Molecular and Genetic Damage from Environmental Tobacco Smoke in Young Children[1]

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Abstract

To assess the risks of early life exposure to environmental tobacco smoke (ETS), we tested whether four biomarkers in peripheral blood were associated with home ETS exposure in Hispanic and African-American children. The biomarkers included cotinine (a metabolite of nicotine) and three indicators of molecular and genetic damage from mutagens/carcinogens, protein adducts formed by the carcinogens 4-aminobiphenyl (4-ABP) and polycyclic aromatic hydrocarbons (PAHs), and sister chromatid exchanges (SCEs). We also explored possible ethnic differences in biomarkers. The study cohort comprised 109 Hispanic and African-American preschool children (1–6 years of age). Plasma cotinine was analyzed by gas chromatography, 4-ABP-hemoglobin adducts by gas chromatography-mass spectroscopy, PAH-albumin adducts by ELISA, and SCEs by cytogenetic techniques. Data on the amount of smoking by mothers (average 10.5 cigarettes per day) and other household members and regular visitors (average 6.5 cigarettes per day) were obtained by interview-administered questionnaires. Cotinine, 4-ABP-hemoglobin adducts, and PAH-albumin were significantly higher ($P < 0.05$) in the ETS-exposed children compared with the unexposed. SCEs were marginally higher ($P = 0.076$). African–American children had higher levels of cotinine ($P = 0.059$) and PAH-albumin ($P = 0.02$) than Hispanic children, after controlling for exposure to ETS. These results indicate molecular and genetic damage in minority children with relatively low exposure to ETS. They highlight the need for smoking prevention and cessation programs in women of reproductive age and in families with young children.

Introduction

An estimated 9 to 12 million American children under the age of 5 are exposed to ETS[3] by a household member (1). ETS contains over 40 known carcinogens, including PAH, 4-ABP, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nitrosamine derived from nicotine (2). There is growing evidence that infants *in utero* and young children are more vulnerable than adults to genetic damage and other effects of carcinogens, and that carcinogenic exposures during early development can increase the risk of cancer later in life (3, 4). An estimated 17% of lung cancers among nonsmokers have been attributed to high levels of ETS exposure during early childhood and adolescence (5). In addition, young children of smoking parents have an increased incidence of chronic bronchitis, pneumonia, asthma, and middle ear infection as well as impaired development of lung function (2, 6, 7). Cancer risks from ETS, especially to young children and minorities, have not been adequately characterized (8).

ETS is comprised of diluted sidestream smoke from the smoldering of the cigarette between puffs and mainstream smoke exhaled by the smoker. Undiluted sidestream smoke contains higher concentrations of certain carcinogens than mainstream smoke, including the PAH BP (2.5–3.5-fold) and 4-ABP (31-fold; Ref. 2).

In this study, multiple biomarkers were evaluated in a cohort of Hispanic and African-American preschool children with varying exposure to ETS. The markers included plasma cotinine, 4-ABP-Hb adducts, PAH-albumin adducts, and SCEs. Cotinine, a metabolite of nicotine, has a plasma half-life of 21–48 h in nonsmokers (9). PAHs are a class of carcinogens found in ETS (BP, 68–136 ng/cigarette in undiluted sidestream smoke) and in other environmental media (diet, drinking water, and workplace and ambient air; Ref. 10). Certain carcinogen-protein adducts, including 4-ABP-Hb and PAH-albumin, can serve as surrogates for DNA adducts induced by the same chemicals (11, 12). PAH-DNA and other carcinogen-DNA adducts have been correlated with carcinogenic potency in experimental studies; both PAH-DNA and 4-ABP-Hb have been associated with human cancer (13, 14). Thus, PAH- and 4-ABP- protein adducts may be considered to be relevant to the potential risk of cancer (4, 15). We previously reported an effect of ETS on PAH-albumin adducts in an initial subset of this cohort (16); however, another study did not observe the

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[3] The abbreviations used are: ETS, environmental tobacco smoke; PAH, polycyclic aromatic hydrocarbon; 4-ABP, 4-aminobiphenyl; BP, benzo(*a*)pyrene; 4-ABP-Hb, 4-ABP- hemoglobin; SCE, sister chromatid exchange; CPD, cigarettes per day.

same relationship (17). PAH-albumin has a half-life of 21 days. SCE represent reciprocal exchanges between sister chromatids at the same locus after the breakage and rejoining of the DNA strands during replication. They have been correlated with DNA adducts and somatic mutations (18). Although the mechanism and human health risk associated with this marker are still unknown, SCEs have been widely studied as a non-chemical-specific indicator of genetic damage from mutagens/carcinogens (19). An increase in SCEs has not generally been seen in passive smokers (20, 21). Because they are measured in lymphocytes, SCEs provide an integrating dosimeter of previous exposure over a period of several years.

