Analysis of Potential Mutagenic Effects of World Trade Center Dust on in Vitro Systems

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Abstract

Ever since the 1950s, air pollution has been linked to decreased lung function and increased cancer risk. Air pollution can consist of natural or artificial materials that range in chemical as well as physical properties. Inhaled pollutants can have a variety of effects on organisms ranging from minor irritation, to chronic obstructive pulmonary disease, and even cancer or death. Historically, it has been noted that increased levels of air pollution are associated with decreases in human health and increases in mortality.

Following the World Trade Center tragedy, rescue workers as well as New York City residents were exposed to thousands of tons of particulate matter that was inhaled and ingested over an extended period of time. Some of the immediate effects of these pollutants included “World Trade Center Cough,” a condition that resulted in increased coughing, respiratory pathway irritation, and decreases in lung function. Though there are several known carcinogens found within the heterogeneous mixture of particles that make up World Trade Center dust, there is currently no link between World Trade Center dust inhalation and cancer development. Furthermore, the molecular processes responsible for “World Trade Center Cough” are not completely understood.

There are a variety of mechanisms that could be responsible for the decreased lung function reported in World Trade Center rescue workers. It is possible that just the presence of particulate matter in the lungs may lead to an inflammatory response that is powerful enough to induce cell damage and subsequent tissue damage and loss of function. Particles can damage cell membranes, inducing necrosis and resulting in the spilling of cellular contents into the surrounding environment and inducing inflammatory responses. Damage could also occur in response to the chemical properties of inhaled
particles. Particle components can be reactive and interact with DNA to induce mutation and even the occurrence of cancer.

This study investigated the physical and chemical properties of World Trade Center dust using *in vitro* methods. Human lung fibroblast cells (MRC-5 and WI-38) were exposed to various concentrations of World Trade Center dust and controls to simulate the World Trade Center dust exposure in *in vivo* lung systems. Proliferation and apoptosis rates were measured in cells exposed to various concentrations of World Trade Center dust and controls in order to elucidate any physical or chemical interactions between cells and particles that may decrease cell viability and induce injury or mutation. An Ames assay was performed to determine mutagenic effects that various concentrations of World Trade Center dust may have on living systems. Mutations incurred by Salmonella *typhimurium* cells suggested the possibility that World Trade Center dust is mutagenic. Analysis of proliferation and apoptosis in cells exposed to World Trade Center dust, in conjunction with an Ames assay, allowed for a better understanding of the risks faced by rescue workers and other personnel exposed to varying quantities of particulate matter pollution following the collapse of the World Trade Center.
ANALYSIS OF POTENTIAL MUTAGENIC EFFECTS OF WORLD TRADE CENTER DUST ON *IN VITRO* SYSTEMS

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Introduction

Atmospheric Pollution

Sixty years ago, populations in Europe as well as the United States began to link pollution with human health. Though environmental toxicology did not exist at this point, the general public began to notice that air pollution, such as smog, led to increased numbers of hospitalizations and even death (Dockery 1993). Since air pollution encompasses a variety of both natural and man-made pollutants that vary in chemical as well as physical structure, researchers had a hard time pinpointing the causes for increased morbidity in response to decreased air quality (Cohen 2000). However, the general reactions to air pollution usually involved upper respiratory inflammation, coughing, wheezing, and irritation. Furthermore, it has been noted that the risk for mutations within living systems increases with increasing air pollution (Ducatti 2003).

In the 1950s, sharp increases in lung cancer rates throughout the world indicated an epidemic that worried both the general public and epidemiologists. Researchers in the United States as well as Europe began investigating air pollution as a potential causative agent behind an ongoing lung cancer epidemic (Cohen 2000). Though there had been no extensive studies regarding the effects of environmental pollutants on lung health, people began to suspect a link between air quality and lung cancer (Nyberg 2000). Extensive studies commenced and it was eventually determined that cigarette smoking was the main culprit behind the jump in lung cancer rates (Dockery 1993).

The discovery of a link between cigarette smoking and cancer, however, was not the end of inhalation toxicology research. Though air pollution is considered less toxic than cigarette smoke, it is believed to affect more people and interact with other materials
to induce cancer and other health issues (Hemminki 1994). Environmental and occupational exposure studies have since discovered links between air pollution increases and increased instances of upper respiratory illnesses. Today, the World Health Organization has recognized air pollution inhalation as a source of exposure leading to cancer, chronic respiratory diseases, and acute respiratory infections (Ducatti 2003). Increases in traffic-related air pollution in the past 30 years are believed to be responsible for as much as 10% of all lung cancer cases in a study based in Stockholm (Nyberg 2000). In at least one infamous incident, extensive coal burning resulted in dense smog in London that resulted in 4000 deaths (Dockery 1993). Furthermore, it has been shown that increases in atmospheric pollution are linked to heart disease, respiratory disease, and cancers (Mehta 2008). Today, indoor and outdoor air pollution has been identified as contributing factors to lung cancer by a comprehensive collection of sources and various efforts have been made to reduce exposure to air pollutants (Cohen 2004).

A variety of dangerous air pollutants ranging in physical and chemical composition have been identified and characterized in numerous studies (Clarke 1999, Wichmann 2008). Particle size has been especially important in determining the dangers of any substance via inhalation. Smaller particles are more capable to reaching the deepest parts of the respiratory system and lead to chronic inflammation with prolonged exposure (Landrigan 2004). In general, particles that are 10 microns in size (PM₁₀) or lower are considered the most dangerous to human health since they can penetrate deeply into the lungs and cause damage in very sensitive areas (Cohen 2000). Larger inhaled particles may not always penetrate deeply into the lungs. However, they can be trapped in the nasal passages, leading to damage and irritation (Herbstman 2005).
Physiological Response to Inhaled Atmospheric Pollution

The processes that lead to lung cancer in response to inhaled air pollution are still unclear. However, there are a number of physiological responses that may end up increasing the risk for cancer (Cohen 2000). Prolonged exposure to particulate matter pollution has been shown to have adverse effects on lung growth and function, especially in urban areas where atmospheric pollution is prominent (Wichmann 2008). Inhaled particulate matter has been shown to induce bronchospasms in healthy individuals, decrease respiratory function, and lead to inflammation in lungs (Clarke 1999). Inflammation and persistent exposure to materials that cannot be eliminated in lung systems are linked to lung cancers such as mesothelioma (Sanchez 2009).

The physical properties of glass fibers, for example, can lead to lung irritation even though they are not chemically reactive. The epithelial tissue that lines the lungs can be easily torn by inhaled particles, resulting in cell injury (Kodovanti 2000). Regardless of the physical or chemical composition of air pollutants, increased levels of exposure is positively correlated with increased effects (Landrigan 2004). It has been determined that instances of lung cancer mortality increase by 8% for each 10 mg/m³ increase in exposure to air pollution (Mehta 2003). Also, increased exposure may lead to the inhalation of different particles that, though inert on their own, can interact with more reactive particles to enhance their effects (Hemminki 1994). Carcinogenic elements can also be absorbed by small, inert particles, which then become carcinogenic (Ducatti 2003).
Oftentimes, the chemical composition of inhaled air pollutants has a significant effect on human health. The materials most responsible for increases in lung cancer resulting from inhalation are polycyclic aromatic hydrocarbons, asbestos, benzene, and arsenic. These materials are directly linked to lung cancer, leukemia, and mesothelioma (Herbstman 2005). Of these materials, polycyclic aromatic hydrocarbons are considered the most dangerous given their ability to interact with DNA directly to and induce mutations (Mehta 2008). Cadmium, chromium, and nickel are also considered to contribute to cancer formation in fewer instances (Herbstman 2005).

