Adverse health effects of airborne toxicants, especially small respirable particles and their associated adsorbed chemicals, are of growing concern to health professionals, governmental agencies, and the general public. Areas rich in petrochemical processing facilities (e.g., eastern Texas and southern California) chronically have poor air quality. Atmospheric releases of products of incomplete combustion (e.g., soot) from these facilities are not subject to rigorous regulatory enforcement. Although soot can include respirable particles and carcinogens, the toxicologic and epidemiologic consequences of exposure to environmentally relevant complex soots have not been well investigated. Here we continue our physico-chemical analysis of butadiene soot and report effects of exposure to this soot on putative targets, normal human bronchial epithelial (NHBE) cells. We examined organic extracts of butadiene soot by gas chromatography–mass spectrometry (GC–MS), probe distillation MS, and liquid chromatography (LC)–MS–MS. Hundreds of aromatic hydrocarbons and polycyclic aromatic hydrocarbons with molecular mass as high as $1,000$ atomic mass units were detected, including known and suspected human carcinogens (e.g., benzo(a)pyrene). Butadiene soot particles also had strong, solid-state free-radical character in electron spin resonance analysis. Spin-trapping studies indicated that fresh butadiene soot in a buffered aqueous solution containing dimethylsulfoxide (DMSO) oxidized the DMSO, leading to CH$_2$• radical formation. Butadiene soot DMSO-exposed NHBE cells displayed extranuclear fluorescence within $4$ hr of exposure. BSD E was cytotoxic to $>20\%$ of the cells at $72$ hr. Morphologic alterations, including cell swelling and membrane blebbing, were apparent within $24$ hr of exposure. These alterations are characteristic of oncosis, an ischemia-induced form of cell death. BSD E treatment also produced significant genotoxicity, as indicated by binucleated cell formation. The combination of moderate cytotoxicity and genotoxicity, as occurred here, can be pro-carcinogenic. Key words blebbing, BSD E, butadiene soot, fluorescence, free radicals, human bronchial epithelial cells, PAHs. Environ Health Perspect 109:965–971 (2001). [Online 12 September 2001] http://ehpnet1.niehs.nih.gov/docs/2001/109p965-971catallo/abstract.html

Cardiovascular disease and lung cancer are among the leading causes of death both in Louisiana and throughout the rest of the United States. Age-adjusted death rates are higher in Louisiana than in the rest of the country for both groups of diseases (1). Additionally, the death rate from chronic obstructive pulmonary disease is $58\%$ higher in Louisiana than the national average (2).

Increasingly, researchers have focused on airborne particles as causes of respiratory (and more recently, in cardiovascular) disease. Adsorbed organic compounds have been implicated in many of the systemic and molecular responses of respiratory system cells exposed to diesel exhaust particles (3–6). Studies with residual oil fly ash (ROFA) particles have demonstrated a role for particle-associated metals in inducing inflammatory responses in respiratory cells (7,8).

Epidemiologic evidence from the past decade has correlated increased respiratory morbidity and mortality with exposures to small airborne particles. National Ambient Air Quality Standards issued by the U.S. Environmental Protection Agency in 1997 (9), shifting the focus from larger particles (≥ $10\mu m$) to those ≤ $2.5\mu m$ (so called PM $2.5$), reflect this.

Among the most common origins of airborne particles, industrial point sources [i.e., major facilities, each emitting ≥ $10$ tons/yr of specific hazardous air pollutants (HAPs) or ≥ $25$ tons/yr of a mixture of HAPs] are of particular concern in Louisiana.

In 1997, Louisiana ranked 2nd in the United States in total releases of chemicals on the Toxic Resource Inventory, which is an incomplete survey of the thousands of compounds that can be released into the air. By a wide margin, East Baton Rouge Parish exceeds all other Louisiana parishes—and also ranks in the top $10\%$ of all U.S. counties—in “Person Days in Exceedance of National Ambient Air Quality Standards” (10). In Louisiana, the $16$ parishes with the highest noncancer cumulative hazard index are all in the southeastern part of the state, where many large petrochemical facilities are located (11). Fourteen of the $16$ parishes with the highest added cancer risk from hazardous air pollutants also are in this region (11).

