Oxidative Stress Produced by Marijuana Smoke

An Adverse Effect Enhanced by Cannabinoids

Theodore A. Sarafian, Jose Antonio Marques Magallanes, Hungyi Shau, Donald Tashkin, and Michael D. Roth

Division of Pulmonary and Critical Care Medicine, Department of Medicine; and Division of Surgical Oncology, Department of Surgery, University of California at Los Angeles School of Medicine, Los Angeles, California

Marijuana (MJ) smoking produces inflammation, edema, and cell injury in the tracheobronchial mucosa of smokers and may be a risk factor for lung cancer. Because oxidative stress may mediate some of these effects, this study was designed to test the hypothesis that cannabinoids in MJ smoke contribute to cellular oxidative stress. Oxidative stress was evaluated in an endothelial cell line (ECV 304) following exposure to smoke produced from MJ cigarettes containing either 0, 1.77, or 3.95% Δ^9-tetrahydrocannabinol (Δ^9-THC). Brief exposure to smoke from 3.95% MJ cigarettes stimulated the formation of reactive oxygen species (ROS) by 80% over control levels and lowered intracellular glutathione levels by 81%. Smoke-induced ROS generation increased in a dose- and time-dependent manner. In contrast, exposure to smoke from MJ containing 0% Δ^9-THC produced no increase in ROS despite a 70% decline in glutathione levels. Smoke from MJ containing 1.77% Δ^9-THC stimulated intermediate levels of ROS. A brief, 30-min exposure to MJ smoke, regardless of the Δ^9-THC content, also induced necrotic cell death that increased steadily up to 48 h of observation. MJ smoke passed through a Cambridge filter that removed particulate matter was 3.4-fold more active in ROS production compared with unfiltered smoke, suggesting that most of the oxidative effects are produced by the gaseous phase. Alveolar macrophages obtained from habitual MJ smokers displayed low levels of glutathione compared with macrophages from nonsmokers. We conclude that MJ smoke containing Δ^9-THC is a potent source of cellular oxidative stress that could contribute significantly to cell injury and dysfunction in the lungs of smokers. **Sarafian, T. A., J. A. M. Magallanes, H. Shau, D. Tashkin, and M. D. Roth. 1999. Oxidative stress produced by marijuana smoke: an adverse effect enhanced by cannabinoids. Am. J. Respir. Cell Mol. Biol. 20:1286–1293.**

Marijuana (MJ) is one of the most commonly abused substances in the United States, where 3.3% of young adults 19 to 28 yr of age use MJ on a daily basis and 54% of people between 26 and 34 have used marijuana at least once (1). Medicinal uses of cannabis date back thousands of years and both crude smoke and the psychoactive component, Δ^9-tetrahydrocannabinol (Δ^9-THC), have been used for treating migraine headache, glaucoma, nausea, and anorexia (2). Despite this widespread use, little information is available regarding toxic effects of MJ smoke. Persistent efforts to legalize MJ and political movements advocating medicinal uses tend to promote the notion that little or no hazardous risk is associated with MJ smoking.

The vast majority of research on biologic effects of cannabinoids has addressed the neurologic and psychotropic activity of these compounds (3). Some publications, however, have documented detrimental effects on the tracheobronchial mucosa, including mucosal edema and inflammation (4), cellular atypia and dysplasia (5), and molecular dysregulation of genes associated with malignant transformation (6). MJ also appears to alter the function of alveolar macrophages (7), key cells in the lung's immune defenses against infection and malignancy. Moreover, several small case-series reports have suggested an association between regular MJ use and upper aerodigestive-tract cancers (8–13). Approximately 60 different cannabinoids classified as C-21 terpenophenolic compounds can be found in the smoke derived from MJ, and the cannabinoid content of an MJ plant varies considerably depending on the type of plant and conditions of cultivation. Some reports suggest that, over the past 10 to 20 yr, the cannabinoid content in MJ cigarettes may have increased severalfold (14, 15).

(*Received in original form May 15, 1998 and in revised form December 23, 1998*)

Address correspondence to: T. A. Sarafian, Div. of Pulmonary and Critical Care Medicine, Dept. of Medicine, UCLA School of Medicine, Los Angeles, CA 90095.

Abbreviations: analysis of variance, ANOVA; ascorbic acid, Asc; 2,7-dichlorofluorescein, DCF; DCF diacetate, DCF-DA; dimethyl sulfoxide, DMSO; emission, Em; excitation, Ex; fetal calf serum, FCS; reduced glutathione, GSH; glutathione-S-transferase, GST; hydrogen peroxide, H_2O_2; low-density lipoprotein, LDL; monochlorobimane, MCB; marijuana, MJ; pyrrolidinedithiocarbamate, PDTC; peroxiredoxin, Prx; reactive oxygen species, ROS; standard error of the mean, SEM; tetrahydrocannabinol, THC.

Am. J. Respir. Cell Mol. Biol. Vol. 20, pp. 1286–1293, 1999
Internet address: www.atsjournals.org

There is little information on toxicologic effects of individual constituents found in MJ smoke. In the present studies we examined the effects of whole MJ cigarette smoke with and without Δ^9-THC and of the gas phase of the smoke on the generation of reactive oxygen species (ROS) and on levels of antioxidants in the cultured human endothelial cell line, ECV 304. Cellular production of ROS and reduced antioxidant activity were considered to be toxicologic markers of oxidative stress that could lead to cell injury, DNA damage, and ultimately, malignant transformation. Human alveolar macrophages collected from the lungs of habitual MJ smokers were also evaluated for evidence of *in situ* exposure to oxidative stress, and were compared with findings in macrophages from control nonsmokers.

Materials and Methods

MJ cigarettes containing either 0, 1.77, or 3.95% Δ^9-THC were obtained from the National Institute on Drug Abuse (NIDA, Rockville, MD) with characteristics as previously described (16). Cigarettes with 1.77 or 3.95% Δ^9-THC were prepared at NIDA by blending MJ leaves of differing potencies, and cigarettes containing 0% Δ^9-THC were prepared from MJ leaves that had been extracted with ethanol to remove cannabinoids. Cigarettes weighed 700 to 900 mg and were weight-matched to within 20 mg for each experiment. For comparison, tobacco cigarettes weighing 850 mg were purchased commercially (Marlboro Red hard-pack filtered cigarettes; Phillip Morris, Richmond, VA). 2,7-Dichlorofluorescein diacetate (DCF-DA) and monochlorobimane (MCB) were from Molecular Probes (Eugene, OR). Propidium iodide, ascorbic acid, and pyrrolidinedithiocarbamate (PDTC) were from Sigma (St. Louis, MO). The Promega (Madison, WI) Apoptosis Assay Kit was used for cytotoxicity evaluation.

