. The optical density was read at 450 nm and serum immunoglobulin concentration was calculated by extrapolation from the linear portion of the standard curve. All samples were tested in duplicate.

Cytokine Analysis

Cytokine levels were quantitated by use of a Luminex based immunoassay from Linco Research (St. Charles, MO). A 5-plex assay measuring IL-4, IL-10, IL-12, IFN-γ and TNF-α was used with serum and bronchoalveolar lavage fluid from saline and silica treated NZM mice 14 weeks following instillation. The protocol provided by Linco Research was followed and the samples were analyzed on a Luminex 100 instrument (Luminex, Austin, TX).

Flow Cytometry Analysis

Spleens and superficial cervical lymph nodes from 4 saline and 4 silica exposed NZM mice were made into single cell suspension and washed 3 times with PBS. Purified rat anti-mouse CD16/CD32 (FcγII/III receptor) monoclonal antibody (Pharmingen, San Jose, CA) was added to block Fc receptors. To identify the B cell populations, cells were stained with FITC conjugated rat anti-mouse CD45R/B220 (Pharmingen), cy-chrome conjugated rat anti-mouse CD5 (ly-1) (Pharmingen), and R-phycoerythrin conjugated rat anti-mouse CD23 (FcεRII) (Pharmingen) monoclonal antibodies. T cell populations were analyzed using cy-chrome conjugated rat anti-mouse CD3 (Pharmingen), R-phycoerythrin conjugated rat anti-mouse CD4 (Pharmingen) and FITC conjugated rat anti-mouse CD25 (IL-2Rα) (Pharmingen). Antibodies were used at 1 μg/10^6 cells. Cells were acquired (10,000/sample) and analyzed on a Becton Dickenson FACScalibur flow cytometer (Becton Dickenson, San Jose, CA). Cells that were B220+, CD5+ and $CD23^{low}$ were classified as B1a B cells (Mohan et al. 1998). Cells that were CD3+ and CD4+ were classified as T helper cells and cells that were CD3+, CD4+ and CD25+ were classified as regulatory T cells (Sakaguchi et al. 1995).

Statistical Analysis

Differences between saline and silica exposed mice were assessed using analysis of variance (ANOVA) and unpaired *t*-test. All values are reported as mean ± SE; $p \leq 0.05$ was considered significant.

Results

Silica exposure leads to decreased IgG levels in NZM mice

Changes in immunoglobulin levels have been reported in both human silicosis patients and in several rat model of silicosis (Doll et al. 1981; Huang et al. 2001; Weissman et al. 2001). Similar to the previous results at 14 weeks, IgG levels were significantly lower in silica-exposed mice than saline control mice (1639 μg/ml ± 193 vs. 3024 μg/ml ± 501 $p<0.05$) (Fig. 10) (Brown et al. 2003). Therefore, a more detailed analysis of serum immunoglobulin levels was conducted at 14 weeks following silica exposure in NZM mice, which included IgG, IgM, IgG1, IgG2a, IgG2b and IgG3 as shown in Figure 10. IgG and IgM levels were also measured from 2 weeks to 14 weeks following silica exposure, however there was no difference until the 14-week time point (data not shown). IgM levels were slightly elevated in silica exposed NZM mice compared to saline exposed mice (979 μg/ml ± 213 vs. 792 μg/ml ± 104) (Fig. 10). IgG1 levels were significantly reduced in the silica-exposed mice compared to the saline control mice (614 μg/ml ± 204 vs. 1124 μg/ml ± 224 $p<0.05$) (Fig. 10). IgG2a, IgG2b and IgG3 all remained at similar levels between the saline and silica exposed mice (Fig. 10). The resulting IgG2a:IgG1 ratio was 1.36:1 suggesting a silica-induced shift towards a Th1 immune response.

FIGURE 10

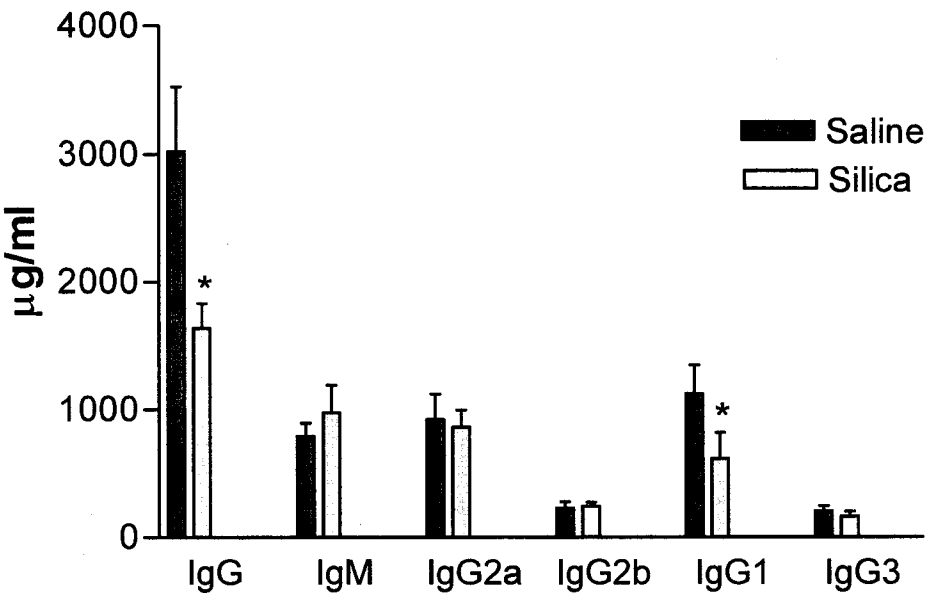

Figure 10:

Immunoglobulin levels measured by ELISA in NZM mice 14 weeks following silica exposure. Serum levels of IgG and IgG1 were significantly decreased in silica exposed NZM mice (n=8) as compared to saline exposed control mice (n=8). IgG2a levels remained at normal levels following silica exposure, suggesting a normal Th1 immune response. The significant decrease in IgG1 suggests a decrease in the Th2 immune response. *($P<0.05$)

Silica exposure leads to increased TNF-α within the lungs of NZM mice

IL-4, IL-10, IL-12, TNF-α and IFN-γ were examined using a Luminex based mouse cytokine assay in order to assess changes in cytokine levels that may indicate inflammation and a shift in the Th1/Th2 balance. We expected to see differences in serum cytokine profiles that would suggest a Th1 type immune response as seen with the immunoglobulin levels. However, only small changes were seen in serum levels of IL-10 (243 ± 7.9 pg/ml vs. 225 ± 4.9 pg/ml) and IL-12 (946 ± 51.9 pg/ml vs. 887 ± 21.1 pg/ml) between saline and silica treated NZM mice (Fig. 11).

Significant differences in TNF-α levels within the bronchoalveolar lavage fluid were observed in the silica exposed NZM mice. The silica treated NZM mice exhibited a 1.5-fold increase in TNF-α compared to the saline control mice 14 weeks following silica exposure (50 ± 0.19 pg/ml in saline exposed mice vs. 75 ± 1.85 pg/ml in silica mice $p<0.05$) (Fig. 12). The increased levels of TNF-α suggest silica is creating an inflammatory environment and may result in excess apoptosis induced through the TNF receptor mediated pathway (Pfeffer 2003).

FIGURE 11

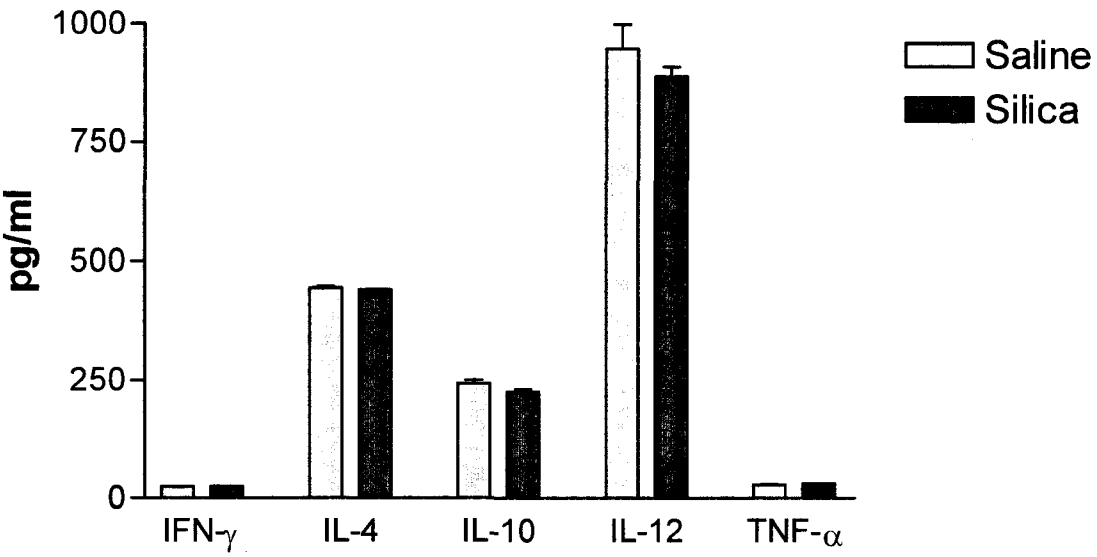

Figure 11:

IL-4, IL-10, IL-12, IFN-γ and TNF-α levels within the serum of saline (n=8) and silica (n=8) exposed NZM mice 14 weeks following silica exposure. Cytokine levels were measured using a Luminex based assay. There were slight differences in the levels of IL-10 and IL-12, however these differences are not biologically relevant.

FIGURE 12

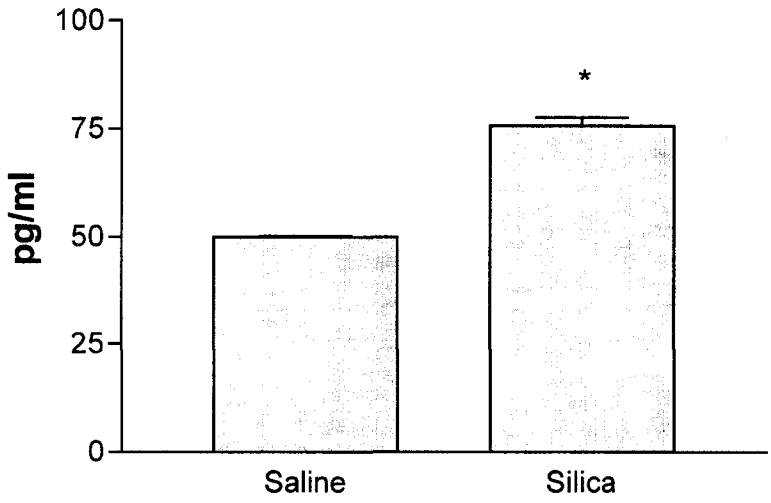

Figure 12:

TNF-α levels measured by a Luminex based assay of bronchoalveolar lavage fluid 14 weeks following silica exposure in NZM mice. TNF-α was significantly increased 1.5 fold in silica exposed NZM mice (n=8) as compared to saline exposed mice (n=8). * ($P<0.05$)

Silica exposure results in an increase in B220+ cells and B1a B cell numbers in NZM mice

Due to the decrease in IgG and the alteration of immunoglobulin isotypes, B cell populations were examined 14 weeks following silica exposure in NZM mice. The numbers of B cells in the superficial cervical lymph node and spleen were analyzed by flow cytometry. As shown in Table 1, the number and percentage of cells expressing B220 increased in the lymph nodes of silica exposed NZM mice compared to saline controls ($5.41x10^6 \pm 0.44$ vs. $1.58x10^6 \pm 0.09$). Figure 13 shows a representative example of B cell staining from a saline (A) and silica (B) instilled mouse by flow cytometry. The number of B220+ B cells also increased within the spleen of silica treated NZM mice from $5.71x10^6 \pm 1.49$ cells in saline exposed mice to $6.82x10^6 \pm 1.08$ B cells in silica-exposed mice (Table 1).

FIGURE 13

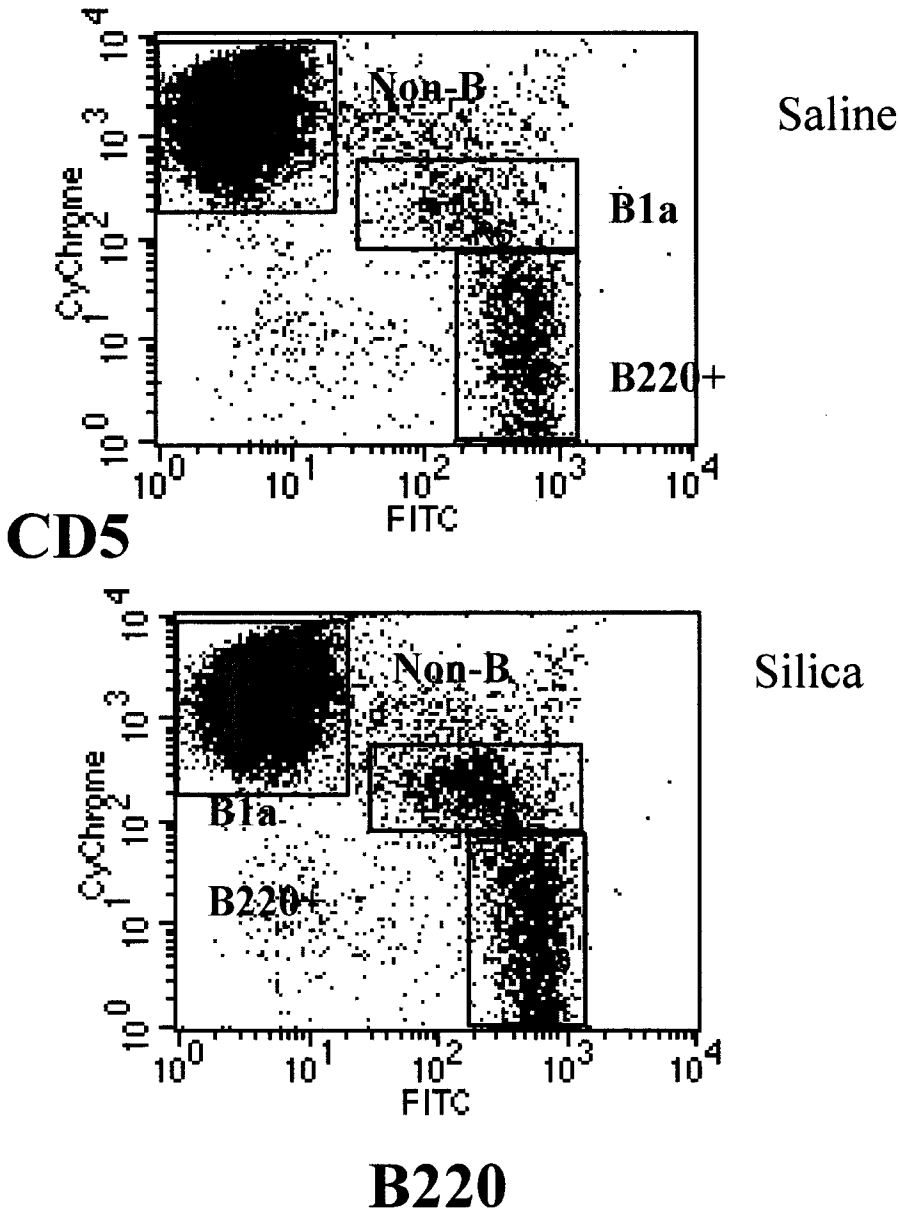

Figure 13:

A representative example of flow cytometric analysis of B cells populations in the superficial cervical lymph node of saline (A) (n=4) and silica (B) (n=4) exposed NZM mice. The number of B220+ B cells increased significantly within the lymph nodes of silica exposed NZM mice as compared with the saline control mice (lower right gate). The number and percentage of B1a B cells significantly increased in lymph nodes of silica exposed NZM mice compared to saline exposed mice (upper right gate).

