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Quantitation of an Acetaldehyde Adduct in Human Leukocyte DNA and the Effect of Smoking Cessation

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Abstract

Acetaldehyde is one of the most prevalent carcinogens in cigarette smoke. It is also a major metabolite of ethanol and is found widely in the human diet and environment. Acetaldehyde DNA adducts are critical for its carcinogenic properties. The role of acetaldehyde DNA adducts in human cancer related to tobacco and alcohol exposure could be investigated with a suitable biomarker. Therefore, in this study we have developed a method for analysis of the major DNA adduct of acetaldehyde, *N*²-ethylidene-dGuo (**1**), in human leukocyte DNA. Leukocyte DNA was subjected to enzyme hydrolysis in the presence of NaBH₃CN, which converts adduct **1** to *N*²-ethyl-dGuo (**2**). [¹⁵N₅]*N*²-ethyl-dGuo was used as internal standard. After solid phase extraction, *N*²-ethyl-dGuo was quantified by LC-ESI-MS/MS-SRM. The method was sensitive, accurate, and precise, and applicable to low microgram amounts of DNA. It was applied to investigate the effect of smoking cessation on levels of adduct **1**, measured as adduct **2**. Twenty-five smokers who were only light drinkers were eligible for the study. Levels of adduct **2** were quantified at two baseline time points separated by one week, and again after four weeks of abstinence from smoking and alcohol consumption. The mean (± S.D.) levels of adduct **2** measured in the leukocytes of the smokers were 1310 ± 1720 (range 124 – 7700) and 1120 ± 1140 (range 138 – 5760) fmol/μmol dGuo at the two baseline points and 705 ± 438 (range 111 – 1530) fmol/μmol dGuo after 4 weeks of cessation. The median level of adduct **2** decreased significantly by 28% upon quitting smoking (*P* = 0.02). These results demonstrate that the major acetaldehyde DNA adduct can be reliably quantified by MS/MS methods in human leukocyte DNA and that cigarette smoking has a modest but significant effect on its levels.

Introduction

Acetaldehyde, found widely in the human environment, is genotoxic and carcinogenic (1). It causes mutations, sister chromatid exchanges, micronuclei, and aneuploidy in cultured mammalian cells, and gene mutations in bacteria (2,3). Inhalation of acetaldehyde produces adenocarcinoma and squamous cell carcinoma of the nasal mucosa in rats, and laryngeal carcinoma in hamsters (2,3). Acetaldehyde is “reasonably anticipated to be a human carcinogen” by the U.S. Department of Health and Human Services and is “possibly carcinogenic in humans” according to the International Agency for Research on Cancer (1,3).

The mutagenic and carcinogenic effects of acetaldehyde are initiated by its reaction with DNA. The major DNA adduct of acetaldehyde is *N*²-ethylidene-dGuo (**1**); several minor adducts as well as an interstrand cross-link have also been identified (4). NaBH₃CN treatment of DNA that has been reacted with acetaldehyde converts adduct **1** to *N*²-ethyl-dGuo (**2**)(4).

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Quantitation of acetaldehyde - DNA adducts in human tissues would provide one approach to investigating its possible role in human cancer. With this goal in mind, we have recently developed an LC-ESI-MS/MS-SRM method for quantitation of *N*²-ethyl-dGuo (**2**) in human hepatic DNA that had been treated with NaBH₃CN (5). Adduct **2** was detected in all human liver DNA samples analyzed, with an average level of about 500 fmol/μmol dGuo. In the present study, we have extended this research by developing a method for the analysis of adduct **2** in NaBH₃CN - treated human leukocyte DNA, a readily available source of DNA for studies investigating the origins of adduct **1**. The method was applied to investigate the effects of cigarette smoking on levels of adduct **1**. Cigarette smoke is one potentially important source of acetaldehyde exposure. Levels of acetaldehyde in mainstream cigarette smoke typically range from 500 – 1000 μg/cigarette (6,7).