In the United States, particularly in southern Louisiana and eastern Texas, there is a substantial and growing processing capacity in C$_2$–C$_6$ hydrocarbon streams for chemical manufacture (~ $150$ billion pounds annually (12)). For economic, regulatory, and technical reasons, flaring of waste or fugitive volatile organic chemicals (VOCs) occurs regularly at many refineries and chemical plants. Light fractions of crude oils are flared routinely, as are purged materials from reforming and cracking plants. VOC releases to the atmosphere are subject to more stringent regulatory reporting than are releases of products of incomplete combustion (PICs, such as soot) including respirable particles and carcinogenic chemicals.

We have been studying combustion product mixtures associated with selected economically and industrially important VOCs (e.g., 1,3-butadiene) and liquid hydrocarbon mixtures (e.g., crude oils, diesel fuel (12)). When 1,3-butadiene and other substrates found in VOC mixtures (e.g., propane, propylene, butane, vinylacetylene) are burned in air, substantial amounts of polynuclear aromatic hydrocarbons (PAHs) and fine particulants (< $2.5\mu m$) are generated (12). Many of the individual chemicals found in VOC combustion mixtures are toxic and mutagenic. Nevertheless, virtually no attention has been paid to the toxicologic and epidemiologic relevance of PICs from VOC burns.

The purpose of this work is to provide additional physico-chemical analysis of combustion residues from 1,3-butadiene (12) and describe the pleiotropic responses of putative targets, normal human bronchial epithelial (NHBE) cells, following exposures to these residues. We evaluated these residues to gain insight into the chemical processes that occur in PICs and to better understand their relationship to adverse health outcomes.

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Materials and Methods

Combustion of 1,3-butadiene and recovery of products of incomplete combustion. Gas-phase 1,3-butadiene (99%; Aldrich, Milwaukee, WI) was introduced at room temperature to a small (20 cm) steel burner at slow feed rates (e.g., 0.5–5 mL/sec). The resulting PICs were trapped on solvent-clean glass fiber filters. These materials were collected and subjected to the treatments and extraction procedures summarized below.

Sample analysis. Weighed soot samples were Soxhlet-extracted for 6 hr using dichloromethane. This extract was dried (Na₂SO₄) and analyzed by gas chromatography–mass spectrometry (GC–MS). We performed semi-quantitation using standard calibration files and/or direct comparisons with internal standards (Ultra Scientific, Manassas, VA). Calibration files and/or direct comparisons were used for selected analyses (below).

Electroanalysis. BSD E was amended with 1 mM tetrabutylammonium perchlorate (Sigma, St. Louis, MO), filtered (0.22 µm), and examined for redox activity at C, Au, and Pt working electrodes (1 mm²) versus Ag/AgCl reference or Pt wire counter electrodes. We performed differential pulse polarography in the physiologic potential range, i.e., +1,100 mV to –1,100 mV versus Ag/AgCl using a Potentiostat (Cypress Systems, St. Louis, MO).

Preparative thin layer chromatography. We applied BSD E to replicate silica gel thin layer chromatography (TLC) plates (Aldrich). The compounds were eluted with hexane mobile phase at room temperature. Fluorescent and colored bands were marked and then collected by scraping. These fractionated samples were used for selected analyses (below).

Electron spin resonance. We examined dry soot and extracts for free radical signals using a Model 109 Electron Paramagnetic Resonance Spectrometer (ESR; Varian Instruments, Palo Alto, CA). We used cylindrical sample tubes for solid soot and the hexane and toluene liquid analyses, and quartz flat cells for the other solvents. The conditions employed were microwave frequency, X-band (~9.35 GHz); microwave power, 10 mW; receiver gain, 10⁻⁴; modulation amplitude, 1.0 G; field modulation, 100 kHz; time constant, 1 sec; sweep width, 100 G; center field, typically 3,360 G; determined using α,α-diphenyl-β-picryl hydrazyl free radical (DPPH) solid or 1 mM solution in DM SO; sweep duration, 16 min.

Ascorbate radical ESR intensity. We extracted soot solids (20 mg) in DM SO and clarified them by centrifugation. We mixed aliquots of this extract with 0.5 mM ascorbate in phosphate buffer, pH 7.4, and determined the intensity of the ascorbate radical by its characteristic ESR doublet at g = 2.0058. In measuring the dose dependence of ascorbate free radical formation by BSD E, we quantified the concentration of the ascorbate radical by double integration of the ESR signal. Freny’s salt was used as a concentration standard.