The endothelial cell line, ECV 304, was obtained from American Type Culture Collection (Rockville, MD). Methods for transfection-mediated overexpression of the human peroxiredoxin (Prx) gene and characterization of antioxidant properties have been described elsewhere (17). The Prx protein confers cellular protection against oxidative stress by consuming hydrogen peroxide (H_2O_2). Cells were cultured in RPMI 1640 media containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin/fungizone mix (GIBCO BRL, Grand Island, NY) on poly-L-lysine dishes and multiwell plates. Cells were passaged every 7 d. Prx-transfected cells were cultured alongside control (vector-only)–transfected cells in 24-well plates.

Lung alveolar macrophages were obtained by bronchoalveolar lavage from human volunteers, including both nonsmokers and habitual smokers of MJ only as previously described (5, 7). Macrophages were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and 1% penicillin/streptomycin/fungizone. Cells were plated at a density of 5×10^4/well in 96-well plates. Cells were analyzed for endogenous ROS generation and reduced glutathione (GSH) content at 1 and 24 h after plating.

ECV 304 cells transfected with either hygromycin-resistance vector DNA (vec) or a human Prx DNA construct were treated for 2 h with various agents (ascorbate, Δ^9-THC, H_2O_2, or control medium) in 24-well culture plates (2×10^4 cells/well) before loading with 40 μM DCF-DA for 20 min in Krebs–Ringer buffer. After washing twice with Krebs–Ringer buffer, agents were reapplied to cells in 200 μl Krebs–Ringer buffer and plates were placed in separate 5,000-ml chambers (Billups-Rothenberg, Del Mar, CA) connected to manually controlled smoking devices (Figure 1). A cigarette holder was attached to a 50-ml sintered glass syringe using 0.7 cm inner-diameter tygon tubing and a three-way stopcock connector. After aspiration of smoke into the syringe, the stopcock valve was turned and smoke expelled into the vented chamber with brief flushing to ensure thorough distribution of smoke. Each chamber received 10 50-ml boluses, equivalent to smoke from a full cigarette, and remained exposed to the smoke for 5 min. Separate chambers exposed to either tobacco or different potencies of MJ smoke were run in parallel and compared with chambers containing room air as a control. Cellular oxidative stress was measured fluorometrically by monitoring the oxidation of intracellular 2,7-dichlorofluorescein (DCF) using a Cytofluor 2300 plate reader (PerSeptive Biosystems, Framingham, MA) at excitation (Ex) = 485, emission (Em) = 530 as previously described (18). Cells were then returned to their respective chambers for a second exposure to the appropriate smoke for a period of 15 min. After a second fluorescence measurement, GSH content, cell viability, and total cell number were measured in a sequential manner as described previously (18). Smoke contains both gaseous and particulate phases. In some experiments, the independent effects of the gaseous phase were determined by passing smoke through a high-efficiency Cambridge filter before venting it into chambers containing the ECV 304 cells.

The capacity for MJ smoke to induce intracellular oxidative stress was compared with its ability to directly oxidize DCF in a cell-free environment. In these studies, 24-well plates were filled with 200 μl of Krebs–Ringer buffer

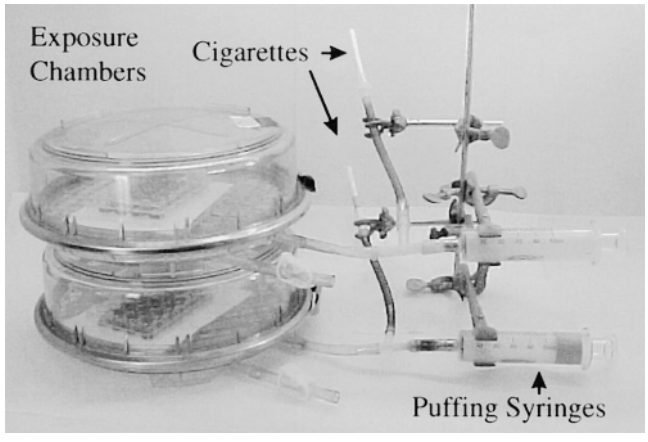

Figure 1. Apparatus used for exposing cultured cells to cigarette smoke. Culture plates (24-well) containing DCF-loaded cells were placed into vented 5,000-ml exposure chambers. MJ or tobacco cigarettes were inserted into holders and lit; 50-ml puffs of cigarette smoke were delivered into the chambers by means of a three-way valve system. The chambers were vented to allow for mixing and equilibration of pressure.

containing either DCF-DA–loaded ECV 304 cells as previously described or 5 μM partially de-esterified DCF-DA in the absence of any cells. DCF-DA was partially de-esterified by diluting DCF-DA to 5 μM in Krebs–Ringer buffer for 1 h at room temperature before smoke exposure. Plates were exposed concurrently to the smoke from one MJ cigarette for 20 min and sealed with Mylar tape, and DCF fluorescence was measured at 30-min intervals. Selected wells were treated with various agents (ascorbate, THC, H_2O_2) immediately before smoke exposure to determine their roles as either pro- or antioxidants.

Long-term viability studies were performed by exposing ECV 304 cells in 96-well plates to MJ smoke with or without Δ^9-THC for 30 min. Control cells were exposed to room air for a similar time period. After smoke exposure, smoke was cleared and cells were confined to chambers containing 10% CO_2 at 37°C for subsequent fluorescent determination of glutathione levels and viability using MCB, propidium iodide, and the Cytofluor 2300. Cells were maintained in serum-containing culture media throughout these studies. Apoptotic and necrotic death was evaluated quantitatively using the Promega Apoptosis Assay Kit. Staining was analyzed by fluorescent microscopy and quantified using the Cytofluor 2300 plate reader.

Data were analyzed in most cases using Student's *t* test for paired data. Data from Figure 1 were analyzed by analysis of variance (ANOVA). Levels of ROS were compared between unexposed cells (control), cells exposed to MJ smoke, and cells exposed to 0% Δ^9-THC smoke by ANOVA, treating all culture plates as independent measurements. Analyses were performed separately for vec and Prx cells, and also with both types of cells combined. Multiple-comparison testing was performed between exposure groups using Tukey's method. Results were considered to be significant at $P < 0.05$. ANOVA was performed using SAS software (SAS Institute Inc., Cary, NC).