Epidemiological studies and assessments of cancer risk from ETS have been limited by inadequate data on individual exposure to ETS and on the range of individual variability in biological response to exposure. Biomarkers can address these gaps in knowledge by providing direct measures of the internal and biologically effective dose of ETS as well as of the biological effects and factors that increase individual susceptibility (22–24).

The goal of this study was to assess whether biomarker levels are associated with ETS exposure in children and to evaluate individual variation among children with similar exposure, including possible ethnic differences in biomarkers. Earlier, we reported the results of cotinine and PAH-albumin on 87 of these children (16). This report summarizes results for a wider array of biomarkers evaluated in the full cohort of 109 children.

Materials and Methods

Subjects and Data Collection

The study cohort was composed of preschool (1–6 years old) children residing in northern Manhattan, New York City. The mothers were the primary caretakers, spending at least 70% of their time in the home with their children. Children were in good health at the time of the study, without chronic diseases such as hypertension, AIDS, or epilepsy and without a history of cancer. Enrollment took place during the fall, winter, and early spring seasons when families spend most of their time indoors. A written consent form approved by the Columbia-Presbyterian Institutional Review Board was signed by each mother. A bilingual interviewer administered a questionnaire concerning environmental and health history of the participants. Information was collected on the child's passive smoking of cigarettes, cigars, and pipes; dietary PAH; and other relevant exposures. The health history of the child was also obtained. After the interview, 15 ml of blood was drawn from each study participant in a heparinized tube. Blood (3 ml) was aliquoted for the SCE assay, and the remaining blood was separated immediately into plasma and cells, aliquoted, and held at −80°C before laboratory analysis. All of the samples were coded and sent to the laboratories without any personal identifiers.

Of the 109 children enrolled, most (88%) were Hispanic, mainly Dominican and Puerto Rican; 13 participants (12%) were African-American. The numbers of subjects with each biomarker vary because of limited amounts of sample and logistical constraints of the laboratories.

Laboratory Analyses

Cotinine. The method involved liquid/liquid extraction of plasma, followed by gas chromatographic separation using a 30-m 0.25-μm megabore column and a nitrogen detector operated in the nitrogen mode. An internal standard, *N*-methyl cotinine, was added to the plasma before extraction. Five-point standard curves were generated for each analytical run, and low- and high-quality control samples were processed each day. The method required cold trapping injection followed by temperature programming to achieve optimal separation, which resulted in an analysis time of 18 min per sample. To facilitate productivity, an autosampler and online automatic data reduction were used so that samples could be processed during the evening or overnight, as needed (25).

4-ABP-Hb Adducts. The procedure used in this study was essentially the same as that reported previously (26). Briefly, the packed RBCs from 10 ml of blood were washed with a saline solution and lysed with a combination of distilled water and toluene. The supernatant after centrifugation was dialyzed against distilled water and was used directly for analysis. Hemoglobin content was determined by Drabkins's assay, and the internal standard (a solution of hemoglobin previously adducted with *N*-hydroxy-4-aminobiphenyl-d_9 and containing 150 pg of hydrolyzable amine) was added. After 30 min at room temperature, sufficient 10 M NaOH was added to make the mixture 0.1 M in NaOH. The amines were extracted with hexane after 1 h and derivatized with pentafluoropropionic anhydride. The hexane solution was concentrated to 20 ml for analysis by capillary gas chromatography with negative ion chemical ionization mass spectrometry. Selected ion monitoring for the derivatives of the amines could detect as little as 1 pg of 4-ABP adduct per 10 ml of blood. The assay has a precision of 4% when the deuterated adduct standard is used.

