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Effect of Kerosene and Its Soot on the Chrysotile-Mediated Toxicity to the Rat Alveolar Macrophages

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In order to examine the pulmonary toxicity of kerosene oil and its combustion product (soot) in asbestos-exposed rats, various biochemical and chemical parameters were assayed. Treatment of rats with a single intratracheal dose of chrysotile asbestos (5 mg) and kerosene (50 μl) or its soot (5 mg) in combination led to an increased number of pulmonary alveolar macrophages (PAM), elevated levels of hydrogen peroxide, and thiobarbituric acid-reacting substances, alterations in the activities of primary (glutathione peroxidase and catalase) and secondary (glutathione reductase and glucose-6-phosphate dehydrogenase) endogenous antioxidant enzymes, and depletion in the levels of glutathione in PAM compared to the chrysotile, kerosene, or soot alone. These changes may indicate the generation of oxidative stress in the macrophages. The resulting oxidative stress may be subsequently critical in collapsing the cellular membrane, which may change the cell membrane permeability and may also damage the phagolysosomal membrane, thereby releasing the membrane bound enzymes as indicated by an increased leakage of intracellular acid phosphatase and lactate dehydrogenase. The injury to macrophages may trigger events that lead to lung fibrosis and/or malignancies in the exposed animals. This study may be helpful in understanding the etiology of certain clinical and pathological disorders in the population exposed simultaneously to both asbestos and kerosene or its combustion products.

INTRODUCTION

It is well established that occupational exposure to asbestos can result in a number of toxic manifestations ranging from simple inflammation to pulmonary fibrosis known as asbestosis, bronchogenic carcinoma, and mesothelioma (Mossman *et al.,* 1990; Rahman *et al.,* 1993). Several studies have suggested the role of reactive oxygen species (ROS) in asbestos-mediated diseases (reviewed in Kamp *et al.,* 1992; Vallyathan, 1994). Asbestos also appears to augment the mutagenic and carcinogenic effects of chemical carcinogens. Epidemiological and experimental studies clearly indicated a multiplicative effect of asbestos and cigarette smoke on the initiation of bronchogenic carcinoma (Selikoff *et al.,* 1980). Besides smoking, several other factors including kerosene oil can also influence the process of disease development in asbestos-exposed subjects (Arif *et al.,* 1992, 1993, 1994).

In a developing country, such as India, a substantial portion of its population continue to use kerosene as a domestic fuel because of its easy availability and subsidized rates; many industries also use kerosene oil as an organic solvent. Incomplete combustion of the kerosene oil generates large volumes of soot which contains various polynuclear aromatic hydrocarbons and aliphatic compounds (IARC, 1985; Johnson *et al.,* 1991; Arif *et al.,* 1992). Exposure to kerosene oil or its soot can cause several biochemical changes in the tissue which may lead to cardiopulmonary disorders (Noa and Illnait, 1987; Arif *et al.,* 1991). The mechanism of soot toxicity is not fully understood at present; however, in a recent report we have shown that soot particles can cause cytotoxicity to the rat pulmonary alveolar macrophages, and reactive oxygen metabolites could play a primary role in the cytotoxic process (Arif *et al.,* 1993). While large number of studies have been conducted on asbestos and cigarette smoke, only a few reports are available on the combined effect of asbestos and kerosene/soot on pulmonary system (Arif *et al.,* 1992, 1994).

The present study was, therefore, designed to investigate the effect of kerosene and its soot on the

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asbestos-mediated cytotoxicity to rat pulmonary alveolar macrophages (PAM) *in vivo* after 1, 4, 8, and 16 days of treatment by monitoring the leakage of cytoplasmic and lysosomal enzymes in the bronchoalveolar lavage. In addition, the oxidative stress in PAM was also assessed by measuring the generation of hydrogen peroxide (H_2O_2), lipid peroxidation (LPO), intracellular reduced glutathione (GSH), and the activities of various primary and secondary antioxidant enzymes known to regulate the cellular oxidative tone. The result of the present study showed that kerosene oil and its soot can exaggerate asbestos-induced toxic effects in rat alveolar macrophages which could alter cell functions leading to the development of pulmonary fibrosis and/or neoplasia.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase, glutathione reductase, hydrogen peroxide, phenol red, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (USA), and GSH, oxidized glutathione (GSSG), and NADPH were obtained from Sisco Research Laboratories (Bombay, India). All the other chemicals were of analytical grade.

Samples

Kerosene (bp 177–288°C) soot preparation and the particle size analysis of both soot and Indian chrysotile asbestos were described elsewhere (Arif *et al.,* 1992, 1993).

Treatment of Animals

Male albino rats from the Industrial Toxicology Research Center animal colony weighing 200 ± 10 g were intratracheally instilled with a single dose of 50 μl of kerosene oil/rat, kerosene soot (5 mg/rat, particle size <10 μm), Indian chrysotile (5 mg/rat, particle size <30 μm), or a mixture of both chrysotile and kerosene/soot in 1 ml of corn oil according to the procedure used in the previous studies (Arif *et al.,* 1992, 1993). These doses were chosen after a dose–response study (data not shown). Vehicle controls received only 1 ml of corn oil in similar fashion. Animals (four or five) were kept in a steel wire cage at a controlled temperature (22 ± 2°C) with a 12-hr light:dark cycle and had free access to the diet (Hindustan Lever Ltd., India) and water.

Isolation of Alveolar Macrophages

Animals were killed 1, 4, 8, and 16 days posttreatment with an ip overdose of pentobarbital and were lavaged *in situ* as described earlier (Arif *et al.,* 1993). Briefly, the lavage was centrifuged to separate the cells from the supernatant containing various surfactants and enzymes. In order to lyse the contaminating erythrocytes, the cell pellet was suspended briefly in cold deionized water, the isotonicity was recovered by adding normal saline (0.15 M), and the cells were centrifuged at 400 g for 10 min. The alveolar macrophages were suspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (0.1 M, pH 7.4). The cells were counted in hemocytometer, and their viability was evaluated with a trypan blue exclusion test (>95% viable cells). The macrophages were suspended in the Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline at the concentration of 1×10^6 cells/ml.

