Introduction of Luminol-dependent Chemiluminescence as a Method to Study Silica Inflammation in the Tissue and Phagocytic Cells of Rat Lung

James M. Antonini, Knox Van Dyke, Zuguang Ye, Michael DiMatteo, and Mark J. Reasor

Department of Pharmacology and Toxicology, Robert C. Byrd Health Sciences Center of West Virginia University, Morgantown, West Virginia

The inhalation of silica has been shown to produce a dramatic inflammatory and toxic response within the lungs of humans and laboratory animals. A variety of cellular and biochemical parameters are used to assess the silica-induced lung injury. The purpose of this paper is to introduce the use of luminol-dependent chemiluminescence as a new method to study inflammation in both phagocytic cells and lung tissue recovered from silica-exposed animals. Chemiluminescence, or the emission of light, accompanies the release of reactive forms of oxygen when phagocytic cells are challenged. In this study, male Fischer 344 rats were intratracheally instilled with either silica (10 mg/100 g bw) or saline vehicle. One day after the instillations, a marked increase in the chemiluminescence was observed in the lung tissue and bronchoalveolar lavage cells recovered from the silica-treated animals when compared with the saline controls. The light reaction was markedly decreased by either superoxide dismutase or N-nitro-L-arginine methyl ester hydrochloride. Superoxide dismutase is involved in the enzymatic breakdown of superoxide anion, while N-nitro-L-arginine methyl ester hydrochloride, a nitric oxide synthase inhibitor, prevents the formation of nitric oxide. When superoxide anion and nitric oxide react, they form the highly oxidizing substance peroxynitrite. This study implicates peroxynitrite as an agent that may be responsible for some of the oxidant lung injury that is associated with silica exposure. The use of luminol-dependent chemiluminescence may prove valuable as a method to measure the earliest events in the inflammatory process, and may be an adjunct in studying the mechanisms that produce inflammation. — Environ Health Perspect 102(Suppl 10):37–42 (1994)

Key words: luminol-dependent chemiluminescence, silica, peroxynitrite, inflammation, alveolar macrophages, neutrophils

Introduction

Research on inflammation has not produced a method that links the initial events of the process to the controlling events. Rather, a variety of parameters are measured that are the result of and not the basis of the inflammatory reaction. Parameters such as tissue weight, edema, blood flow, enzyme and protein release, or production of metabolites (prostaglandins, leukotrienes) have been used extensively. A method is needed that could be applied to cells of a particular type, groups of cells of different types, and even tissue, in an attempt to study the kinetics of the inflammation as well as being sensitive enough to monitor the early events of the process. Since inflammation is so complicated, the relationship between the control of the initial events and the oxidation process has never been explained nor studied in any detail.

We have been studying inflammation by the intratracheal instillation of silica into the lungs of rats. Exposure of the respiratory epithelium to silica particles results in inflammation, damage to the respiratory epithelium and interstitial matrix, and fibrosis (1). The cytotoxic and fibrogenic activity appears related to the rupture of the lysosomal membrane of the alveolar macrophage and the release of lysosomal enzymes into the cytoplasm. Following lysis of the macrophage, the free silica particles are once again released to be ingested by fresh macrophages, some of which are in turn also killed (2). Thus, unlike most other particulates, silica is not completely detoxified by macrophage phagocytosis. Instead, silica initiates a cycle of ingestion and cytotoxicity thought to be the basis for the chronic toxicity associated with silica exposure (3).

Studies in our laboratory indicate that the negative surface of silica binds calcium (4). Excessive calcium is carried into the phagocytic cells during phagocytosis leading to a dramatic loss of cell viability. If calcium is chelated internally in the phagocytic cell, the toxicity is decreased. This may be evidence indicating that the silica-induced inflammation is associated with an increase in intracellular calcium. This process may occur within minutes to hours of the initial insult.

When phagocytic cells (macrophages and neutrophils) are challenged, they release reactive forms of oxygen, accompanied by chemiluminescence, or the emission of light. Initial techniques of cellular luminescence required a large number of cells. To study the oxidative processes using smaller numbers of cells, our group developed a method in the mid-1970s known as luminol-dependent chemiluminescence. Luminol (3-aminoephahalhydrizide), a cyclic hydrazide, chemiluminesces upon reaction with some oxidizing species. In our laboratory, this luminol-dependent chemiluminescence has been useful in studying a variety of dis-
ease states including chronic granulomatous disease, opsonization defects in humans, and cellular defects in cells associated with leukemia and chronic bacterial infection (5).