PAH-Albumin Adducts. Albumin was isolated from the plasma after precipitation of immunoglobulins with saturated ammonium sulfate. The supernatant was acidified with acetic acid and left at 4°C overnight. The precipitated albumin was collected by centrifugation and resuspended in 0.05 M phosphate buffer (pH 7.6). The sample (5 mg in 500 μl) was adjusted to 0.1 N HCl and heated at 96°C for 3 h to release BP tetrols and possibly other PAH metabolites. After neutralization with NaOH and dilution with 500 μl of 0.05 M phosphate buffer, the sample was extracted twice with isoamyl alcohol. The combined extracts were washed with 2 ml of water, evaporated under a vacuum, and dissolved in 500 μl of PBS. The sample was analyzed by a competitive ELISA on 96-well polystyrene microwell plates that were coated with 5 ng of BPDE-I-DNA by drying PBS solutions overnight at 37°C. The plates were washed with PBS containing 0.05% Tween 20 using an automatic plate washer. A similar wash step was completed after each incubation. Monoclonal antibody 8E11 (27), which recognizes BPDE-I-DNA and protein adducts, was used at a 1:30000 dilution in 1% FCS. For the standard curve, serial dilutions of BPDE-I-tetrols in PBS from 5 to 2500 fmol in 50 μl were added to the coated wells followed by 50 ml of antibody. Albumin extracts (50 μl) were also mixed with 50 μl of diluted antibody. After the incubation of the mixture on the plate for 90 min and washing, goat antimouse IgG-alkaline phosphatase was added to the wells, followed by the substrate *p*-nitrophenyl phosphate (100 μl of 1:500 dilution). After 90 min, 100 μl of the substrate *p*-nitrophenyl phosphate (1 mg/ml) was added. For statistical analysis of the data, absorbance at 405 nm was measured after 2 h on a Dynatech MR 5000 recorder (Alexandria, VA) connected to an IBM computer.

SCEs. The SCE assay was performed on lymphocytes according to a variation of the method of Carrano and Moore (28). Lymphocyte cultures were initiated with 0.5 ml of plasma added to RPMI containing 15% FCS and 0.1 μg of phytohe-

Table 1 Cotinine, 4-ABP-Hb, PAH-albumin, and SCEs in children with and without ETS exposure

Passive exposure	Cotinine (ng/ml)	4-ABP-Hb (pg/g)	PAH-albumin (fmol/μg)	SCE (per cell)
No: no smoker in household				
Mean	0.264	23.8	0.185	8.82
Number	25	10	24	11
SD	0.596	9.22	0.142	1.78
Yes: mother or other household smoker(s)				
Mean	2.87	34.3	0.437	10.03
Number	84	41	82	53
SD	5.61	16.9	0.542	2.32
Total				
Mean	2.27	32.3	0.380	9.82
Number	109	51	106	64
SD	5.05	16.1	0.493	2.27
No < Yes, adjusting for ethnicity	$P < 0.001$	$P < 0.05$	$P = 0.04$	$P = 0.076$

Table 2 Biomarkers in children by three ETS exposure groups

Passive exposure	Cotinine (ng/ml)	4-ABP-Hb (pg/g)	PAH-albumin (fmol/μg)	SCE (per cell)
A: no: no smoker				
Mean	0.264	23.8	0.185	8.82
Number	25	10	24	11
SD	0.596	9.22	0.142	1.78
B: yes: household smoker				
Mean	0.869	32.4	0.315	9.98
Number	39	18	38	21
SD	1.13	13.4	0.449	2.42
C: yes: mother smokes				
Mean	4.61	35.9	0.543	10.07
Number	45	23	44	32
SD	7.19	19.31	0.597	2.28
A < B	$P = 0.002$	0.066	[a]	[a]
B < C	$P < 0.001$	[a]	$P = 0.017$	[a]
A < C	$P < 0.001$	$P = 0.073$	$P < 0.001$	$P = 0.078$

[a] $P > 0.1$, all values adjusted for ethnicity.

magglutinin (PHA by Burroughs-Wellcome) per 10 ml of culture medium. Two cultures were prepared for each sample. The mixture was incubated at 37°C for 72 h. Thirty μg/ml bromodeoxyuridine (BrdUrd) was added 40 h before harvest. Cells were arrested in metaphase with colcemid for 45 min. After hypotonic treatment and fixation in 3:1 methanol:glacial acetic acid, the cells were dropped onto cold wet slides. The staining method of Perry and Wolff (29) was used. A total of 50 metaphases were examined for each individual; 25 cells were scored by each of two observers to control for technician variability. The number of SCEs were counted in 50 cells, and results were reported as the average number of SCEs.