Spin-trapping. We prepared a solution of the spin-trap α-(4-pyridyl-1-oxide)-N-tetraakis(4-POBN; Aldrich (13)) in phosphate buffer, pH 7.4, adding 10% DM SO as an oxidizable substrate. Butadiene soot was bubbled into the spin-trap solution immediately after generation under vacuum. The mixture was allowed to separate into an upper phase containing soot solids and an aqueous phase containing trapped radicals. ESR signals were recorded over 24 hr.

Cell culture. NHBE cells from never-smokers (Strains #2129 & 2505) and bronchial epithelial cell growth medium (BEGM) were purchased from Clonetics (San Diego, CA). The cryopreserved cells (passage 1) were plated on Corning 60 mm plastic tissue culture dishes (Fisher Scientific, Springfield, NJ) precoated with FNC Coating Mix (BRFF, Ijamsville, MD), expanded to about 90% confluence and subcultured.

Figure 1. Schematic representation of combustion of 1,3-butadiene showing the genesis of oxidized (A,B) and reduced products (C).
**Exposure of NHBE cells to BSDE.** NHBE cells (passage 3 or 4) were cultured and expanded to approximately 50% confluence. We added BSDE to fresh BEGM just before exposure. For each treatment group, we prepared dosing solutions by adding BSDE from the stock solution to the total volume of medium (1:100) for the whole group, mixing, and then adding 5 mL of dosing solution to each dish of cells. The treatment groups were vehicle control, 0.1%–1.0% DMSO added to the media; low-dose, 1 µL BSDE (stock = 2.5 mg soot/mL DMSO) added/mL of media; high-dose, 1 µL BSDE (stock = 25 mg soot/mL DMSO) added/mL of media. After treatment, the cells were incubated for 72 hr at 35.5°C in an atmosphere of 95% air/5% CO2.

**Intracellular fluorescence.** At periods ranging from 4 to 72 hr after exposure, BSDE- and vehicle control-exposed NHBE cells were viewed at 200× with an inverted phase-contrast fluorescent microscope (Nikon, Garden City, NY) and photographed immediately. Excitation and emission wavelengths were 365 nm and >420 nm, respectively.

**Cytotoxicity.** At 72 hr, we stained cells with 0.4% Trypan Blue solution (Sigma) and then fixed them in 2% formaldehyde (pH 7.4). Three fields of 500 cells/dish were scored at 600× to determine the number of white (viable) and blue (nonviable) cells.

**Binucleated cells (BNCs).** After 72 hr, we washed the cells twice with HEPES-buffered saline, fixed them in 2% formaldehyde (pH 7.4), treated them with 10 µg ribonuclease A (Sigma) (30 min, 37°C), and then stained them with 5 µg propidium iodide (Sigma) overnight at 4°C. Three fields of 500 cells per dish were scored at 600× to determine the number of binucleated cells (14). We considered cell cultures to have significantly elevated levels (p < 0.05) of BNCs if the number of BNCs per 500 cells was greater than the mean number of BNCs per 500 cells + 2 standard deviations (SDs) for the vehicle control group.

**Statistical analysis.** For both the cytotoxicity and BNC assays, we compared means of control and BSDE-exposed groups by analysis of variance (ANOVA). We compared differences between group means by the Student-Newman-Keuls test (alpha = 0.05).

**Transmission electron microscopy.** We fixed NHBE cell monolayers in situ with a mixture of 1.25% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate for 1 hr at room temperature. We washed the cells in several changes of 0.1M sodium cacodylate containing 5% sucrose; postfixation occurred in 1% Oso4 in distilled water for 1 hr. Following postfixation, cell monolayers were dehydrated through a graded ethanol series and embedded in situ with Epon-Araldite (Electron Microscopy Sciences, Fort Washington, PA). After polymerization, portions of the embedded monolayer were excised and sectioned. We stained the resulting sections with uranyl acetate and lead citrate and examined them with a Zeiss EM-10C transmission electron microscope (Zeiss, Oberkochen, Germany).