Experimental Procedures

Chemicals and Enzymes

*N*²-Ethyl-dGuo (**2**) and [¹⁵N₅]*N*²-ethyl-dGuo ([¹⁵N₅]**2**) were prepared as described (5). [¹³C₂]Acetaldehyde was procured from Cambridge Isotope Laboratories (Andover, MA). Ethanol was obtained from AAPER Alcohol and Chemical Co. (Shelbyville, Ky). Isopropanol was purchased from Acros Organics (Morris Plains, NJ). Puregene DNA purification solutions were obtained from Gentra Systems (Minneapolis, MN). Calf thymus DNA was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Alkaline phosphatase (from calf intestine) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). All other chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

DNA isolation from Human Leukocytes

DNA was isolated using the DNA purification from buffy coat protocol (Gentra Systems) with several modifications. Briefly, 9 mL of RBC cell lysis solution was added to 3 mL of buffy coat prepared from 30 mL of whole blood. The white blood cell pellet was collected by centrifugation and treated with 20 mL of cell lysis solution and 150 μL RNase A (4 mg/mL). To the cell lysate was added 10 mL of protein precipitation solution and the mixture was centrifuged to remove protein. DNA was precipitated from the supernatant by addition of 20 mL of isopropanol. The DNA pellet was dissolved in 3 mL of 10 mM Tris -1mM EDTA buffer, pH 7, and further purified by extraction with 3 mL chloroform/isoamyl alcohol (24:1). The DNA was precipitated from the aqueous phase by addition of 0.3 mL of 5M NaCl and 6 mL of 100% ice cold ethanol sequentially and washed with 5 mL of 70% ethanol in H₂O, and then 100 % ethanol. DNA was dried in a stream of N₂ and stored at -20 °C until use. Buffy coat (3 mL) from 30 mL of blood typically yielded 0.3 – 0.5 mg DNA.

For the artifact study, the samples were initially lysed with 15 mL of cell lysis solution containing 134 mM NaBH₃CN, followed by addition of 5 mL cell lysis solution containing 0.57 mM [¹³C₂]acetaldehyde. For this study, the isopropanol, Tris -EDTA buffer, ethanol and 70% ethanol contained 100 mM NaBH₃CN.

The purity of the DNA was determined by measuring its UV absorption at 230 nm, 260 nm and 280 nm. The A₂₆₀/230 and A₂₆₀/280 ratios were greater than 2.3 and 1.7, respectively.

Quantitation of dGuo was carried out using Waters Associates (Milford, MA) instruments and a UV detector (Shimadzu Scientific Instruments, Columbia, MD), operated at 254 nm. A 4.6 mm $\times$ 25 cm Luna 5 μ m C18(2) column (Phenomenex, Torrance, CA) was used with a gradient from 5 to 40% CH₃OH over the course of 35 min at a flow rate of 0.7 mL/min.

Sample Enrichment and MS/MS Analysis

For enzyme hydrolysis, DNA (0.1 – 1.2 mg) was dissolved in 400 μ L of 10 mM Tris/5 mM MgCl₂ buffer containing [¹⁵N₅]*N*²-ethyl dGuo (250 fmol) and NaBH₃CN (30 mg). After the pH was adjusted to 7 with 0.1N HCl, the DNA was initially digested overnight at room temperature with 1300 units of DNase I (type II, from bovine pancreas). Then to the resulting mixture were added 1300 additional units of DNase I, 0.07 units of phosphodiesterase I (type II, from *Crotalus adamanteus* venom), and 750 units of alkaline phosphatase. The mixture was incubated at 37 °C for 70 min and then allowed to stand overnight at room temperature. Enzymes were removed by centrifugation using a centrifree MPS device (MW cutoff of 30,000; Amicon, Beverly, MA). The hydrolysate, after removal of a 10 μ L aliquot for dGuo analysis, was desalted and purified using a solid phase extraction cartridge [Strata-X 33 μ m, 30 mg/1 mL (Phenomenex)]. The cartridge was washed with 1 mL of H₂O, 1 mL of 10% CH₃OH in H₂O, and 1 mL of 70% CH₃OH in H₂O. The 70% CH₃OH fraction was collected and evaporated to dryness, dissolved in 1 mL of H₂O, and purified using a mixed mode, anion exchange reversed phase extraction cartridge (Oasis MAX, 30 mg/cartridge, Waters). The pH of the sample was adjusted to >12 by addition of 300 μ L of 0.2 N NaOH, and it was applied to the cartridge, which had been equilibrated with 0.2 N NaOH. The cartridge was washed with 1 mL of 0.01 N NaOH, 1 mL of 0.01 N KOH in CH₃OH, 0.5 mL of H₂O, 1 mL of 1 M ammonium acetate (pH 6.8), 0.5 mL of H₂O, and 1 mL of 10% CH₃OH in H₂O. Adducts were eluted with 1 mL of 70% CH₃OH, and the solution was evaporated to dryness. The residue was dissolved in 20 μ L of H₂O, and 8 μ L aliquots were analyzed by LC-ESI-MS/MS.