Statistical Analysis

Questionnaire-derived variables were used to calculate an ETS exposure index score for each child as follows. For children of smoking mothers, the exposure score was based on the mothers' average daily consumption of cigarettes, plus the average daily consumption of other household members and regular visitors, accounting for the amount of time the visitors were in the home. The score for children of nonsmoking mothers was based on the daily average smoked by household members and regular visitors. In addition to the total passive exposure score as a continuous variable, the exposed group was dichotomized (high/low) using the average score for the exposed group. Differences in biomarkers between children exposed to ETS by their mothers, children exposed only via others in the home, and children without home ETS exposure were also analyzed.

Levels of cotinine, 4-ABP-Hb, PAH-albumin, and SCEs were log (ln) transformed to normalize the distribution and to stabilize the variance. Regression analysis was used to determine whether the following variables were associated ($P < 0.1$) with the biomarkers: gender, ethnicity (Hispanic *versus* African-American), and season when the samples were collected. Consumption of charbroiled meat as an index of dietary PAH was infrequent and was not included. On this basis, only ethnicity was retained in the final regression model.

Differences between exposure groups (ETS yes/no) and between the three exposure groups (mother smokes, other household members smoke, no one in home smokes) were initially analyzed by Student's *t* test. Differences in biomarkers between African-American and Hispanic subjects were tested by ANOVA, adjusting for exposure status. Multiple regression was used to examine biomarker differences between exposure groups as well as dose-response relationships between the ETS exposure index and biomarkers, adjusting for ethnicity (Hispanic *versus* African American) as appropriate. Correlations between biomarkers were evaluated by Pearson's product moment correlation.

Results

Tables 1 and 2 summarize the distribution of children according to the exposure to ETS. Most of the smoking mothers and household members were light smokers (mothers: average 10.5 CPD, range 1–30; other household smokers: average 6.5 CPD, range 1–30). A few mothers reported only occasional exposure to cigar or pipe smoke.

All of the biomarkers were higher in exposed children than in unexposed children (Table 1 and Fig. 1). The differences were statistically significant for cotinine ($P = <0.001$), 4-ABP-Hb ($P < 0.05$), and PAH-albumin ($P = 0.04$) and of borderline statistical significance for SCEs ($P = 0.076$), after adjusting for ethnicity in the regression model. Differences between biomarkers in the low and high exposure groups—dichotomized by using the average score for the exposed group—were not statistically significant.

All of the four biomarkers increased across the three exposure groups (A, no ETS exposure; B, exposure by household members other than the mother; C, exposure via maternal smoking), as shown in Table 2. Regression analysis showed marked differences between exposure groups for cotinine (A<B<C, $P \leq 0.002$) and PAH-albumin (A<B, $P = 0.017$; A<C, $P = 0.001$). There were modest intergroup differences for 4-ABP-Hb (A<B, $P = 0.066$; A<C, $P = 0.073$) and SCEs (A<C; $P = 0.078$).

Regression of biomarkers against the continuous index of total passive smoking showed a significant association only for cotinine ($P < 0.001$). Cotinine and PAH-albumin were higher in the African-American children compared with the Hispanic children, after adjusting for exposure group ($P = 0.059$ and $P = 0.021$, respectively; see Table 3). Cotinine, 4-ABP-Hb, and SCEs were significantly correlated with PAH-albumin ($P < 0.02$).

Discussion

This study demonstrates for the first time that ETS exposure of young children via smoking by their mothers and other house-