Although a variety of phagocytic cells could be triggered to produce cellular light, a scientific weakness of this method was the failure to determine the chemistry of the chemicals which initiate the chemiluminescent process. A number of substances such as singlet oxygen, superoxide anion, hypochlorous acid, hydroxyl free radical, and hydrogen peroxide had been studied, but many questions still remain unanswered concerning the role of these agents in the process of chemiluminescence.

However, in a study by Wang et al. (6) using phagocytic Kupffer cells from the liver, they discovered that luminol may produce light upon reaction with peroxynitrite (OONO'). It is formed from two free radical gases, superoxide anion and nitric oxide. Neither of these free radicals is particularly toxic alone, although the product of these two free radicals is a very strong oxidizing agent. Nitric oxide can be generated by the cytosolic enzyme nitric oxide synthase. This enzyme exists in two forms, i.e., a constitutive and an inducible form. In pulmonary phagocytes, the activity of the constitutive form of nitric oxide synthase is low. However, the enzyme can be induced in macrophages and neutrophils by a variety of stimulants such as lipopolysaccharide, interferon gamma, chemotactic peptide, platelet activating factor, or leukotriene B(4) (7,8). Once generated, nitric oxide can combine with superoxide anion to form peroxynitrite. Peroxynitrite possesses 1000 times the oxidative ability of hydrogen peroxide (9). Peroxynitrite also has been shown to oxidize sulphydryl groups and to inactivate α1-proteinase inhibitor making the lung more susceptible to damage caused by proteolytic enzymes (10). It is reasonable to believe that phagocytic cells activated by silica may produce large amounts of peroxynitrite, that in turn may lead to oxidant lung injury.

In this investigation, we introduce the use of luminol-dependent chemiluminescence as a method to study inflammation in both phagocytic cells and particularly lung tissue. Male Fischer 344 rats were intratracheally instilled with silica or saline (vehicle control). We compared a variety of cellular and biochemical parameters 24 hr after the instillations with luminol-dependent chemiluminescence to demonstrate the usefulness of this technique in examining the initial events of cellular and tissue inflammation. An attempt also was made to determine the oxidizing agent(s) that may be responsible for the silica-induced light generation.

Materials and Methods

Drugs and Chemicals

Crystalline min-U-sil silica (US Silica Corp., Berkeley Springs, WV) was a gift from Val Vallyathan (National Institute for Occupational Safety and Health, Morgantown, WV). Purity of the silica was determined by automated X-ray diffractometry and was 99.5% α-quartz. Size fraction < 5 μ in diameter was made by a centrifugal airflow particle classifier. Ninety-eight percent of this fraction was < 5 μ in size with a median area equivalent diameter of 3.5 μm as estimated by automated scanning electron microscopic image analysis. These are particles of respirable size. Enzyme reagents were purchased from Sigma Chemical Company (St. Louis, MO). Other chemicals used in this study were from Fisher Chemical Company (Pittsburgh, PA).

Animals

Male Fischer 344 rats weighing 200 to 250 g were obtained from Hilltop Laboratories (Scottsdale, PA). Rats were given a conventional laboratory diet (Purina Chow pellets) and tap water ad libitum during a 5-day acclimation period.

Silica Treatment

Before silica was instilled in the animals, it was cleaned by boiling in 1.0 M HCl for 60 min. The silica was suspended and sonicated for 15 min in 0.9% sterile saline at a concentration such that each rat received a constant dose volume of 0.5 ml. One half of all rats used in this study were dosed intratracheally with a single instillation of 10 mg/100 g bw of silica with a mean particle size of < 5 μm. As a result of some preliminary studies, this dose was shown to cause significant acute pulmonary inflammation and toxicity. The remaining animals were instilled with an equal volume of sterile saline (vehicle control).