LC-ESI-MS/MS analysis was carried out with an Agilent 1100 capillary flow HPLC (Agilent Technologies, Palo Alto, CA) with a 0.5 mm $\times$ 25 cm 5 μ m C18 column (Agilent Zorbax SB-C18) and a Discovery Max (Thermoelectron, San Jose, CA) triple quadrupole mass spectrometer. The solvent elution program was a 10 μ L/min gradient from 5 to 40% CH₃OH in 35 min at 30 °C. The ESI source was set in the positive ion mode as follows: voltage, 3.7 kV; current, 3 μ A; and heated ion transfer tube, 275 °C. The collision energy was 12 eV, and the Ar collision gas pressure was 1.0 mTorr.

Adducts were quantified by MS/MS with selected reaction monitoring (SRM) at m/z 296 $\rightarrow$ m/z 180 ([M + H]⁺ $\rightarrow$ [BH]⁺ of *N*²-ethyl-dGuo (2)), and the corresponding transitions, m/z 298 $\rightarrow$ m/z 182 for [¹³C₂]*N*²-ethyl-dGuo, and m/z 301 $\rightarrow$ m/z 185 [¹⁵N₅]*N*²-ethyl-dGuo.

A calibration curve was constructed before each analysis using a standard solution of *N*²-ethyl-dGuo and [¹⁵N₅]*N*²-ethyl-dGuo. A constant amount of [¹⁵N₅]*N*²-ethyl-dGuo (40 fmol) was mixed with differing amounts of *N*²-ethyl-dGuo (4, 8, 16, 32, 128 and 512 fmol) and analyzed by LC-ESI-MS/MS-SRM.

Accuracy and precision were determined by adding *N*²-ethyl-dGuo (25, 50, 100, 200 or 400 fmol) to 0.3 mg calf thymus DNA and analyzing samples in triplicate. *N*²-ethylidene dGuo already present in calf thymus DNA (130 fmol/0.3 mg) was subtracted from each value. Sensitivity was determined by estimation of the LOD of the adduct in the DNA matrix with S/N ~4. Recovery was determined by adding [¹⁵N₅]*N*²-ethyl-dGuo (250 fmol) to 0.35 mg calf thymus DNA and processing as described above. These samples were compared to the same amount of calf thymus DNA processed without [¹⁵N₅]*N*²-ethyl-dGuo, which was then added just before the LC-ESI-MS/MS step.

Buffer blanks containing internal standard were processed as above and analyzed to check the MS instrument baseline and possible contamination. Calf thymus DNA (0.2 mg) with internal standard added as above was used as a positive control to determine inter-day precision and accuracy. Each set of samples was run together with buffer blanks and positive controls.

Smoking Cessation Study

This study was approved by the University of Minnesota Research Subject's Protection Programs Institutional Review Board Human Subjects Committee. The purpose of this study was to examine the effects of 4 weeks of smoking abstinence on levels of adduct **1**. Smokers were recruited by newspaper, radio and television advertisements and word-of-mouth from the Minneapolis and St. Paul metropolitan areas. Interested subjects called our research clinic and underwent initial screening over the telephone. Subjects who met the screening criteria were asked to come into the clinic to obtain consent and to undergo a more thorough screening procedure including completion of questionnaires related to smoking, drinking and health history. Subjects were included in this study if they were: 1) smoking at least 10 cigarettes per day; 2) motivated to quit; 3) drinking alcohol only occasionally or not at all (e.g., drinking less than or equal to 6 alcoholic drinks per month and willing to abstain from alcohol during the study; 4) not using marijuana on a regular basis (greater than once a month); 5) not diagnosed with a history of alcohol dependence; 6) in stable and good physical and mental health; and 7) not pregnant.