We next examined levels of B1a B cells since the NZM mouse model has been reported to exhibit increased numbers of B1a B cells as compared to other strains of mice (Mohan et al. 1998). B1a B cells have been implicated to play a role in the development and progression of autoimmune disease (Murakami et al. 1995; Mohan et al. 1998; Berland et al. 2002). Following silica exposure, NZM mice significantly increased the number of B1a B cells within the superficial cervical lymph node while decreased B1a B cells were seen in the spleen. The number of B1a B cells in the draining lymph nodes increased from $0.305 \times 10^6 \pm 0.04$ cells in the saline treated mice to $1.75 \times 10^6 \pm 0.36$ cells in the silica treated mice ($p<0.05$) (Table 1). Since B1a B cells have been reported to be involved in autoimmune disease, the significant increase in B1a B cells in the lymph nodes suggest an additional mechanism for the exacerbation of autoimmune disease following silica exposure (Murakami et al. 1995; Mohan et al. 1998; Berland et al. 2002).

Table 1. Distribution of B cell subsets in the superficial cervical lymph node and spleen 14 weeks following saline and silica exposure in NZM mice.

Site, Treatment	Total B220+ cells $x10^6$ (%)	Total B1a B cells $x10^6$ (%)
Lymph Node		
Saline	1.58 ± 0.09 (12.15 ± 0.85)	0.305 ± 0.04 (2.35 ± 0.51)
Silica	5.41 ± 0.44 (18.12 ± 2.65)*	1.75 ± 0.36 (5.75 ± 1.08)*
Spleen		
Saline	5.71 ± 1.49 (40.81 ± 11.86)	1.27 ± 0.18 (9.68 ± 1.19)
Silica	6.82 ± 1.08 (35.81 ± 4.37)	0.952 ± 0.12 (5.01 ± 0.62)

* ($P<0.01$)

Silica exposure results in increased CD4 T cell numbers and an altered regulatory T cell:T helper cell ratio in NZM mice

Results from the immunoglobulin data suggest a shift towards a Th1 or T cell mediated immune response; therefore, we examined increases in the CD4+ T helper cell population 14 weeks following exposure to silica in NZM mice. The number of CD4+ T cells within the superficial cervical lymph node increased 6 fold following silica exposure. The lymph nodes of the saline control mice contained $6.37x10^6 \pm 0.49$ CD4+ T cells, while the silica treated mice contained $17.55x10^6 \pm 1.68$ CD4+ T cells ($p<0.05$) (Table 2). We previously reported an increase in the size of the superficial cervical lymph node following silica exposure (Brown et al. 2003) and now confirm that this increase is mainly due to an influx of CD4+ T helper cells. The number and percentage of CD4+ T cells within the spleens of silica exposed NZM mice were not statistically different as compared to saline exposed mice (data not shown). Figure 14 is a representative example of T cell staining in the superficial cervical lymph node of a saline (A) and silica (B) instilled NZM mouse showing an increase in the number the number of CD4+ T cells in the upper right gate.

We further measured the number and percentage of CD4+CD25+ regulatory T cells to assess any changes that may be occurring within the regulatory capacity of the significant T cell mediated immune response observed within the lymph nodes of silica exposed mice. The percentage of regulatory T cells decreased from 9.5% in the saline exposed mice to 7.6% in the silica-exposed mice (Table 2). However, the numbers of regulatory T cells were minimally increased from $1.24x10^6 \pm 0.12$ cells in the saline exposed mice to $2.28x10^6 \pm 0.34$ cells in the silica exposed mice (Table 2). The

significant increase of CD4+ T cells with a minimal increase in CD4+CD25+ regulatory T cells alters the ratio of T regulatory cells to T helper cells from a ratio of 1:5 in the saline exposed mice to 1:8 in the silica exposed mice (Table 2). This alteration of the T regulatory:T helper cell ratio may further allow the immune system to break tolerance within the NZM mouse model following silica exposure.

FIGURE 14

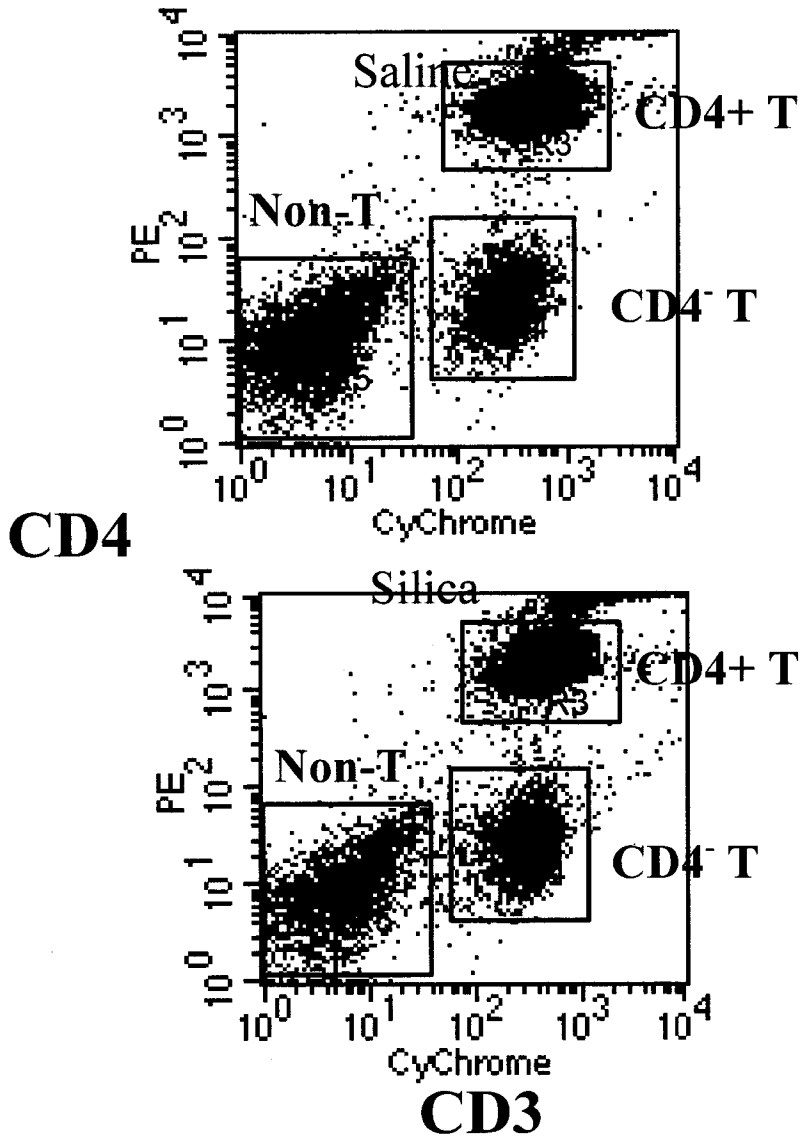

Figure 14:

A representative example of flow cytometric analysis of T cell populations within the superficial cervical lymph node of saline (A) (n=4) and silica (B) (n=4) exposed NZM mice. The number and percentage of CD4+ T helper cells significantly increased following silica exposure in NZM mice (upper right gate). The CD4+ T helper cell population was further analyzed for expression of CD25 or the regulatory T cell population. There was a slight increase in the number of CD4+CD25+ regulatory T cells in the silica-exposed mice. However the ratio of regulatory T cells to T helper cells was altered suggesting lack of T cell regulation following silica exposure.

Table 2. T cell populations in the superficial cervical lymph node 14 weeks following saline and silica exposure in NZM mice.

Site, Treatment	Total CD4+ T cells $x10^6$ (%)	Total CD4+CD25+ T cells $x10^6$ (%)
Lymph Node		
Saline	6.37 ± 0.49 (49.6 ± 3.87)	1.24 ± 0.12 (9.5 ± 0.71)
Silica	17.55 ± 1.68 (58.5 ± 1.22) *	2.28 ± 0.34 (7.6 ± 0.75)

* ($P<0.05$)

Discussion

The increased incidence of autoimmune disease following exposure to silica has previously been reported, however, very few mechanistic studies have been done to explore the mechanisms involved in this disease process. In this study, we utilized the New Zealand mixed mouse model to examine changes in immunoglobulin and cytokine levels as well as changes occurring within the B and T cell populations to try and bridge the gaps of knowledge existing between epidemiology studies and mechanistic studies of silica-induced autoimmunity.

Silica exposure has been associated with an increased incidence of systemic autoimmune disease and these patients have exhibited increased autoantibodies, immunoglobulins and immune complexes (Doll et al. 1981; Wilke et al. 1996; Parks et al. 1999; Parks et al. 2002). Following silica exposure, NZM mice exhibit an exacerbated autoimmune response as measured by increases in autoantibodies, glomerulonephritis and pulmonary fibrosis (Brown et al. 2003). Due to these changes, we examined immunoglobulins, cytokines and lymphocyte numbers to provide further insight into mechanisms that may be involved in the exacerbation of autoimmune disease following silica exposure.

Previous results, examining immunoglobulin levels following silica exposure, have reported an increase in Th2 type immunoglobulins in a rat model of pulmonary fibrosis (Huang et al. 2001; Weissman et al. 2001). However, in the present study we examined alterations in immunoglobulin levels in a mouse model of silica induced autoimmune disease. Following silica exposure we previously reported that there was a significant decrease in IgG levels 14 weeks following silica exposure in NZM mice

(Brown et al. 2003). Due to this decrease in overall IgG levels, we examined several IgG isotypes to determine if specific IgG isotypes account for the decrease in IgG. Following silica exposure, IgG2a, indicative of a Th1 immune response, remained at the same levels as saline control mice. However, IgG1, indicative of a Th2 immune response was significantly decreased in the silica instilled NZM mice as compared to the saline instilled mice. The balance of IgG2a and IgG1 seen following silica exposure in NZM mice suggests an alteration in the balance of the Th1/Th2 immune response in favor of a Th1 or T cell mediated immune response.

Due to the immunoglobulin suggested alterations in the Th1/Th2 balance, we examined cytokine levels within serum and bronchoalveolar lavage fluid (BALF) from saline and silica treated NZM mice. However, there were only slight changes in serum levels of IL-4, IL-10 IL-12, IFN-γ and TNF-α fourteen weeks following silica exposure. The levels of IL-4, IL-10, IL-12 and IFN-γ were too low to detect within the BALF. However, we saw a 1.5 fold increase in the levels of TNF-α within the BALF of silica-instilled mice as compared to the saline instilled mice. TNF-α has clearly been defined has a mechanism of inducing apoptosis (Pfeffer 2003). Apoptosis has been reported to play a role in the development of autoimmune disease, therefore a silica induced increase in TNF-α may play a role in the exacerbation of autoimmune disease we have proposed by induction of apoptosis in lung cells (Mevorach et al. 1998; Rosen et al. 1999; Greidinger 2001; Brown et al. 2003).

We next examined B cell populations to explain the decrease in overall IgG levels. We examined numbers of B220+ B cells and B1a B cells within the spleen and superficial cervical lymph nodes. NZM mice have been reported to have an increased

number of B1a B cells as compared to several other strains of mice (Mohan et al. 1998). Further, the B1a B cell has been reported to play a role in the development and progression of autoimmune disease (Murakami et al. 1995; Mohan et al. 1998; Berland et al. 2002). Fourteen weeks following silica exposure, NZM mice had significantly increased numbers of B220+ cells within the spleen and superficial cervical lymph node. However, the numbers of B1a B cells were decreased within the spleen following silica exposure as compared with the saline control mice. At the same time point the number of B1a B cells increased 3 fold within a draining lymph node of the lung following silica instillation. This suggests a pattern of migration of B1a B cells from the spleen to the superficial cervical lymph node. This increase in the number of B1a B cells may play a major role in the development of autoantibodies due to silica exposure. B1a B cells have been reported to have increased antigen presenting cell activity as measured by phagocytosis and high expression levels of CD80 and CD86 on their cell surface (Mohan et al. 1998). Therefore, the B1a B cells may aid in the development of autoimmune disease through phagocytosis of apoptotic cellular material and subsequent presentation of autoantigens to T cells. Due to the reported increased expression of CD80 and CD86 on the surface of B1a B cells, these cells potentially have an increased capacity to activate autoreactive T cells further aiding the silica exacerbated immune response in NZM mice (Mohan et al. 1998).

The number of CD4+ T helper cells increased 6 fold within the superficial cervical lymph node 14 weeks following silica exposure. The number of CD4+CD25+ T regulatory cells increased minimally within the lymph node following silica exposure. It is expected that as an increase in CD4+ T cells occurs, there should be a concomitant

increase in CD4+CD25+ T regulatory cells to control the immune response and to prevent autoreactivity (Sakaguchi et al. 1995; Takahashi et al. 1998; Shevach 2000; Shevach 2002). However, following silica exposure, the balance of regulatory T cells to T helper cells was altered. It has been reported that the regulatory T cell population constitutes approximately 10% of the T cell population and this was true within our saline exposed mice (Sakaguchi et al. 1995). However, following silica exposure, the regulatory T cell population decreased within the lymph node from 9.5% to 7.6% of the total CD4+ T cell population. The change in the regulatory T cell and T helper cell populations following silica exposure resulted in an altered ratio of regulatory T cells to T helper cells. Within the lymph nodes of saline instilled mice, the ratio of regulatory T cells to T helper cells was 1:5, while the ratio in the silica exposed mice changed to 1:8, suggesting a decreased ability to regulate T cell autoreactivity.

This significant increase in CD4+ T cells and altered balance of regulatory T cells following silica exposure, combined with a significant increase in B1a B cells, may provide an environment of excess immune activation. This environment could then become autoreactive by the addition of excess apoptotic cellular material provided by previously reported silica induced apoptosis of macrophages and excess TNF-α induced cellular apoptosis (Hamilton et al. 2000). The alterations in immunoglobulin, cytokine and lymphocyte numbers reported in this study provide additional insights into possible mechanisms that may be occurring within silica as well as other environmentally induced autoimmune diseases.

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CHAPTER 3

Silica-exposed mice generate autoantibodies to apoptotic cells

Abstract

Silica exposure has been associated with development of autoantibodies and systemic autoimmune disease, but the mechanisms leading to these events are unknown. This study was undertaken in order to test the hypothesis that apoptotic cells may provide the epitopes for the exacerbated autoimmune responses in New Zealand Mixed (NZM) mice following silica exposure. Apoptotic cells, but not live cells, were shown by immunofluorescence microscopy and flow cytometry to stain brightly with serum from ANA-positive mice, but they did not stain with ANA negative serum. Confocal microscopy colocalized staining by the serum antibodies with annexin V on apoptotic blebs suggesting recognition of epitopes on apoptotic cells by autoantibodies. In addition, binding of antibodies from ANA-positive mice was shown to be significantly greater on cellular lysates from apoptotic cells, but not necrotic or live cell lysates using an ELISA based assay. Finally, inhibition of apoptosis with a caspase inhibitor, Boc-D-FMK, blocked the increased binding by the autoantibodies. These results suggest that apoptosis plays an important role in the development of autoimmune disease in New Zealand mixed mice by exposing antigenic epitopes to the immune system, and silica is able to exacerbate the disease process by induction of excessive apoptosis.