Results

Intracellular Effects of Smoke Exposure

In vitro studies in which ECV 304 cells were exposed to whole unfiltered MJ or tobacco smoke revealed rapid oxidation of intracellular DCF. Although not statistically different, the effect of MJ smoke was generally of greater magnitude than that of tobacco on a per-cigarette basis. After 20 min total exposure of vector-transfected cells to two MJ cigarettes, values for DCF oxidation were 1.8-fold greater than control cells exposed to room air ($P < 0.05$) (Figure 2). In Prx-transfected cells, MJ smoke increased DCF oxidation 1.6-fold. At the same time, intracellular GSH levels were decreased to 19% of control values ($P < 0.001$) regardless of the presence of the Prx gene (Figure 3). MJ cigarette smoke devoid of Δ^9-THC produced no sig-

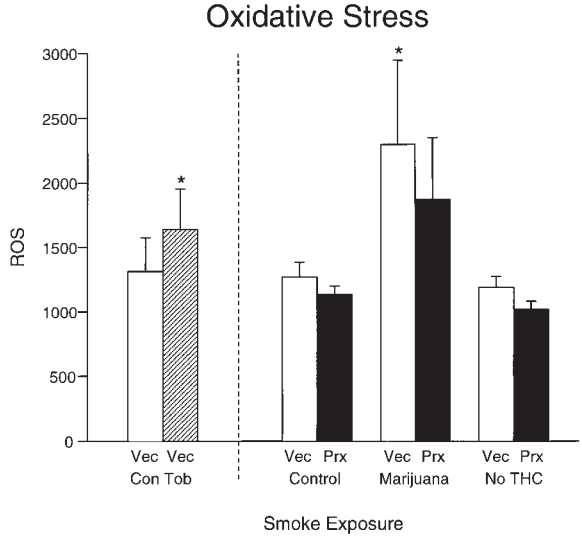

Figure 2. Accumulation of ROS in cultured endothelial ECV 304 cells exposed to smoke from tobacco (Tob) or MJ cigarettes. Cells transfected with either hygromicin-resistance vector DNA (Vec) or human peroxiredoxin-B (Prx) DNA were loaded with DCF before smoke exposure. After exposure to smoke from two cigarettes with porthole ventilation, culture chambers were sealed at room temperature for 20 min. DCF fluorescence was then measured at Ex = 485, Em = 530, subtracting a background value from a well containing no cells. These values were then divided by values reflecting total cell number per well, derived from propidium iodide fluorescence (Ex = 530, Em = 560) in the presence of 160 μM digitonin to permeabilize all cells. These normalized fluorescence values were multiplied by 1,000 to give relative measures of ROS. *Left:* Comparison of untreated control cells (Con) with cells exposed to tobacco cigarette (Marlboro) smoke (Tob) ($n = 6$; $^*P < 0.05$ using Student's *t* test). *Right:* Separate experiments comparing unexposed control cells with cells exposed to smoke from MJ cigarettes with or without Δ^9-THC ($n = 12$; $^*P < 0.05$ comparing MJ smoke to control or to MJ without Δ^9-THC using ANOVA). *Error bars* indicate standard error of the mean (SEM).

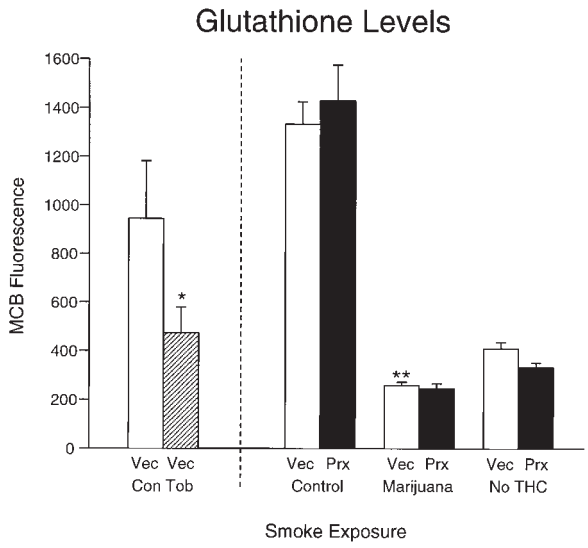

Figure 3. GSH levels in cultured endothelial cells exposed to smoke from tobacco (Tob) or MJ cigarettes as described in Figure 1. MCB fluorescence (Ex = 395, Em = 460) was measured as described in MATERIALS AND METHODS and normalized to cell number per well as in Figure 1. *Left:* $n = 6$; $^*P < 0.05$ comparing tobacco with control. *Right:* $n = 6$; $^{**}P < 0.001$ comparing control with MJ or with MJ without Δ^9-THC using Student's *t* test. *Error bars* indicate SEM.

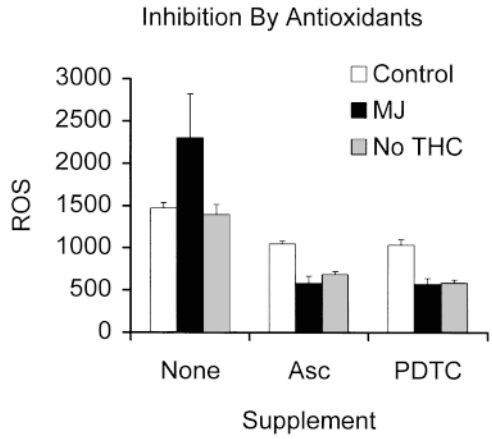

Figure 4. ROS accumulation (expressed as relative normalized DCF fluorescence) inhibited by 1 mM Asc or 1 mM PDTC. Values are means of five determinations ± SEM. *P* values were < 0.05 comparing ascorbate- and PDTC-treated samples with corresponding untreated samples by Student's *t* test.

nificant increase in DCF oxidation relative to controls (Figure 2), despite a drop in GSH levels comparable to that caused by the Δ^9-THC containing MJ smoke (Figure 3). Treatment of cells with ascorbic acid (1 mM) or PDTC (1 mM) suppressed the MJ-induced DCF oxidation by 100 and 99%, respectively (Figure 4), without appreciably affecting GSH levels (data not shown). Similar results were obtained using cultures of lung alveolar macrophages obtained from bronchoscopy from nonsmoking subjects (data not shown). Exposure of ECV 304 cells to synthetic purified Δ^9-THC (0.5 mg/ml) produced no significant DCF oxidation above that of vehicle control (ethanol) (data not shown).