The study protocol called for a six week study with two weeks of baseline smoking data collection and four weeks of data collection during abstinence from all tobacco products. After two weeks of baseline smoking, subjects were asked to quit smoking after the second clinic visit. In order to facilitate smoking abstinence, subjects were provided brief behavioral counseling and the nicotine patch and/or the nicotine lozenge. During this study, subjects attended weekly clinic visits with the exception of two clinic visits during the quit week. Subjects were also asked to keep a daily diary of smoking events while in the study. At the clinic visits, subjects were assessed for smoking status by reviewing the diaries and were measured for alveolar carbon monoxide levels to verify abstinence ($\text{CO} < 8 \text{ ppm}$). Other measurements included vitals, concomitant medications, diet, and adverse events. Blood (30 mL) and first void morning urine samples which were assessed for adduct **1** and total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL plus its glucuronides), respectively, were obtained two times during the baseline smoking period (one week apart) and at the end of the 4 week abstinence period. Subjects were asked to refrain from all alcohol consumption, which was verified by breath alcohol testing. They were paid for participation. They were paid \$10 per visit (5 visits during abstinence treatment) and a bonus of \$150 for remaining abstinent for the four week period and complying with the study protocol, for a total of \$200.

Leukocyte DNA was isolated from buffy coat as described above. The lifetime of the majority of the leukocytes is considerably less than the 4 week smoking cessation period used in our study. Total NNAL was determined as described previously (8). DNA was isolated and processed for quantitation of N^2 -ethyl-dGuo as described above. The mean amount of DNA was $610 \pm 342 \mu\text{g}$ (range 50 – 1270 μg , with 3 samples yielding $<100 \mu\text{g}$), as determined by measurement of dGuo. Each set of 3 samples from the same smoker was analyzed in the same assay group.

Statistical Analysis

Since the distribution of adduct **2** was skewed toward the larger values, we applied natural log transformation to the data. Because observations on the same subject are usually correlated, we used the linear mixed model, which appropriately accounts for within subject correlations. Restriction Maximum Likelihood Methods were used to estimate the regression coefficients.

SAS version 9.1.3 was used for model estimation. The indicator variable (quit or not quit) was fitted into the model, which implicitly assumed that the two measurements taken before a subject quit were comparable to each other. The estimated coefficients from the regression were exponentiated such that the effect of quitting could be interpreted on the original measurement scale as percent change in the median level of adduct **2**. Potential confounders were analyzed by multivariate regression.

Results

The method is outlined in Figure 1. [$^{15}\text{N}_5$] N^2 -Ethyl-dGuo ([$^{15}\text{N}_5$]**2**) was added to the DNA as internal standard. Enzyme-catalyzed hydrolysis of the DNA was carried out in the presence of NaBH_3CN . The hydrolysate was purified by solid phase extraction and the appropriate fraction was analyzed by LC-ESI-MS/MS-SRM at m/z 296 $[\text{M} + \text{H}]^+ \rightarrow m/z$ 180 $[\text{BH}]^+$ for **2** and the corresponding transition for [$^{15}\text{N}_5$]**2**.

An LC-ESI-MS/MS-SRM chromatogram obtained upon analysis of NaBH_3CN -treated leukocyte DNA (0.34 mg) is illustrated in Figure 2A. A clear peak for N^2 -ethyl-dGuo (**2**) was observed and it co-eluted with [$^{15}\text{N}_5$]**2**. This sample contained 1000 fmol/ μmol dGuo of adduct **2**. When NaBH_3CN treatment was omitted from the procedure, no peak corresponding to N^2 -ethyl-dGuo (**2**) was observed (Figure 2B), consistent with the instability of N^2 -ethylidene-dGuo (**1**) at the deoxyribonucleoside level. These data provide strong evidence for the presence of N^2 -ethylidene-dGuo (**1**), or possibly its hydrate, in human leukocyte DNA. Although in this study the average amount of DNA used per analysis was 610 μg , the analysis was also feasible with much smaller amounts of DNA. As shown in Figure 2C, N^2 -ethyl-dGuo was detectable in a sample containing only 2 μg of DNA, of which the equivalent of 0.8 μg was injected on the HPLC column. This sample contained 1000 fmol/ μmol dGuo of adduct **2**.