Biochemical Assays

Acid phosphatase (AP) (EC 3.1.3.2) and lactate dehydrogenase (LDH) (EC 1.1.1.27) in the extracellular lavage fluid were assayed by the methods of Moss (1984) and Wotton (1964), respectively. Glutathione peroxidase (GPx) (EC 1.11.1.9), glutathione reductase (GR) (EC 1.6.4.2), and catalase (CAT) (EC 1.11.1.6) in the cells were measured by the procedures of Lawrence and Burke (1976), Carlberg and Mannervik (1975), and Claiborne (1985), respectively. Glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) was also performed in the cells by the method of Zaheer *et al.* (1965).

Chemical Estimations

Lipid peroxidation. Thiobarbituric acid-reactive substances (TBARS) of lipid peroxidation were estimated by the method of Hunter *et al.* (1963) by incubating the alveolar macrophages (1×10^6 cells) with or without NADPH (0.4 mM), and Fe^{2+} (2.5 mM) in 0.1 M phosphate buffer, pH 7.4, for 1 hr at 37°C. The reaction was terminated by the addition of trichloroacetic acid (15%), 2-thiobarbituric acid (0.375%), and HCl (0.25 N) followed by heating at 90°C for 20 min. The samples were cooled down on ice and centrifuged at 10,000*g* for 5 min. The amount of malondialdehyde (MDA) was measured at 532 nm, and the level was calculated by using molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ for MDA.

Hydrogen peroxide. H_2O_2 production was measured by H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red as described by Pick and Keisari (1981). The treated and untreated

macrophages were incubated with 0.28 nM phenol red, 8.5 units horseradish peroxidase, 5.5 nM dextrose in 0.05 M phosphate buffer (pH 7.0). After 1 hr of incubation at 37°C, the reaction was stopped by adding 0.1 N NaOH and centrifuged at 800*g* for 5 min. The absorbance of the supernatant was observed at 610 nm against a reagent blank. The H_2O_2 concentration was measured according to the standard curve.

Reduced glutathione and protein. Intracellular reduced glutathione and protein were measured by the methods of Sedlak and Lindsay (1968) and Lowry *et al.* (1951), respectively.

Statistical Analysis

Statistical significance was calculated by Student's *t* test (two-tailed) and a value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSIONS

The present study demonstrates that kerosene and its soot increased the toxic potential of chrysotile in PAM measured through enhanced release of lactate dehydrogenase and acid phosphatase from the macrophages. Interestingly, chrysotile-induced imbalances in the levels of oxidants–antioxidants, e.g., lipid peroxidation, H_2O_2 production, GPx, GR, G6PD, and GSH, were further modulated by kerosene and its soot.

Exposure to chrysotile, kerosene, soot, and their combinations led to a gradual increase of PAM populations which was highest at Day 4 (Fig. 1). The maximum increase was noted with chrysotile and kerosene/soot combinations. This effect is considered to be a typical defensive lung reaction to a variety of particulate materials (Bitterman *et al.*, 1984). Various explanations have been attributed for this effect, which include (i) chemotaxis and migration of cells into lung alveoli, (ii) local proliferation, and (iii) decreased cell efflux or turnover. Attention has recently been paid on the local proliferation of macrophages as a predominant source of phagocytic cells. However, the relative contribution of the above factors to the overall macrophage population has yet to be established.

Our results showed an approximately twofold increase in the production of H_2O_2 in the case of combined exposures with a gradual increase in the ab-

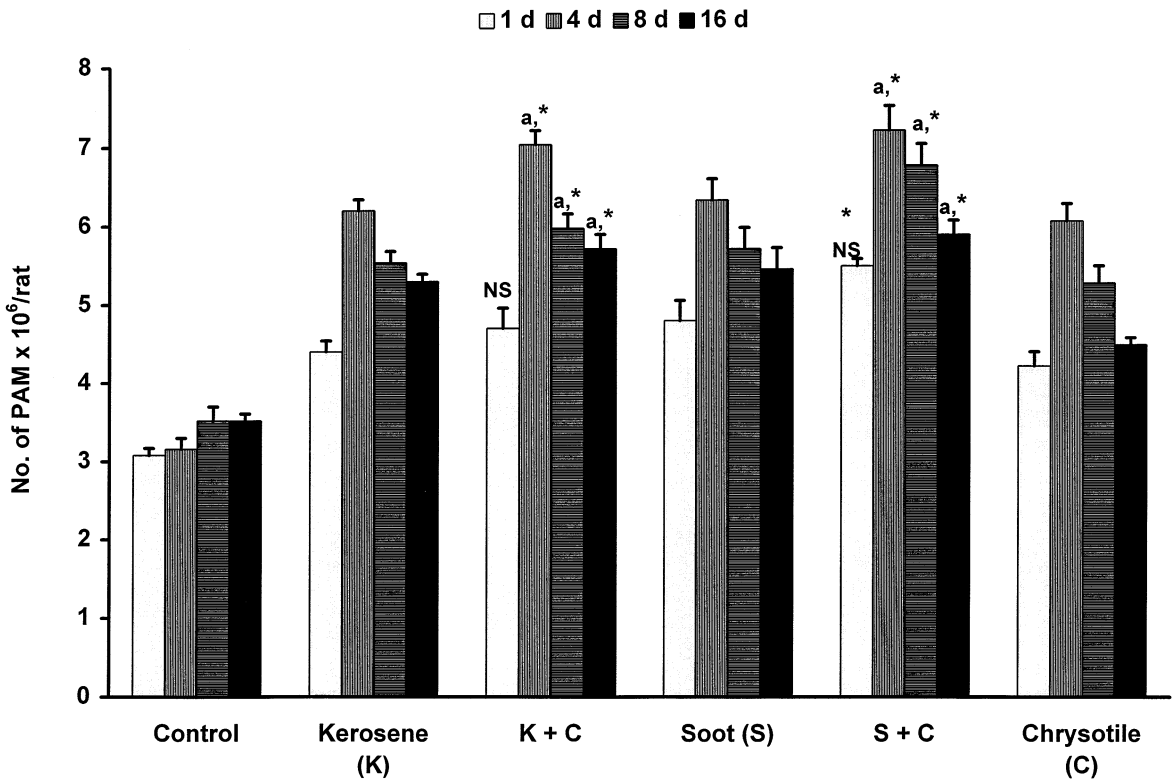

FIG. 1. Pulmonary alveolar macrophage populations in the experimental rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SE ($n = 6$). $^aP < 0.05$ when compared to respective kerosene or soot controls, and $^*P < 0.05$ when compared to chrysotile alone. NS, not significant compared to kerosene or soot controls.