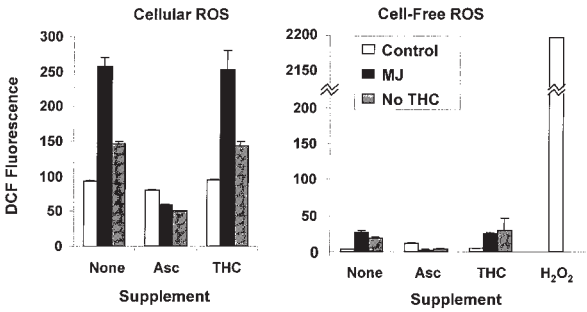

Figure 5. Smoke-induced ROS generation in DCF-loaded ECV 304 cells (Cellular ROS) and in Kreb–Ringer buffer containing 5 μM DCF (Cell-Free ROS). Paired cellular and cell-free plates were enclosed together in chambers infused with room air (control; *open columns*), with 3.95% Δ^9-THC MJ smoke (*filled columns*), or with 0% Δ^9-THC MJ smoke (No THC; *shaded columns*). After 20 min exposure, plates were sealed with Mylar tape; and DCF flourescence was measured after 2 h at room temperature in the dark. Specific wells were supplemented with 0.4 mM Asc, 0.2 mg/ml synthetic Δ^9-THC (THC), or 30 mM H_2O_2 in triplicate. Values represent means ± SEM from a single experiment that was representative of four experiments. Addition of 30 mM H_2O_2 to DCF-loaded cells produced a DCF fluorescence value of 530.

Extracellular Oxidation by Smoke

The capacity for MJ smoke to produce oxidative stress in ECV 304 cells (cellular ROS) was compared with its oxidative effects on media alone (cell-free ROS; Figure 5). In the absence of any smoke exposure, DCF fluorescence was 30-fold higher in wells containing DCF-loaded cells as compared with wells containing DCF alone, suggesting basal generation of ROS by ECV 304 cells. After exposure to smoke from 3.9% MJ cigarettes, there was an increase in cell-free DCF fluorescence ($P < 0.02$), but it was only 10% of the value observed for cellular ROS. Both the cellular and cell-free oxidation produced by smoke exposure were inhibited by ascorbic acid (Asc), but the addition of Δ^9-THC directly to the wells had no effect on either basal or smoke-induced oxidation. In contrast to the pattern of ROS that resulted after smoke exposure, the addition of 30 mM H_2O_2 directly into the wells produced a rapid increase in DCF fluorescence that was 4-fold higher in cell-free wells than in wells containing ECV 304 cells. Similar results were observed following exposure to tobacco smoke (data not shown).

Smoke Dose–Response

Dose–response studies for MJ smoke were performed by varying the amount of smoke delivered to ECV 304 cells *in vitro* over a fixed interval of time. Either two, six, or 10 injections of 50 ml each were delivered into chambers with 5-s intervals between injections. The chambers were sealed for 10 min starting from exposure to the final bolus. Although the increases in DCF oxidation observed over the first 25 min of exposure were not statistically different between groups, dose- and time-dependent increases in DCF oxidation were significant by 45 min of exposure (Figure 6). After 60 min, the cells exposed to six and 10 50-ml injec-

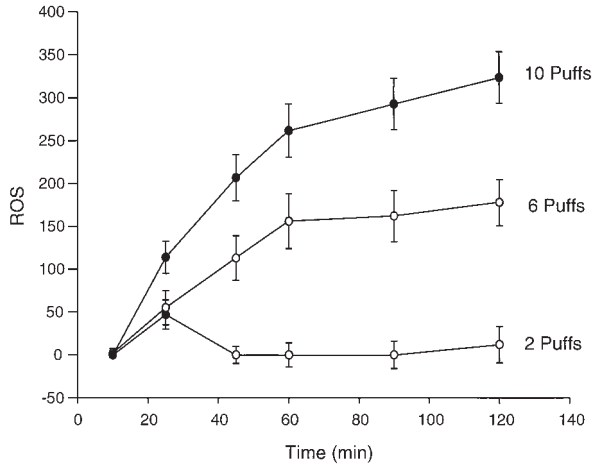

Figure 6. Time course and dose–response for MJ smoke–induced ROS accumulation. ECV 304 cells were exposed to indicated number of 50-ml smoke puffs and chamber was then sealed. Total time for smoke exposure was 10 min for each sample. Normalized DCF fluorescence was measured and values from untreated control cells were subtracted. Values represent means of five or six determinations ± SEM. The experiment was repeated twice with similar results.

TABLE 1
THC content and ROS generation

THC (%)	ROS
0	1,260 ± 53
1.77	1,520 ± 88*
3.95	3,578 ± 260†

DCF-loaded ECV 304 endothelial cells were exposed to whole smoke from MJ containing 0, 1.77, or 3.95% THC for 30 min before reading normalized DCF fluorescence. Values represent means ± SEM of 20 to 32 determinations from seven experiments.
*$P < 0.01$ compared with 0% THC, using Student's *t* test.
†$P < 0.005$ compared with 1.77% THC.

tions displayed 25 and 38% higher levels, respectively, of ROS than did unexposed control cells. Exposure to only two 50-ml injections had no significant effect on ROS generation at any time after exposure.

Comparison of smoke from 0, 1.77, and 3.95% Δ^9-THC MJ cigarettes revealed a dose-dependent relationship between cannabinoid content and ROS generation (Table 1). However, injection of pure synthetic Δ^9-THC in ethanol directly into 0% Δ^9-THC cigarettes 24 h before smoking failed to increase ROS generation significantly (data not shown).

Effects on Viability

The effect of MJ smoke on cell viability was examined by exposing cells to smoke for 30 min in chambers at room temperature. Cells were exposed in the presence of complete culture media and viability was monitored at periodic intervals. MJ smoke caused a time-dependent increase in cell death that reached 78% at 2 d (Figure 7). Control cells consistently displayed low (3 to 10%) death throughout this period. Cells exposed to 0% Δ^9-THC smoke also displayed high levels of death (70%) at 2 d. MJ smoke caused a rapid and sustained decrease in cellular GSH level of 83% after 10 min exposure and 77% after 6 h, with little further change up to 48 h (Figure 8). Smoke lacking Δ^9-THC lowered GSH levels by only a slightly lesser degree (71 and 69%, respectively) at these same times. (Differences were not significant.)