Introduction

Epidemiological studies have reported strong associations between silica exposure and several autoimmune diseases, especially scleroderma (Reviewed in (Parks, Conrad et al. 1999)). Increased levels of serum immunoglobulins, immune complexes, and autoantibodies have been demonstrated in humans with silicosis (Doll, Stankus et al. 1981; Nigam, Suthar et al. 1993). However, neither the mechanisms leading to humoral changes nor their relationship to subsequent pathology have been elucidated. Scleroderma (systemic sclerosis) is a chronic multisystem disease characterized by fibrotic changes in affected tissues including the lung, and leading to a variety of symptoms that can be severely disabling. Silica-exposed individuals can develop a systemic sclerosis that is very similar to idiopathic scleroderma (Haustein and Anderegg 1998). They also show increased prevalence of antinuclear antibodies (ANA), including antibodies to topoisomerase (Scl70) and centromere proteins which appear to be associated with subsequent development of scleroderma (Haustein and Anderegg 1998). The presence of specific antibodies associated with silica exposure provides evidence for an immunologic etiology related to alterations in immune responses following silica exposure (McHugh, Whyte et al. 1994).

Models of autoimmunity suggest that at least two events must occur to generate an autoimmune response: immune activation and the loss of peripheral T cell tolerance leading to self recognition. This study was based on the hypothesis that silica exposure alters the immune status in ways that meet both of these criteria. Cells of the lung efficiently clear most respired particulates without residual damage. Silica, however, leads to chronic inflammation and subsequent fibrosis, possibly due to disruption of

alveolar macrophage (AM) function and excess production of pro-inflammatory cytokines (Davis, Pfeiffer et al. 1998). This adjuvant like effect of silica has long been recognized to have an effect on antibody production (Pernis and Paronetto 1962). Therefore, exposure to silica clearly meets the first criterion in terms of establishing an immune activated/inflammatory environment.

Silica is quite cytotoxic, leading to both necrosis and apoptosis (Iyer, Hamilton et al. 1996; Leigh, Wang et al. 1997), but it seems unlikely that cytotoxicity alone would lead to a loss of tolerance since the ingestion of apoptotic bodies by phagocytes normally down-regulates production of inflammatory cytokines (Fadok, Bratton et al. 1998). Instead, we have previously shown that silica appears to shift the population of human AM to a more immune active phenotype, through selective apoptosis of the normally immune suppressive AM population (Holian, Uthman et al. 1997; Hamilton, Pfau et al. 2001). Therefore, silica induces a highly inflammatory state in the exposed lung, in addition to apoptosis of alveolar macrophages. In this environment, excess apoptosis and the inability to clear the apoptotic cells could lead to excess presentation of self-antigens therefore exacerbating an autoimmune response.

We have previously shown that silica exposure leads to exacerbated autoimmune disease in the New Zealand Mixed (NZM) autoimmune-prone mouse, including increased autoantibody production, immune complexes, proteinuria, pulmonary fibrosis and glomerulonephritis (Brown, Archer et al. 2003). In order to test the hypothesis that this autoimmune disease exacerbation may be associated with silica-induced apoptosis and presentation of apoptotic antigens, serum from silica-exposed NZM mice was used to demonstrate that antibodies were present which recognize epitopes on apoptotic

macrophages.

Materials and Methods

Mice

Male and female New Zealand mixed (NZM 2410) mice were obtained from Taconic (Germantown, NY) and maintained in microisolation containers in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council. The animal room was set on 12-hour dark/light cycles with food and water provided *ad libitum*. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee (IACUC).

Silica-instillation

Silica was obtained from Pennsylvania Glass Sand Corp. (Pittsburgh, PA) and was acid washed, dried and determined to be free of endotoxin by Limulus assay (Cambrex, Walkersville, MD). At six weeks of age, mice were instilled intranasally with either 30 μL saline or 30 μL saline suspensions of 1 mg crystalline silica as previously described (Brown, Archer et al. 2003). All mice received 2 instillations 2 weeks apart in order to represent several exposures over a period of time. Control and experimental groups were matched for the number of male and female mice. After 14 weeks, a time point when the majority of mice developed a positive ANA, the animals were euthanized with a lethal injection of Nembutal (200 μl ip), and blood was collected by cardiac puncture. Clotted blood was centrifuged, and serum was collected and frozen at –20°C until use.

ANA

All serum samples were diluted 1:40 in PBS and tested by indirect immuno-fluorescence

(IF) on commercially prepared and fixed HEp-2 cells (ImmunoConcepts, Sacramento, CA). The staining pattern and relative fluorescence intensity were compared to known positive and negative controls using a fluorescence microscope, and recorded as positive (1+ to 4+) or negative. Anti-nuclear antibodies were also detected using ELISA kits for mouse ANA (Alpha Diagnostics, San Antonio, TX) according to manufacturer's protocol.

Cells

MH-S cells, an alveolar macrophage Balb/C derived cell line (ATCC, Manassas, VA) was used to generate apoptotic cells for IF and for ELISA cellular lysates. Cells were cultured in RPMI with 10% FCS on tissue-culture treated multiwell slides (Cel-Line, Portsmouth, NH) or multi-well culture plates, with cycloheximide (0.1mM, Calbiochem, San Diego, CA) or silica (200 μg/ml) for 4 hr to induce apoptosis. Boc-D-FMK, a broad-spectrum caspase inhibitor (Calbiochem) was used at 10 μM for 1 hr prior to addition of silica. This inhibitor was shown to block silica-induced apoptosis in MH-S cells effectively to control levels, as detected by microscopic visualization of apoptotic morphology and by quantitation of caspase 3 activity in cells grown on multi-well slides (data not shown). Caspase 3 activity was measured on a laser scanning cytometer (CompuCyte, Cambridge, MA) using rabbit anti-active caspase 3 (Promega, Madison, WI) and goat anti-rabbit IgG conjugated with Alexafluor 488 (Molecular Probes, Eugene, OR).

Staining for fluorescence/confocal microscopy and flow cytometry

Cells were either plated on cell culture–treated slides for microscopy, microtiter plate wells, or 6-well plates at $5x10^5$ cells/ml. The cells were treated with media or various

treatments, blocked with PBS containing 2% BSA and Fc Block (5 μg/ml, Pharmingen, San Jose, CA), then covered with PBS containing diluted serum (1:50) from ANA-positive silica exposed mice or ANA negative saline exposed mice. After washing, anti-mouse IgG secondary antibody (2^{o} Ab) conjugated to FITC was added (Pharmingen). Then cells were counter-stained with either propidium iodide (PI, 5 μg/ml, Molecular Probes) or annexin V conjugated to phycoerythrin (PE)(Pharmingen), to visualize apoptotic cells by DNA fragmentation or phosphatidyl-serine exposure, respectively. For fluorescence microscopy, the slides were washed, coverslipped, and analyzed on a Zeiss fluorescence microcope. For microtiter assays, plates were centrifuged 5 min at 1500 rpm to prevent cell loss, washed gently 2 times and analyzed using a Spectra-Max microtiter plate reader (Molecular Devices, Sunnyvale, CA) set on 485 excitation and 530 emission. For flow cytometry, the cells were loosened from the wells and suspended in PBS for analysis on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). A minimum of 10^4 cells were analyzed in each sample.

Generation of Apoptotic Cell Lysates

MH-S cells were grown to confluence in RPMI1640 and 10% FCS and either received no treatment, repeated freeze-thaw cycles, 1 μg/ml cycloheximide (Calbiochem) or 400 μg/ml silica. The cells were examined under light microscopy for morphological changes indicative of apoptosis before lysis. The cells were counted and a percentage of total cells undergoing apoptosis was recorded. MH-S cell pellets (10^7 cells) were incubated in lysis buffer containing 150 mM NaCl, 1mM $MgCl_2$, 80 mM Tris-HCl, 0.1% NP-40 and a protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were passed several times sequentially through 18 gauge to 25 gauge needles to ensure lysis of the cell

nuclei. Protein concentrations were determined after lysis using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) to ensure equal coating on a 96-well ELISA plates.

Cell Lysate ELISA

Ninety-six well Maxisorp Nalge-Nunc ELISA plates (Fisher) were coated with 100 μL of 1 μg/mL cell lysates in carbonate coating buffer. Plates were blocked with 200 μL PBS-1% bovine serum albumin (BSA) for 2 hours at room temperature followed by 3 washes with PBS-0.1% Tween 20. Serum was diluted 1:100 in PBS-0.1% Tween 20/1% BSA. Diluted sera were added to wells and incubated in duplicate for 1 hour at room temperature followed by 3 washes with PBS-0.1% Tween 20. HRP-conjugated goat anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories, Philadelphia, PA) was diluted 2000-fold in PBS-0.1% Tween 20/1%BSA and incubated for 1 hour at room temperature followed by 3 washes with PBS-0.1% Tween 20. One hundred μL One Step TMB substrate (Zymed, San Francisco, CA) was added and incubated for 15 minutes followed by the addition of 100 μL 2N H_2SO_4 to stop the reaction. The optical density was read on a SpectraMax microtiter plate reader at 450 nm. All samples were tested in duplicate or triplicate wells.

Statistics

Statistics were performed using GraphPad Prism software, and sets of data were analyzed against controls using an unpaired 2-tailed *t* test. A p value of ≤ 0.05 was considered significant, and is represented as an asterisk. Error bars represent standard error of the mean of a minimum of 3 replicate samples. All experiments were performed independently at least twice, with similar results.

Results

Enhanced staining of apoptotic cells with ANA-positive NZM mouse serum

In order to demonstrate autoantibody binding to apoptotic cells, NZM mouse serum was first determined to be ANA positive using HEp-2 ANA slides and a mouse ANA ELISA, compared to positive controls. Silica-treated MH-S cells were used as the target cell, representing an alveolar macrophage undergoing apoptosis after exposure to silica. Immunofluorescent analysis of the staining of apoptotic macrophages with ANA-positive serum showed an enhancement of FITC intensity on cells undergoing nuclear blebbing as visualized by propidium iodide staining of the nucleus. Figure 15 shows an identical field stained with both ANA-positive serum and FITC-conjugated anti-mouse IgG secondary antibodies (A), and propidium iodide to visualize the nucleus (B). The arrow identifies a silica-induced apoptotic cell, showing nuclear fragmentation as well as intense FITC staining. Moderate staining of adjacent cells was more intense than that of cells stained with the secondary antibody alone (not shown), which could represent exposure of target epitopes on early apoptotic cells. Several fields were similarly analyzed, and all cells with nuclear fragmentation showed similar increases in intensity of FITC staining. This suggests that cells undergoing this stage of silica-induced apoptosis may be exposing target epitopes for autoantibodies present in the autoimmune serum of the silica-treated mice.

FIGURE 15

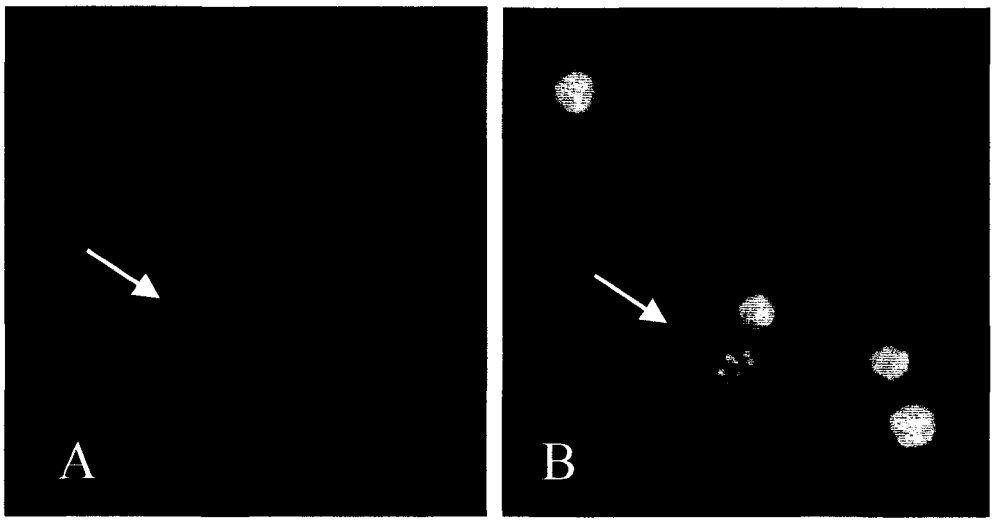

Figure 15.

Visualization of apoptotic cells stained with ANA-positive serum. MH-S cells plated on tissue-culture treated slides were treated with silica (200 μg/ml for 4 hr) to induce apoptosis, fixed on the slides with 1% paraformaldehyde, then stained with serum from the ANA-positive silica-exposed mice (A). The cells were counter-stained with propidium iodide to visualize the nucleus. The arrow identifies an apoptotic cell, showing nuclear fragmentation with propidium iodide in the identical field (B). 400X

Quantification of apoptosis and serum antibody staining

To obtain a more quantitative measure of the staining of apoptotic cells, cells were treated to undergo apoptosis, and then stained with ANA positive serum or annexin V. Annexin V can be used to identify apoptotic cells, due to its ability to bind to phosphatidylserine, which moves to the outer membrane leaflet during apoptosis. Cells were treated with cycloheximide, which induces apoptosis through a caspase-dependent pathway (Blom, de Bont et al. 1999; Tang, Lahti et al. 1999), and then stained with either autoimmune serum (and FITC conjugated anti-mouse IgG secondary antibody) or annexin V conjugated to phycoerythrin (PE). Figure 16 shows the staining of cells treated with media alone (filled) or containing cycloheximide (open). Approximately 25% of the untreated cells and 60% of the cycloheximide treated cells stained with annexin V (Fig 16A), and similar percentages (30% and 68%, respectively) stained with the autoimmune serum (Fig 16B). This suggested that the same cells undergoing apoptosis were also staining with the autoimmune serum, and demonstrated that another inducer of apoptosis other than silica (cycloheximide) could expose autoantigens on the apoptotic cells.

FIGURE 16

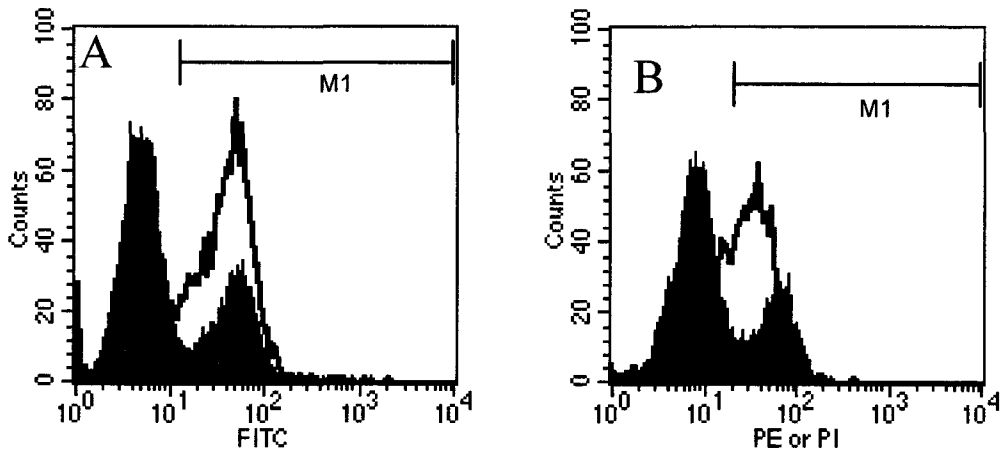

Figure 16.