Though transition metals are not considered carcinogenic, their presence in the lungs can contribute to cancer through the promotion of Fenton reactions that may result in reactive oxygen species that can damage tissue (Mehta 2008). The World Trade Center dust samples analyzed by Lioy et al. contained a variety of known carcinogens such as cadmium, asbestos, and polycyclic aromatic hydrocarbons (Lioy 2002a).

As seen in Figure 1, the World Trade Center dust samples also included elements that may aid in
cancer development, such as arsenic, chromium, nickel, aluminum, and zinc (Herbstman 2005, Yang 1997, Sauvant 1998, Oh 2004)

Today, there are several known carcinogens, such as asbestos and cadmium, which are known to increase cancer risk in humans with increased exposure and inhalation. Particles such as asbestos and cadmium are known to irritate lung tissue when inhaled, causing tissue damage (Herbstman 2005). In some cases, prolonged exposure to toxicants can lead to decreased alveolar permeability and increased hemorrhaging, which ultimately lead to decreased respiratory ability as a result of injured tissue (Kodovanti 2000). This damage to lung tissue incites an inflammatory response that will lead to the production of cytokines and other signaling molecules that will lead to the repair of tissue (Lag 2010). This process is normally self-limiting and ends once the tissue is repaired (Balkwill 2001). Inflammation can be signaled by necrosis which can, in turn, be prevented by cell apoptosis. Though environmental stimuli such as inhaled pollutants have the ability to trigger programmed cell death in lung systems, they also have the ability to inhibit apoptosis. Inhibition of apoptosis can, in turn, allow damaged cells to continue proliferating, increasing chances for mutations that may lead to cancer (Jacobson 1997).

**The World Trade Center Cough and Related Health Issues**

Following the tragic collapse of the World Trade Center on September 11, 2001, thousands of tons of particulate and gaseous air pollution were released into the surrounding environment (Berrios-Torres 2003). The gaseous and solid atmospheric pollution that resulted, enveloped Manhattan varied in size and chemical composition from site to site (Lioy 2002a). In addition to this release of pollutants, the fires within the
remaining 6-story pile of rubble continued to burn for several months, releasing even more particulate matter into the surrounding environment (Landrigan 2004). It is estimated that over 5000 rescue workers were exposed to this dust as cleaning operations took place around the clock for several months. Though these workers managed to clean 100 millions of tons of debris from ground zero, they were exposed to various pollutants throughout the cleanup (Berrios-Torres 2003).

The levels of particulate air pollution present in the surrounding air environment fluctuated in the days following the attack (Landrigan 2004). The level of particles suspended in the atmosphere peaked at numbers that threatened to surpass current National Ambient Air Quality Standards. Furthermore, levels of exposure varied among New York City residents, depending on occupation, distance from Ground Zero, and other factors. Though it has been difficult to pinpoint the exact level of exposure for each individual in Manhattan following the World Trade Center attacks, it is estimated that at least 1/5 of Manhattan’s population was exposed to PM$_{2.5}$ concentrations of at most 60micrograms/m$^3$. Though these levels are not high enough to surpass the current National Ambient Air Quality Standards value of 65micrograms/m$^3$, they are concerning (Ng 2005). Previous studies, have demonstrated that increases in ambient air pollution, in general, have been linked with 30-50% increases in lung cancer (Nyberg 2000). An increase of 10 micrograms per cubic meter, for example, has been associated with increased rates of cardiovascular deaths (Pope III 2003).

Immediately following clean-up efforts, rescue workers expressed some concern regarding the air quality (Herbstman 2005). Respiratory-related injuries were the second-most highest category of issues reported by firefighters, police, EMS, and other members
of search and rescue teams. Respirators and goggles were given to workers to at the
initiation of clean-up, but were not always used by each individual throughout their shifts
(Berrios-Torres 2003). These issues persisted throughout the rescue efforts with many
workers developing new coughs and other respiratory issues (Landrigan 2004). Though it
is difficult to assess just how much particulate air pollution was inhaled by each
individual, it has been noted that length of exposure was related to the instance of health
issues among rescue workers (Herbstman 2005). Furthermore, decreases in lung function
among workers persisted for years following the rescue efforts (Aldrich 2010).

In the time following rescue and clean-up, hundreds of rescue workers and
firefighters were diagnosed with what became known as “World Trade Center Cough.”
Symptoms of World Trade Center Cough consisted of coughing as well as mild to severe
decreases in respiratory function. The prevalence of World Trade Center Cough increased
as time of exposure increased, high levels of particulate matter present in sputum
(Landrigan 2004). In the years that followed the World Trade Center attacks, the
chemical composition of World Trade Center Dust has been analyzed and its components
identified (Lioy 2002a). Several carcinogenic materials have been identified in World
Trade Center dust. These materials include asbestos, polycyclic aromatic hydrocarbons,
lead, mercury, and cadmium (Lambroussis 2009, Landrigan 2004, Lioy 2002a, Mehta
2008). The presence of asbestos in inhaled World Trade Center dust is especially
alarming, as it may lead to mesothelioma (Landrigan 2004).

Though various health issues have been directly linked to the inhalation of World
Trade Center dust by Manhattan residents and rescue workers, it is still unclear if World
Trade Center dust inhalation is linked to increases in cancer risk (Berrios-Torres 2003).
Furthermore, the molecular processes leading to the decreased respiratory function linked to World Trade Center dust inhalation are still unknown (Cohen 2000). As a result, it is still unclear if World Trade Center dust is solely responsible for the decreases in respiratory function found in rescue workers. It is possible that these issues are linked solely to powerful immune responses leading to increases in cytokines, free radical oxygen species production, and other responses that may cause cell damage and eventual cancer (Balkwill 2001. Coussens 2002).

In general, inhaled air pollutants have been shown to induce inflammatory responses in respiratory systems (Clarke 1999). In 1863 Rudolf Virchow suggested that cancer could be caused by chronic inflammation (Balkwill 2001). Since this hypothesis was made, studies have found that several elements of a typical inflammatory response can increase the likelihood for cancer. The presence of DNA damage-inducing agents, growth factors, inflammatory cells, and injury repair associated with wounding, the likelihood for cancer increases (Coussens 2002). As other studies have noted, increased or prolonged exposure to particulate matter in inhaled air leads to increased numbers of leukocytes, and increased inflammation that may lead to bronchoconstriction and even chronic obstructive pulmonary disorder (COPD) (Clarke 1999). Furthermore, biopersistence of materials that cannot be broken down in the lungs naturally will continue to induce inflammation (Sanchez 2009). Together these conditions can lead to increased tissue damage, decreased lung function, and even cancer development over time.

As has been noted in previous studies, there are a variety of mechanisms that may lead to cancer and other diseases in people exposed to increased air pollution (Dockery
1993, Ducatti 2003, Hemminki 1994, Sanchez 2009, Nyberg 2000). These mechanisms may be due to the direct DNA damage-inducing carcinogenic present in pollutants, indirect interactions that may lead to the production of reactive oxygen species, or prolonged exposure which may lead to prolonged inflammatory response and subsequent cancer. Thousands of individuals exposed to World Trade Center dust during clean-up and rescue missions were exposed to increased levels of air pollution for prolonged periods of time (Balkwill 2001, Clarke 1999, Dockery 1993). This exposure has been linked to decreased lung function and various respiratory health issues that have persisted since the World Trade Center tragedy (Aldrich 2010). The identification of any link between World Trade Center dust and mutagenicity would lead to more specific and better preventive care for rescue workers and New York City residents who could be at risk for cancer development or the emergence of other health issues resulting from cell damage or DNA mutation.