The rats were lightly anesthetized by ip injection of 0.5 ml of a 1% solution of sodium methohexitol (Brevital, Eli Lilly and Co., Indianapolis, IN). Once anesthetized, the intratracheal instillations were performed according to the method of Brain et al. (11). The rat was placed on a slanted board and was supported by a wire under its upper incisors. The tongue of the animal was moved aside with a hemostat, and the larynx was illuminated by a modified laryngoscope. Each rat then was intratracheally instilled with either the saline containing silica or the saline vehicle using a No. 20 gauge, 2 in animal feeding needle (Popper and Sons, Inc., New Hyde Park, NY).

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed on the animals 24 hr after the intratracheal instillations. The rats were deeply anesthetized with an overdose of sodium pentobarbital (Butler Co., Columbus, OH) and exsanguinated by severing the abdominal aorta. The lungs of each rat were first lavaged with one separate aliquot of warm, calcium- and magnesium-free Hanks' balanced salt solution (HBSS), pH 7.4, that was left in the lungs for 30 sec, aspirated, and replaced for an additional 30 sec and then withdrawn. A volume of 2.0 ml/100 g of animal body weight was used for this lavage to take into account any variations in body weights of the rats lavaged. This lavage sample was centrifuged at 500g for 7 min, and the resultant cell-free supernatant was analyzed for various biochemical parameters. Additionally, the lungs were further lavaged 10 times with 5-ml aliquots of HBSS. These samples were also centrifuged for 7 min at 500g and the cell-free lavage fluid discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 2 ml of HBSS. The cells were then subjected to counting and differentiation.

Cellular Evaluation

Total cell numbers were determined using a hemacytometer. Using a Shandon cytospin centrifuge, 1.5 x 10⁶ cells were spun for 4 min at 400 rpm and pelleted onto a slide. Cells (200/rat) were differentially stained on the cytospin-prepared slides after staining with Wright-Giemsa Sure Stain (Fisher Scientific, Pittsburgh, PA). Alveolar macrophages, neutrophils, and lymphocytes were counted.

Biochemical Assays

The protein content of cell-free bronchoalveolar lavage fluid samples was determined by the method of Hartree (12) using bovine serum albumin as standard. Enzyme activities in the cell-free bronchoalveolar lavage fluid were assayed as follows: β-glucuronidase by the method of Lockard and Kennedy (13) and lactate dehydrogenase according to Wroblewski and LaDue (14).

Chemiluminescence

Chemiluminescence was measured with a Berthold LB9505C Luminometer (Wildbad, Germany). Chemiluminescence was fol-
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Figure 1. The phorbol myristate acetate (PMA)-stimulated chemiluminescence of lung tissue recovered from rats 1 day after intratracheal instillations of silica (10 mg/100 g bw) or saline. N-nitro-L-arginine methyl ester hydrochloride (L-NAME) and superoxide dismutase (SOD) were used as inhibitors of the light reaction. Values are mean ± SEM (n = 4). Mean value of the PMA-stimulated chemiluminescence of the silica-treated lungs was significantly greater than the mean value of the saline-treated lungs (*p < 0.05). Mean value of the PMA-stimulated chemiluminescence after the addition of L-NAME of the silica group was significantly less than the mean value of the PMA-stimulated chemiluminescence in the absence of L-NAME (*p < 0.05). Mean values of the PMA-stimulated chemiluminescence after the addition of SOD in the silica and saline groups were significantly less than the mean value of the PMA-stimulated chemiluminescence in the absence of SOD (*p < 0.05).

Figure 2. The phorbol myristate acetate (PMA)-stimulated chemiluminescence of bronchoalveolar lavage fluid cells recovered from rats 1 day after intratracheal instillations of silica (10 mg/100 g bw) or saline. N-nitro-L-arginine methyl ester hydrochloride (L-NAME) and superoxide dismutase (SOD) were used as inhibitors of the light reaction. Values are mean ± SEM (n = 4). Mean value of the PMA-stimulated chemiluminescence of the silica-treated animals was significantly greater than the mean value of the saline-treated animals (*p < 0.05). Mean values of the PMA-stimulated chemiluminescence after the additions of L-NAME or SOD in the silica group were significantly less than the mean value of the PMA-stimulated chemiluminescence in the absence of L-NAME and SOD (*p < 0.05).