Flow cytometric quantitation of apoptosis and serum antibody staining of cycloheximide-treated cells. Macrophages were treated with media alone (solid curves) or 0.1 mM cycloheximide (open curves) to induce apoptosis. Gates (M1) were set on FITC- or PE-conjugated secondary antibody (Ab) staining alone, allowing <1% of the cells in the positive region of the histogram. A: FITC (FL1) analysis of media- and CHX-treated cells stained with ANA-positive serum and FITC-conjugated secondary Ab. B: Annexin V:PE (FL2) staining of media- and CHX-treated cells. Histograms are representative of 3 experiments.

Co-staining of apoptotic cells with autoimmune serum and annexin V

In order to quantify the apoptotic cells and demonstrate the specificity of the enhanced staining of the apoptotic cells by the serum, MH-S cells were induced to undergo apoptosis by exposure to silica, stained with ANA-positive or negative serum followed by FITC-conjugated anti-mouse secondary antibodies, and then counter-stained with annexin V:PE to identify the apoptotic cells. Figure 17 is a histogram which shows that apoptotic cells (annexin V:PE+) stained with serum from ANA-positive mice (B) but not from ANA-negative mice (A). In Figure 17, approximately 38% of the cells were apoptotic in both samples, above the horizontal quadrant marker set on live (untreated) cells. Only 2% of the total cell sample also stained with ANA-negative serum, whereas 27% were dual positive in the ANA-positive serum-stained sample (upper right quadrant). This suggests that approximately 71% of the apoptotic cells stained with the serum antibodies above secondary antibody background staining and confirms the specificity of the staining to apoptotic cells. The data also shows that the staining was not due to non-specific binding of serum antibodies to the cells, since ANA negative serum did not stain apoptotic cells.

FIGURE 17

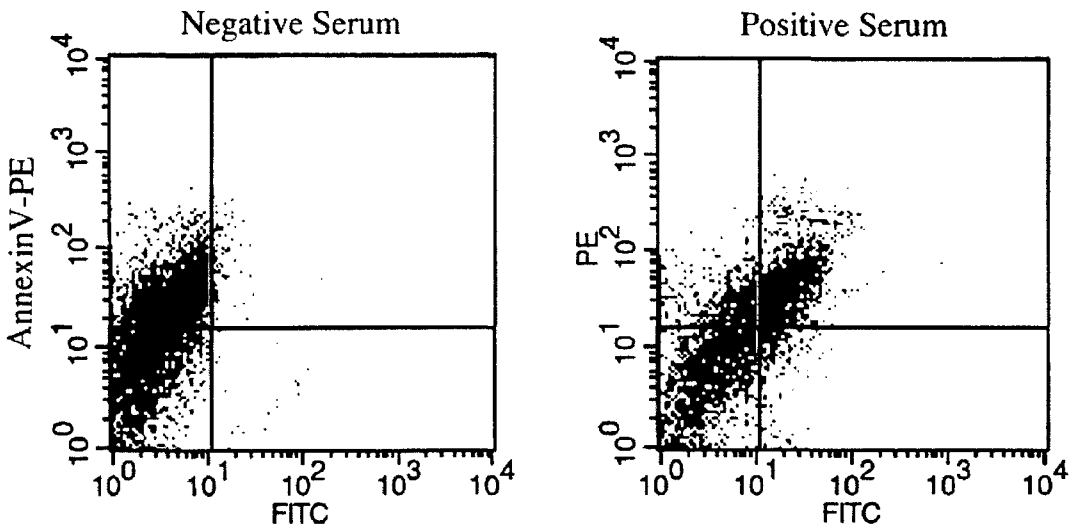

Figure 17.

Flow cytometric analysis of dual staining of apoptotic cells with the ANA-positive serum. Apoptosis was induced with cycloheximide in both samples, and then stained with ANA-positive serum as the primary Ab and anti-mouse FITC-conjugated 2^{o} Ab, and counterstained with Annexin V:PE. Serum from an ANA-negative mouse did not co-stain the apoptotic cells (A). Co-staining of apoptotic macrophages (Annexin V conjugated to PE) is seen with serum from ANA-positive silica-exposed mouse (B). Gates were set on 2^{o} Ab alone.

Co-localization of staining by serum and annexin V on apoptotic bodies and blebs

Because many autoantigens are cellular proteins exposed on apoptotic bodies and blebs, confocal microscopy was used to determine whether autoimmune serum from silica-exposed NZM mice recognized apoptotic blebs. Figure 18 shows an apoptotic cell (MH-S) that has been stained with ANA-positive serum and annexin V:PE as in Figure 16. Although some staining of the whole cell occurred with both serum and annexin V, the apoptotic bleb and bodies show the most intense staining by both stains consistent with the proposed idea that the autoantibodies within serum from silica exposed NZM mice recognize the silica-induced apoptotic MH-S cell.

Effect of inhibition of caspase activity on the staining with autoimmune serum

In order to further support the above evidence of apoptosis-specific antigens being exposed on the surface of cells staining with autoimmune serum, apoptosis was blocked with the broad-spectrum caspase inhibitor, Boc-D-FMK. Confirmation of caspase 3 inhibition of silica-induced apoptosis by Boc-D-FMK was performed as described in Materials and Methods (data not shown). To measure the binding of serum antibodies to cells with and without inhibitor, cells were plated, treated with Boc-D-FMK at 10 μM 1 hr prior to the addition of silica, and stained in 96-well plates after 4 hr. Figure 19 shows significantly more fluorescence of the silica-treated cells stained with autoimmune serum compared to untreated cells, and a return to control levels with addition of the caspase inhibitor. This data shows that the staining of cells by autoimmune serum is due to recognition of apoptosis-specific antigens on the surface of the cells undergoing apoptosis.

FIGURE 18

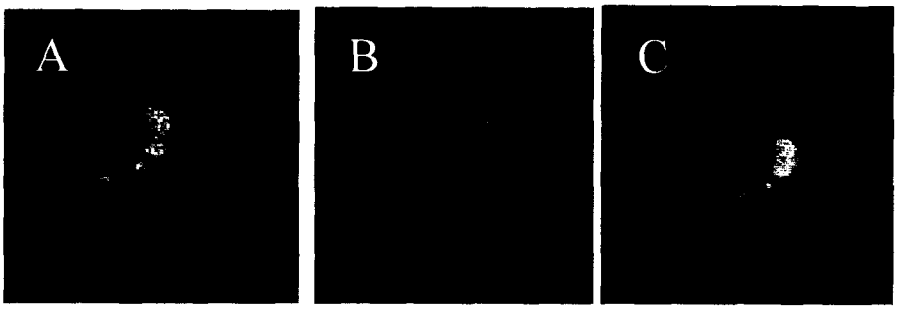

Figure 18.

Visualization of staining of apoptotic blebs with Annexin V and ANA-positive serum. Using confocal microscopy, an apoptotic cell with associated blebs is shown stained with serum from the ANA-positive silica-exposed mouse, where green is staining by serum & FITC 2^{o} Ab (A) and red is Annexin V-PE (B). Co-staining of the apoptotic blebs with serum antibodies is shown in C as an orange color. Oil emersion, 600X.

FIGURE 19

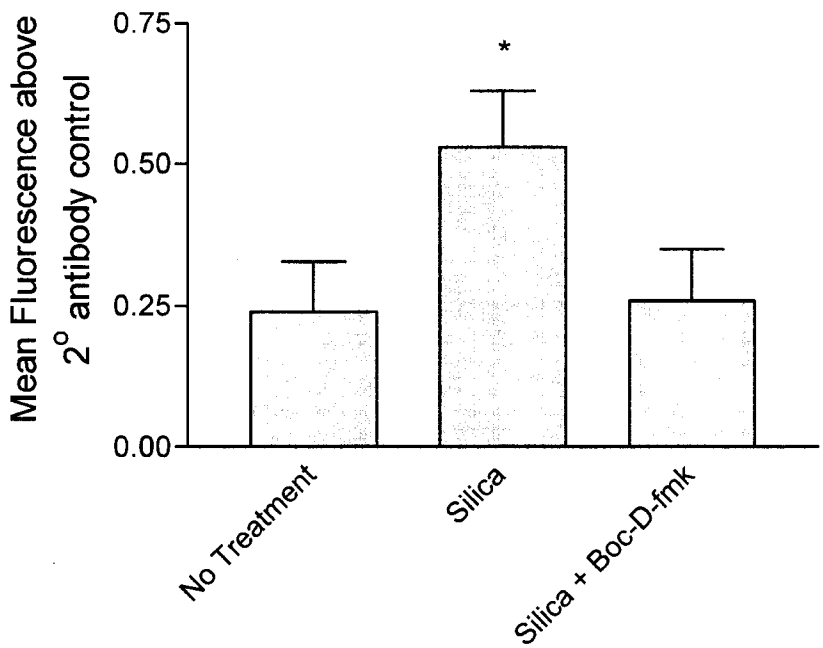

Figure 19.

Blockage of serum antibody binding by caspase inhibitor Boc-D-FMK. MH-S cells plated in 96-well plates were treated with media alone, silica, or Boc-D-FMK plus silica (with caspase inhibitor added 1 hr prior to silica). Plates were centrifuged to prevent cell loss, and then stained with ANA-positive serum from silica-treated NZM mice. Counting of 40x microscope fields in the wells following staining showed that significant cell loss had not occurred in any of the wells. The plates were analyzed for FITC fluorescence on a microtiter plate reader. Data is presented as mean fluorescence above staining by secondary antibody control. Error bars represent standard error, $n = 8$, and * = $p<0.05$.

Specificity of the staining to antigens of apoptotic, but not necrotic, cells

Both necrosis and apoptosis could potentially expose self-antigens to the immune system, but the unique target epitopes seen in many autoimmune diseases suggest that the self-proteins are specifically altered during the process of apoptosis to become antigenic. To demonstrate the specificity of the autoimmune serum antibodies to apoptotic cell antigens, a cell lysate-based ELISA was developed. MH-S cells were either untreated or treated with silica or cycloheximide to induce apoptosis. Another sample of cells was frozen and thawed, to induce necrotic cell death. These samples were lysed and the proteins were used to coat the ELISA plates. Binding of serum antibodies from autoimmune sera to the lysate proteins was then quantified as described in Methods. The results shown in Figure 20 demonstrates significantly higher levels of autoantibodies bound the apoptotic cell lysates compared to untreated cells. Although there was an increase in binding to the cycloheximide-treated cell lysate, this increase was not significant. This interesting result suggests that the silica-induced apoptosis exposed antigens that were not detected on the cycloheximide-treated cells, or were exposed to a lesser degree. Overall, the data demonstrate that the major targets for autoantibodies from the mice are antigens exposed on apoptotic, but not necrotic or live, cells.

FIGURE 20

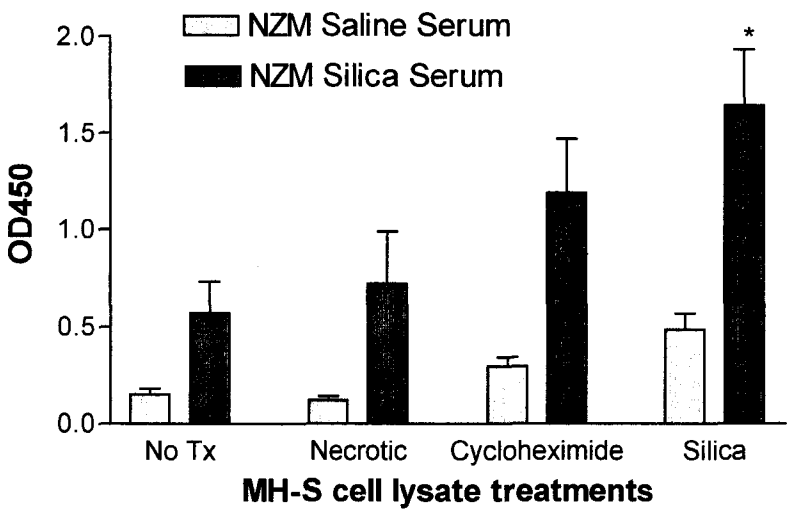

Figure 20.

ANA-positive serum binding to cellular targets specific for apoptotic, not necrotic cells. MH-S cells were treated as described in Materials and Methods to induce apoptosis or necrosis. Cells were lysed, protein quantified and coated on ELISA plates. Serum from ANA-positive mice was used as primary antibody, and goat anti-mouse IgG conjugated to horseradish peroxidase was added as the detection reagent. Color development after addition of substrate was analyzed by plate reader at 450 nm. Serum from silica-exposed mice (solid bars, n=6) and serum from saline-only exposed mice (hatched bars, n=8). Error bars represent standard error and * = $p < 0.05$ between saline and silica-treated mice.

Discussion

Although silica exposure is primarily associated with lung fibrosis, silica has also been reported to trigger autoimmune responses and is associated with an increased incidence of systemic autoimmune disease. However, the mechanisms leading to these autoimmune responses are not clear. A better understanding of this process might elucidate mechanisms that lead to other environmentally exacerbated autoimmune diseases. Several mechanisms have been proposed whereby silica might break peripheral tolerance. The reported excess production of sFas in silicosis could lead to self-recognition by inhibiting Fas-mediated apoptosis of self-reactive immune cells (Otsuki, Sakaguchi et al. 1998). However, this would not explain the source of antigen that would stimulate proliferation of the self-reactive cells. One source of self antigen that might break tolerance would occur with necrosis or apoptosis leading to excess self antigen presentation on antigen presenting cells (APC). Rosen, et al., have shown that several major autoantigens, including nucleosomal components, are exposed on apoptotic cells, providing a possible mechanism of presentation of self antigens to the immune system (Rosen and Casciola-Rosen 1999). Because apoptotic cells are normally cleared effectively, immune responses against these antigens would not occur unless a condition also existed that supported active antigen presentation activity. In the normal lung, most inhaled particles and any damaged cells are cleared by alveolar macrophages (AM) without chronic inflammation. However, silica exposure can alter the lung environment in ways that lead to chronic inflammation and fibrosis. In humans, the effects of silica include a switch in phenotype to a much more active and pro-inflammatory phenotype of AM, possibly through selective apoptosis of the more quiescent normal AM (Holian,

Uthman et al. 1997; Hamilton, Pfau et al. 2001). Accumulation of apoptotic AM plus the switch to a more immune active environment may provide the necessary conditions for an autoimmune response to occur.

In order to explore the mechanism of the silica-exacerbated autoimmune disease, this study used several techniques to demonstrate the presence in mice of autoantibodies in serum that recognize antigens on apoptotic macrophages. We have previously reported that autoimmune-prone NZM mice exposed to crystalline silica exhibited significantly higher titers of antibodies to nucleosomal components and overall exacerbated systemic autoimmune disease compared to saline or titanium dioxide ($Ti0_2$, control particle) instilled NZM mice (Brown, Archer et al. 2003). Because NZM mice develop autoantibodies even without silica exposure, saline-exposed mice were selected that did not produce positive ANA tests at 14 weeks of age. This provided control samples that gave background levels for non-specific staining. Serum antibodies from silica-exposed, ANA-positive NZM mice bound to epitopes on cells that had been induced to undergo apoptosis with silica, and stained more brightly than serum from saline-exposed ANA-negative NZM mice. This staining was prominent on apoptotic blebs and bodies, suggesting that antigenic targets were exposed during the apoptotic process. The results suggest that the exacerbation of autoimmune disease by silica may be due to an increased immune response to apoptotic antigens.