**Project Description**

This study will identify and analyze any effects World Trade Center dust may have on *in vitro* cultures of human lung fibroblasts and Salmonella *typhimurium* bacteria. This study will analyze chemical as well as physical properties of World Trade Center dust to determine the effects of World Trade Center dust on MRC-5 and WI-38 cell lines. An Ames Mutation assay will then be conducted to determine if World Trade Center has the ability to induce DNA mutations, which may result in decreased cell viability and may lead to cancer.

To date, the possible link between World Trade Center dust inhalation and the respiratory issues and cancers experienced by rescue workers has not been explored in
depth. The collapse of the World Trade Center resulted in the release of thousands of tons of debris into the surrounding environment (Herbstman 2005, Landrigan 2004). New York City residents and rescue workers, therefore, were exposed to large increases in pollutants suspended in the air (Landrigan 2004). As previous research has showed, increases in air pollution are linked to increases in cancer rates due to the inhalation and ingestion of compounds that may have a detrimental effect on physiological systems (Ducatti 2003, Hemminki 1994, Lioy 2002a).

Some of the gaseous and solid compounds characterized in World Trade Center dust mixture have been previously characterized as being carcinogenic or mutagenic. These materials include and are not limited to cadmium, lead, mercury, polycyclic aromatic hydrocarbons, and asbestos (Lioy 2002a). On their own, each of these materials is considered a health hazard and various precautions are usually implemented to minimize occupational exposure to these materials. It is possible, however, that these materials can work in conjunction with one another to induce damage to cells and tissues (Hemminki 1994). Even seemingly innocuous elements, such as aluminum, can become more hazardous when they interact with other molecules on the molecular level (Dong 1994, Sauvant 1998). These interactions can increase affinity to DNA and cause subsequent mutation and even cancer (Dong 1994, Lioy 2002a).

Furthermore, it must be noted that chemical composition of World Trade Center dust, in itself, may not be the only factor responsible for decreases in respiratory health. Particles size determines how far particulate air pollution can penetrate into the lungs once inhaled. Particles that are 10 microns or smaller are capable of entering deep into the lungs, blocking gas exchange taking place in the alveoli and decreasing respiratory
function (Herbstman 2005, Kodovanti 2000). Furthermore, the mere presence of these particles may trigger an immune response from the host that results in increased mucous secretion, the release of cytokines, and increased inflammation at these sites (Lag 2010). As Virchow noted, the risk for cancer increases with increased inflammatory responses from the host. Inflammatory responses resulting in reactive oxygen species can lead to self-inflicted protein, tissue, and DNA damage, especially in severe cases (Clarke 1999, Coussens 2002, Hart 1995). In this way, World Trade Center dust may be the indirect cause of some of the respiratory issues faced by rescue workers that inhaled large concentrations of particulate and gaseous air pollution over time during the clean-up process.

The purpose of this study will be to better characterize potential effects of World Trade Center dust on the molecular scale using in vitro systems. Through the utilization of human lung cell as well as bacterial in vitro systems, this study will attempt to elucidate potential mutagenicity due to the chemical composition of World Trade Center dust using a reverse-mutation Ames assay. World Trade Center dust particle size will also be analyzed in order to better determine the degree of penetration that may have occurred through the inhalation of this dust on the days and months that followed September 11, 2001. Particle size analysis will allow a better understanding of what respiratory system organs may have been most affected by World Trade Center dust inhalation.

Cell response to World Trade Center dust will be analyzed using apoptosis as well as proliferation assays. Cells exposed to World Trade Center dust may experience damage in response to physical or chemical interactions with different particles. If any of these interactions result in irreversible protein, membrane, or DNA damage, affected cells
may undergo programmed cell death. Furthermore, apoptosis may occur due to a physical interaction between the cell and a component of the World Trade Center dust. Inhaled shards of glass, for example, though inert, may puncture lung tissue or damage cells due to its abrasive surface properties. Physical contact, therefore, may lead to irreversible damage that may result in either apoptosis or necrosis. Ideally, apoptosis is a safer route for cells than necrosis since it does not result in the spilling of reactive cell contents into the surrounding environment (Majno 1995).

The Promega Caspace-Glo 3/7 assay will measure the levels of caspase-3 and caspase-7 activity in cells exposed to WTC, HHD, and GYP. Caspase-7 and caspase-3 are proteases that play a role in the induction of apoptosis in mammalian cells. The luminogenic substrate included in this assay is cleaved by any caspase-3 and caspase-7 proteases present in cell cultures. This reaction will free tetrapeptide sequence DEVD. This luciferin will then react with the UltraGlo luciferase included in this assay to produce light. The level of light released by this reaction will be directly proportionate to the level of caspase present in a well of cells. Since caspases are present in cells undergoing apoptosis, increased light production will also indicate increased rates of apoptosis in cells. The implementation of this apoptosis assay will determine if the caspase apoptosis pathway has been induced in cells exposed to WTC, HHD, and GYP.

Figure 2. Caspase-3/7 cleavage of substrate containing DEVD tetrapeptide sequence and resulting luciferase reaction between cleaved amino luciferin and UltraGlo Luciferase (Promega 2005)
The activation of this pathway would suggest that cells had been irreversibly damaged in some way by the World Trade Center dust or controls (Promega 2005).

A proliferation assay will be implemented to determine the overall effects of World Trade Center dust on the growth of cells. Proliferation levels will be compared among cells grown in different concentrations of WTC. Healthy proliferation of fibroblast cells within an in vitro system is usually characterized by the formation of a confluent monolayer. Once cells have grown into a monolayer, they will cease growing due to contact inhibition. Continued growth may indicate uncontrolled growth, such as that of cancer cells. Increased growth in the presence of a potential toxin may increase the risk of cancer cell formation every time the DNA is replicated the likelihood for mutations increases with increased levels of replication. Decreases in proliferation in response to materials may suggest decreased cell viability and possible growth inhibition due to cell damage, mutation, or cell death. In *in vivo* systems, decreased proliferation and viability may result in decreased wound repair and possible persistence of inflammation due to injury.

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay will be implemented to analyze proliferation and cell viability within this experiment. This assay will measure proliferation based on the ability of cells to reduce MTS tetrazolium salt into formazan. The chemical transformation of MTS tetrazolium to formazan leads to a color change which, in turn, results in increased levels of absorbance at 490nm.
absorbance. The dehydrogenase enzymes required for the reduction of MTS tetrazolium to formazan are present and functional in healthy, viable cells. Therefore, increased levels of 490nm absorbance in cell cultures will indicate increased numbers of healthy, living cells (Promega 2009).

The Ames reverse-mutation assay has been implemented in the industrial and laboratory settings as a standard tool to measure potential mutagenicity of a variety of materials for over thirty years. This test implements mutated strains of salmonella bacteria that are incapable of forming histidine. When these bacteria are exposed to mutagens, their DNA may undergo mutations that will enable the bacteria to develop histidine (Ducatti 2003, Mortelmans 2000). The Muta-Chromoplate assay will implement two strains of bacteria (TA98 and TA100) with mutations in the operon for histidine synthesis. Mutations to DNA will be distinguished by the bacteria’s newly developed ability to synthesize histidine. This, in turn, will be noted by a change in the color of the bromocresol purple dye in the assay (EBPI 2009).