Figure 3. Total number of each cell type recovered from the lungs of rats 1 day after the intratracheal instillations of silica (10 mg/100 g bw) or saline. Alveolar macrophages (AMS), neutrophils (PMNS), and lymphocytes (LYMPHS) were differentiated. Values are mean ± SEM (n = 4). Mean values of the silica-treated animals were significantly greater than mean values of the saline-treated animals (*p < 0.05).

Cellular Chemiluminescence of Bronchoalveolar Cells. Bronchoalveolar cells were recovered from the lungs as described above. Cell number was adjusted to 10^6/ml. Luminol (10^-4 M) was first dissolved in DMSO and then diluted to 10^-3 M in physiologic HEPES buffer (0.1 M, pH 7.4). Zymosan (0.2 mg/ml) and phorbol myristate acetate (PMA, 10^-5 M) were used as stimulants for the chemiluminescence reaction. Zymosan particles were opsonized with rat serum by incubating the particles and serum for 30 min at 37°C, then centrifuged, and resuspended in HEPES buffer. Nonopsonized zymosan was suspended in the HEPES buffer. The final volume in each cuvette was 500 μl (100 μl cells, 100 μl luminol, 100 μl PMA or zymosan, and 200 μl HEPES buffer). Inhibitors of chemiluminescence were also used in some of the reactions. Superoxide dismutase (SOD, 5 mg/ml) or N-nitro-L-arginine methyl ester hydrochloride (L-NAME 5 x 10^-3 M) were added in a volume of 100 μl in place of 100 μl of HEPES buffer. The final concentrations then were 1 mg/ml for the SOD and 1 mM for the L-NAME. When these inhibitors were used, they were incubated with the cells for 10 min at 37°C before stimulation with the PMA.

Nonlavaged Chopped Lung Chemiluminescence. Nonlavaged lungs were removed by surgical technique post-exsanguination of the abdominal aorta 24 hr after the instillations. The lungs were placed on a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Brinkmann, Westbury, NY). The tissue micrometer was set at 5.0, that produced slices about 0.5 millimeters thick, and the lungs chopped. The base was turned 90°, chopped, and repeated again. The cut lung portions (150 mg) were placed in luminometer cuvettes containing HEPES buffer (150 μl), luminol 10^-3 M (100 μl), and PMA 10^-5 M (100 μl). When inhibitors (SOD, 5 mg/ml; L-NAME, 5 x 10^-3 M) of the light reaction were studied, 100 μl of each was used in place of an equal volume of HEPES buffer, and they were incubated for 10 min at 37°C with the lung tissue samples before the PMA was added. The final concentrations for the inhibitors in the cuvette were 1 mg/ml for the SOD and 1 mM for the L-NAME.

Statistical Analysis

A comparison was made between the silica and saline treatment groups using the Student's t-test to analyze cellular, biochemical, and chemiluminescence data. For Figures 1 and 2, an analysis of variance (ANOVA) was used. If significance was observed among the treatment groups in these two figures, the post-hoc, Student-Newman-Keuls test was used to determine significantly different groups. For all comparisons in this investigation, p < 0.05 was used as the level of significance.

Results

In these studies, male Fischer 344 rats were intratracheally instilled with silica (10 mg/100 g bw) or saline vehicle. Bronchoalveolar lavage was performed on the animals 24 hr after the instillations. To assess the inflammation of the lungs, the cells recovered from the animals of the two treatment groups were counted and differentiated. Significant elevations in the total number of cells, neutrophils, and lymphocytes were observed for the silica group when compared with the saline group (Figure 3). Within the acellular lavage fluid, a number of biochemical parameters indicative of lung damage were assessed. The silica instillation caused significant increases in total protein, lactate dehydrogenase activity, and β-glucuronidase activ-
Figure 4. Total protein of the cell-free bronchoalveolar lavage fluid 1 day after the intratracheal instillations of silica (10 mg/100 g bw) or saline. Values are means ± SEM (n = 4). Mean values of the silica-treated animals were significantly greater than mean values of the saline-treated animals (*p < 0.05).

Figure 5. Lactate dehydrogenase (LDH) activity of the cell-free bronchoalveolar lavage fluid 1 day after the intratracheal instillations of silica (10 mg/100 g bw) or saline. Values are means ± SEM (n = 4). Mean values of the silica-treated animals were significantly greater than mean values of the saline-treated animals (*p < 0.05).