The staining of apoptotic cells occurred not only when apoptosis was induced with silica, but also with cycloheximide. Both silica and cycloheximide have been shown to activate caspase-dependent apoptotic pathways in myeloid cells (Zhu, Fearnhead et al. 1995; Chao, Hamilton et al. 2001). The ability of autoimmune serum to stain cells

undergoing cycloheximide-induced apoptosis suggested that the autoantigens in these mice might be due to caspase activity that provided unique epitopes to the immune system. Rosen *et al* (Rosen and Casciola-Rosen 1999) demonstrated that several nuclear antigens are cleaved by caspases during apoptosis and are exposed on apoptotic cells and blebs. Because many of these nuclear antigens are common targets for autoantibodies, their results suggested a mechanism of unique self-antigen presentation. More recently, data has accumulated that support both the ability of apoptotic cells to serve as antigens for autoantibody development (Mevorach, Zhou et al. 1998), as well as to involve cleavage and re-localization of important cellular components to which autoantibodies are developed (Greidinger 2001; Nozawa, Casiano et al. 2002). The possibility that silica leads to autoantigen exposure through caspase-dependent cleavage during apoptosis was approached in two ways. First, if autoantibodies from silica-exposed mice were specific for caspase-cleaved peptides, they should not recognize proteins on live or necrotic cells to the same degree as apoptotic cells. In the current study, only the cell lysates from cells undergoing silica-induced apoptosis showed significantly more binding by serum antibodies than live cell lysates, although the binding to cells treated with cycloheximide was also increased somewhat. This is consistent with results on surface staining of cycloheximide treated MH-S cells, and suggests that although some of the autoantibodies generated in silica-treated mice recognized antigens on cells triggered by different apoptosis inducers, some of the autoantibodies appear to recognize unique epitopes only exposed on cells undergoing silica-induced apoptosis. This may provide an explanation for some of the specific autoantibodies found in silicosis patients. Second, if the autoantibodies were binding to proteins uniquely cleaved by caspases or some

downstream apoptotic event, caspase inhibition should reduce antibody binding by blocking the apoptotic pathway leading to the protein cleavage. To test this hypothesis, a broad-spectrum caspase inhibitor, Boc-D-FMK, was shown to prevent both silica-induced apoptosis and the increased binding of serum autoantibodies from silica exposed NZM mice to silica-treated cells.

Taken together, these data demonstrate that silica treatment of NZM mice developed autoantibodies that recognize caspase-cleaved proteins on apoptotic cells. The presence of antibodies to apoptotic cells provides clues to a possible mechanism leading to autoimmune responses following silica exposure, in which accumulation of apoptotic cells in an inflammatory environment leads to presentation of self-antigens and antibody production.

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CHAPTER 4

Silica-induced apoptosis and systemic autoimmune disease is protein kinase Cδ dependent

Abstract

Silica exposure has been associated with formation of autoantibodies and development of systemic autoimmune disease, but mechanisms leading to these events are unknown. Silica exposure in autoimmune prone New Zealand mixed (NZM) mice results in a significant exacerbation of systemic autoimmunity as measured by increases in autoantibodies and glomerulonephritis. This study examined the role of alveolar macrophage apoptosis and protein kinase Cδ (PKCδ) in silica exacerbation of systemic autoimmune disease in NZM mice. Fourteen weeks following silica exposure, RNA and protein levels of PKCδ were significantly elevated in alveolar macrophages. To further assess the role of PKCδ *in vivo*, silica instilled NZM mice were treated with rottlerin, a selective PKCδ inhibitor, once a week. Fourteen weeks following silica exposure, NZM mice receiving only silica had increased levels of anti-histone autoantibodies, proteinuria and glomerulonephritis. However, silica instilled mice that received weekly instillations of rottlerin had significantly lower levels of proteinuria, anti-histone autoantibodies and complement C3 deposition within the kidney. Weekly instillations of rottlerin in silica instilled NZM mice also completely inhibited the upregulation of PKCδ in alveolar macrophages. Silica exposure in BALB/c bone marrow derived macrophages led to increased protein levels of PKCδ within 2 hours and by inhibiting PKCδ silica-induced apoptosis was reduced. Here we report that silica induces PKCδ upregulation leading to

apoptosis of alveolar macrophages and by inhibiting this process in silica exposed NZM mice, we significantly inhibited the exacerbation of systemic autoimmune disease.

Introduction

Silica is ubiquitous in the environment as an abundant mineral found in rock, sand and soil. Silicosis is an occupational disease resulting from acute or chronic high levels of silica exposure that leads to decreased pulmonary function and increased susceptibility to other diseases of the respiratory tract (1997). Silicosis has also been associated with increased incidence of systemic autoimmune disease, such as systemic lupus erythematosus (SLE) and scleroderma (Wilke 1996; Parks 1999; Parks 2002). Patients with silicosis have increased autoantibodies, immunoglobulins and immune complexes (Doll 1981). Although some of the enhanced humoral response could be attributed to a nonspecific adjuvant effect, the tendency towards select autoimmune diseases suggests a more specific effect. Using autoimmune prone New Zealand mixed (NZM) mice, we have previously reported that silica exposure significantly exacerbates the progression of autoimmune disease (Brown 2003). Following silica exposure, NZM mice develop high levels of autoantibodies to nuclear antigen including histones, as well as developing glomerulonephritis and pulmonary fibrosis within 14 weeks (Brown 2003).

Apoptosis has been reported to play a role in the initiation and progression of systemic autoimmune disease and silica has been reported to induce a caspase specific apoptotic response within alveolar macrophages (AM) (Iyer 1996; Mevorach 1998; Rosen and Casciola-Rosen 1999; Lorenz 2000; Greidinger 2001; Gabler 2003; Thibodeau 2003). We have previously reported a possible role for apoptosis in the exacerbation of autoimmune disease in New Zealand mixed mice (Pfau 2004). Autoantibodies from silica-exposed mice specifically recognize apoptotic cells and there appears to be greater recognition of silica induced apoptotic cells compared to live, necrotic or cycloheximide

induced apoptotic cells (Pfau 2004). Therefore we identified several apoptotic genes induced in AM following silica exposure and focused on pro-apoptotic protein kinase Cδ.

Protein kinase Cδ is a novel PKC family member that has been reported to play a role in the initiation of apoptosis in many cell types (Pongracz 1999; Webb 2000; Bertho 2002; Lounsbury 2002; Brodie and Blumberg 2003; Shukla 2003). PKCδ is activated by diacylglycerol/phorbol esters in a calcium-independent manner. PKCδ activation and translocation are induced by a variety of apoptotic stimuli in many different cellular systems. PKCδ is activated in neutrophils undergoing spontaneous apoptosis, in H_2O_2 induced apoptosis, TNF-α induced apoptosis, Fas mediated apoptosis and asbestos induced-apoptosis of alveolar epithelial cells (Pongracz 1999; Webb 2000; Bertho 2002; Lounsbury 2002; Brodie and Blumberg 2003; Shukla 2003). PKCδ has been reported to translocate to the nucleus and mitochondria following activation by the above-mentioned mechanisms thereby inducing caspase activation (Gschwendt 1999; Kikkawa 2002; Brodie and Blumberg 2003). Using rottlerin, a PKCδ selective inhibitor, these apoptotic processes have been reported to be blocked (Gschwendt 1994; Gschwendt 1999; Kikkawa 2002; Brodie and Blumberg 2003).

The overall objective of this study was to test the hypothesis that PKCδ is upregulated in alveolar macrophages following silica exposure leading to apoptosis. Furthermore, we propose that by inhibiting the apoptotic process through PKCδ we can decrease the severity of silica exacerbated systemic autoimmune disease in NZM mice. This hypothesis was tested *in vitro* using silica treated bone marrow derived macrophages and *in vivo* using silica exposed NZM mice.

Materials and Methods

Mice

Male and female New Zealand mixed (NZM 2410) mice were obtained from Taconic (Germantown, NY) and maintained in microisolation containers in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council. The animal room was set on 12-hour dark/light cycles with food and water provided *ad libitum*. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee.

Cells

BALB/c bone marrow derived macrophages were generated for *in vitro* use. BALB/c mice were euthanized with a lethal injection of Nembutal (200 μl ip) and the femur bones were collected. Recovery of the bone marrow cells was done within a sterile hood and the cells were flushed from the bones using a 25-gauge needle. The cells were counted and plated at $3x10^7$ cells in 75 mm^2 flasks. The cells were incubated overnight at 37°C then the non-adherent cells were collected allowing removal of stromal cells. The non-adherent cells were then re-plated in RPMI-1640/10% FCS and 10 ng/ml recombinant mouse macrophage colony stimulating factor (R&D Systems, Minneapolis, MN). The cells were allowed to differentiate into macrophages for 5-7 days before use. Cell viability was determined to be >90% by trypan blue exclusion before treatments. Silica was added at 250 μg/ml or 500 μg/ml for 2, 4 and 8 hours, untreated cells were used as a control as well as TiO_2 at 250 μg/ml and 500 μg/ml as a control particle. Rottlerin (1 μg/ml) was added to bone marrow cell cultures 1 hour before addition of 500 μg/ml of

silica. Cells were collected by scraping for generation of protein lysates for use in Western immunoblotting.

Apoptosis Assay

Apoptosis was determined by DNA fragmentation in bone marrow derived macrophages using a TiterTacs Assay (R&D Systems, Minneapolis, MN). Manufacturers protocol was followed. Equal numbers of cells ($1x10^5$/well) were plated in a 96-well plate and allowed to adhere overnight. Silica was added for 8 hours at 500 μg/ml with or without the addition of 1 μg/ml rottlerin 30 minutes prior. Non-treated cells were used as a negative control and a nuclease generated positive control was used. Experiments were repeated 3 times. The reported values are mean optical density (OD) values from each treatment.

Silica-Instillation

Silica was obtained from Pennsylvania Glass Sand Corp. (Pittsburgh, PA) and was acid washed, dried and determined to be free of endotoxin. At six weeks of age, mice were instilled intranasally with either 30 μL saline (n=6) or 30 μL saline suspensions of 1 mg crystalline silica (n=6) or 500 μg TiO_2 (n=6) as previously described. All mice received 2 instillations 2 weeks apart. Control and experimental groups were matched for the number of male and female mice. Alveolar macrophages were collected at 14 weeks for use in microarray experiments. A second cohort of mice was used to measure changes in protein levels of PKCδ and received 30 μL saline (n=4) or 30 μL saline suspensions of 1 mg crystalline silica (n=4) or 500 μg TiO_2 (n=4). A third cohort of mice was used for *in vivo* inhibition of PKCδ using rottlerin (A.G. Scientific, San Diego, CA), a PKCδ selective inhibitor (Gschwendt 1994; Davies 2000). Rottlerin was given at 10 μg/instillation and diluted in sterile saline; a volume of 30 μL was used for intranasal

instillation. Mice received either saline (n=5), silica (1mg x 2 instillations) (n=5), silica (1mg x 2 instillations) + rottlerin (10 μg/weekly instillation) (n=5) and rottlerin only (10 μg/weekly instillation) (n=5). Rottlerin was given intranasally 1 day before addition of saline or silica and then again with the instillation of saline or silica at six weeks of age. Rottlerin was then given once a week until the mice were sacrificed at 14 weeks. The saline and silica only treated animals were given saline instillations once a week for 14 weeks as a control instillation to match the rottlerin instillations. After 14 weeks, a time point when the majority of silica exposed mice developed autoimmune disease as measured by proteinuria and autoantibodies, the mice were euthanized with a lethal injection of Nembutal (200 μl ip), and blood was collected by cardiac puncture. Clotted blood was centrifuged, and serum was collected and frozen at –20°C until use. Alveolar macrophages were harvested from the lung by bronchoalveolar lavage for generation of protein lysates for use in Western immunoblotting and kidneys were removed and placed in Histochoice fixative (Amresco, Solon, OH).

RNA Extraction and Amplification

RNA was extracted from 6 mouse bronchoalveolar lavage samples per condition (saline, silica or TiO_2 treatment) using Trizol (Invitrogen, Carlsbad, CA) with an extra chloroform extraction following the initial chloroform step to further remove organics from the final RNA product. Equal amounts of the resulting RNA from each sample within a condition were pooled and further purified and concentrated using RNeasy mini-preps (Qiagen, Valencia, CA). Production of amplified RNA (aRNA) was performed using a RiboAmp kit (Arcturus, Carlsbad, CA).

Microarray Target Labeling and Hybridization

For each sample, 5 μg of aRNA and 10 μg of Universal Mouse Reference RNA were indirectly labeled with Alexa-fluor 647 or Alexa-fluor 555 respectively using Superscript II (Invitrogen) reverse transcriptase according to the ARES DNA labeling kit protocol. Labeled cDNA from reference RNA and aRNA were combined and hybridized overnight in a 25% formamide hybridization buffer to an in-house cDNA microarray with 1178 different cDNA probes specific to mouse toxicology and immunology genes.

Microarray Analysis

Hybridized arrays were scanned with an Axon Genepix 4000B laser slide scanner and visualized with GenePix Pro 4.0 software. Resulting result and image files were imported into GeneTraffic Duo Microarray Data Management and Analysis Software (Iobion Informatics, La Jolla, CA) and normalized to a total 1:1 red to green ratio for the entire slide. Data was filtered based on replicate quality, signal intensity and signal to background ratios of 1.2 or higher. An arbitrary cutoff of 1.5-fold up- or down-regulation was used to determine genes of interest.

Histology

Kidneys were removed and placed into Histochoice fixative then routinely processed using an automated processor (ThermoShandon, Pittsburgh, PA). The kidneys were embedded in paraffin wax and sectioned 5-7 μM thick, then collected on poly-L-lysine coated slides (Sigma Chemical, St. Louis, MO). The kidney sections were boiled in a 0.01 M sodium citrate buffer for 10 minutes followed by washes in distilled water and phosphate buffered saline. The kidney sections were then blocked with 4% fetal bovine serum in phosphate buffered saline. Goat anti-mouse IgG-FITC antibody (1:100) (ICN Biomedicals, Irvine, CA) and a goat anti-mouse C3-FITC (1:100) (ICN Biomedicals,

Irvine, CA) were added for 4 hours for the detection of immune complexes and complement deposition. A goat anti-rat IgG antibody (1:100) (ICN Biomedicals, Irvine, CA) was used as an isotype control. Samples were blinded and examined using a confocal microscope.

Urinary Protein

Proteinuria was measured by Chemstrip 2 GP test strips as described by the manufacturer (Boehringer Mannheim Diagnostics, Indianapolis, IN). Milligram protein per deciliter was measured between groups following the provided scale (0 = negative, trace, 1+ = 30 mg/dL, 2+ = 100 mg/dL, 3+ = 500 mg/dL).