Combined, these assays should elucidate potential mutagenic activities of the World Trade Center dust and better determine whether decreases in respiratory health of 9/11 rescue workers was the result of physical properties such as particles size, the chemical composition of World Trade Center dust, or due to indirect causes such as a host inflammatory response. The results of this in vitro study may better explain any potential mutagenic effects of this dust in vivo. The results achieved may be of great significance as they may identify a possible route responsible for the decreased respiratory health in rescue workers. Furthermore, determination of mutagenic or carcinogenic properties of World Trade Center dust may lead to better identification of
health hazards incurred by rescue workers as well prevention of health hazards that may manifest themselves in the future without proper care or medical attention.

**Materials/Methods**

**Cell Culture Maintenance**

MRC-5 and WI-38 human lung fibroblast cells were ordered from ATCC. Once cells arrived, they were warmed to room temperature and then suspended in 5-6mL of fresh media that had been warmed to room temperature using a water bath. Cells were dispensed into sterile T-25 flasks using aseptic technique. Caps to T-25 remained loose at all times to allow for proper gas exchange to occur to ensure proper cell growth. Upon subculturing, cells were placed in an incubator set at 37 degrees Celsius with 5% Carbon dioxide levels.

Both cells lines were grown in Eagles Minimum Essential Medium (MEM) that had been supplemented with about 6mL of Penicillin-Streptomycin, 6mL of Glutamine, 6mL of Kanamycin and 50mL fetal bovine serum per 500mL of media, unless otherwise indicated. Cell growth was carefully monitored using an ACCU-SCOPE phase contrast microscope with attached Micrometrics digital camera and Micrometric SE Premium software.

Once confluent monolayers had formed, cells were scraped aseptically using sterile Costar model 3010 cell scrapers under a ventilated hood. Cells were then spun at 10,000 rpm for 5-7 minutes until the cells had separated from the media, to form a pellet.
at the bottom of 15mL centrifuge tubes. The supernatant was removed from the centrifuge tube and the cells were suspended in 10mL of fresh media using a sterile pipette. Once cell pellet was dispersed throughout the media included in the centrifuge tube, the mixture was equally allotted into 2-3 T-25 flasks and allowed to grow to a confluent monolayer until needed.

**World Trade Center Dust and Controls**

A sample of World Trade Center dust (WTC) was generously donated to our laboratory by Dr. Paul Lioy of Rutgers University. The sample was collected a few days following the World Trade Center attacks from the hood of a car parked on Market Street. Wallboard was acquired from a hardware store. The wallboard was broken into smaller pieces after the paper layers were removed. The remaining gypsum pieces (GYP) were then crushed into small grains using a mortar and pestle until their size was similar to that of the larger World Trade Center dust particles. Household dust (HHD) was acquired using a hand collection method using a previously described surface wipe sampling method (Lioy 2002b). This dust did not require any further preparation as particles were already minute enough to be comparable to the finer particles of the World Trade Center dust sample.

World Trade Center dust, gypsum, and household dust were stored in separate, airtight containers away from direct sunlight. All dust was sterilized via ultraviolet germicidal radiation by placing dust to be sampled under UV light exposure for 24 hours prior to use.
**Media Preparation**

Prior to making stock solutions, MRC-5 cells were exposed to media containing various concentrations of WTC to determine the LD$_{50}$ due to exposure. Once LD$_{50}$ was determined, WTC concentrations in media were adjusted to allow for cell proliferation. Concentrations of HHD and GYP mirrored concentrations of WTC used in experiments.

WTC, HHD, and GYP to be added to growth media were exposed to UV radiation upon aseptic removal from their respective containers. After 24 hours of UV exposure, dusts were weighed aseptically in sterile weighing boats or paper using a Sartorius scale. Once desired amounts of GYP, HHD, and WTC were weighed out, they were added to 10% fetal bovine serum-enriched media to make a 1000ppm stock solution. Each stock solution was then diluted accordingly to make 2.5ppm, 25ppm, and 250ppm working solutions of WTC, GYP, and HHD media. WTC, HHD, and GYP media stock solutions were stored in airtight containers within a freezer set at about -18 degrees Celsius. 50mL aliquots of each media were stored in airtight flasks within a refrigerator set at 4 degrees Celsius.

**Particle Size Analysis**

Particle size analysis was conducted to ensure that crushed gypsum particles and household dust were of comparable size to the WTC sample obtained from Rutgers University. Before analysis, 100 milligrams of GYP, WTC, and HHD were massed aseptically using a Sartorius electronic scale and sterile weighing boats. Each sample was then placed in an airtight and labeled container for analysis. Before size analysis, each
sample was suspended in 50mL of Millipore-filtered water using sodium pyrophosphate as a dispersal agent and then placed in a Cole-Parmer 8890 ultrasound water bath for 10 minutes. Beakers containing the samples were then placed on hot plates at 400 degrees Celsius until boiling. This allowed for proper dispersal of samples.

Once the particles in each sample were dispersed, size analysis was conducted using a Malvern Instruments Mastersizer 2000 unit with a Hydro 2000 MU Unit Accessory by Malvern Instruments. This instrument utilized blue and red light sources to measure the sizes of particles based on diffraction of light from suspended particles. Before each sample was analyzed, background measurements were taken using Millipore water. These background measurements were then cancelled out from the final sample readings. Analysis of World Trade Center dust and household dust samples were performed using the carbon standard operating procedure. The gypsum sample particle size analysis was performed using the gypsum standard operating procedure. Particle size analysis for each sample was repeated three times and the instrument was cleaned after each sample reading. Background measurements were also taken before each sample analysis. Results were measured using the Mastersizer 2000 program by Malvern.

**Proliferation Assay**

Cell proliferation was measured using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay manufactured by Promega. Before the assay was run, MRC-5 cell cultures were scraped, collected, centrifuged, and then suspended in fresh MEM media. Cell concentrations were calculated using a hemacytometer and microscope to ensure that the final concentration of cells was $1 \times 10^5$ cells/mL. Once this concentration of
cells was achieved for MRC-5 cells, a multichannel pipette was implemented to aseptically transfer 100 microliters of cells and media into each well of a clear 96 well plate. WI-38 and MRC-5 cells were allowed to settle and grow in each respective 96 well plate.

Once cells had been given ample time to attach and grow, the old media was removed and replaced with media containing 1.25ppm, 2.5ppm, 12.5ppm, 25ppm, 125ppm, and 250ppm WTC concentrations. Media containing 2.5%FBS and 1%FBS were implemented alongside 10%FBS media to simulate stressful cell environments. All media were vortexed prior to use to ensure thorough dispersal of particulate matter. Cells were then placed in the incubator overnight. Cell proliferation analysis took place after cells had been exposed to GYP, HHD, or WTC for at least 24 hours.

On day of cell proliferation analysis tetrazolium MTS solution was constituted aseptically according to methods described in Promega Technical Bulletin #TB169. 100 microliters of electron coupling reagent (PMS) was added to 2 milliliters of MTS to create reaction solution. 20 microliters of MTS/PMS solution was added to each well of
the 96 well plates and each plate was then incubated for 1 hour. Absorbance of this
colorimetric assay was recorded at 490nm using an ELISA plate reader.

**Apoptosis Assay**

Apoptosis was measured using a Caspace-Glo 3/7 assay manufactured by
Promega. Before the assay was run, both MRC-5 and WI-38 cell cultures were scraped,
collected, centrifuged, and then suspended in fresh MEM media. A multichannel pipette
was implemented to aseptically transfer 100 microliters of cells and media into each well
of a 96 well plate. WI-38 and MRC-5 cells were allowed to settle and grow into confluent
monolayers before old media was removed. Cells were then exposed to 100 microliters of
media containing HHD, GYP, or WTC in concentrations 2.5ppm, 25ppm, or 250ppm. All
cells were exposed to 10% fetal bovine serum media concentrations since stress situations
were not implemented for this assay. 96-well plates were placed in an incubator at 5%
carbon dioxide at 37 degrees Celsius.