**Results**

**Combustion of 1,3-butadiene and recovery of products of incomplete combustion.** The 1,3-butadiene vapor fire used for generating BSDE had large thermal gradients with temperatures measured in situ ranging from 430°C at the edges of the flame to 1,100°C at the cone (12). Soot yields varied with fuel flow rate and ranged from 0.1–1.0%, but were constant within the specified flow regime (see "Materials and Methods"). This soot contains a broad and unique range of aromatic hydrocarbons (AHS) and PAHs. In the semivolatile range, the mixture contained hundreds of AHSs and PAHs ranging from benzene to coronene. Cyclopenta-fused PAHs also were well represented. Among these compounds were numerous known and suspected human carcinogens, e.g., benzo(a)pyrene [BaP] (Figure 1). We detected PAHs with higher molecular mass, ranging as high as 1,000 amu (not shown), with compounds in the range 228–366 amu well represented (Figure 2; M+H++ masses > 500 not shown) and in large amounts [1,000–5,000 cigarette equivalents of benzo(ghi)perylene + benzo(a)pyrene + perylene].
per gram substrate burned; m/z 252; M+H•+ masses (i.e., 253.1) shown]. At least four dibenzopyrene isomers [C24H14; m/z 302; M+H•+ masses (i.e., 303.1) shown] also were tentatively identified. Dibenzopyrene isomers are of particular interest because they are found in urban air and most are mutagenic (15).

The presence of NO2 vapors either in the flame or in contact with combustion products produced assemblages of nitrated compounds including nitro- and polynitrotoluenes, -C2-benzenes, -phenols, and PAHs, e.g., -naphthalenes and -fluorene (data not presented). Cocombustion of mixtures of 1,3-butadiene/NO2 produced more nitration of selected chemicals than did post-combustion NO2 exposure, but cocombustion also provided much less soot yield than the latter (data not shown).

Electrochemistry. Electrochemical measurements indicated that BSDE was redox active at solid electrodes within an approximate physiologic potential range. Differential pulse polarography showed at least two reduction systems in the positive potential range (+600; +1,100 mV) plus another at ~750 mV (Figure 3). These data indicated that butadiene soot constituents may be expected to undergo redox reactions in solution or at adsorption sites in exposed cells. Additional findings showed that these redox systems were not classically reversible (data not presented).

Electron spin resonance (ESR). Soot particles from combustion of pure 1,3-butadiene also had strong solid-state free-radical character in ESR analysis (g factor = 2.0028; ~4% spin/weight vs. weak pitch and DPPH standards (Figure 4A)). Based on the weak pitch standard, the soot contained 1,018 spins/g, comparable to the values for cigarette tar. The radicals present in soot powder were stable for more than one year and could be extracted in organic solvents, although in relatively low yields. The lack of discernible hyperfine structure suggested the presence of a mixture of radical species with overlapping ESR spectra. Alternatively, this could indicate the presence of a solid-state radical.

As a result of these electrochemical and ESR findings, we investigated the effects of several dissolved redox systems on the stable butadiene soot radical signal. Reducing agents including ascorbic acid, glutathione, sodium dithionite, chlorogenic acid, and NADPH had no effect on the intensity or line shape of the soot radical ESR signal (data not presented). Although ascorbic acid was unable to quench the soot radical signal, BSDE did effect the one electron oxidation of ascorbic acid to the semidehydro-ascorbate free radical (Figure 4B). The signal was quasi-stable, suggesting that a redox cycle maintains a steady-state concentration of the ascorbate radical (Figure 4C).

Spin trapping. We examined the oxidizing properties of butadiene soot further using the spin trap, 4-POBN. A 15-line ESR spectrum (Figure 5) arose from two radical...
species, the methyl (CH₃•) adduct of 4-POBN (hyperfine splittings: aN = 16.0 G and aH = 2.8 G) and the hydrogen atom adduct of 4-POBN (aN = 15.8 G and aH (2H) = 10.2 G). The CH₃• adduct signal intensity increased steadily over the course of the experiment (Figure 5), indicating that the DMSO was being oxidized continuously to form the CH₃• radical, which is trapped by 4-POBN in the aqueous phase.

Intracellular fluorescence in BSD E-exposed NHBE cells. Within 4 hr after exposure to BSD E, extranuclear cell fluorescence was apparent. The fluorescence intensified up to 24 hr, and still was prominent at 72 hr. The increase in intracellular fluorescence was dose-related in BSD E-exposed cells (Figure 6B,D,F). About 30% of NHBE cells in the high-dose group were fluorescent.

BSD E cytotoxicity. We measured Trypan Blue exclusion in NHBE cells 72 hr after a single exposure to BSD E (Table 1). BSD E treatment elicited significant increases in cytotoxicity relative to vehicle controls (ANOVA, F = 54.04). Both low dose (2.5 µg soot/mL) and high dose (25.0 µg soot/mL) BSD E-treated cells exhibited increased cytotoxicity compared with vehicle control cells. In addition, there was significantly greater cytotoxicity in high-dose than in low-dose BSD E-treated NHBE cells (Student Newman-Keuls test, alpha = 0.05).