A calibration curve for N^2 -ethyl-dGuo and [$^{15}\text{N}_5$]**2** is illustrated in Figure 3. It was linear in the range measured ($R^2 = 1.0$). The accuracy of the method was tested by adding various levels of N^2 -ethyl-dGuo to 0.3 mg calf thymus DNA. The amount of N^2 -ethyl-dGuo present in NaBH_3CN -treated calf thymus DNA was subtracted from each value. The results are summarized in Table 1. There was an excellent correlation ($R^2 = 1.0$) between the added and measured amounts of N^2 -ethyl-dGuo. The reproducibility of the measurements was excellent, with RSD ranging from 20% at the lowest levels added to 0.73% at the highest. Recoveries averaged 48%. The limit of detection of N^2 -ethyl-dGuo injected on column was 0.05 fmol ($S/N = 6$). The estimated limit of quantitation of N^2 -ethyl-dGuo in DNA was 10 fmol/mg DNA, about 3 adducts per 10^9 nucleotides, or about 15 fmol/ μmol dGuo, starting with 500 μg DNA, and $S/N = 4$. When the assay was run with 1 μg DNA, the estimated limit of quantitation was 0.4 fmol of adduct **2**.

It was possible that N^2 -ethylidene-dGuo could be formed as an artifact from cellular acetaldehyde during DNA isolation. We tested this possibility by adding [$^{13}\text{C}_2$]acetaldehyde to the leukocyte preparation after cell lysis (Table 2). When NaBH_3CN was also added to all solvents used in the cell lysis, DNA isolation, and hydrolysis steps, artifact formation was minimal (41 fmol/ μmol dGuo), but when NaBH_3CN was omitted from these solvents, considerable artifact formation occurred, as shown by the increased amount (816 fmol/ μmol dGuo) of [$^{13}\text{C}_2$] N^2 -ethyl-dGuo detected, as summarized in Table 2. Levels of N^2 -ethyl-dGuo (**2**) under these two conditions were similar however demonstrating that there was no artifactual formation of this analyte, because if there had been, the levels of adduct **2** would have been reduced to the same extent as those of [$^{13}\text{C}_2$]**2** by NaBH_3CN treatment of all solvents.

The method was then applied to investigate the effect of cessation of cigarette smoking on levels of adduct **1** in DNA. Of the 618 smokers who were screened over the telephone, 107

were eligible, 59 attended the orientation visit, 59 consented, and 46 were still eligible after the orientation screening. Of these individuals, 3 dropped out during baseline evaluation, and 13 dropped out during the abstinence phase due to an inability to quit smoking or to remain abstinent. Two participants were terminated for smoking during the study. This resulted in 28 subjects who completed the study. Three of these were excluded from the data analysis because their total NNAL and CO levels showed that they did not remain abstinent.

The mean age of the 25 subjects in the analysis was 40 ± 11 years (range 25 – 59). Nineteen were female, 16 were white, 7 were black, 1 was American Indian, and 1 was of mixed racial heritage. They smoked an average of 17.4 ± 4.3 cigarettes per day.

The data from the smoking cessation study are summarized in Table 3. In each case, there was a substantial decrease in urinary total NNAL after 4 weeks of cessation (mean $\pm$ S.D., $88.5 \pm 7.9\%$), confirming abstinence from smoking in these 25 subjects. The mean ($\pm$ S.D.) levels of adduct **2** measured in the leukocytes of the smokers were 1310 ± 1720 (range 124 – 7700) and 1120 ± 1140 (range 138 – 5760) fmol/ μ mol dGuo at the two baseline points and 705 ± 438 (range 111 – 1530) fmol/ μ mol dGuo after 4 weeks of cessation. The corresponding median values were 895 and 806 fmol/ μ mol dGuo at the two baseline points and 602 fmol/ μ mol dGuo after cessation. Fifteen of the 25 subjects had decreases in levels of adduct **2** greater than 20% (mean decrease, $49 \pm 21\%$) and 4 had increases of greater than 20% (mean increase, $108 \pm 63\%$). The log transformed levels of adduct **2** after four weeks of cessation were lower than the baseline levels (Figure 4). The ratio of adduct **2** after vs. before quitting was 0.72 ($P = 0.02$, 95% CI, 0.56 – 0.93). Thus, the median level of adduct **2** significantly decreased by 28% upon quitting smoking.