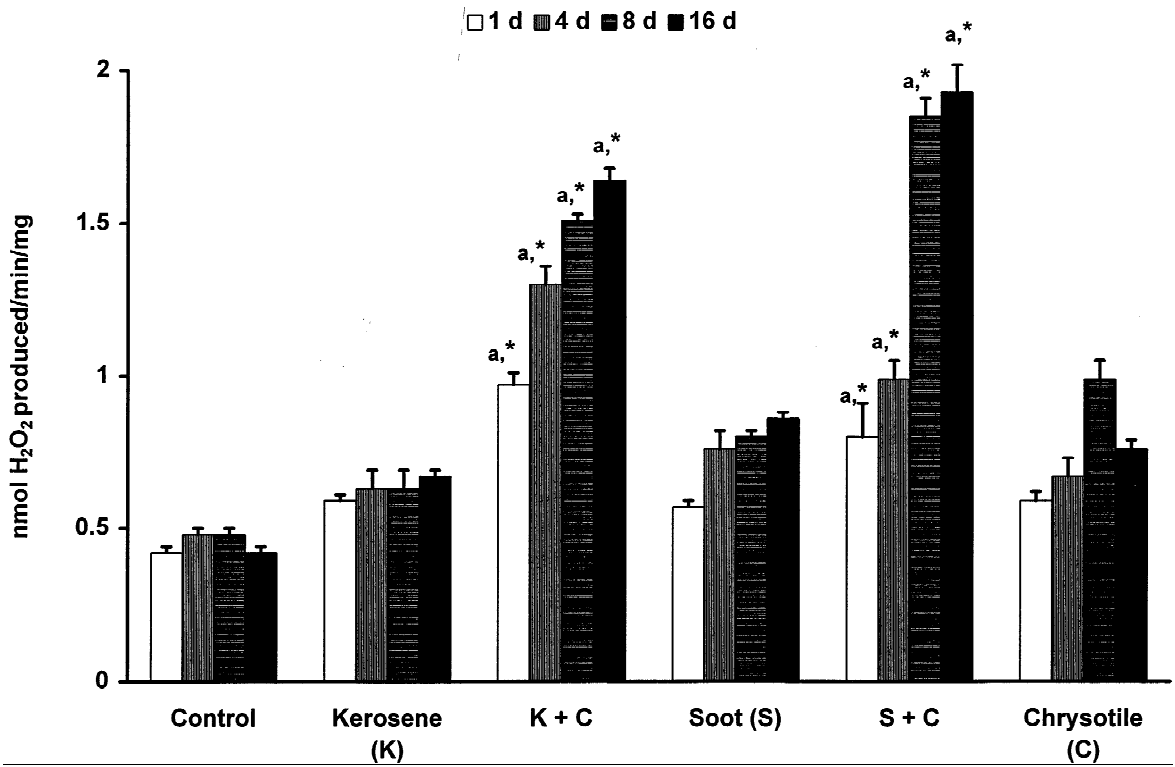

FIG. 2. Changes in the profile of H_2O_2 generation in the PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SE ($n = 6$). $^aP < 0.05$ when compared to respective kerosene or soot controls, and $^*P < 0.05$ when compared to chrysotile alone.