After 24 hour exposure to WTC, HHD, and GYP media, Caspace-Glo 3/7
substrate and Caspace-Glo 3/7 buffer were defrosted using a water bath. Bottles were
sprayed with ethanol to minimize contamination after removal from water bath. 100
milliliters of Caspace-Glo 3/7 buffer was aseptically added to Caspace-Glo 3/7 substrate
under a ventilated hood. The bottle containing the resultant reagent (Rg) was then swirled
to ensure proper mixture of contents. 100 microliters of this reagent was added to all
wells of each 96 well plate and plates were placed on a plate shaker for 30 seconds. Plates
were then placed in an incubator for half an hour before luminescence was read using a
plate-reading luminometer.
Reverse-Mutation Assay

Mutagenic activity of WTC, GYP, and HHD was measured using the Muta-ChromoPlate™ Bacterial Strain Kit manufactured by Environmental Biodetection Products Inc (EBPI). HHD, GYP, and WTC to be used in this assay was measured out and placed under UV light radiation for 24 hours. Lyophilized Salmonella *typhimurium* TA100 and TA98 strains were rehydrated with growth medium and incubated at 37 degrees Celsius in an incubator overnight. Once sufficient bacterial growth was confirmed, test solutions were made using HHD, GYP, and WTC. The reaction mixture was made according to the volumes and solutions dictated in the Muta-ChromoPlate Version 3.3 protocol. Under a ventilated hood, 43.24ml of Davis-Mingoli salts, 95.ml of D-glucose, 4.76ml of Bromocresol Purple, 2.38ml of D-Biotin, and .12ml L-Histidine were mixed together in a sterile flask using aseptic technique.

Some alterations to the Muta-ChromoPlate Version 3.3 protocol provided by EBPI in the making of the test solutions. Mainly, the test solutions were not filtered before use. However, UV-radiation of WTC, HHD, and GYP as well as utilization of aseptic technique prevented contamination. 20mL of reaction mixture was dispensed into 18 sterile and labeled 50ml centrifuge tubes. 25ppm and 250ppm WTC, HHD, and GYP test solutions were created for each bacterial strain to be tested. Control test solutions were created by aseptically adding .1ml of sodium azide to one centrifuge tube and .1ml of 2-nitrofluorene to another tube of test solution. 5 microliters of either TA98 or TA100
bacterial suspension was allotted into appropriate tubes. No bacteria were added to reaction mixture that was to be used in “Blank” plates.

The contents of each tube were poured into a sterile reagent boat one at a time to allow for even uptake by a multichannel pipette. 100 microliters of reaction mixture was dispensed into each well of a sterile, white polystyrene 96 well plate using a multichannel pipette. Once media was dispensed into all the plates, plates were placed in an airtight plastic bag to prevent evaporation of sample. These sealed bags were then placed in an incubator at 37 degrees Celsius for 5 days. Plates were then removed from the incubator and scored after the 5 day incubation period.

Results

Particle Size Analysis Results

![Particle Size Analysis Graph](image)

*Figure 5. GYP particle size analysis reading* Particle sizes within the GYP sample examined ranged from 1-3000 microns in size. The peak in this figure indicates that the majority of GYP particles ranged from 100-1000 microns in size.
Figure 6. HHD Particle Size Analysis Reading  Particle sizes within the HHD sample examined ranged from 1-2000 microns in size. The peak in this figure indicates that the majority of HHD particles ranged from 10-100 microns in size.

Figure 7. WTC Particle Size Analysis Reading  Particle sizes within the WTC sample examined ranged from 1-3000 microns in size. The tallest peak in this figure indicates that most particles ranged from 10-100 microns. However, the smaller peak indicates that a significant fraction of the sample analyzed was composed of particles that ranged from 100-300 microns in size.

The analysis shows that most of the particles present in all three samples ranged from 10 to 1000 microns in size. The WTC reading shows one larger peak between 10 and 100 micrometers, with a smaller peak between 100 and 1000 microns. This indicates
that the majority of particles in this sample were between 10 and 100 microns in size, with a smaller, but still significant portion of the sample ranging from 100 to 1000 microns in size. The majority of particles in the HHD sample ranged from 10 to 100 microns in size with a smaller amount of particles ranging between 100 and 1000 microns. The majority of particles in the GYP sample ranged from 100 to 1000 microns.

**Proliferation Assay Results**

The proliferation assay results in Figure 4 indicated that WTC is capable of inhibiting cell viability. In comparison to controls (not shown), cells exhibited decreased levels of 490nm absorbance, indicating decreased reduction of MTS tetrazolium to formazan. This indicates a decrease in dehydrogenase enzyme activity, which is indicative of decreased cell viability and subsequent proliferation. As demonstrated, increases in WTC concentration lead to decreases in proliferation, regardless of FBS concentration.
**Luminescence (RLU)**

**Apoptosis Assay Results**

- **Figure 10** Normalized apoptosis assay results from initial reading for MRC-5 cells

- **Figure 9** Normalized apoptosis assay results from initial reading for WI-38 cells
Figure 11 Normalized apoptosis assay results at 1.5 hours for MRC-5 cells

Figure 12 Normalized apoptosis assay results at 1.5 hours for WI-38 cells
As indicated in Figures 9-12, MRC-5 and WI-38 cells exposed to various concentrations of WTC generated the most light compared to HHD and GYP. Luminescence increased with increasing concentrations of WTC. GYP and HHD exposure, for the most part, did not generate high levels of luminescence in relation to the light generated in cells exposed to 25ppm and 250ppm concentrations of WTC. The results from the initial readings were comparable to the results obtained at 1.5 hours, with little variation. In general, the luminescence levels noted in both MRC-5 and WI-38 apoptosis assays suggest that caspase-3 and caspase-7 activity increases with increasing WTC concentrations. These results, therefore, imply that increasing levels of WTC are correlated with increased levels of apoptosis.
Reverse Mutation Assay Results

Figure 13 Reverse-mutation Assay Results I (a) 9 mutated colonies in TA100 strain exposed to 25ppm HHD; (b) 1 mutated colony in TA98 strain exposed to 25ppm HHD; (c) 8 mutated colonies in TA100 strain exposed to 250ppm HHD

Figure 14 Reverse-mutation Assay Results II (a) 4 mutated colonies in TA98 strain exposed to 250ppm HHD; (b) 5 mutated colonies in TA100 strain background; (c) 0 mutated colonies in TA98 strain background

Figure 15 Reverse-mutation Assay Results III (a) 7 mutated colonies in TA100 strain exposed to 25ppm GYP; (b) 1 mutated colony in TA98 strain exposed to 25ppm GYP; (c) 9 mutated colonies in TA100 strain exposed to 250ppm GYP
Figure 16 Reverse-mutation Assay Results IV
(a) 0 mutated colonies in TA 98 strain exposed to 250ppm GYP; (b) 0 mutations in Blank 1
(c) 0 mutations in Blank 2

Figure 17 Reverse-mutation Assay Results V
(a) 10 mutated colonies in TA100 strain exposed to 25ppm WTC; (b) 6 mutated colonies in TA98 strain exposed to 25ppm WTC; (c) 35 mutated colonies in TA98 strain exposed to 250ppm WTC

Figure 18 Reverse-mutation Assay Results VI
(a) 23 mutated colonies in TA100 strain exposed to 250ppm WTC; (b) 96 mutated colonies in TA100 strain exposed to sodium azide (c) 82 mutated colonies in TA98 strain exposed to 2-nitrofluorene
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Figure 19 Summary of Data Collected from Reverse-mutation Assay

Figure 20 Comparison of mutation percentages for TA100 strain exposed to 25ppm and 250ppm concentrations of WTC, HHD, and GYP
Figure 21 Comparison of mutation percentages for TA98 strain exposed to 25ppm and 250ppm concentrations of WTC, HHD, and GYP

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Figure 22 Statistical significance data for 96 well fluctuation tests as calculated using Muta-Chromoplate Version 3.3 statistical analysis14
As shown in Figure 15, color change did not occur in “blank” plates, indicating that contamination did not occur during the mixture and plating of reagents. This also implies that mutations in other plates were the result of the chemical and/or physical properties of the contents present in the remaining reagent mixtures. The large numbers of mutations noted in the positive 2-nitrofluorene (2NF) and sodium azide (NaN₃) controls, indicates that bacteria were viable and that mutation could be induced in the experimental setup and conditions. These mutations also demonstrate that TA98 and TA100 strains are capable of undergoing reverse mutations can be induced and can lead to the synthesis of histidine by mutated colonies.