Induction of BNCs. Unexposed controls, vehicle controls, and NHBE cells exposed to BSD E for 72 hr were stained with propidium iodide and assayed for the presence of BNCs (Table 2). The background level was 18 ± 1 BNCs/500 cells (mean ± SD, n = 3) in unexposed controls, so the significance level (mean + 2 SD s, p < 0.05) was established as > 20 BNCs/500 cells. There were 20 BNCs/500 cells in the vehicle control group. The ANOVA (F = 52.17) revealed differences between the group means. The high-dose NHBE group had significantly more BNCs than either the low-dose or vehicle-treated groups. There were no differences between the vehicle-control and low-dose groups (Student Newman-Keuls test, alpha = 0.05).

Morphologic alterations. The cytotoxicity measured at 72 hr was preceded by pronounced morphologic changes. These alterations, detected by light microscopy as early as 24 hr after exposure, included cell swelling and rounding as well as apparent plasma membrane irregularities. These changes were examined further by transmission electron microscopy. At 72 hr, DMSO-treated cells displayed normal-sized nuclei with nucleoli, a number of small, mostly perinuclear vacuoles, prominent endoplasmic reticulum (ER), and numerous surface microvilli-like projections (Figure 7A). In contrast, high-dose BSD E-exposed NHBE cells were rounded, with swollen nuclei, no visible nucleoli, vesiculated cytoplasm with large, peripheral cytoplasmic vacuoles, swollen ER cisternae, and loss of surface microvilli-like projections (Figure 7B). The membrane irregularities seen by light microscopy were revealed as numerous rounded surface blebs, some of which had budded off from and were located adjacent to the plasma membrane (Figure 7B). These blebs enclosed amorphous cytoplasmic material.

Discussion

The increased interest in the roles of PM₂.₅ in respiratory and cardiovascular morbidity and mortality has been accompanied by efforts to develop model systems for studying in vivo and in vitro responses to airborne particulates. ROFA studies have provided data on inflammatory responses of airway epithelial cells to particles containing primarily trace metals (7,8). The National Institute
of Standards and Technology (Gaithersburg, MD) urban dust mixture (16) is a good model for particulates containing low levels of adsorbed organic compounds. On the other hand, pyrogenic yields of hydrocarbons and their product distribution differ dramatically among combustion sources as well as regionally. Thus, additional models of urban/industrial PM mixtures reflecting these differences would be valuable. This is particularly true of pollutant-enriched particulate mixtures that could represent worst cases or upper limits of potential exposure, as well as provide novel toxicologic insights.

The butadiene soot described here is a model for a “real-world” flame product. In the real world, the combustion substrate for uncontrolled burns of low molecular weight petrochemicals generally is a mix of industrial grade C₉–C₉ hydrocarbons, primarily, but not exclusively, 1,3-butadiene. Thus, the levels we describe here of PAHs generated from open flaring of a single pure (>99%) chemical, 1,3-butadiene, represent a pollutant-enriched situation. The range of products generated from combustion of pure 1,3-butadiene is wider than that produced by combustion of a mix of C₂–C₈ hydrocarbons.

Here we continue the physico-chemical characterization of butadiene soot (12) and report our initial observations on the effects of BSDE, a DM SO extract of this soot, on putative targets—NHBE cells.

The combustion product analyses revealed that butadiene soot contains a broad and concentrated range of PAHs and high molecular weight species, including reactive cyclopenta and methylene-bridged species and a series of known and suspected mutagens/carcinogens. The wide range of products found in BSDE, including AHs, PAHs, and free radicals, combined with the small size of the generated particles, indicates that inhalation of BSDE could have deleterious health consequences.

The data presented here were derived from one stock preparation of BSDE. In experiments not reported here, with other stocks prepared under identical burn conditions, no differences were found in either the range or yield of PAHs, as analyzed in two different laboratories by GC/MS. If, during combustion of 1,3-butadiene, the same conditions (e.g., substrate flow rate) are maintained from run to run, then the soot properties remain constant. If, for example, flow rates are increased dramatically, the same products are formed, but at lower yields.