Detection of Serum Autoantibodies

Anti-histone autoantibodies were detected using an ELISA kit (Alpha Diagnostics, San Antonio, TX). Sera were diluted 100-fold before assay and the manufacturer's protocol was followed. The reported values are mean optical density (OD) values from each treatment group.

Western Immunoblots

Western immunoblots were performed using lysates generated from alveolar macrophages collected by bronchoalveolar lavage and from bone marrow derived macrophages. Lavage cells were collected and determined to be between 75-90% alveolar macrophages and an equal number of cells were lysed using a 0.5% Nonidet P-40 detergent (Sigma, St. Louis, MO) with freshly added complete protease inhibitors: 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotonin and 1mM phenylmethylsulfonyl fluoride (PMSF) (Roche, Indianapolis, IN). Bone marrow cells were collected by scraping followed by the same lysis treatment. Samples were kept on ice during lysis and

frozen immediately at –20°C until use. Protein concentrations of lysates were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) and equal concentrations of lysates were loaded onto Invitrogen 12% Nu-PAGE gels (Carlsbad, CA) followed by transfer onto nitrocellulose (Bio-Rad). Nitrocellulose blots were probed using an antibody to PKCδ (1:300) (BD Transduction Laboratories, San Diego, CA). Purified PKCδ (Oxford Biomedical Research, Oxford, MI) was used as a positive control. Detection of antibody to PKCδ was performed using goat anti-mouse IgG-HRP (1:2000) (Jackson Immunoresearch, West Grove, PA). Detection of positive bands was done using an enhanced chemiluminesence reagent (Amersham, Piscataway, NJ) and visualized using a VersaDoc imaging system (Bio-Rad). Experiments using BMDM were repeated 3 times. Densitometry was performed using Quantity One software (Bio-Rad) to quantitate differences between bands. Numbers reported are density (intensity/mm^2).

Statistical Methods

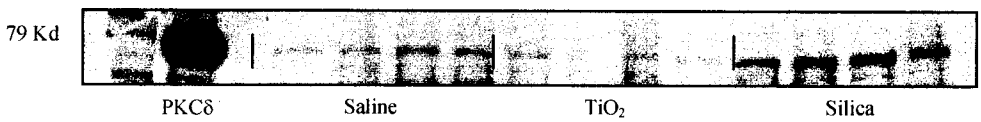
Statistical analysis was done using the software package PRISM, v. 3.03 (GraphPad, San Diego, CA). Differences between saline, TiO_2 and silica treated mouse groups was assessed using an unpaired two-tailed *t*-test. Differences between controls, silica treatments and rottlerin treatments of bone marrow derived macrophages were assessed using a one-way analysis of variance (ANOVA). All values are reported as mean ± SEM; $p \leq 0.05$ was considered significant.

Results

PKCδ is upregulated in alveolar macrophages from silica exposed NZM mice

Silica exposure has been reported to induce apoptosis in several cell types, however, the signaling cascade leading to the apoptotic response is not fully defined (Hamilton 2000; Chao 2001; Thibodeau 2003). Therefore, alveolar macrophages were collected from silica exposed NZM mice to screen for changes in apoptotic genes using microarray technology. Using an in-house microarray chip with 1200 immunology and toxicology genes, we identified several genes involved in apoptosis that were upregulated 1.5 fold or higher following silica exposure of which PKCδ was the most prominent. Fourteen weeks following 2 instillations of 1 mg silica, the RNA levels of PKCδ were elevated 2.4-fold as compared to saline instilled mice. TiO_2 did not elevate the levels of PKCδ RNA as compared to the saline control mice. Similar to the RNA results, PKCδ protein levels were also found to be significantly elevated in silica exposed mice compared to saline and TiO_2 exposed mice (4921 ± 357 int/mm^2 versus 2571 ± 333 int/mm^2 and 1805 ± 256 int/mm^2; $p \leq 0.05$, respectively) (Fig. 21). These results indicate that PKCδ RNA and protein levels were both increased long term in alveolar macrophages following silica exposure in NZM mice. Consequently, the rest of the study examined the potential role of PKCδ in silica-induced autoimmune disease in NZM mice.

FIGURE 21



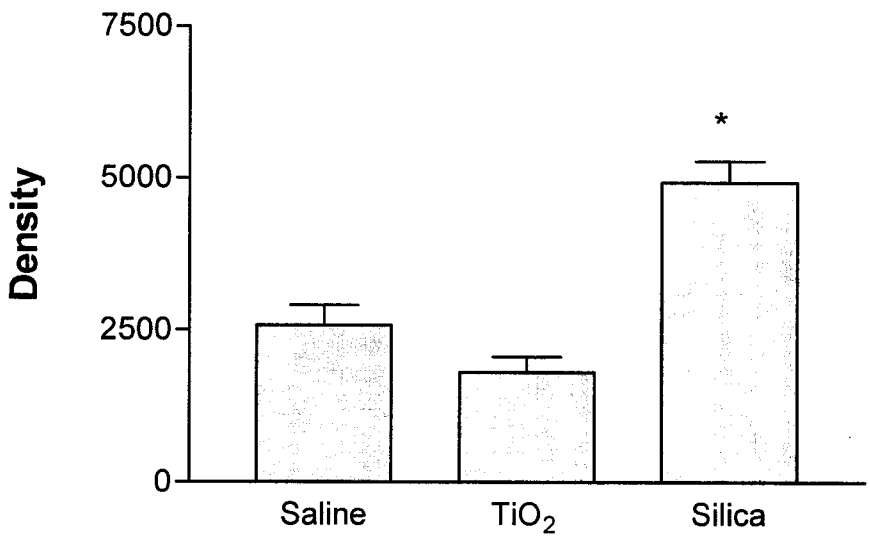


Figure 21.

Western blot analysis for PKCδ in alveolar macrophage cellular lysates from saline (n=4), TiO_2 (n=4) and silica (n=4) instilled NZM mice 14 weeks following exposure. Densitometry shows a 2-fold increase in PKCδ levels as compared to saline and TiO_2 exposed mice. Equal concentrations of protein were added per lane. * Significant differences between silica exposed mice and saline or TiO_2 controls ($p \leq 0.05$).

PKCδ levels are increased following silica exposure in bone marrow derived macrophages

To further examine the role of PKCδ in alveolar macrophage apoptosis, an *in vitro* model using bone marrow derived macrophages (BMDM) was utilized. PKCδ levels were significantly increased 5-fold within 2 hours following treatment with 500 μg/ml silica as compared to an untreated control (1766 ± 177 int/mm^2 versus 311 ± 13 int/mm^2; $p \leq 0.001$, respectively) (Fig. 22). TiO_2 (250 and 500 μg/ml) did not increase the levels of PKCδ as compared to an untreated control (404 ± 93 int/mm^2 and 359 ± 40 int/mm^2 versus 311 ± 13 int/mm^2, respectively) (Fig. 22). The levels of PKCδ began to decrease within 4 and 8 hours after silica exposure, a time point at which cells began to die and float into the media (Fig. 22). To inhibit PKCδ, rottlerin was added to the media of the BMDM culture 1 hour before the addition of silica. Figure 22 demonstrates that addition of 1 μg/ml rottlerin inhibited the induction of PKCδ by silica at 2 hours for the 500 μg/ml silica exposure (302 ± 40 int/mm^2 versus 1766 ± 177 int/mm^2; $p \leq 0.001$, respectively). Treatment of BMDM with rottlerin alone did not have an effect on the levels of PKCδ at 2 hours (Fig. 22). Apoptosis of silica treated BMDM was determined by measuring DNA fragmentation. Figure 23 shows a 500 μg/ml silica treatment induced a 3-fold increase in apoptotic cells within 8 hours as compared to an untreated control and was significantly reduced by the addition of rottlerin (1.074 ± 0.084 versus 0.325 ± .073 versus 0.663 ± 0.091; $p \leq 0.05$, respectively). However, if cells are treated with 500 μg/ml silica for greater than 12 hours, rottlerin does not inhibit apoptosis (data not shown). These results demonstrate that BMDM provide a good *in vitro* model to

examine the role of PKCδ in silica-induced apoptosis of alveolar macrophages and that rottlerin can delay apoptosis in silica treated cells.

FIGURE 22

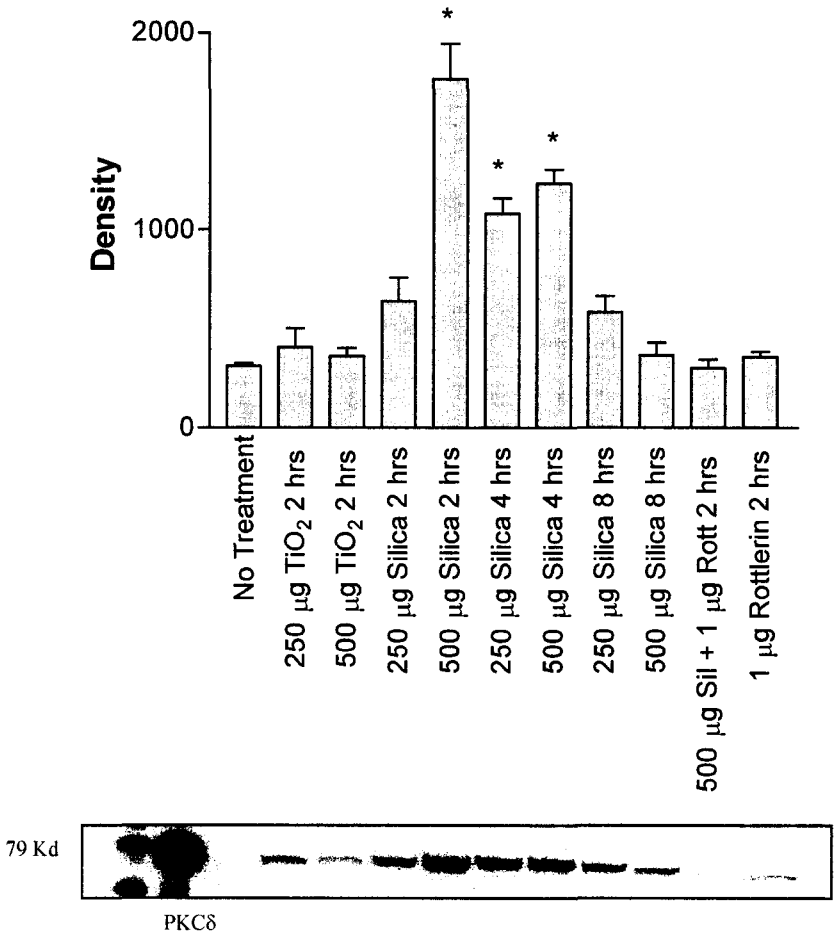


Figure 22.

Western blot analysis for PKCδ in bone marrow derived macrophages. Cells received no treatment, 250 and 500 μg/ml TiO_2, 250 and 500 μg/ml silica from 2, 4 and 8 hours, 500 μg/ml silica + 1 μg/ml rottlerin for 2 hours or 1 μg/ml rottlerin only for 2 hours. Silica exposure increases PKCδ levels within 2 hours and decreases around 8 hours following exposure. Densitometry shows a significant increase in the level of PKCδ with 500 μg/ml silica exposure at 2 hours, adding rottlerin completely inhibited the silica induced upregulation of PKCδ. Equal concentrations of protein were added per lane. Representative example from 1 of 3 separate experiments. * Significant differences between silica treated BMDM and no treatment, TiO_2 treatments, silica + rottlerin treatments or rottlerin only treatments ($p \leq 0.001$).

FIGURE 23

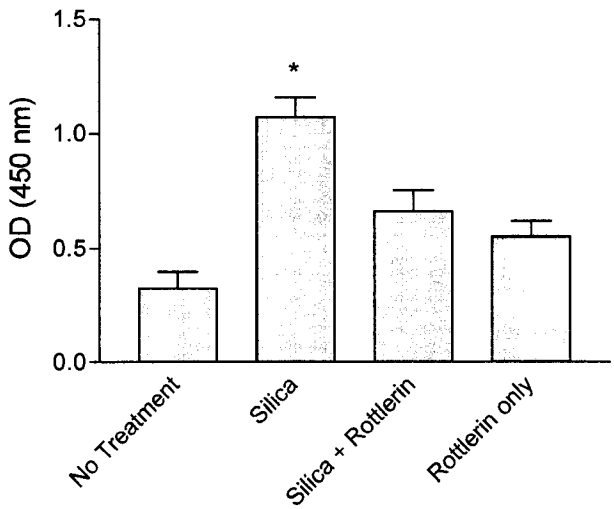


Figure 23.

Determination of apoptosis measuring DNA fragmentation. BMDM received no treatment, 500 μg/ml silica for 8 hours, 500 μg/ml silica + 1 μg/ml rottlerin or 1 μg/ml rottlerin only. Rottlerin reduced the amount of apoptosis in silica treated BMDM. * Significant differences between silica treated BMDM and untreated BMDM and silica + rottlerin treated BMDM ($p \leq 0.05$).

Rottlerin treatment decreases severity of silica-exacerbated systemic autoimmune disease in NZM mice

As previously described, silica exposure in NZM mice results in a significant exacerbation of systemic autoimmune disease within 14 weeks, however, the role that silica-induced apoptosis may play in the exacerbation of the autoimmune disease has not been elucidated (Brown 2003). Based on the results above demonstrating the role of PKCδ in silica-induced apoptosis *in vitro* and the ability of rottlerin to inhibit this process, the role of silica-induced apoptosis was examined *in vivo*. Therefore, NZM mice were exposed to saline, 2 instillations of 1 mg silica, 2 instillations of 1 mg silica and 10 μg rottlerin or 10 μg rottlerin alone. Rottlerin instillations were given one day prior to silica instillation then once a week for 14 weeks. Similar to the results in Figure 21, PKCδ levels in alveolar macrophages lavaged from silica only treated NZM mice at 14 weeks were significantly increased compared to saline control mice (2887 ± 643 int/mm^2 versus 249 ± 44 int/mm^2; $p \leq 0.001$, respectively) (Fig. 24). However, instillation of rottlerin once a week for 14 weeks significantly decreased the levels of PKCδ expression in silica treated mice as compared to mice receiving only silica (606 ± 124 int/mm^2 versus 2887 ± 643 int/mm^2; $p \leq 0.001$, respectively) (Fig. 24).