No mutation was noted in the background plate for the TA98 strain. However, 5 mutated colonies were found in the background plate for the TA100 strain. This low level of mutations for both background plates indicated that mutations in these plates were due to chance and not contamination. Furthermore, these low rates of mutation indicate that the higher rates of mutation present in bacteria colonies exposed to HHD, GYP, and WTC were due to the chemical properties of these materials. As shown in Figures 19 and 20, mutations occurred in the presence of HHD, GYP, and WTC, the media containing 250ppm WTC had significantly larger instances of mutation than the rest of the materials.

**Discussion**

The chemical composition of the wallboard used to make the GYP particle used in this study was not characterized. However previous research has determined that pulverized wallboard was a major component of the dust that covered Manhattan in the days following the World Trade Center collapse (Lioy 2002a). As a result, GYP can be considered a reasonable control since there is a strong possibility that it was inhaled by workers who were exposed to
World Trade Center dust. As seen in Figure 5, the GYP particles ranged in size from 1-3000 microns with most sample particles ranging from 100 to 1000 microns in diameter. The size of these particles was comparable to the size of the larger particles within our World Trade Center dust sample. A portion of the GYP sample consisted of particles that were equal to or less than 10 microns in size. Therefore, it can be assumed that a portion of the GYP sample tested could have been inhaled into the deepest parts of the lung (Cohen 2000). A comparison of the GYP and WTC particles size analyses shown in Figure 5 and Figure 7, demonstrate that the majority of particles in the GYP sample were within the same size range as a portion of particles within the WTC sample analyzed. Therefore, the size as well as the chemical composition of the GYP sample makes it a suitable control for WTC.

The analyses shown in Figure 6 and Figure 7 demonstrate that both the WTC and the HHD samples contained particles that were 10-1000 microns in size. Figure 6 demonstrates that though most HHD particles were 10-100 microns in size, there were some particles that were 10 microns and smaller. In terms of size, HHD falls within the same range as WTC, making it a suitable control in terms of size. As has been suggested by previous studies, household dust is inhaled daily and small enough in size to penetrate the deepest parts of the lung (Lioy 2002b, Cohen 2000). Though chemical analysis was not performed on HHD, it has been determined that the dust present in households contains some of the same elements that have been identified in WTC. However, unlike WTC, HHD is mainly composed of fibers as well as skin, hair, pollen, crumbs, and other innocuous materials (Lioy 2002b).

The results of these particle size analyses imply that any changes in apoptosis or mutation noticed in assays employing WTC and controls, will be the result of the chemical composition of the materials rather than the size. Previous studies demonstrate that components of both GYP and HHD are found in WTC. However, there are a variety of potential
mutagens and carcinogens present only in WTC. Therefore, the effects of WTC in relation to the effects of GYP and HHD in vitro may determine if WTC is potentially hazardous to the individuals that inhaled it following the World Trade Center collapse.

The proliferation assay results shown in Figure 8 indicate that increasing WTC concentrations lead to decreased levels of 490nm absorbance. As Figure 8 demonstrates, increasing levels of WTC lead to decreases in reduction of MTS tetrazolium to formazan. Since the reduction of this salt is accomplished by dehydrogenase enzymes present in viable cells, the results of this test demonstrate that increased levels of WTC lead to decreases in cell viability. Compared to cells grown in control conditions, cells grown in media containing WTC were less viable (Promega 2009). This indicated that WTC exposure was, in some way, detrimental to cells. Though the results of this assay did not explain how WTC dust was detrimental to cells, they were in concordance to the effects resulting from WTC exposure in vivo. Though the exact pathways involved in WTC exposure-induced decreases in health are unknown, there is evidence suggesting that WTC is linked to decreased lung function (Aldrich 2010). The results of this experiment support this observation.

Decreased cell viability could be detrimental in vivo, especially if it persists. Decreased growth and metabolic activity may make it more difficult for cell proliferation to heal wounds and break down xenobiotics. This could, in turn, enable xenobiotics to persist in sensitive lung environments and affect cellular processes. If cells are unable to properly breakdown compounds found in WTC, the persistent presence of xenobiotics may lead to chronic inflammation (Clarke 1999, Sanchez 2009). As previously mentioned, even the most inert materials can induce a powerful inflammatory response that could be damaging to lung tissue. Chronic inflammation can, in turn, result in DNA
damage, reductions in repair, and the development of cancer (Balkwill 2001, Coussens 2002). Furthermore, the results noted in Figure 8 also agree with observations made in the days during World Trade Center rescue and clean-up. In general, it was noted that increased exposure to WTC led to increased rates of health issues in rescue workers (Herbstman 2005). This could be due, in part, increases in WTC concentration within lungs due to continuous inhalation of WTC over time.

Higher concentrations of FBS provide cells with a variety of proteins needed for survival and growth. The cells exposed to 10% FBS, therefore, have access to more proteins that allow for optimal growth. Cells grown in 1% and 2.5% FBS, however, are grown in more stressful environments than cells grown in 10% FBS. The results of this experiment are consistent with the results expected when comparing cells growing in optimal growth environments to those grown in physiologically stressful environments.

Stressful situations are evident for cells grown in 1% and 2.5% FBS since there are, in general, lower levels of absorbance noted in cells grown in all WTC concentrations. The decreases in absorbance noted in cells grown in lower FBS concentrations indicate that lower concentrations of FBS can have a negative impact on cell growth at various concentrations of WTC. The small decreases in cell viability noted in cells grown in lower concentrations of WTC indicate that stress may be a factor for damage susceptibility in response to WTC exposure, as has been suggested by researchers monitoring the health of rescue workers (Herbstman 2005).

Figures 9 through 12 show the level of luminescence generated by cell cultures grown in various concentrations of HHD, WTC, and GYP. The levels of luminescence levels shown are directly proportional to the level of caspase-3 and -7 generated by
MRC-5 and WI-38 cells in each culture. Since caspase 3 and 7 are generated when cells begin to undergo apoptosis, it can be assumed that increases in caspase 3 and 7 activity reflect increases of apoptosis taking place in each cell culture. Figures 9 and 10 display normalized readings taken right after immediately after incubation in Caspace-Glo 3/7 Assay reagents, whereas Figures 11 and 12 show normalized readings taken 1.5 hours after the initial reading. There are some fluctuations in the overall luminescence emitted for each HHD, GYP, and WTC concentration tested. However, there were no major changes within this time span. In general, the readings taken at 1.5 hours were similar to those taken immediately after incubation.