Results of electrochemical studies and those from ESR analysis of ascorbate radicals generated in a dose-dependent manner by BSDE confirmed the presence of pro-oxidant activity. This activity, however, was not related to the solid-state soot radical (Figure 4). This radical was unaltered by treatment with ascorbate and was unaffected by aging (18 months) of the soot. The stability and longevity of the solid-state radical and its presence in filtered, clarified BSDE suggests its possible utility as an environmental and biologic probe (i.e., of soot production and exposure). Conversely, POBN spin-trap results confirmed the presence of a very strong, reactive oxidant. This oxidant system was temporally dynamic, with radical products evolving and being trapped for >24 hr. This time frame of oxidant evolution is on the same order as that of the toxicologic endpoints reported here. These data indicate that BSDE has strong oxidative properties that persist over biologically relevant time frames.

By virtue of their anatomic location, NHBE cells are putative targets in vivo for inhaled submicron particles that are present in urban and/or industrial particulate mixtures. Therefore, we selected NHBE cells as the targets for toxicologic studies with BSDE. Cytoplasmic fluorescence was the earliest and most striking response of the cells to BSDE exposure. Fluorescence was evident in the low-dose cells, very prominent in the high-dose group and absent from controls (Figure 6). These effects could be detected as early as 4 hr after exposure. There was no evidence of nuclear fluorescence up to 72 hr.

Cytoplasmic fluorescence has been attributed to accumulation of both aromatic hydrocarbons (17) and oxidized macromolecules, including age-associated fluorescent pigments, e.g., lipofuscin (18). However, the absence of cytoplasmic fluorescence in control NHBE cells suggests that their response to BSDE is distinct from the prominent, punctate fluorescence that has been described in mammalian cells and that has been ascribed to lysosomal lipofuscin accumulation (19). The cytoplasmic fluorescence does not appear to be caused by intrinsic fluorescence of a BSDE component. The fluorescence emission spectrum of a whole-cell lysate from BSDE-treated cells differed from

Table 1. Dose-dependent cytotoxicity of BSDE in NHBE cells.

| Treatment group | Mean no. of viable cells | Percent viable cells | Percent cytotoxicity |
|-----------------|--------------------------|----------------------|---------------------|
| Vehicle control | 435 ± 13                 | 87 ± 3               | 9.2                 |
| Low dose        | 395 ± 10                 | 79 ± 2               | 9.2                 |
| High dose       | 354 ± 2                  | 71 ± 1               | 18.6                |

*See “Materials and Methods” for description of treatment groups. *Values shown are mean ± SD; these were determined by dividing the mean number of viable cells by the total number of cells scored. *Values are relative to the control group.

Figure 7. Low-magnification transmission electron micrographs of a vehicle-control (DM SO) NHBE cell (A) and a high-dose BSDE-exposed NHBE cell (B) 72 hr after a single acute exposure to BSDE. The cell surface in (A) is characterized by many microvillus-like surface projections. The cell surface in (B) is characterized by large blebs containing amorphous cytoplasmic material. The numerous microvilli seen in (A) are not apparent in (B). In (B) the cytoplasm is vesiculated and contains large peripheral vacuoles and swollen cisternae of the endoplasmic reticulum. Magnification = 2,500 x.
those of BSD E alone and of BSD E added to a lysate from vehicle-control NHBE cells (data not presented). It is more likely that NHBE fluorescence can be accounted for largely by protein denaturation, which begins early in cell death, continues even after cells have died, and is detected readily in unfixed tissue samples by ultraviolet microscopy. Fluorescence of altered (e.g., denatured, covalently modified) proteins as an early marker of cell death has been discussed in detail elsewhere (20).

Here, cellular fluorescence was detectable as early as 4 hr after treatment and varied with dose. Cell populations within treatment groups exhibited a range of fluorescence intensity, possibly corresponding to degrees of cell damage (Figure 6D,F). The presence in BSD E of a wide range of AHs and PAHs and of at least one strongly oxidizing component is consistent with these observations—the NHBE cell responses reflect acute oxidative damage. Quantitation of the cell damage indicated by the fluorescence results was provided by the dye exclusion and BNC assays. Dose-dependent cytotoxicity was demonstrated in both strains of NHBE cells, 72 hr after exposure to BSD E. Although wide differences (1,000-fold) have been reported in the survival of different NHBE strains treated with chemical carcinogens (21,22), here both strains responded very similarly to the single BSD E challenge. The BSD E was only mildly cytotoxic, (~20% in the high-dose group) as estimated by Trypan Blue exclusion. This suggests that more subtle forms of damage than large-scale denaturation of proteins (e.g., genetic damage) might be produced in vivo and retained in otherwise viable BSD E-exposed cells, as the BNC results indicate.