The majority of dead cells after 2 d exposure to MJ smoke had died by necrosis. While the terminal uridine nucleotide end-terminal labeling apoptosis assay revealed sporadic cells with strongly positive staining, condensed nuclei, and fragmentation into apoptotic bodies, the majority of cells were unstained and slightly swollen. In addition, most cells showed positive staining with propidium iodide, indicating loss of membrane integrity. GSH levels displayed biphasic changes, initially declining after smoke exposure and subsequently tending to increase slightly, a pattern characteristic of many oxidation-mediated effects on the cellular antioxidant.

Filtered Smoke

To evaluate independently the gaseous and particulate phases of smoke for their ability to generate cellular ROS, smoke was first passed through high-efficiency Cambridge filters that remove > 98% of particulate components but allow passage of gas-phase components. Exposure of cells to gas-phase MJ smoke resulted in an approximately 2-fold increase in DCF oxidation relative to whole-smoke exposure (Figure 9). DCF oxidation caused by exposure to filtered smoke from ethanol-extracted MJ (0% Δ^9-THC) was also elevated 3-fold relative to that from exposure to unfiltered THC-free smoke. Filtered smoke from THC-containing cigarettes caused approximately 30% higher DCF oxidation than did filtered smoke from ethanol-extracted cigarettes. When particulate matter on filters was extracted with dimethyl sulfoxide (DMSO) and applied to cultured cells, DCF oxidation by whole smoke was

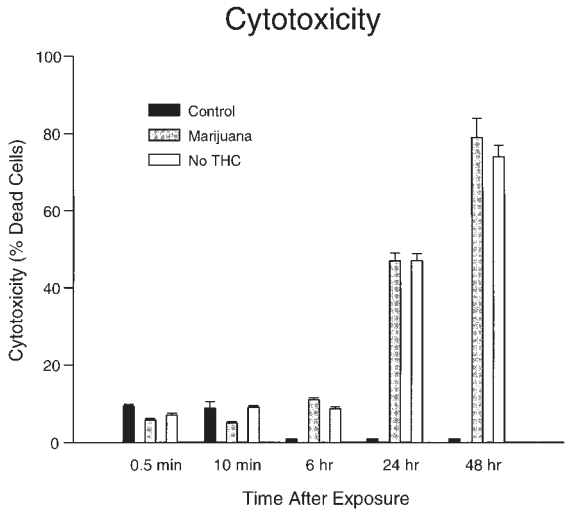

Figure 7. Time course for ECV 304 cell death following 10 min exposure to smoke from a single MJ cigarette. Cytotoxicity was quantitated as described in MATERIALS AND METHODS. Values represent means of six determinations ± SEM. The experiment was repeated twice with similar results.

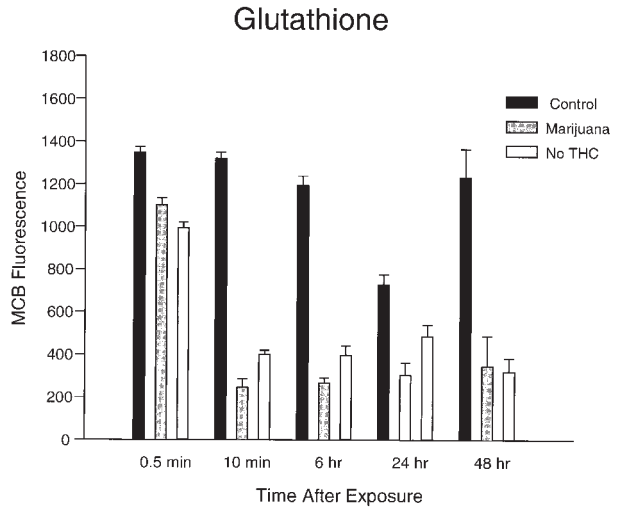

Figure 8. Time course for GSH levels measured as MCB fluorescence following 10 min exposure to smoke from a single cigarette. Values represent means of six determinations ± SEM. Experiments were repeated twice with similar results.

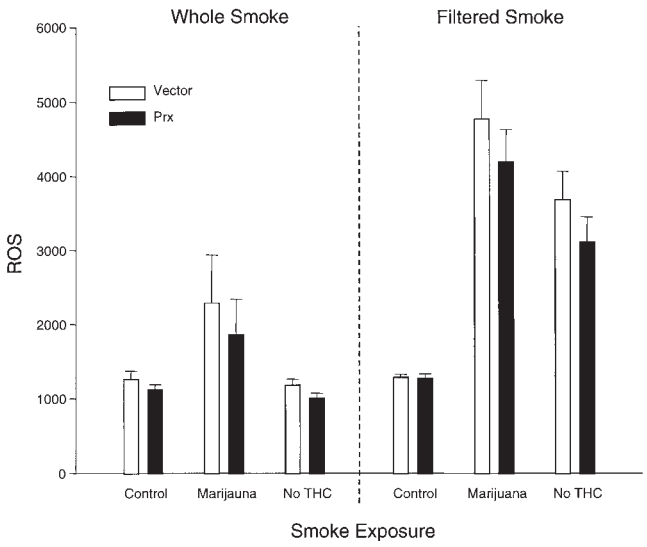

Figure 9. ROS accumulation following exposure to gaseous-phase MJ cigarette smoke. Smoke from two cigarettes was filtered through Cambridge filters to remove particulates before exposure to ECV 304 cells. Values represent means of 34 determinations ± SEM. $P < 0.005$ comparing filtered smoke with whole smoke using Student's *t* test

attenuated by 50 to 70% (data not shown). DMSO alone had no effect on either basal or MJ smoke-induced DCF oxidation.

Alveolar Macrophages

Cultured lung alveolar macrophages obtained by bronchoalveolar lavage from habitual MJ smokers revealed GSH levels 31% lower than levels in cells obtained from nonsmokers ($P < 0.025$) (Table 2). However, incubation of these cells with DCF revealed a lower rate of ROS production of borderline statistical significance ($P = 0.05$) compared with cells from nonsmokers. Cells from both MJ and tobacco smokers contained high amounts of dense intracellular inclusions.