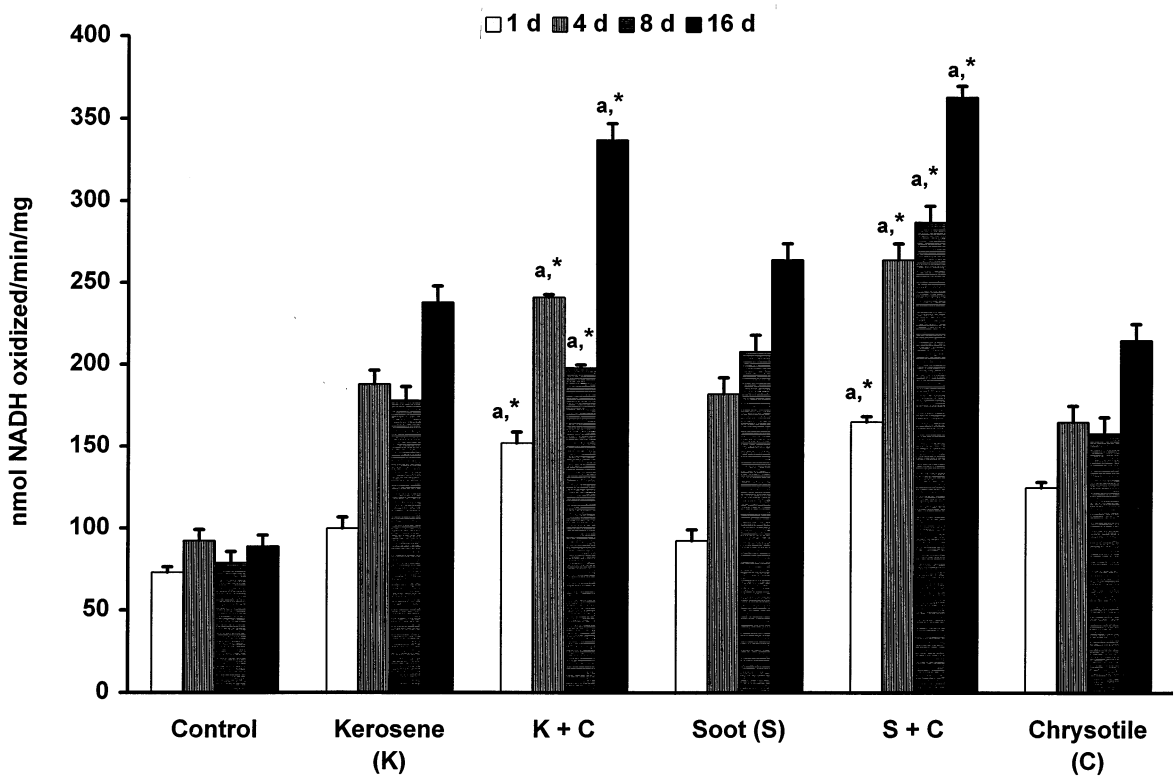

FIG. 3. Changes in the activities of lactate dehydrogenase in the bronchoalveolar lavage fluid of rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SE ($n = 6$). $^aP < 0.05$ when compared to respective kerosene or soot controls, and $^*P < 0.05$ when compared to chrysotile alone.

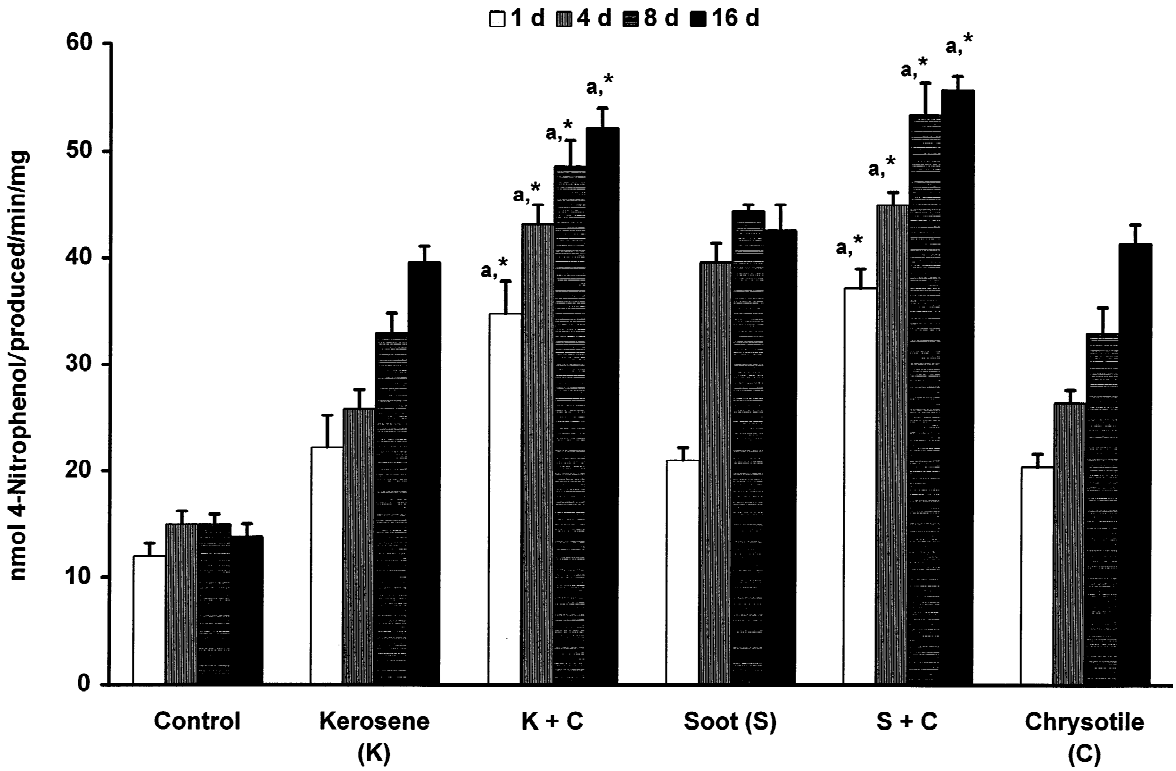

FIG. 4. Changes in the activities of acid phosphatase in the bronchoalveolar lavage fluid of rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SD ($n = 6$). $^{a}P < 0.05$ when compared to respective kerosene or soot controls, and $^{*}P < 0.05$ when compared to chrysotile alone.

solute amount of H_2O_2, reaching a maximum at Day 16 (Fig. 2). Several studies conducted in man and animals have shown that cigarette smoke increases pulmonary mineral dust retention as well as their penetration (McFadden *et al.,* 1986), thereby enhancing the inflammatory reactions in the lung (Filipenko *et al.,* 1985; Wright *et al.,* 1988). Similarly, presence of kerosene and its soot in the macrophage may also slow down the clearance of asbestos fibers, which ultimately might increase the functional dose of asbestos to the tissue and thus provide asbestos more time to stay in the lung. It might subsequently enhance the frequency of phagocytosis, which may in turn activate the respiratory burst mechanism, leading to the increased H_2O_2 production. The increased production of H_2O_2 by chrysotile asbestos in the presence of kerosene and soot may lead to enhanced tissue inflammatory reactions which could subsequently cause more damage to the lung tissue. Measurement of LDH activity in the cell free supernatant of rat lungs exposed to kerosene, soot, or chrysotile alone showed increased leakage of

TABLE 1

Protein Content in Bronchoalveolar Lavage (BAL) of Rat Lungs Exposed to Kerosene, Soot, Chrysotile, and Their Combinations

Groups	Posttreatment period (day)			
	1	4	8	16
Control	690 ± 3	630 ± 45	510 ± 24	600 ± 18
Kerosene	1790 ± 14	1500 ± 15	940 ± 49	1080 ± 32
Kerosene + chrysotile	2080 ± 22*,**	1760 ± 86*,**	880 ± 51^{NS,NS}	880 ± 37^{*,NS}
Soot	2310 ± 99	1930 ± 114	1030 ± 104	1310 ± 35
Soot + chrysotile	2950 ± 49*,**	2230 ± 56*,**	1430 ± 33*,**	1110 ± 78*,**
Chrysotile	1140 ± 12	1090 ± 34	640 ± 61	560 ± 25

Note. Values are represented as μg/ml BAL and were means ± SE ($n = 6$). NS, not significant.