Figure 6. Beta-glucuronidase (BETA-GLU) activity of the cell-free bronchoalveolar lavage fluid 1 day after the intratracheal instillations of silica (10 mg/100 g bw) or saline. Values are means ± SEM (n = 4). Mean values of the silica-treated animals were significantly greater than mean values of the saline-treated animals (*p < 0.05).

Figure 7. A typical phorbol myristate acetate (PMA)-stimulated chemiluminescence 20-min tracing of lung tissue recovered from rats 1 day after the intratracheal instillations of silica (10 mg/100 g bw) or saline.

Figure 8. A typical phorbol myristate acetate (PMA)-stimulated chemiluminescence 20-min tracing of bronchoalveolar cells recovered from rats 1 day after the intratracheal instillations of silica (10 mg/100 g bw) or saline.

Figure 9. The zymosan-stimulated chemiluminescence of bronchoalveolar cells recovered from rats 1 day after the intratracheal instillation of silica (10 mg/100 g bw) or saline. Values are means ± SEM (n = 4). Mean values of the zymosan-stimulated chemiluminescence of the silica-treated animals were significantly greater than the mean values of the saline-treated animals (*p < 0.05).

From these results, an inflammatory and toxic response within the lungs was demonstrated with this dose of silica 24 hr after the instillation exposure.

The luminol-dependent chemiluminescence of cells and tissue recovered from the lungs of rats 24 hr after the instillation of silica and saline vehicle then was measured. When the soluble stimulating agent PMA was added to 150 mg tissue samples from animals of the two treatment groups, a significant increase in the chemiluminescence was demonstrated in the tissue from the silica animals when compared with the saline group (Figure 1). If the lung samples were incubated for 10 min with L-NAME, a nitric oxide synthase inhibitor, a 33% and 37% reduction in the PMA-stimulated light reaction was seen for the silica and saline groups, respectively. When the samples were incubated with SOD, a 94% decrease in chemiluminescence was observed for the silica treatment group, while the saline group showed a 96% reduction.

A significantly greater chemiluminescence also occurred when PMA was added to cells lavaged from the lungs of the animals instilled with silica as compared to the group receiving saline (Figure 2). The addition of L-NAME and SOD to the cells recovered from the silica groups caused a 60% and 67% reduction in the PMA-stimulated light emission, respectively. The incubation of the cells removed from the saline-treated animals with L-NAME caused a 37% decrease in PMA-stimulated chemiluminescence, while SOD caused a 65% attenuation.

In Figure 7, a representative 20-min tracing from one experiment depicts the kinetics of the PMA-stimulated chemiluminescence of lung tissue recovered from a silica-treated and a saline-treated animal. From this figure, a substantially elevated light reaction is observed in the tissue of the silica-exposed animal when compared with the tissue from the animal instilled with saline. In Figure 8, a similar response is demonstrated when the chemiluminescence of bronchoalveolar lavage cells are compared in silica- and saline-treated animals.

In Figure 9, bronchoalveolar lavage cells recovered from the lungs of the animals from the two treatment groups were activated with the insoluble stimulant, zymosan. Zymosan is an extract of a yeast cell wall, Saccharomyces cerevisiae. In the unopsonized state, zymosan is readily phagocytized by macrophages, but the zymosan needs to be opsonized for neutrophil phagocytosis. When the cells then are stimulated with either opsonized or unopsonized zymosan, the individual contribution of the macrophages and the neutrophils to the chemiluminescence can be estimated. The light generated when unopsonized zymosan is used represents the
macrophage-activated chemiluminescence, while the stimulation of the cells with the opsonized zymosan represents the neutrophil- and macrophage-activated chemiluminescence. The cells then recovered from the silica-treated animals had a major and significant elevation in chemiluminescence when stimulated with either opsonized or opsonized zymosan as compared with cells from saline-instilled animals.