FIGURE 24

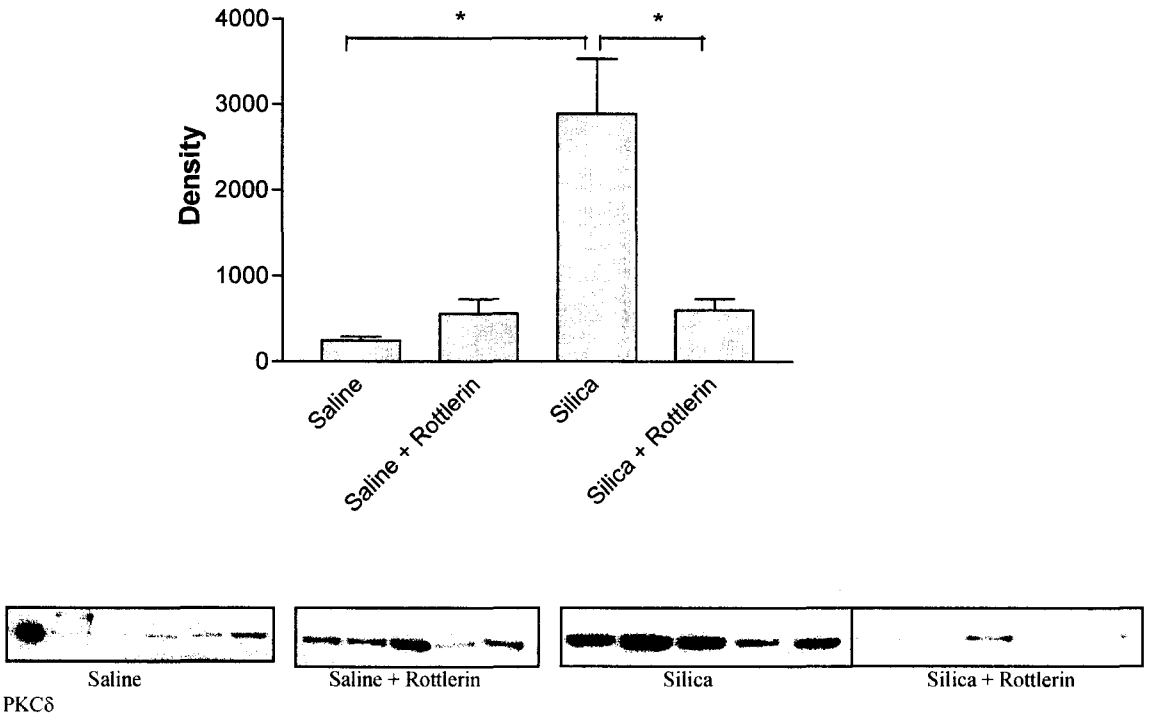


Figure 24.

Western blot analysis for PKCδ in alveolar macrophages collected from saline (n=5), silica (1mg x 2 instillations) (n=5), silica (1mg x 2 instillations) + rottlerin (10 μg/weekly instillation) (n=5) and rottlerin only (10 μg/weekly instillation) (n=5) treated NZM mice 14 weeks following exposure. Silica exposure upregulated the levels of PKCδ 14 weeks following exposure, however, weekly instillation of rottlerin completely inhibited PKCδ activation by silica. Equal concentrations of protein were loaded per lane. * Significant differences between silica exposure and saline, silica + rottlerin or silica + rottlerin ($p \leq 0.001$).

As previously reported, silica exposure in NZM mice resulted in increased proteinuria and severe immune complex and complement deposition within the kidney (Brown 2003). Proteinuria levels greater than 500 mg/dl were seen in 3 out of 5 (60%) of the silica treated mice 14 weeks post-instillation; however, no proteinuria was observed in silica-instilled mice that were treated with rottlerin once a week for 14 weeks (Fig. 25). Proteinuria was observed in 1 out of 5 mice receiving rottlerin only treatments (Fig. 25). Kidneys from these mice were examined for IgG immune complex and complement C3 deposition. Figure 26 shows increased complement C3 deposition within the glomerulus of silica-exposed mice as compared to the saline control mice as previously reported (Brown 2003). Silica exposed mice receiving rottlerin instillations once a week for 14 weeks showed less extensive C3 deposition within the glomerulus (Fig. 26). IgG immune complex deposition was also examined and similar staining as the C3 deposition was seen (data not shown).

As previously reported, silica exposure in NZM mice results in a significant increase in the levels of anti-nuclear autoantibodies, including anti-histone autoantibodies (Brown 2003). Development of anti-histone autoantibodies was significantly increased in silica exposed NZM mice at 14 weeks as compared to saline control mice (0.392 ± 0.086 versus 0.187 ± 0.034; $p \leq 0.05$, respectively) (Fig. 27). However, instillation of rottlerin significantly decreased the levels of anti-histone autoantibodies within silica exposed NZM mice as compared to mice that received only silica (0.159 ± 0.045 versus 0.392 ± 0.086; $p \leq 0.05$, respectively) (Fig. 27). The levels of anti-histone autoantibodies in NZM mice treated with rottlerin only were also significantly lower than saline controls (0.106 ± 0.007 versus 0.187 ± 0.034; $p \leq 0.05$, respectively) (Fig. 27).

Therefore, instillation of rottlerin in silica exposed NZM mice significantly reduced the levels of PKCδ in AM and decreased proteinuria, C3 deposition in kidneys and the levels of anti-histone autoantibodies. Taken together, silica exacerbation of systemic autoimmune disease was significantly inhibited by blocking PKCδ *in vivo*.

FIGURE 25

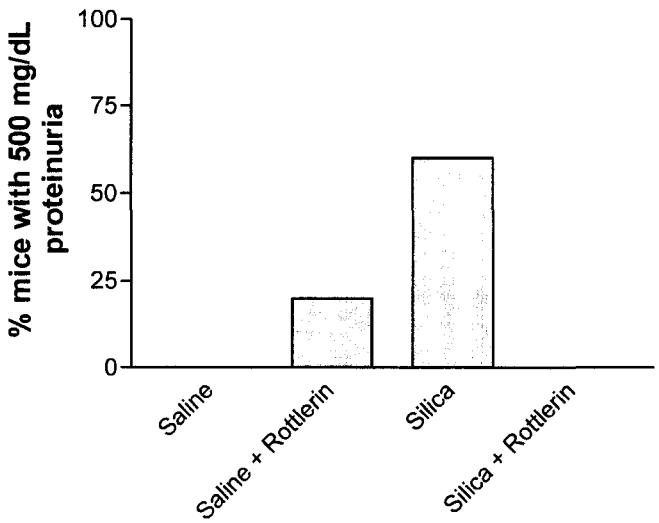


Figure 25.

Percentage of mice with 500 mg/dL proteinuria levels in saline (n=5), silica (1mg x 2 instillations) (n=5), silica (1mg x 2 instillations) + rottlerin (10 μg/weekly instillation) (n=5) and rottlerin only (10 μg/weekly instillation) (n=5) treated NZM mice 14 weeks following exposure as measured by use of ChemStrip 2GP glucose/proteinuria strips. Sixty percent of silica exposed mice developed high proteinuria, while none of the saline or silica + rottlerin instilled mice developed high proteinuria.

FIGURE 26

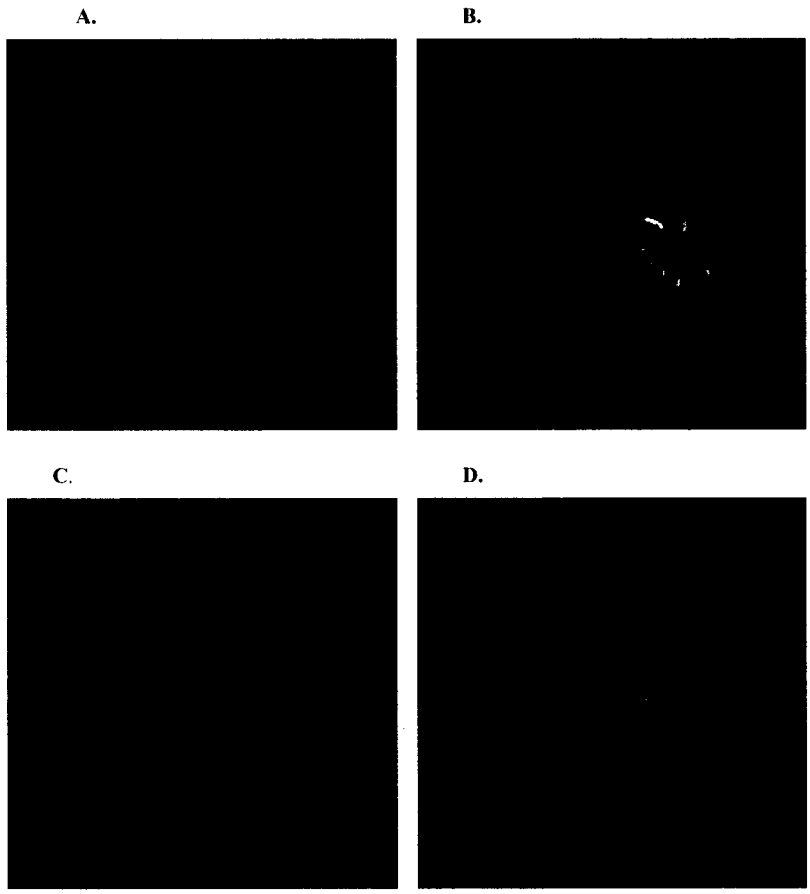


Figure 26.

Representative examples of immunohistochemical staining of kidney sections for complement C3 deposition from (A) saline (n=5), (B) silica (1mg x 2 instillations) (n=5), (C) silica (1mg x 2 instillations) + rottlerin (10 μg/weekly instillation) (n=5) and (D) rottlerin only (10 μg/weekly instillation) (n=5) treated NZM mice 14 weeks following exposure. A goat anti-mouse C3-FITC antibody was used to stain for complement deposition within the glomerulus of the kidney. The silica exposed mice showed complement deposition within the glomeruli (b), however, weekly rottlerin instillations inhibited the silica-induced complement deposition (c). Saline and rottlerin only instilled mice showed very little staining for complement deposition. Representative example of 1 out of 5 mice per group. Magnification 20X.

FIGURE 27

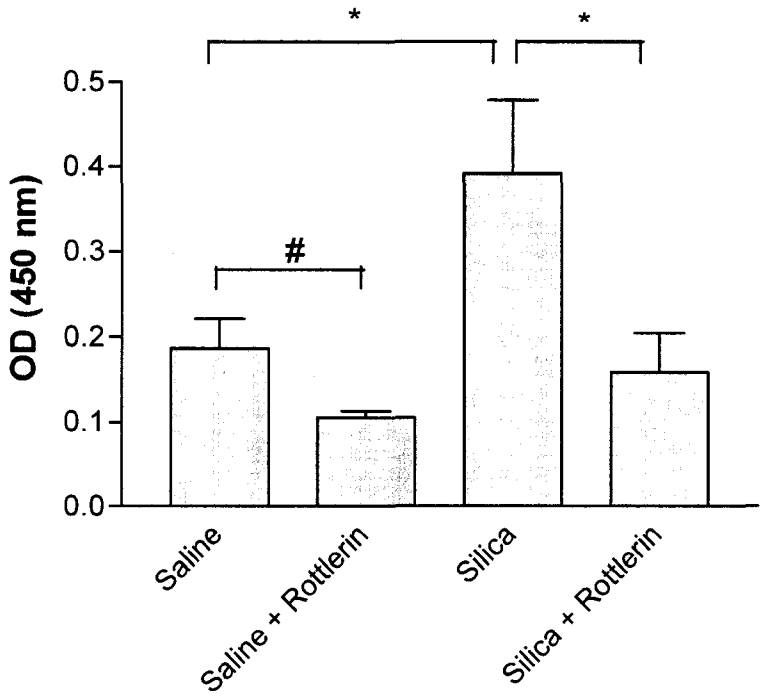


Figure 27.

Development of anti-histone autoantibodies in saline (n=5), silica (1mg x 2 instillations) (n=5), silica (1mg x 2 instillations) + rottlerin (10 μg/weekly instillation) (n=5) and rottlerin only (10 μg/weekly instillation) (n=5) treated NZM mice 14 weeks following exposure as measured by ELISA. Silica exposure significantly elevated the levels of anti-histone autoantibodies in NZM mice 14 weeks following silica exposure. However, weekly instillations of rottlerin in silica-instilled mice inhibited the development of high levels of anti-histone autoantibodies. The values reported are mean OD values for each treatment group. * Significant differences between silica exposed mice and saline or silica + rottlerin exposed mice ($p \leq 0.05$). # Significant differences between saline exposed mice and saline + rottlerin exposed mice ($p \leq 0.05$).

Discussion

Silica exposure has been reported to induce autoimmune responses and is associated with an increased incidence of systemic autoimmune disease (1997; Parks 1999; Parks 2002). However, the mechanisms leading to these autoimmune responses are not understood. Elucidating these mechanisms may provide a better understanding of other environmentally relevant exposures implicated in triggering autoimmune responses as well as providing directions for novel therapies.

Possible mechanisms of silica induced autoimmune disease may involve the alveolar macrophage, an immune cell of the lung that is the first line of defense. It has previously been reported that silica exposure leads to a caspase dependent apoptosis of alveolar macrophages (Iyer 1996; Thibodeau 2003). Silica induced apoptosis of alveolar macrophages leads to release and uptake of silica by other alveolar macrophages, producing a cyclical process of inflammation and cell death (Cooper 2002). This constant inflammation and cellular death may provide excess antigen that is being presented to the immune system, thereby breaking immune tolerance.

Studies by Rosen, *et al* have reported concentrated autoantigens within the blebs of apoptotic cells (Rosen and Casciola-Rosen 1999). Lupus autoantigens are represented by structures that are chemically cleaved or modified during apoptosis and if this material is not removed by non-inflammatory processes, apoptotic material could be presented by specialized antigen presenting cells to induce immune responses (Utz and Anderson 1998; Ronchetti 1999). In an *in vitro* system, macrophages were found to prevent immunity to apoptotic material by competing with dendritic cells for uptake of apoptotic blebs, demonstrating the importance of the cell population silica is targeting for injury

(Albert 1998). Consistent with the hypothesis, mice intravenously exposed to apoptotic cellular material have been reported to develop autoantibodies (Mevorach 1998).

In this study, a cDNA microarray specific for mouse toxicology and immunology genes was used with RNA from alveolar macrophages collected from silica exposed NZM mice to analyze for expression changes in genes involved in the apoptotic process. Silica exposure in NZM mice induced several genes involved in apoptosis, including thymosin β10, PKCδ, TNF-α induced protein-6, and Bcl-2 like protein. PKCδ RNA levels were increased more than the other genes; therefore, we focused on the role PKCδ plays in silica-induced apoptosis and autoimmune disease. It appears that PKCδ is being increased by silica exposure and remains increased long term, possibly through release of TNF-α (Emoto 1995). Concurrent with this hypothesis, we have previously reported a significant increase in TNF-α levels within the lung lavage fluid of silica treated NZM mice 14 weeks following silica exposure (Brown).

To further assess the role of PKCδ in alveolar macrophage apoptosis, we utilized an *in vitro* model using BALB/c bone marrow derived macrophages. Using bone marrow derived macrophages we demonstrated a significant increase in the levels of PKCδ within 2 hours following 500 μg/ml silica exposure, while untreated cells and TiO_2 treated cells had low levels of PKCδ. Measuring DNA fragmentation we demonstrated that silica induces apoptosis of BMDM and this process can be delayed by the addition of rottlerin. Silica exposure has previously been reported to induce mitochondrial cytochrome c release leading to a caspase-mediated apoptosis of macrophages (Thibodeau 2003). Further, PKCδ has been reported to migrate to the mitochondria following exposure to asbestos and H_2O_2 resulting in altered mitochondrial membrane permeability, thereby

activating caspases (Majumder 2001; Shukla 2003). Silica has also been reported to mediate it's apoptotic effects through the scavenger receptor, therefore it appears silica can induce apoptosis by several different pathways and by inhibiting one pathway via PKCδ we can delay the onset of apoptosis in macrophages (Hamilton 2000).