$^{*}P < 0.05$ when compared to kerosene and soot controls, and $^{**}P < 0.05$ when compared to chrysotile alone.

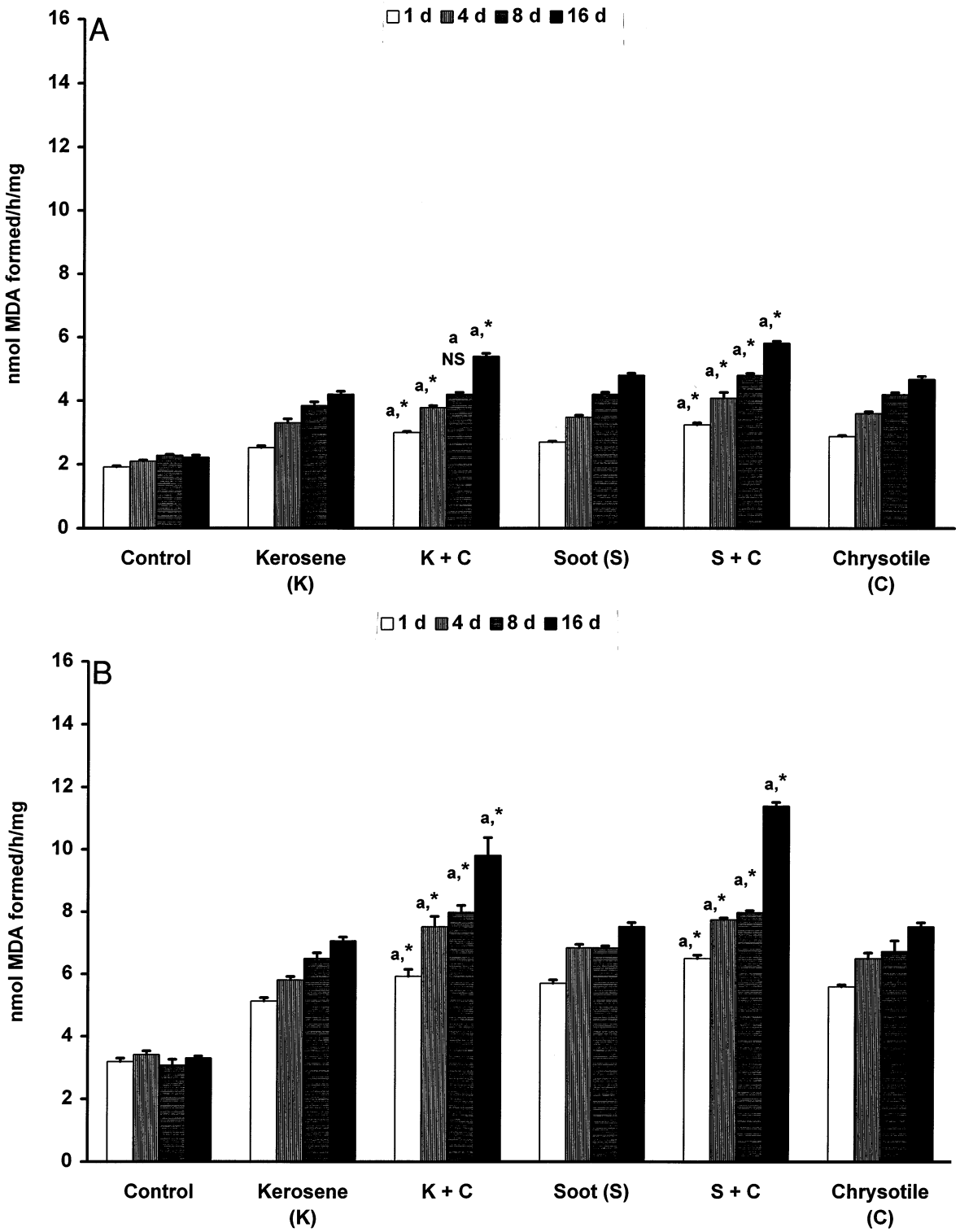

FIG. 5. (A) Changes in the profile of lipid peroxidation in PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. (B) Changes in the profile of NADPH-induced lipid peroxidation in PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. (C) Changes in the profile of Fe^{2+}-induced lipid peroxidation in PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SE ($n = 6$). $^{a}P < 0.05$ when compared to the respective kerosene or soot controls, and $^{*}P < 0.05$ when compared to chrysotile alone. NS, not significant.