**Discussion**

Inflammation has been defined as a tissue irritative process that produces the classic signs of swelling, pain, redness, and heat with possible loss of function (15). The actual basis for these symptoms has never been delineated. Certainly, the phagocytic cells (macrophages, neutrophils, eosinophils, and basophils) play a major role in this process in the blood, attached to blood vessels, and in tissues. Furthermore, the process of oxidation, defined by the addition of oxygen, the loss of hydrogen, or the loss of electrons from an organic molecule, plays a definitive role in inflammation as attested by the production of prostaglandins and leukotrienes from arachidonic acid and release from phospholipids.

A method is needed to measure the cellular and tissue events related to the oxidative process. Such a method is luminol-dependent chemiluminescence. The major weakness in measuring the luminol-based light emitted from cells was that it was unclear what produced the blue light (425 nm) emitted from the oxidation of luminol. The substances proposed to produce chemiluminescence were singlet oxygen, superoxide anion, hydrogen peroxide, hypochlorous acid, and hydroxyl free radical. A complete analysis of the various oxidants mentioned has revealed that none of these substances or any free radical tested causes luminol to produce blue light at the physiologic pH of 7.4 (9,16).

Wang et al. (6) have identified the first substance to produce chemiluminescence directly without any enzyme involved. They noted that peroxynitrite anion could react directly with luminol and produce light at physiologic pH. Ral et al. (9) further defined the reaction and showed a quantitative relationship between peroxynitrite and excess luminol in the production of light. The peroxynitrite is formed from two free radicals, superoxide anion, and nitric oxide. Peroxyxinitrite has a very short half-life at physiologic pH, but has been shown to effectively kill bacteria.

It then can be hypothesized that active peroxides, such as peroxyxinitrite, play a significant role in the inflammatory process, and the luminol-dependent chemiluminescence produced at physiologic pH may measure this process. If this is the case, an increase in oxidation caused by the active peroxides would be detectable as an ongoing process in both early and late inflammation. It would occur in both cells and tissues. Luminol is a very lipid-soluble substance that can penetrate cells and tissue easily. Possibly, the luminol-dependent chemiluminescence could be used in various ways to measure luminescence in single phagocytic cells, groups of cells, and cells bound to or located within tissue.

To test the usefulness of luminol-dependent chemiluminescence, a substance would be needed that could create constant inflammation. For our investigation, silica was used as the irritating agent. The cytotoxic and inflammatory nature of inhaled silica has been well studied and reviewed (17). In a number of preliminary studies, we noted a tremendous discrepancy in light production between *in vivo* and *in vitro* experiments where silica was added to macrophages lavaged from normal animals versus intratracheally instilling silica in the lungs of animals and evaluating the lavage cells that were removed 24 hr later. The PMA-stimulated chemiluminescence of the cells removed from the animals instilled with silica was raised 10-fold when compared with adding silica to cells that were removed from normal animals.

To further study this interesting observation, we then set up a complete set of experiments in which luminol-dependent chemiluminescence was used to study the silica-induced inflammatory response. Male Fischer 344 rats were instilled with either silica (10 mg/100 g bw) or saline vehicle. The animals were lavaged 24 hr after the instillation, and a variety of methods were then used to measure the pulmonary inflammation. When a number of parameters indicative of lung injury were assayed from the acellular bronchoalveolar lavage fluid, significant elevations were seen in total protein and lactate dehydrogenase and β-glucuronidase activities in the samples from the silica-treated animals as compared to the saline-treated group. The silica instillation also caused increases in the recovery of total cells, neutrophils, and lymphocytes when compared to animals which received saline. Clearly, substantial inflammation was present in the lungs when evaluated by these classical techniques.

Luminol-dependent chemiluminescence was demonstrated to correlate with the cellular and biochemical parameters of inflammation. In measuring the luminol-dependent chemiluminescence of the bronchoalveolar lavage cells of the two treatment groups, an enhanced PMA-stimulated light reaction was demonstrated in the cells from the silica-treated group.