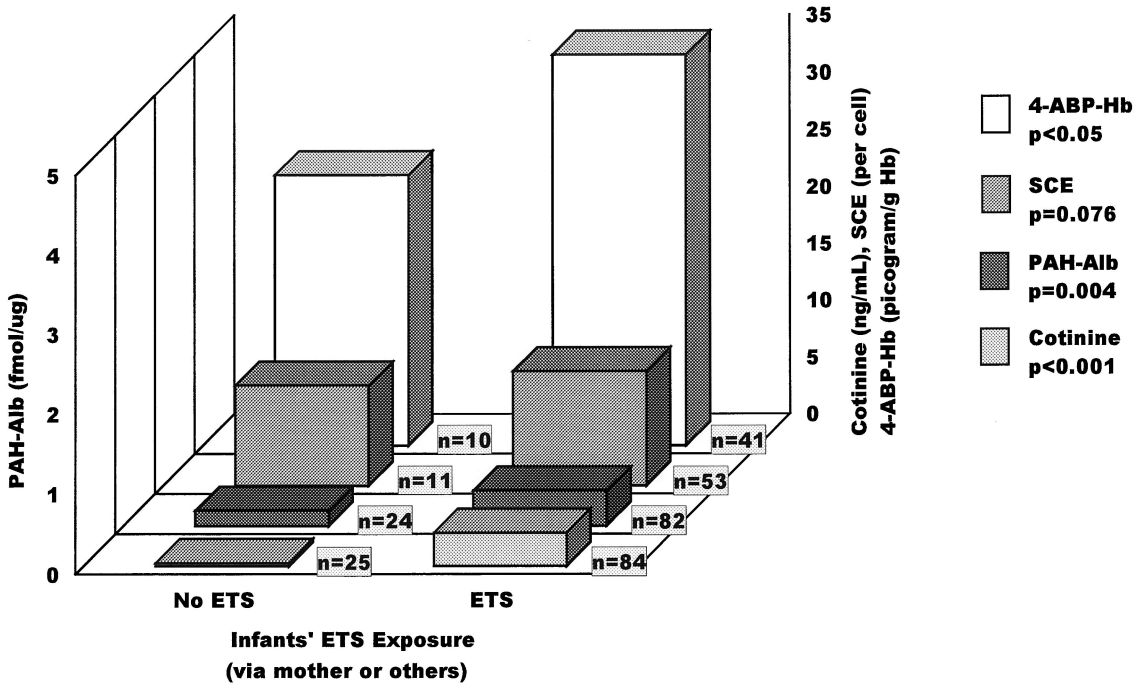

Fig. 1. Biomarkers are compared in the ETS-exposed and ETS-unexposed children. By regression analysis adjusting for ethnicity, all of the biomarkers were increased with exposure (see Table 1 for SDs and other details).

Table 3 Biomarkers in children stratified by ethnicity

	Cotinine (ng/ml)	4-ABP-Hb (pg/g)	PAH-albumin (fmol/μg)	SCE (per cell)
A. African-American				
Mean	5.64	26.3	0.550	9.80
Number	13	4	13	9
SD	12.9	5.08	0.369	1.86
B. Hispanic				
Mean	1.82	32.8	0.356	9.83
Number	96	47	93	55
SD	2.50	16.7	0.504	2.34
A > B, adjusting for ETS exposure	$P = 0.059$	$P > 0.1$	$P = 0.021$	$P > 0.1$

hold members is associated with increases in the internal dose of ETS (cotinine), the biologically effective dose of two known carcinogens (protein adducts with 4-ABP and PAH), and a general measure of genetic damage (SCEs). As mentioned, experimentally these particular carcinogen-protein adducts have been correlated with their corresponding DNA adducts, which in turn have been associated in a number of studies with increased cancer risk (2, 4, 11, 13, 30). ETS has been linked to 4-ABP-Hb (31, 32), and PAH adducts (16) in different populations. However, no studies have jointly measured all of the markers here, nor has this array of biomarkers been evaluated in minority children. Taken together, these results indicate that there is no point in the life cycle when ETS exposure is not capable of incurring molecular or genetic damage.

It is also noteworthy that the exposure to ETS in this cohort of children was relatively low; the mothers and other household members consumed an average of 10.5 and 6.5 CPD, respectively. The lack of a significant dose-response with the continuous index of ETS exposure for markers other than cotinine probably reflects the modest exposure to ETS in this study and/or the large interindividual variability in biological response to carcinogen/mutagens in tobacco smoke.

Limitations of the study include: (*a*) the lack of complete data on ETS exposure outside of the home; (*b*) the absence of personal monitoring data on air concentrations of nicotine and the other chemicals evaluated; and (*c*) the lack of information on exposure to ambient PAH. However, strengths of the study are detailed information on the level of home ETS exposure and the use of biomarkers that offer a significant advantage over air monitoring through their ability to register individual variability in the biological handling of these constituents.

The finding of increased biomarkers (cotinine and PAH-albumin) in African-American children compared with Hispanic children, after adjusting for exposure, is limited by small numbers and the possibility of misclassification of exposure. However, it is consistent with other data showing ethnic variation in the internal or molecular dose and effects of ETS (4, 33, 34). Possible ethnic differences in susceptibility require further investigation, particularly in light of sales campaigns for cigarettes targeting minorities (35). The results of this study highlight the need for policies and programs to prevent and reduce smoking by mothers and other members of households with young children.

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