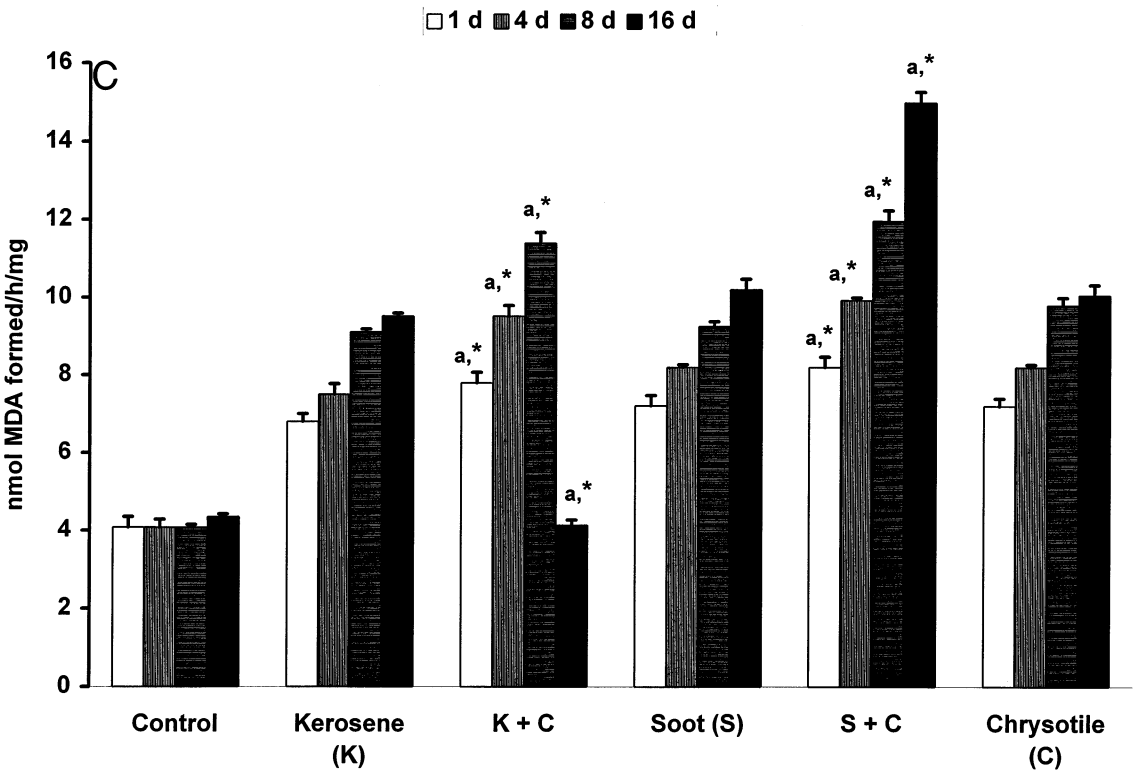

FIG. 5—*Continued*

LDH at all time points, reaching a maximum, i.e., 257, 280, and 230% of the control value, respectively, at Day 16 postexposure (Fig. 3). However, coexposure to chrysotile with kerosene/soot showed a further increase in the LDH activity, but the inducing potential was higher in the group exposed simultaneously to chrysotile and soot. An exactly similar pattern was also observed for AP activity in the cell-free supernatant of the experimental groups (Fig. 4). These results specifically indicated injury to the macrophages (Henderson *et al.,* 1979, 1981). In addition, the increase of protein in the acellular lavage fluid also appears to be due to leakage from PAM injured during phagocytosis (Table 1).

The H_2O_2 produced by the macrophages under combined exposure of chrysotile and kerosene/soot may interact with the iron (≈2700 ppm as measured by atomic absorption spectroscopy) present on the surface of asbestos to produce highly reactive hydroxyl radicals which could cause structural and functional disorders in the biomolecules and plasma membranes (Halliwell and Gutteridge, 1984; Aust *et al.,* 1985; Meneghini, 1988) as evident from increased microsomal lipid peroxidation in the exposed rat alveolar macrophages (Fig. 5A). The data in Figs. 5B and 5C showed a synergistic increase in the TBARS upon addition of NADPH (or iron) to the reaction system containing chrysotile and kerosene/soot-exposed macrophages. Since additional ferrous ions have a marked effect on lipid peroxidation, it is possible that the iron present on the asbestos surface as a contaminant may enhance the production of lipid peroxides (Lund and Aust, 1991).

The increase in oxidative stress may also impair the cellular antioxidant defense systems which attenuate the effects of various oxidants (Heffner and Repine, 1989; Quinalan *et al.,* 1994). In the present study, GPx was significantly inhibited at Day 1 after exposure to kerosene, soot, and chrysotile (Fig. 6). Coexposure of chrysotile with kerosene further inhibited the enzymatic profile, whereas this effect was not observed after coexposure with soot. The exact mechanisms for such changes are not yet clear. At Days 4, 8, and 16, the decreased GPx level gradually tends to acquire the normal basal level. The inhibition of GPx activity may occur through the oxidation of the enzyme itself since GPx utilizes selenium in its active sites and under stress conditions due to perturbance of the cellular oxidation–reduction cycle may result in accumulation of the selenic acid and hence the inactivation of enzyme (Loeb *et al.,* 1988). Thus, the increased accumulation of H_2O_2 in conjunction with the decreased activity of

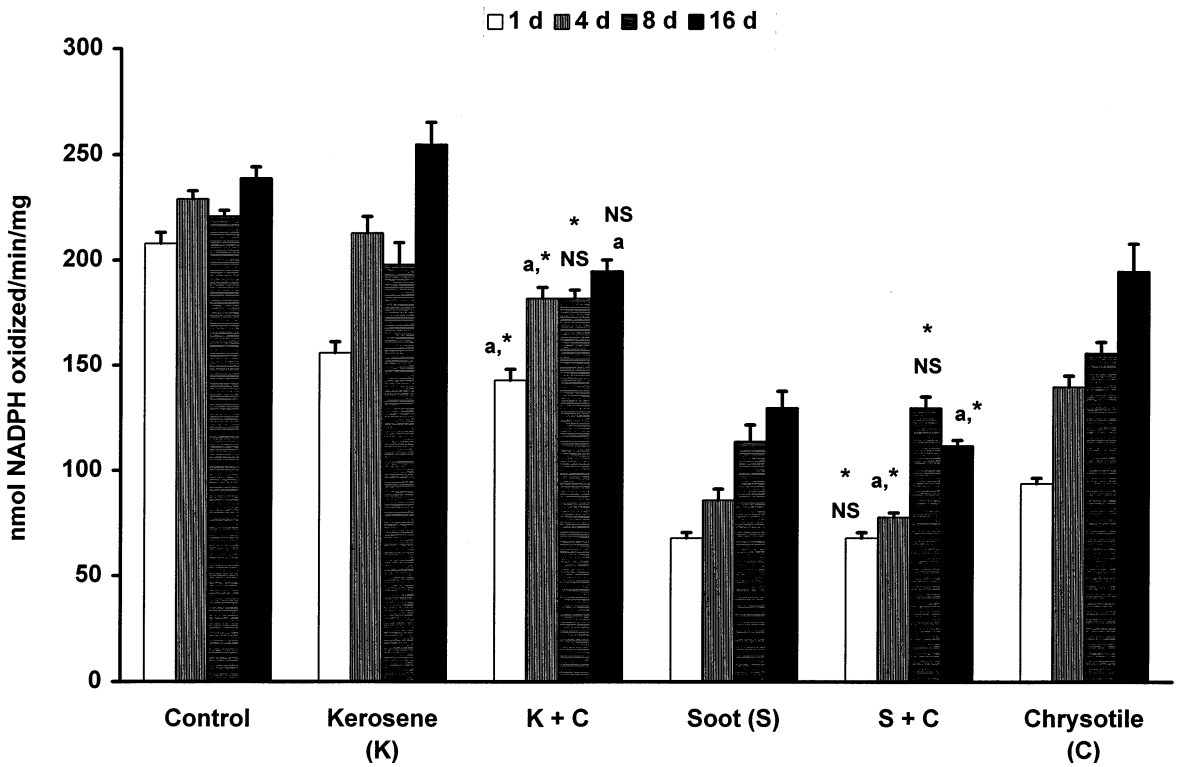

FIG. 6. Changes in the activities of glutathione peroxidase in PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SE ($n = 6$). $^{a}P < 0.05$ when compared to the respective kerosene or soot controls, and $^{*}P < 0.05$ when compared to chrysotile alone. NS, not significant.