Since the differential of the cells lavaged from the lungs of the two treatment groups was different, a method to separate the response of the individual groups of cells was needed. It is known that for neutrophil phagocytosis, opsonization is required, while macrophages can phagocytize opsonized as well as unopsonized particles (18). Thus, by stimulating the cells with opsonized and unopsonized zymosan, the chemiluminescence response of neutrophils and macrophages can be separated. Our results indicate that both the macrophages and neutrophils are stimulated to produce light, and this light production is greatly enhanced in the lungs exposed to silica as compared to saline. It is of interest to note that the alveolar macrophage to neutrophil ratio 24 hr after silica instillation was 1:4. However, the ratio of the chemiluminescence in response to unopsonized versus opsonized zymosan is 2:3. These data suggest that after silica exposure more light is generated per cell from the macrophages than from the neutrophils. In contrast, with saline controls, neutrophils seem to generate more chemiluminescence per cell than alveolar macrophages. It may be possible that neutrophils harvested from silica-treated rats are spent and less able to respond to zymosan *in vitro*.

When the luminol-dependent chemiluminescence of the nonlavaged lung tissue was measured, an increase in PMA-stimulated light also was demonstrated in the samples from silica-treated animals. Tissue chemiluminescence is not only a measure of the phagocytic cells within the airways and airspaces, but also a measure of the phagocytic cells that are activated and cannot be removed by bronchoalveolar lavage or are located within the interstitium of the lungs.

The light from both phagocytic cells and lung tissue was remarkably decreased by the inhibitors SOD and L-NAME, a nitric oxide synthase inhibitor. The enzymes nitric oxide synthase and NADPH oxidase are responsible for the production of nitric oxide and superoxide anion, respectively. These two chemicals have been shown to produce peroxyxinitrite. The inhibition of the light reaction indicates that the enzyme activity responsible for the formation of peroxyxinitrite may be markedly increased or induced during an inflammatory insult. It is known from earlier studies that the
inflammatory mediators gamma interferon and lipopolysaccharide increase lumi- noin-dependent chemiluminescence of cultured phagocytic cells (19). There then may be a coordinate induction of nitric oxide synthase and NADPH oxidase in the phago-
cytic cells and tissues recovered from the lung of animals treated with silica. In addition, it is known that a variety of in-
flammatory and fibrogenic factors, such as interleukins, tumor necrosis factor, platelet-
derived growth factor, macrophage-derived growth factor, and fibronectin are all dra-
"matically increased in the presence of silica (17,20,21). Clearly, the possibility exists for a common induction mechanism of the inflammatory cascade itself.

We believe that the NF-Kappa B trans-
fraction protein within macrophages may be responsible for the coordinate induction of a variety of inflammatory genes. NF-
Kappa B is formed of three elements: p50 protein, p65 protein, and i Kappa B (an inhibitory factor). When oxidative metabo-
lism is activated, the inhibitory factor sepa-
rates from the complex in the cytoplasm and the p50-p65 heterodimer migrates into the nucleus and turns on the genes of the inflammatory cascade (22). In mature macrophages, this mechanism is activated without protein synthesis, so it can act very quickly (23). We are currently using lumio-

nol-dependent chemiluminescence to study this process. In some preliminary results, an increase in the emission of light from lungs can be seen as early as 4 hr after the instillation of silica when compared to untreated or saline-treated animals (24).

From the findings of these experiments, the mechanism of activation of the inflammatory reaction within the lungs after exposure to silica may be hypothesized. The silica particles interact with the macrophages in the lungs, and the i Kappa B protein sepa-
rates from the NF-Kappa B complex, lead-
ing to an induction of enzymes and protein synthesis in addition to increases in the vari-
ous inflammatory mediators. Peroxynitrite is then formed resulting in the production of oxidant lung damage. The increase in damage caused by oxidation then may lead to cellular and tissue injury characterized by the appearance of vascular protein and cyto-
plasmic enzymes within the airspaces. Since the silica is not removed or detoxified by phagocytosis, the inflammation persists within the lungs leading to the eventual development of fibrosis.

The purpose of this study was to intro-
duce lumi-nol-dependent chemilumines-
cence as a method to measure some of the earliest and continuous events in both acute and chronic inflammation. Our study demonstrates that the results using this technique correlate qualitatively with cellular and biochemical indices of inflammation. While these latter measures are static reflections of the source and results of the inflammatory process, lumino-

nol-dependent chemiluminescence is a dynamic reflection of the ongoing inflammatory reaction. Luminol-depen-
dent chemiluminescence can be applied to both cells and tissues, and may be an important adjunct in studying the mecha-
nisms that produce inflammation.

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