There were some minor differences in the levels of luminescence generated by each cell type for the same concentrations of HHD, GYP, and WTC. However, both cells exhibited the same types of responses to each HHD, GYP, and WTC concentration. For the most part, there was no distinguishing pattern of luminescence generated by cultures exposed to 2.5ppm, 25ppm, and 250ppm concentrations of GYP and HHD as well as cells exposed to control settings with no dust. Both MRC-5 and WI-38 cell cultures generated very low levels of luminescence for each of these dust concentrations and in conditions where any form of dust was omitted. Even so, there was a larger and unexpected level of luminescence generated by MRC-5 and WI-38 cells exposed to media containing 2.5ppm HHD as well as MRC-5 cells exposed to media with no dust added. Though HHD is seemingly innocuous, both cell MRC-5 and WI-38 cell cultures generated a generous level of luminescence when exposed to 2.5ppm HHD. Since the same 2.5ppm HHD media was used for the testing for both cultures, it can be assumed that this increase in luminescence is due a component of the HHD present in this media.
As noted in previous studies, HHD can contain trace levels of toxins from household cleaners and pesticides (Lioy 2002b). It is, therefore, possible that the 2.5ppm HHD media contained toxins that caused increases in apoptosis in exposed cells. In general, however, both HHD and GYP were shown to induce low levels of apoptosis in comparison to WTC.

Both MRC-5 and WI-38 cell cultures emitted a substantial level of luminescence after exposure to 250ppm and 25ppm concentrations of WTC. Levels of luminescence emitted by MRC-5 and WI-38 were too inconsistent to be considered significant. However, levels of luminescence generated by both MRC-5 and WI-38 cell cultures exposed to 25ppm and 250ppm WTC were substantially higher than any other control. This demonstrates that the levels of caspase-3 and -7 present in these cultures was significantly higher than the levels of caspase 3 and 7 generated in other cultures as noted in Figures 9 through 12. The results of this assay, therefore, demonstrate that 25ppm and 250ppm concentrations of WTC are able to induce substantial levels of apoptosis in exposed cells. Furthermore, the levels of caspase in cells increased with increased concentrations of WTC.

Since increased levels of caspase are linked to apoptosis, it can be assumed that increased levels of WTC exposure are linked with increased rates of cell apoptosis. Cell apoptosis is induced by cells in response to damage or mutation in order to prevent necrosis. Though apoptosis and necrosis result in the death of cells, necrosis is much more harmful to surrounding cells since it leads to the release of cellular contents that signal an inflammatory response from the body (Majno 1995). The induction of apoptosis in this in vitro system suggests that the chemical properties of WTC are damaging.
enough to irreversibly damage cells. Apoptosis is an essential mechanism in living systems because it prevents the formation of cancer by limiting a damaged cell’s ability to reproduce. The replication of damaged cells and damaged DNA increases the likelihood for more DNA mutations to occur, a phenomenon which may result in cancer formation. Though WTC contains known carcinogens, they are present in trace amounts and current research has been unable to determine if WTC, as a whole, is carcinogenic. Though previous research has suggested that WTC induces apoptosis and inhibits proliferation, potential mutagenicity or carcinogenicity is unclear based on the results of this apoptosis assay alone (Lambroussis 2009).

Figures 13 through 18 show the results of the liquid-based reverse mutation assay. Each well shown in each of the plates represents a bacterial colony of Salmonella typhymurium. The TA100 and TA98 Salmonella typhymurium strains used contained mutations in the operon coding for histidine synthesis. Wells that remained purple following treatment with WTC, HHD, and GYP did not undergo reverse mutation since histidine synthesis would result in a color change of the media used. Reverse mutation resulting in histidine synthesis occurred in wells that changed color from purple to yellow or brown. The data collected from all plates featured in Figures 14 through 18 is summarized in Figure 19.

As noted in Figure 19, no reverse mutations occurred in the “blank” plates. Since all wells remained purple, it is safe to assume that no contamination occurred during the plating or storage of the reagents and assay. There was a very low instance level of mutations in the “background” plates, indicating that any reverse mutations present in these plates were due to chance rather than environmental conditions, contamination, or
bacteria viability. TA 100 bacteria exposed to sodium azide experienced very high levels of reverse mutation, as did the TA 98 strain exposed to 2-nitrofluorene. This indicates that both strains used were viable. The results for the background plates also demonstrate that the conditions in which the bacteria were incubated were suitable for growth (EBPI 2009).

The liquid-based Ames assay utilized in this experiment was modified to best mimic the conditions present in the lungs of rescue workers and New York City residents that inhaled WTC in the months following the World Trade Center collapse. Therefore, materials did not undergo filter sterilization. Instead, HHD, GYP, and WTC were exposed to UV light for 24 hours to allow for sterilization. The low level of mutations noted in the bacteria cultures containing HHD, GYP, and WTC indicate that the UV sterilization method was successful in killing any bacteria present on the samples. In general, there were low levels of reverse mutation taking place in bacteria exposed to 25ppm and 250ppm concentrations of HHD and GYP.

As noted in Figure 19, exposure to 250ppm WTC resulted in the highest levels of mutation occurring among TA98 and TA100 strains. The levels of mutations noted in these well were, on average, significantly higher than those noted in wells treated with various concentrations of HHD or GYP. In addition to this, the levels of mutation occurring after 250ppm WTC exposure were significantly higher than those that occurred in background plates that weren’t treated. The level of mutations present in bacteria exposed to 25ppm WTC were also larger than those encountered in HHD and GYP-treated cells. HHD, GYP, and WTC samples were handled in the same manner throughout the weighing of the materials, UV sterilization, and the making of reagent
solutions. Therefore, any differences noted in the rate of mutation taking place in WTC in relation to HHD and GYP would be due to the chemical properties of WTC rather than contamination.

Figures 20 and 21 each show the percentage of mutated colonies per 96 well plate used in this assay. Figure 20 compares the total percentage of mutations that occurred in wells containing the TA100 strain exposed to various concentrations of HHD, GYP, and WTC. Figure 21, on the other hand, compares the total percentage of mutations that occurred in wells containing the TA98 strain exposed to HHD, GYP, and WTC. The significance of mutations occurring in TA98 and TA100 colonies exposed to HHD, GYP, and WTC was based on the frequency of reverse mutation occurring in the background plate. Since the background plate contained 5 mutated TA100 colonies, these colonies were subtracted from the total number of mutated colonies for the statistical analysis shown in Figure 22. As the background place for TA98 did not contain any mutated colonies, all mutated colonies present in plates containing TA98 were considered statistically significant.

As shown in Figure 22, statistical analysis determined that reverse-mutations occurring in 25ppm and 250ppm WTC plates were most likely due to mutagenic effects of the heterogeneous WTC mixture rather than chance. Results and analysis of HHD and GYP plates indicate that GYP and HHD are unlikely to be mutagenic. The small increase in mutagenicity noted in TA-98 bacteria exposed to 250ppm HHD is not statistically significant. However, according to previous studies, it is still possible that HHD has some mutagenic properties. This can be attributed to the presence of trace amounts of cleaning products and pesticides present in common households (Lioy 2002b).
The statistical analysis shown in figure 22 was performed according to methods described in the Muta-Chromoplate Version 3.3 protocol, indicate a very low likelihood that the mutations taking place in the presence of 25ppm and 250ppm WTC were due to chance. The same can be said for the mutations occurring in 25ppm WTC. As noted in Figure 21, the possibility of mutations occurring due to chance in 250ppm concentrations in TA98 and TA100 colonies was significantly lower than other compounds, save for the known mutagens 2-nitrofluorene and sodium azide. Mutations to colonies exposed to 250ppm WTC were more consistent with mutations occurring in the presence of these known mutagens than they were for all HHD, all GYP, or 25ppm WTC. This suggests that 250ppm concentrations of WTC may be mutagenic.