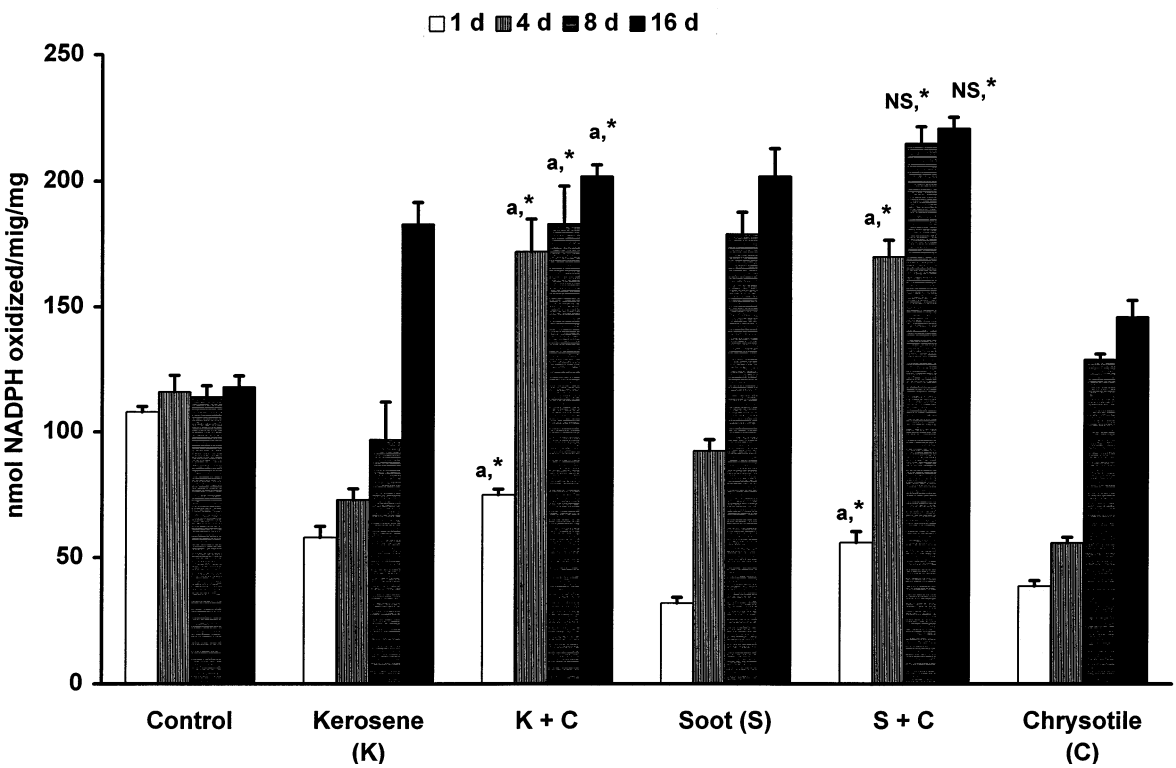

FIG. 7. Changes in the activities of glutathione reductase in PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SD ($n = 6$). $^{a}P < 0.05$ when compared to the respective kerosene or soot controls, and $^{*}P < 0.05$ when compared to chrysotile alone.