Discussion

Very little research has been devoted to the cytotoxic effects of direct exposure to MJ smoke. Alterations that have been found in the tracheobronchial mucosa of habitual MJ smokers include mucosal edema and inflammation (4), cellular atypia and dysplasia (5), and molecular dysregulation of genes associated with malignant transformation (6). *In vitro* and whole-animal studies suggest that Δ^9-THC has a direct immunosuppressive effect on a variety of immune cells, including macrophages, natural killer cells, and T lymphocytes (11–13, 19). Habitual MJ smoking has also been shown to alter alveolar macrophage morphology (20, 21), phagocytic function (7), fungicidal and bactericidal activity (7, 22), and oxidative burst superoxide production (22).

In the present studies we examined the effects of short-term (5 to 30 min) exposure to MJ smoke on generation of ROS, levels of reduced GSH, and cell viability *in vitro*. Exposure to MJ smoke caused a dramatic increase in ROS

TABLE 2

Lung alveolar macrophage oxidative stress

	Nonsmoker	MJ Smoker
MCB fluorescence	806 ± 109	524 ± 33*
ROS	1,930 ± 237	1,290 ± 252†

Lung alveolar macrophages from bronchoscopic lavage of volunteer subjects were cultured for 24 h in 10% FCS in DMEM and 1% penicillin/streptomycin/fungizone before assays. Values represent means of five determinations ± SEM.

*$P < 0.025$ compared with nonsmoker.

†$P = 0.05$ compared with nonsmoker, using Student's *t* test.

over control levels, an increase that was as much as 3-fold higher than the increment resulting from exposure to a similar amount of tobacco smoke. The attenuation of DCF oxidation in cells overexpressing the antioxidant gene, Prx, supports the notion that pro-oxidants such as H_2O_2 were responsible for some of the MJ-induced effects. Prx is a novel antioxidant cytoplasmic enzyme that appears to eliminate peroxides, one of the several classes of ROS known to be generated intracellularly. In the present study, Prx-overexpressing cells displayed consistently lower DCF oxidation than did vector-only–transfected cells. However, the number of experimental determinations for each exposure group was not sufficiently high to achieve statistical significance.

The MJ-induced ROS appeared to be cannabinoid-dependent because smoke from cigarettes lacking Δ^9-THC produced no increase in ROS compared with control cells exposed to room air only. Although the alcohol extraction procedure used to deplete MJ leaves of cannabinoids could have removed other tar components essential for generating oxidative stress, methanol extraction of Marlboro cigarettes did not alter ROS generation produced by equivalent volumes of smoke (data not shown). Further, MJ cigarettes containing 1.77% Δ^9-THC stimulated intermediate levels of ROS, suggesting a direct dose–response relationship. The particulate phase of MJ smoke is qualitatively similar in composition to that of tobacco smoke, with the major exception being that MJ tar contains Δ^9-THC and approximately 60 other cannabinoid compounds not found in tobacco. Conversely, tobacco tar contains nicotine not found in MJ (7). Because purified Δ^9-THC added to cells failed to produce significant changes in ROS, GSH, or cell viability, it is likely that pyrrolysis products produced in the presence of cannabinoids, rather than Δ^9-THC itself, were responsible for the observed oxidative injuries. The strong effects of MJ smoke on GSH levels and cell viability were not appreciably influenced by Δ^9-THC content. This disparity suggests that DCF oxidation and GSH depletion are affected to some extent by different components in MJ smoke.

After intracellular loading and de-esterification, dihydro-DCF can be oxidized to its fluorescent derivative, DCF, by a variety of agents including hydrogen peroxide, hydroxyl radical, and peroxynitrite (23, 24). Evidence indicates that the fluorescent compound is not permanently retained within cells as originally proposed (25), but is gradually released into the surrounding medium. This slow release of dihydro-DCF could diminish the signal caused by oxidants of intracellular origin and increase signal from

extracellular oxidants adsorbed directly from smoke. However, our studies comparing cellular and noncellular oxidation of DCF by MJ smoke revealed that DCF in buffer solution is poorly oxidized by direct smoke exposure, in contrast to results obtained with cellular DCF measurements (Figure 5). These results suggest that most of the DCF fluorescence results from smoke exposure generated by cellular mechanisms. Smoke-induced disruption of mitochondrial or endoplasmic reticular electron transport is among the possible mechanisms for such ROS generation.

Our studies revealed that, compared with smoke generated from MJ cigarettes containing 0% Δ^9-THC, unmodified MJ smoke deposited 50% higher amounts of nitrates ($NO/NO_2^-/NO_3^-$) into culture wells (data not shown). Nitrates can generate peroxynitrite in the presence of superoxide anion. This effect may partially account for the difference in ROS produced from MJ with or without Δ^9-THC. However, smoke from a tobacco cigarette of equivalent weight contained nearly twice as much nitrate as smoke from 3.9% Δ^9-THC MJ cigarettes, yet produced somewhat lower ROS. Thus, smoke nitrate levels did not correlate directly with ROS generation.

Loss of cellular GSH can occur by two major pathways (26). Free radical–mediated oxidative activity results in generation and/or efflux of oxidized glutathione, which we did not measure in this study. Alternatively, nucleophiles, including aldehydes known to be prevalent in cigarette smoke (e.g., formaldehyde), form covalent conjugates with GSH. These reactions, catalyzed by glutathione-S-transferase (GST) enzymes, result in lower MCB-detectable GSH levels. These reactions do not necessarily reflect oxidative stress per se, but would partially impair cellular defenses and inhibit the removal of ROS. MCB has been used extensively to estimate intracellular levels of reduced GSH (27–30). Although fluorescence can also be generated by protein-MCB conjugate formation (31), the rate of this reaction at low (10 to 100 μM) concentrations of MCB is lower than that for the complex with GSH. Thus, limiting the reaction time to 20 min allows for a more accurate estimation of GSH. Recent studies suggest that reactivity of MCB with GSH is low in human peripheral blood monocytes compared with reactivity with other low molecular-weight compounds (32). This low reactivity is apparently due to the low affinity of some forms of GSTs for MCB. In the present study, the human alveolar macrophages displayed 20 to 60% lower levels of MCB fluorescence than did the ECV 304 cell line. However, in both cell types, MCB fluorescence was inhibited 80 to 90% by 10 min pretreatment with 2 mM diethylmaleate, which rapidly removes cytoplasmic GSH (33).