To test our hypothesis that PKCδ mediated apoptosis of alveolar macrophages plays an important role in the exacerbation of systemic autoimmune disease by silica, we utilized weekly intranasal instillations of rottlerin to inhibit PKCδ and delay silica induced apoptosis. We have previously reported that intranasal instillation of silica in autoimmune-prone NZM mice results in a significant exacerbation of autoimmunity 14 weeks following instillation as measured by increases in mortality, proteinuria, autoantibodies and glomerulonephritis (Brown 2003). NZM mice typically do not develop high levels of autoantibodies and glomerulonephritis until around 6 months of age (Rudofsky and Lawrence 1999). However, following silica exposure, NZM mice develop high levels of autoantibodies and severe immune complex and complement deposition within the kidney around 14 weeks (Rudofsky and Lawrence 1999; Brown 2003). As previously reported, NZM mice in this study that received only silica instillations developed high proteinuria (60% of the mice), high levels of anti-histone autoantibodies and excess immune complex and complement C3 deposition in the kidneys (Brown 2003). Silica exacerbated systemic autoimmune disease was significantly reversed by blocking PKCδ with weekly rottlerin instillations. Silica exposed NZM mice receiving weekly instillations of rottlerin did not develop high levels of proteinuria, anti-histone autoantibodies or severe immune complex or complement C3 deposition within the kidney. Weekly rottlerin instillations decreased PKCδ levels in

alveolar macrophages from silica exposed NZM mice down to levels seen in saline instilled mice. These significant findings suggest that one pathway of silica-induced apoptosis mediated by PKCδ plays a major role in silica exacerbated autoimmune disease.

Taken together, these data demonstrate that silica exposure induces the upregulation of PKCδ within alveolar macrophages thereby inducing apoptosis. More importantly, inhibiting PKCδ upregulation *in vivo* significantly decreases the exacerbation of systemic autoimmune disease by silica exposure. These findings may provide additional mechanisms for other relevant xenobiotics that induce apoptosis and provide additional insight into the importance of apoptosis in autoimmune disease. More importantly, these results may provide possible therapeutic approaches to the treatment of environmentally mediated autoimmune diseases.

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CONCLUSIONS

These studies examined mechanisms by which silica is able to exacerbate the progression of systemic autoimmune disease in New Zealand mixed mice. As discussed earlier, silica exposure has been associated with several autoimmune diseases in human populations including systemic lupus erythematosus, rheumatoid arthritis and scleroderma (Steenland and Brown 1995; Parks, Conrad et al. 1999; Parks, Cooper et al. 2002; Parks, Cooper et al. 2003). However, very few studies have examined mechanisms by which silica may initiate or exacerbate these autoimmune diseases. Further, many animal studies with silica exposure have focused on the development of fibrosis and lung inflammation and while these mechanisms may be relevant for the development of autoimmune disease, very little data exist specifically examining silica-induced autoimmunity. Therefore, this project begins to fill the gaps of knowledge that exist in understanding mechanisms of silica exposure and the development of autoimmune disease. The goals of this project were to characterize the exacerbation of autoimmune disease by silica and to examine the role of silica-induced apoptosis in the exacerbation of autoimmunity.

Silica exposure resulted in the exacerbation of systemic autoimmune disease in NZM mice. The most pronounced response to silica in NZM mice was decreased survival. The silica exposed mice died significantly earlier than saline or TiO_2 exposed mice. Further, mortality corresponded to the development of high levels of proteinuria suggestive of renal failure. Silica particles have been reported to accumulate within the kidney, however, the increase in autoantibodies and immune complexes within our model suggested immune complex and complement mediated glomerulonephritis (Slavin,

Swedo et al. 1985). Therefore, kidneys were examined for immune complex and complement C3 deposition within the glomerulus of silica exposed NZM mice. Fourteen weeks following silica exposure, glomeruli from silica exposed NZM mice had higher levels of immune complex and complement C3 deposition suggesting that decreased survival in silica-exposed NZM mice was due to renal disease. These results further provide evidence that a localized exposure to silica in the lung can lead to and have detrimental effects systemically.

This study found increased levels of autoantibodies to nuclear antigen, which included increased levels of anti-dsDNA and anti-histone autoantibodies in silica-exposed NZM mice as compared to saline and TiO_2 exposed mice. These autoantibodies have also been reported in silicosis patients with autoimmune disease (Parks, Cooper et al. 2002). The autoantibody repertoire within silica exposed NZM mice included autoantibodies that preferentially recognized apoptotic cells rather than live or necrotic cells. Several studies have reported an important role for apoptosis in the development and progression of autoimmune disease (Huggins, Todd et al. 1999; Rosen and Casciola-Rosen 1999; Lorenz, Herrmann et al. 2000; Greidinger 2001; Gabler, Kalden et al. 2003). Therefore, it appears the apoptotic process may play a major role in silica-induced exacerbation of autoimmunity by exposing self-antigens that are not normally presented to the immune system.

Following silica exposure, NZM mice had lower levels of IgG as well as alterations in immunoglobulin isotypes suggestive of a Th1 type immune response. These findings were similar to studies done in rat silicosis models in which a shift to a Th1 response was reported (Huang, Hubbs et al. 2001; Weissman, Hubbs et al. 2001).

Due to the alterations in IgG and immunoglobulin isotypes, lymphocytes were examined in silica exposed NZM mice. B cell numbers were not decreased as suggested by the decreased IgG levels, thereby indicating that IgG is being deposited in tissues as was evidenced by deposition within the kidneys. There was an increase in the number of B1a B cells in the superficial cervical lymph node in silica exposed NZM mice. NZM mice have been reported to have an expanded population of B1a B cells within the peritoneal cavity, therefore it appears silica is inducing the migration of these cells to regional lymph nodes of the lung (Mohan, Morel et al. 1998). Further, B1a B cells are reported to be very potent antigen presenting cells, therefore, these cells may aid in the progression of autoimmune disease by presenting self-antigen to T cells (Mohan, Morel et al. 1998). T helper cells were also examined within lymph nodes of silica-exposed mice and were significantly increased in number as compared to saline exposed mice. These results were similar to what has been reported in human silicosis patients and other animal models (Watanabe, Shirakami et al. 1987; Friedetzky, Garn et al. 1998). The percentage of regulatory T cells was decreased in lymph nodes of silica exposed NZM mice mainly due to the large increase in T helper cells. Taken together, these data suggest silica exposure led to the migration of B1a B cells and T helper cells to local lymph nodes as well as a decrease in regulatory T cells, therefore allowing for excess antigen presentation which may aid in the progression of systemic autoimmunity.

To further examine the role of macrophage apoptosis in silica exacerbated systemic autoimmune disease, a microarray analysis of 1200 genes was performed on RNA collected from alveolar macrophages that were harvested from saline, TiO_2 and silica exposed NZM mice. Several genes involved in apoptosis were upregulated in

alveolar macrophages in response to silica exposure in NZM mice. These increases in RNA included; protein kinase Cδ (PKCδ), thymosin β10, TNF-α, Bcl-2 like protein and several MAP kinases. Protein kinase Cδ was the most prominent increase in silica exposed NZM mouse alveolar macrophages, therefore, the role of PKCδ in apoptosis and autoimmunity was further examined. Fourteen weeks following silica exposure, the levels of PKCδ RNA and protein were significantly increased as compared to saline and TiO_2 exposed mice. Using bone marrow derived macrophages as an *in vitro* model representing alveolar macrophages, we demonstrated that silica induced an increase in PKCδ within 2 hours and declined by 8 hours. Using rottlerin, a selective inhibitor of PKCδ, the levels of PKCδ were decreased in silica-exposed cells. Further, treatment of the silica-exposed bone marrow derived macrophages with rottlerin reduced the level of apoptosis as compared to cells exposed only to silica. Therefore, it appears PKCδ is contributing to the induction of apoptosis following silica exposure in macrophages. These results were similar to the reported effects of asbestos on the activation of PKCδ in alveolar epithelial cells followed by caspase-9 activation and subsequent apoptosis (Shukla, Stern et al. 2003). Due to the ability of rottlerin to reduce the level of apoptosis in silica exposed cells and the possible role of apoptosis in the progression of autoimmune disease, rottlerin was used *in vivo* to inhibit silica-induced apoptosis of alveolar macrophages. Silica exposed NZM mice receiving weekly rottlerin instillations for 14 weeks had decreased proteinuria, kidney disease and anti-histone autoantibodies as compared to mice receiving only silica. Therefore, it appears that by reducing silica-induced apoptosis via PKCδ of alveolar macrophages within NZM mice, we significantly reduced the exacerbation of systemic autoimmune disease by silica exposure.

Summary

Using autoimmune-prone New Zealand mixed mice, this study investigated the exacerbation of systemic autoimmune disease by crystalline silica exposure. Results from silica exposure in NZM mice demonstrated that silica is able to exacerbate systemic autoimmune disease within a genetically susceptible murine model. Silica exposure in NZM mice resulted in increased mortality due to immune complex and complement deposition within the kidney. Silica exposed mice had increased levels of autoantibodies as compared to saline and TiO_2 exposed mice. Further, autoantibodies from silica-exposed mice preferentially recognized apoptotic cells. Silica exposure was shown to increase levels of pro-apoptotic protein kinase Cδ within alveolar macrophages which played a major role in the exacerbation of autoimmunity. Taken together, these results suggest that silica-induced apoptosis of alveolar macrophages plays a significant role in the exacerbation of systemic autoimmune disease.

Our proposed model, shown in Figure 28, suggests that inhalation of crystalline silica results in concurrent activation and apoptosis of the alveolar macrophage resulting in an environment of inflammation and recurrent apoptosis. This environment may provide excess antigen that is further ingested by activated macrophages or dendritic cells which are able to migrate to local lymph nodes. Within the lymph nodes, these antigen-presenting cells laden with apoptotic material activate T cells and B cells thereby inducing an autoimmune response. The poor ability of the body to clear silica from the lung prolongs this cycle of macrophage apoptosis and lymphocyte activation, thereby progressing from an autoimmune response into a systemic autoimmune disease.

Significance

Very little data exist in regards to mechanisms of silica exposure and the development of autoimmune disease, therefore these results are significant in that they represent one of the first identified mechanisms by which silica exposure may be inducing or progressing the development of autoimmune disease. As discussed previously, the concordance of autoimmune disease among twins is only 25-40%, therefore, along with genetics, environmental factors may play a large role in the initiation and progression of autoimmunity. This study examined the ability of silica to exacerbate systemic autoimmune disease, however, similar mechanisms may be relevant in other environmental exposures such as asbestos, mercury, organic solvents and pharmaceuticals. In addition to environmentally induced autoimmune disease, these results further define the importance of the regulation of apoptosis in the immune system.

Future Directions

We filled several gaps of knowledge in regards to mechanisms by which silica can initiate or progress the development of systemic autoimmunity, however, several future directions need to be addressed. We demonstrated increased recognition of apoptotic cells by autoantibodies from silica exposed NZM mice, however, the antigens involved have not been identified. It is possible that silica-induced apoptosis may result in the specific cleavage or alteration of antigen that is then recognized as foreign by the immune system. Therefore, identification of the antigens in apoptotic cells that we demonstrated are recognized by the immune system may provide unique biomarkers to identify autoimmune diseases that may have an environmental origin.

We demonstrated that PKCδ is involved in silica-induced apoptosis of alveolar macrophages, however, it remains unknown how PKCδ is being activated by silica exposure. Since blocking PKCδ only delayed the onset of apoptosis and did not completely inhibit it *in vitro*, it appears several pathways of apoptosis are activated by silica exposure. Further, it remains unknown how these pathways may interact or if additional apoptotic pathways are involved. Understanding the complete mechanisms by which silica induces apoptosis provides a means to further define cellular signaling pathways and provides insight into mechanisms of other xenobiotics which induce apoptosis.

FIGURE 28

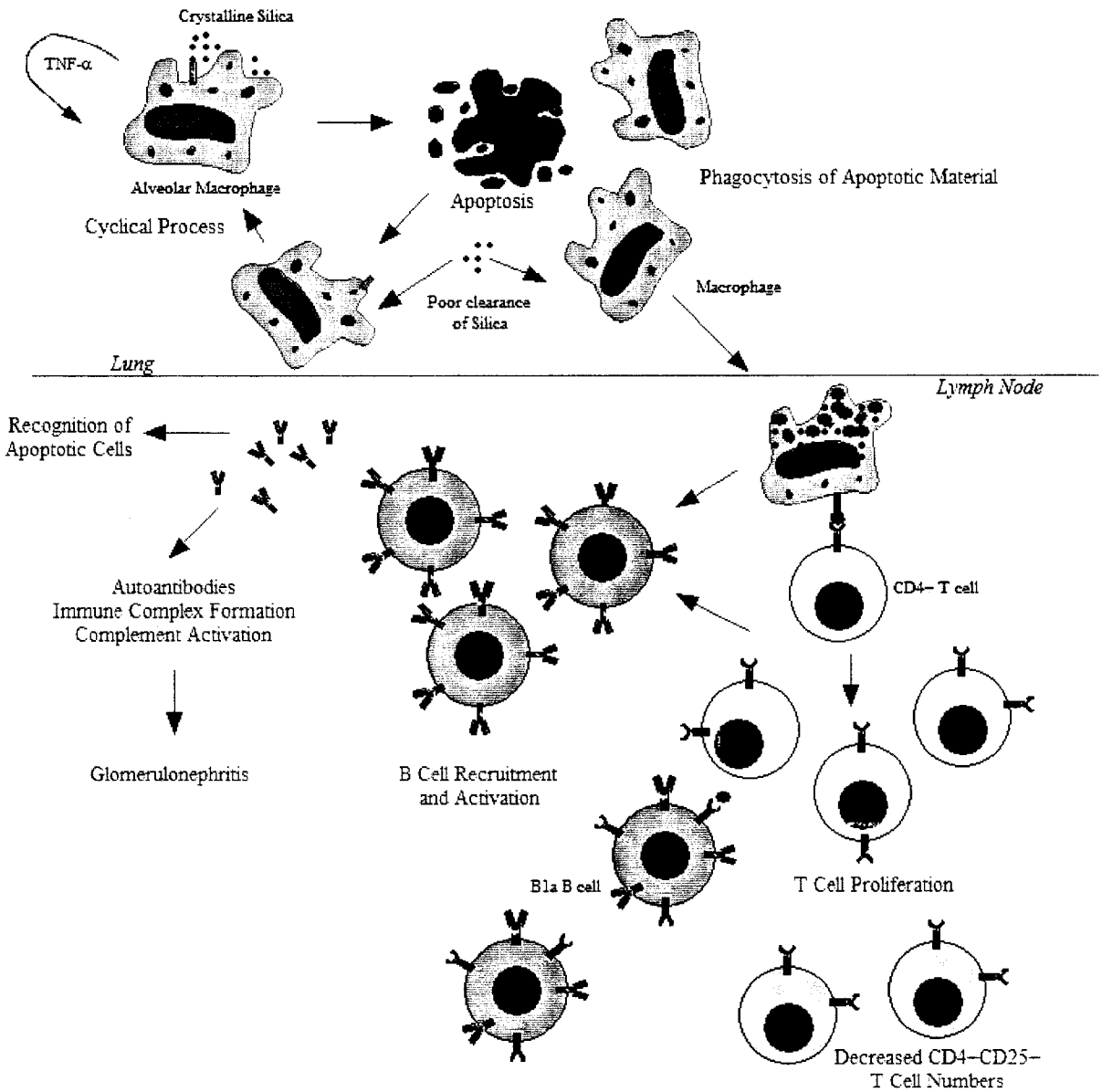

Figure 28.

A diagram of the proposed model of silica-exacerbated systemic autoimmune disease in the NZM mouse.

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