BN Cs appear spontaneously both in vivo and in vitro (23). They can result from an arrest in the G2 phase of the cell cycle (24) and can be induced by chemicals (14,25) and radiation (26). BSD E elicited a dose-dependent increase in BN Cs in NHBE cells (Table 2) similar to that observed in the phenotypically altered progeny of NHBE cells exposed to fractionated doses of α-particles (26). In addition, BN C formation has been proposed to be a form of genomic instability leading to altered DNA content in tumor cells (27). These independent studies (26,27) combined with the presence of known genotoxic agents in the BSD E lead us to postulate that the BNC formation observed here likely is a genotoxic response. A fraction of BSD E-treated cells exhibited major morphologic alterations, including swelling both of the cytoplasm and endoplasmic reticulum, cytoplasmic vacuolization, and extensive plasma membrane blebbing. Three changes are characteristic of oncosis, an ischemia-induced process of accidental cell death (20,28,29).

The chemical analyses here reveal the presence of known carcinogens/mutagens in high amounts and of highly reactive oxidizing species. The combination of these findings with the moderate cytotoxicity and genotoxicity in NHBE cells exposed to BSD E is provocative. BSD E, or a similar complex mixture, would be expected to be procarcinogenic by virtue of its interference with normal cell division combined with its relatively mild cytotoxicity to target cells along the respiratory tract (i.e., NH BE cells). Thus, in vivo investigations of the potential carcinogenicity, as well as of other respiratory and cardiovascular effects, of BSD E and similar complex mixtures are warranted.

REFERENCES AND NOTES

1. Louisiana Office of Public Health. 1999 Louisiana Health Report Card, Vol. 38, New Orleans, LA:Louisiana Office of Public Health, 1999.

2. Kodavanti UP, Costa DL, Bromberg PA. Rodent models of carcinogenicity, as well as of other respira-

3. Bayram H, Devalia J, Sapsford R, Ohtoshi T, Miyabara Y, Sagai M, Davies R. The effect of diesel exhaust particles on cell function and release of inflammatory mediators from human bronchial epithelial cells in vitro. Am J Respir Cell Mol Biol 19:98–106 (1998).

4. Boland S, Baeza-Squiban A, Fornier T, Houcine O, Sagai M, Davies R. The effect of diesel exhaust particles on cell function and release of inflammatory mediators from human bronchial epithelial cells in vitro. Am J Respir Cell Mol Biol 19:98–106 (1998).

5. Kennedy C, Mitchell C, Fukushima N, Neft R, Lechner J. Induction of genomic instability in normal human bronchial epithelial cells by 238 Pu α-particles. Am J Physiol Lung Cell Mol Physiol 278:L25–32 (2000).

6. Hashimoto S, Gon Y, Takeshita I, Matsumoto K, Ibi iki, Takizawa H, Kudoh S, Horie T. Diesel exhaust particles activate p38 MAP kinase to produce interleukin-8 and RANTES by human bronchial epithelial cells and N-acetylcysteine attenuates p38 MAP kinase activation. Am J Respir Crit Care Med 161:280–285 (2000).

7. Carter J, Ghio A, Samet J, Devlin R. Cytokine production by human airway epithelial cells after exposure to air pollution particles is metal-dependent. Toxicol Appl Pharmacol 146:180–188 (1997).

8. Quay J, Reed W, Samet J, Devlin R. Air pollution parti-

9. U.S. Environmental Protection Agency. National ambient air quality standards for particulate matter—final rule. Fed Reg 62:138 (1997).

10. Pollution Locator-Criteria Air Pollutants in 1998, Rank Counties by Health Risks. Environmental Defense Fund: In Your Community. Available: http://www.scorecard.org/scorecard/index [cited 11 January 2001].

11. Info Louisiana: LA Maps & Geographical Information. Available: http://www.state.la.us/state/map.htm [cited 11 January 2001].

12. Catallo WJ, Polycyclic aromatic hydrocarbons in combustion residues from 1.3-butadiene. Chemosphere 16:1053–1067 (1988).

13. Pou S, Ramos C, Gladwell T, Renks E, Centra M, Young D, Cohen M, Rosen G. A kinetic approach to the selection of a sensitive spin trapping system for the detection of hydroxyl radical. Anal Biochem 217:76–83 (1994).