Cannabinoids, including THC, contribute substantially to the particulate mass of MJ smoke comprising 20 to 30% of the total tar weight for cigarettes containing 3.9% Δ^9-THC (13). To determine whether the increased particulate material in MJ smoke was responsible for the enhanced DCF oxidation, MJ cigarette smoke was passed through Cambridge filters before exposure to cells. Such filters trap $>$ 98% of particulate material but permit passage of all gaseous elements. This procedure not only failed to attenuate DCF oxidation, but also greatly stimulated oxidation in both unmodified MJ and THC-deficient smoke. This stimulation was consistent with reports of strong oxidizing activity of the gaseous phase of tobacco cigarette smoke after removal of particulates by filtration. For example, in studies on the role of tobacco smoke in atherosclerosis, oxidation of low-density lipoprotein (LDL) has been observed with filtered smoke but not whole smoke (34). Filter-trapped particulates can inhibit LDL oxidation induced by cupric chloride or azo-bis (2-amidinopropane), and it has been suggested that antioxidants, such as polyphenolic compounds found in smoke particulate fractions, may be responsible for inhibition of LDL oxidation (35, 36). In the present studies, the concept that particulate components of MJ smoke had antioxidant properties was further supported by the finding that DMSO extracts of a Cambridge filter pad absorbed with MJ smoke particulates suppressed ROS generation. Thus, the level of smoke-induced cellular ROS appears to be a function of the relative amounts of gaseous-phase pro-oxidants and particle-phase antioxidants.

The cannabinoids present in the particle phase of MJ smoke, including Δ^9-THC and cannabinol, have been reported to have antioxidant properties as measured by cyclic voltametry and by their ability to prevent H_2O_2-mediated oxidation of a fluorescent probe (37). This is consistent with their known structure, which includes hydroxyl groups and aliphatic rings. However, the addition of Δ^9-THC to our assays before smoke exposure did not provide any measurable antioxidant protection, suggesting that this effect is relatively weak compared with the pro-oxidant activity induced by smoke.

Preliminary studies with cultured lung alveolar macrophages from human MJ smokers demonstrated lower levels of GSH in these cells than in alveolar macrophages from nonsmokers. These results suggest that habitual exposure to MJ smoke causes a sustained decrease in GSH-dependent oxidative defenses. Such a decrease could be due to inhibition of GSH synthetic or recycling enzymes concomitant with depletion of cytoplasmic GSH. Inhibition of GST could also contribute to diminished MCB fluorescence because this enzyme accelerates MCB-GSH conjugation. The observed decrease in rate of ROS production in cells from MJ smokers relative to nonsmokers is, seemingly, paradoxical. One explanation consistent with the cytotoxic effects of MJ smoke is that chronic *in vivo* exposure of cells to this smoke produced general metabolic impairments diminishing either mitochondrial electron transport or oxidative burst capacity.

The generation of ROS has several undesirable consequences, including the impairment of cellular energetic (38) and defense (39) systems and the promotion of malignant transformation (40). Cell death induced by MJ smoke is largely necrotic. These deleterious effects of MJ smoke could have serious implications for tissues in direct contact with cannabinoid-containing smoke, including lung macrophages and surface epithelial cells in the upper aerodigestive tract and the tracheobronchial mucosa. Such effects need to be taken into consideration when evaluating risk–benefit factors associated with MJ consumption.

Acknowledgments: The authors are grateful to Bianca Grigorian, Priya Pilutla, Angela Chang, Nancy Pashayan, Amy Ling, Pramod Patel, and Ruth Chang for technical assistance; and they thank Donna Crandall and Carol Gray for illus-

trations, Fernando Casimiro for word processing, and Mike Simmons for statistical analyses. This work was supported by grant #DA 03018 from the National Institute on Drug Abuse.