The results of this assay, though a strong indication that WTC may be mutagenic in *in vivo* systems was limited by its use of prokaryotic cells. Eukaryotic systems, unlike prokaryotic systems, contain enzymes that are capable of metabolizing xenobiotics and either detoxifying them or activating them. Though the liver is the primary site of xenobiotic metabolism, lung cells contain a significant level of metabolizing enzymes as well (Hodgson 2004). Rat liver extract was not used in this assay. Therefore, it is possible that some components that would have been activated within human lung systems were not activated or detoxified in this assay.

WTC is a heterogeneous mixture containing components, such as polycyclic aromatic hydrocarbons which can become more reactive following activation (Ducatti 2003, Lioy 2002a). Once activated, these compounds can act as tumor activators and lead to cancer. However, there are also components of WTC that may become less reactive following activation. Though metabolism continually occurs in the lungs, there is also a
possibility that inhaled materials can damage cells and interfere with function before metabolism can occur (Hodgson 2004). The results of this assay demonstrate that WTC has the potential to induce mutation before it is metabolized in the lungs, as opposed to HHD and GYP.

**Conclusions**

Combined, these assays demonstrated that WTC may have a physiological impact on living systems and possibly induce DNA mutation *in vivo*. The particle size analysis conducted in this study included WTC, HHD, and GYP samples that contained particles small enough to penetrate deeply into the lung. Therefore, it is reasonable to conclude that at least a portion of the HHD, WTC, and GYP samples used in this study could have been inhaled or ingested had they been suspended in air (Cohen 2000). Though WTC contains a number of volatile and carcinogenic agents, it is possible that the sole physical presence of inhaled WTC within the lungs was enough to induce the health issues experienced by rescue workers. However, the results of this study demonstrated that the chemical composition of WTC may be a factor that led to the decreases in respiratory health experienced by rescue workers.

The results of the proliferation assay demonstrated that WTC has a negative impact on cell viability and that cell viability decreases with increasing WTC concentrations. This decrease in cell viability may decrease the ability for lung cells to repair damage incurred by inhaled particles. This, in turn, may lead to a possible prolongation of inflammatory response and subsequent increase in cancer risk (Clarke 1999). The direct link between increases in WTC exposure and decreases in cell viability mirrored *in vivo* observations that correlated increasing exposure to World Trade Center
dust to decreasing respiratory health (Herbstman 2005). The results of this study suggest that WTC has a negative effect on cell function and proliferation in vitro and that this effect is magnified with increasing WTC exposure. This may prove deleterious in in vivo systems since it implies that increased exposure to WTC may inhibit cell repair.

The apoptosis, proliferation, and reverse-mutation assays demonstrated that the chemical composition of WTC may be an important factor in causing cellular damage and mutation. Therefore, the health issues experienced by rescue workers may be due to damage on the DNA level. The apoptosis assay demonstrated that WTC induced apoptosis in cells. Since cells commit apoptosis in response to damage, it can be implied that WTC damages cells to the point where they can no longer function normally. Such damage could easily disrupt normal cell function and could be severe enough to induce necrosis or aid in the development of cancer. Extreme cellular damage can prevent apoptosis from occurring, which may lead to necrosis and even cancer. In the event that necrosis does occur, prolonged exposure to WTC may continue result in the continual release of cell contents into the surrounding tissue environment over time (Majno 1995). This may, in turn, eventually lead to chronic inflammation which could, in turn, lead to the development of cancer over time (Coussens 2002).

The mechanisms behind the health issues experienced by rescue workers and New York City residents are still unknown. Chronic inflammation can result in an increased risk for cancer, as can increases in inhaled air pollution. In both instances, the body’s immune system can be the most antagonizing agent in the development of cancer. As was noted in the proliferation assay, physiological stress can magnify any potential harmful effects of a xenobiotic in in vivo systems by either suppressing immune system function
or inhibiting replication and repair in cells. By inhibiting replication, a system may decrease the incidence of cancer and increased mutation. However, decreased levels of replication may make it harder for torn or damaged lung tissue to be replaced by newly formed cells. Following the collapse of the World Trade Center, rescue workers inhaled abrasive particles, such as pulverized glass, which could have torn cell membranes and punctured lung tissue (Lioy 2002a). In normal lungs, undamaged cells will replicate until the wounds are covered up. However, increased rates of apoptosis and decreased rates of proliferation, as seen in this study, would inhibit the growth of healthy cells and subsequent wound healing.

Previous studies have demonstrated that increases in air pollution such as World Trade Center dust is linked with cancer (Rayne 2005). Though a link between WTC and cancer has yet to be made, the results of the assays implemented in this study imply that WTC may induce DNA damage. The health issues experienced by rescue workers and New York City residents varied in severity and are not yet fully understood. However, the increased rates of apoptosis noted in human cells exposed to WTC supports the possibility that human cells are susceptible to damage induced by WTC. The reverse-mutation assay employed in this study supports the possibility that WTC damages cells. More importantly, however, the reverse-mutation assay demonstrates that mutations to DNA can occur in the presence of WTC.

Though more research is needed to properly identify the potential risk of WTC to exposed rescue workers and residents, the results in this study suggest that WTC may damage cells in a way that leads to decreased cell viability and induces DNA damage. This is especially relevant to rescue workers and residents who were exposed to and
inhaled vast quantities of WTC since this exposure may lead to latent damage that may one day lead to cancer or other upper respiratory diseases. Though more research is needed to fully characterize the mutagenic effects of World Trade Center dust, this study’s identification of WTC as a potential mutagen may lead to better identification of health hazards incurred by rescue workers as well prevention of health hazards that may manifest themselves in the future without proper care or medical attention.

**Future Research**

Though *S. typhymurium* reverse mutation assays have been used by medicine and industry for several decades to determine mutagenicity, bacterial systems do not perfectly mimic eukaryotic systems and more research is needed to determine the level of DNA damage that occurs in human cell cultures exposed to WTC. Though these results suggest that WTC may be a mutagen, more research is needed to understand cell responses to the various materials present in WTC. One of the limitations of the reverse mutation assay implemented in this study was the lack of rat liver extract. Future reverse mutation studies should implement rat liver extract to best mimic the metabolism induced by eukaryotic lung systems. Utilization of rat liver extract would allow for a more accurate idea of how mutagenic WTC may be in human lung systems where some metabolism of xenobiotics occurs.

Future research should also focus on better understanding the effects of WTC, HHD, and GYP on the cellular and molecular level. Protein expression in cells exposed to WTC, HHD, and GYP should be explored in order to determine how gene expression changes in response to WTC. This may lead to a better understanding of the different
pathways that are induced when cells come in contact with WTC. This will, in turn, elucidate how the health of rescue workers and residents was impacted by the inhalation of WTC over time. Therefore, a microchip array may be useful in determining protein expression in cells exposed to WTC and controls.

Though HHD and GYP were similar in structure to WTC, any differences and similarities in chemical composition in comparison to WTC must be determined. Therefore, a chemical analysis should be performed to better ensure that these controls are suitable for this study. Since particles larger than 10 microns are normally not inhaled into the lungs, GYP chemical analysis may not be necessary. However, most of the HHD particles analyzed are within a range comparable to the smallest particles of WTC that were inhaled by rescue personnel.
Bibliography


10. EBPI. 2009. Muta-Chromoplate Bacterial Strain Kit Version 3.3 Instructions for Use. 1-16.