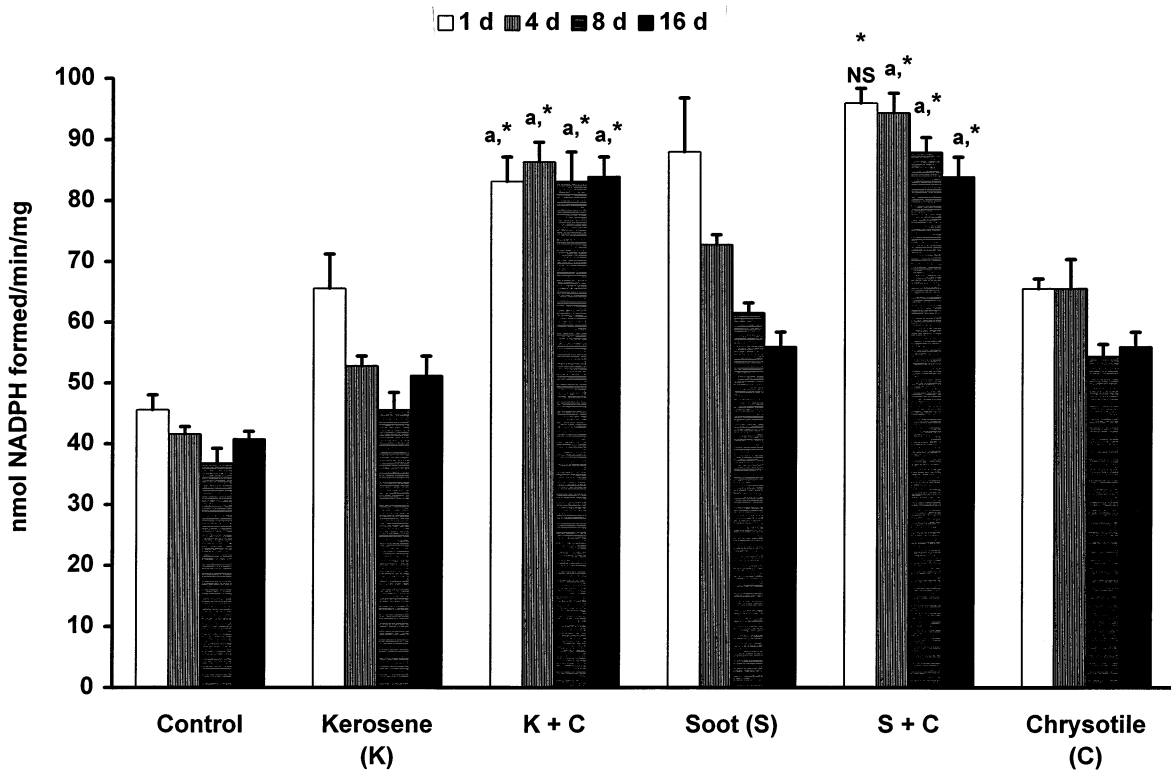

FIG. 8. Changes in the activities of glucose-6-phosphate dehydrogenase in PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SE ($n = 6$). $^{a}P < 0.05$ when compared to the respective kerosene or soot controls, and $^{*}P < 0.05$ when compared to chrysotile alone. NS, not significant.

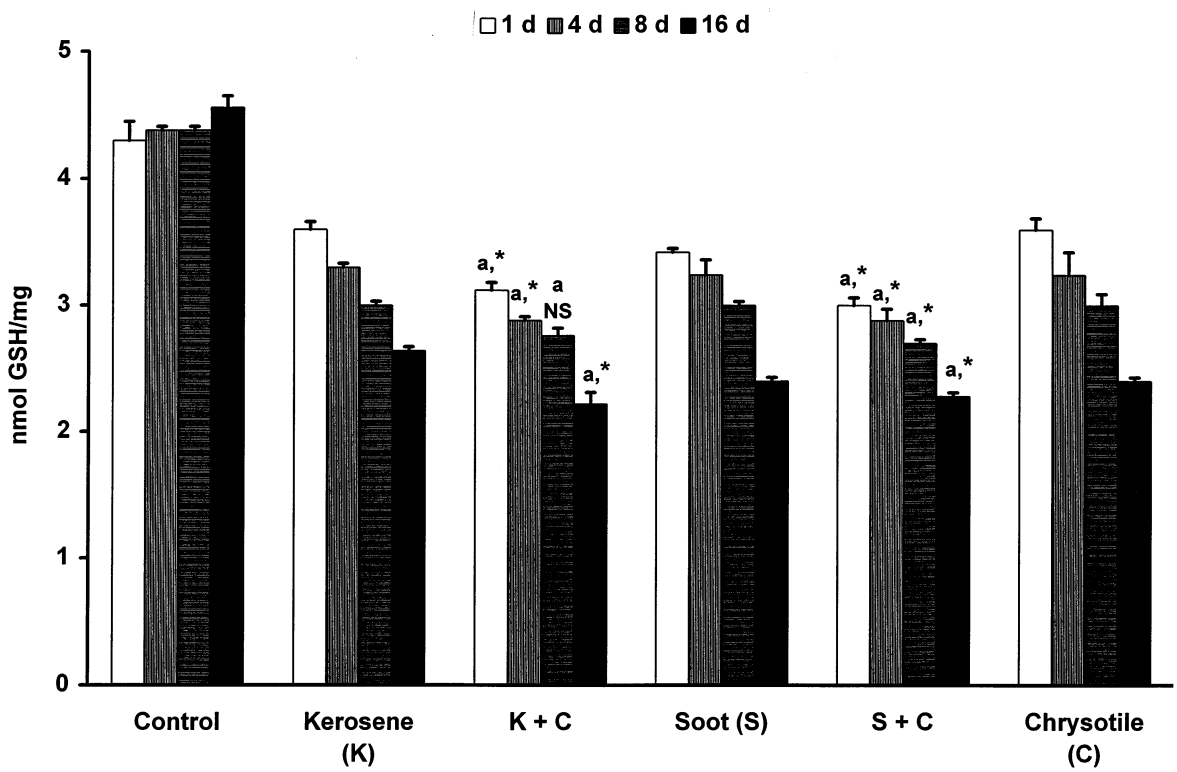

FIG. 9. Changes in the reduced glutathione contents in PAM from rats exposed to kerosene, soot, chrysotile, and their combinations. Values are means ± SE ($n = 6$). $^{a}P < 0.05$ when compared to the respective kerosene or soot controls, and $^{*}P < 0.05$ when compared to chrysotile alone. NS, not significant.

GPx may lead to a cellular proxidant state. In addition, an initial decrease followed by an increase in the catalase activity was also observed in PAM population exposed to the combination mixture indicating its involvement in the metabolism of H_2O_2 produced under the exposure conditions (data not shown). It was also observed that kerosene, soot, chrysotile, and their combinations initially inhibited the activities of GR in PAM populations, whereas the enzymatic profiles were gradually enhanced afterward (Fig. 7). Unlike GR, G6PD activities were increased throughout the exposure (Fig. 8). These observations appear to be interesting in that they meet the enhanced requirement of reducing equivalents needed for the protection of PAM from damaging species, such as H_2O_2 and lipid peroxides.

Furthermore, exposure of lungs to kerosene, soot, and chrysotile resulted in decreased intracellular GSH content in PAM (Fig. 9). GSH content was further decreased on coexposure of chrysotile with kerosene/soot. Notably, GSH was gradually depleted throughout the exposure. GSH is well known for its direct as well as indirect roles in protecting the cells against a variety of insults (Cantin and Begin, 1991). Accordingly, lower levels of GSH in exposed PAM populations may in part play a role in protecting the macrophages from damaging reactive species.

In conclusion, the above results indicate that alterations in the cellular enzymatic and nonenzymatic balance due to combined exposure of asbestos and kerosene/soot may expose the macrophages to increased toxicity and oxidative stress which may eventually lead to pulmonary fibroproliferative changes and/or malignancies in the asbestos-exposed subjects simultaneously exposed to kerosene and its soot.

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