References

1. Johnston, L. D., P. M. O'Malley, and J. G. Bachman. 1997. National survey results on drug use from the Monitoring the Future Study, 1975–1995, Vol II. College students and young adults. USDHHS, NIH Publication No. 98-4140, Washington, DC. 35–74.
2. Grinspoon, L., and J. B. Bakalar, 1997. Marijuana: The Forbidden Medicine, Revised and Expanded Edition, Yale University Press, New Haven, CT.
3. Adams, I. B., and B. R. Martin. 1996. Cannabis: pharmacology and toxicology in animals and humans. *Addiction* 91:1585–1614.
4. Roth, M. D., A. Arora, S. H. Barsky, E. C. Kleerup, M. Simmons, and D. P. Tashkin. 1998. Visual and pathologic evidence of injury to the airways of young marijuana smokers. *Am. J. Respir. Crit. Care Med*. 157:928–937.
5. Fligiel, S. E., M. D. Roth, E. C. Kleerup, S. H. Barsky, M. S. Simmons, and D. P. Tashkin. 1997. Tracheobronchial histopathology in habitual smokers of cocaine, marijuana, and/or tobacco. *Chest* 112:319–326.
6. Barsky, S. H., M. D Roth, E. C. Kleerup, M. Simmons, and D. P. Tashkin. 1998. Histopathologic and molecular alterations in bronchial epithelium in habitual smokers of marijuana, cocaine, and/or tobacco. *J. Natl. Cancer Inst.* 90:1198–1205.
7. Baldwin, G. C., D. P. Tashkin, D. M. Buckley, A. N. Park, S. M. Dubinett, and M. D. Roth. 1997, Habitual smoking of marijuana and cocaine impairs alveolar macrohage function and cytokine production. *Am. J. Respir. Crit. Care Med.* 156:1606–1613.
8. Taylor, F. M. 1988. Marijuana as a potential respiratory tract carcinogen: a retrospective analysis of a community hospital population. *South Med. J.* 81:1213–1216.
9. Caplan, G. A., and B. A. Brigham. 1990. Marijuana smoking and carcinoma of the tongue: is there an association? *Cancer* 66:1005–1006.
10. Donald, P. J. 1991. Advanced malignancy in the young marijuana smoker. *Adv. Exp. Med. Biol.* 288:33–46.
11. Klein, T. S., Y. Kawakami, C. Newton, and H. Friedman. 1991. Marijuana components suppress induction and cytolytic function of murine cytotoxic T cells in vitro and in vivo. *J. Toxicol. Env. Health* 32:465–477.
12. Kusher, D. I., L. O. Dawson, A. C. Taylor, and J. Y. Djeu. 1994. Effect of the psychoactive metabolite of marijuana, Δ^9-tetrahydrocannabinol (THC), on the synthesis of tumor necrosis factor by human large granular lymphocytes. *Cell. Immunol.* 154:99–108.
13. Hoffman, D., D. K. Brunneman, G. B. Gori, and E. L. Wynder. 1975. On the carcinogenicity of marijuana smoke. *Rec. Adv. Phytochem*. 9:63–81.
14. ElSohly, M. A., and S. A. Ross. 1994. Quarterly Report, NIDA Potency Monitoring Project, Report No. 50. National Institute on Drug Abuse, Rockville, MD.
15. Zimmer, L., and J. P. Morgan. 1997. Marijuana Myths, Marijuana Facts. Lindesmith Center, New York. 135–141.
16. Matthias, P., D. P. Tashkin, J. A. Marques Magallanes, J. N. Wilkins, and M. S. Simmons. 1997. Effects of varying marijuana potency on deposition of tar and delta-9-THC in the lung during smoking. *Pharmacol. Biochem. Behav*. 58:1145–1150.
17. Sarafian, T. A., N. Rajper, B. Grigorian, A. Kim, and H. Shau. 1997. Cellular antioxidant properties of human natural killer enhancing factor B. *Free Rad. Res.* 26:281–289.
18. Sarafian, T. A., L. Vartavarian, D. J. Kane, D. E. Bredesen, and M. A. Verity. 1994. bcl-2 expression decreases methyl mercury-induced free-radical generation and cell killing in a neural cell line. *Toxicol. Lett.* 74:149–155.
19. Burnette-Curley, D., and G. A. Cabral. 1995. Differential inhibition of RAW264.7 macrophage tumoricidal activity by Δ^9-tetrahydrocannabinol. *Proc. Soc. Exp. Biol. Med*. 210:64–76.
20. Davies, P., G. C. Sornberger, and G. L. Huber. 1979. Effects of experimental marijuana and tobacco smoke inhalation on alveolar macrophages. *Lab. Invest.* 41:220–223.
21. Beals, T. F., S. E. G. Fligiel, S. Stuth, and D. P. Tashkin. 1989. Morphological alterations of alveolar macrophages from marijuana smokers. *Am. Rev. Respir. Dis.* 139:A336. (Abstr.)
22. Sherman, M. P., L. A. Campbell, H. Gong, Jr., M. D. Roth, and D. P. Tashkin. 1991. Respiratory burst and microbicidal characteristics of pulmonary alveolar macrophages recovered from smokers of marijuana alone, smokers of tobacco alone, smokers of marijuana and tobacco and nonsmokers. *Am. Rev. Respir. Dis.* 144:1351–1356.
23. Lebel, C. P., H. Ischiropoulos, and S. C. Bondy. 1992. Evaluation of the probe 2′,7′-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* 5:227–231.
24. Possel, H., H. Noack, W. Augustin, G. Keilhoff, and G. Wolf. 1997. 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. *FEBS Lett.* 416:175–178.
25. Bass, D. A., J. W. Parce, L. R. Dechatelet, P. Szejda, M. C. Seeds, and M. Thomas. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* 130: 1910–1917.
26. Bellomo, G., H. Thor, and S. Orrenius. 1990. Modulation of cellular glutathione and protein thiol status during quinone metabolism. *Methods Enzymol.* 186:627–635.
27. Rice, G. C., E. A. Bump, D. C. Shrieve, W. Lee, and M. Kovacs. 1986. Quantitative analysis of cellular glutathione by flow cytometry utilizing monochlorobimane: some applications to radiation and drug resistance *in vitro* and *in vivo*. *Cancer Res.* 46:6105–6110.
28. Shrieve, D. C., E. A. Bump, and G. C. Rice. 1988. Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J. Biol. Chem*. 263: 14107–14114.
29. Kavanagh, T. J., A. Grossman, J. C. Jinnerman, S. B. Kanner, C. C. White, D. L. Eaton, J. A. Ledbetter, and P. S. Rabinovitch. 1993. The effect of 1-chloro-2,4-dinitrobenzene exposure on antigen receptor (CD3)-stimulated transmembrane signal transduction in purified subsets of human peripheral blood lymphocytes. *Toxicol. Appl. Pharmacol.* 119:91–99.
30. Reichelt, W., J. Stabel-Burow, T. Pannicke, H. Weichert, and U. Heinemann. 1997. The glutathione level of retinal Muller glial cells is dependent on the high-affinity sodium-dependent uptake of glutamate. *Neuroscience* 77:1213–1224.
31. Cook, J. A., H. I. Pass, S. N. Iype, N. Friedman, W. DeGraff, A. Russo, and J. B. Mitchell. 1991. Cellular glutathione and thiol measurements from surgically resected human lung tumor and normal lung tissue. *Cancer Res.* 51:4287–4294.
32. van der Ven, A. J. A. M., P. Mier, W. H. M. Peters, H. Dolstra, P. E. J. van Erp, P. P. Koopmans, and J. W. M. van der Meer. 1994. Monochlorobimane does not selectively label glutathione in peripheral blood mononuclear cells. *Anal. Biochem*. 217:41–47.
33. Ku, R. H., and R. E. Billings. 1986. The role of mitochondrial glutathione and cellular protein sulfhydryls in formaldehyde toxicity in glutathione-depleted rat hepatocytes. *Arch. Biochem*. 247:183–189.
34. Frei, B., T. M. Forte, B. N. Ames, and C. E. Cross. 1991. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma: protective effects of ascorbic acid. *Biochem. J.* 277:133–138.
35. Chen, C., and G. Loo. 1995. Cigarette smoke extract inhibits oxidative modification of low density lipoprotein. *Atherosclerosis* 112:177–185.
36. Church, D. F. and W. A. Prior. 1985. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.* 64:111–126.
37. Hampson, A. J., M. Grimaldi, J. Axelrod, and D. Wink. 1998. Cannabidiol and (-)Δ^9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc. Natl. Acad. Sci. USA* 95:8268–8273.
38. Wallace, K. B., J. T. Eelss, V. M. C. Madeira, G. Cortopassi, and D. P. Jones. 1997. Mitochondria-mediated cell injury. *Fundam. Appl. Toxicol.* 38:23–37.
39. Repine, J. E., A. Bast, I. Lankhorst, and The Oxidative Stress Study Group. 1997. Oxidative stress in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med*. 156:341–357.
40. Bankson, D. D., M. Kestin, and N. Rifai. 1993. Role of free radicals in cancer and atherosclerosis. *Clin. Lab. Med*. 13:463–480.