The possible modifiers – age, gender, race, body mass index, and cigarettes per day – were individually investigated. Only race significantly modified the effect of quitting on levels of adduct **2**. A significant 57% decrease in the median level of adduct **2** was found in the black plus other racial group ($P < 0.01$) while there was no effect of quitting in the white group. Since it was possible that this result could have been due to the unusually high baseline values, 5830 and 7700 fmol/ μ mol dGuo at the first sampling, for subjects 10 and 19, respectively, the analysis was performed again excluding these subjects. A significant 39% decrease in the median level of adduct **2** in the black plus other racial group was still observed ($P = 0.01$) while no decrease was seen in the white group. The median level of adduct **2** was 1.32 times higher at baseline in the black plus other racial group than in the white smokers, but this was not statistically significant.

Discussion

We have developed a practical method for measurement of N^2 -ethylidene-dGuo (**1**) in human leukocyte DNA. Our method quantifies this major acetaldehyde DNA adduct as its NaBH_3CN reduction product, N^2 -ethyl-dGuo (**2**). A major advantage of this method is its applicability to relatively small amounts of DNA, resulting from the sensitivity of the MS/MS detection method combined with the relatively high levels of adduct **1** in DNA. The method should be useful for analysis of small blood or leukocyte samples of the type which are frequently stored in molecular epidemiology studies. Another advantage is the specificity of LC-ESI- MS/MS-SRM detection, insuring analyte identity. The accuracy and precision of the method, which uses a ^{15}N -labelled internal standard, were established in this study. This is the first demonstration of N^2 -ethylidene-dGuo in human leukocyte DNA. We have used a similar method to quantify N^2 -ethylidene-dGuo in human liver DNA (5). A previous study quantified N^2 -ethyl-dGuo in granulocytes and lymphocytes of alcoholic patients and controls using ^{32}P -postlabelling (9). Levels in alcoholic patients were 2–3 adducts per 10^7 nucleotides, but the

adduct was not detected in control subjects. Since NaBH_3CN reduction was not used in that study, it is unclear whether the measured N^2 -ethyl-dGuo originated from N^2 -ethylidene-dGuo.

The results of the smoking cessation study demonstrate that there was a modest but significant 28% decrease in levels of adduct **1** four weeks after cessation. In each case, abstinence from tobacco was confirmed by quantifying levels of urinary total NNAL, a tobacco-specific metabolite, and by measurements of exhaled CO at each clinic visit. The decrease in NNAL levels of about 89%, four weeks after cessation, is consistent with our previous study, in which we showed that NNAL and its glucuronides were only slowly eliminated from the body (10). In that study, a 90% decrease was observed after 4 weeks of cessation. The relatively modest 28% overall decrease in levels of adduct **1** was not surprising considering that there are numerous sources of acetaldehyde other than cigarette smoking. Alcohol consumption may be the most important of these, since a typical drink contains 10 – 15 g of ethanol, much of which may be metabolized to acetaldehyde, while exposure to acetaldehyde from smoking would be about 20 mg per day. In our study, subjects were eligible only if they consumed less than six alcoholic beverages per month and abstained during the study, but occasional drinking might have been undetected and could potentially contribute to acetaldehyde DNA adducts. It would not have been practical to find a sufficient number of smokers who did not drink alcohol at all. Another important source of acetaldehyde is the diet (1). Consistent with ubiquitous exposure to acetaldehyde, we have yet to find a sample of liver, lung or leukocyte DNA lacking substantial levels of adduct **1**, in this and unpublished work.

The modest decrease in levels of adduct **1** observed here could be due in part to our use of leukocytes. While most leukocyte cell types have a lifetime shorter than 4 weeks, some lymphocytes have relatively long lifespans (> 4 weeks) and adducts in this subpopulation would not be expected to decrease. Further studies using DNA from individual white blood cell types are necessary to address this question.

An unexpected and potentially important result of this study was the effect of race on changes in levels of adduct **1** upon smoking cessation. When the data were stratified by race, there was no change in adduct levels in whites, but a significant 57% decrease was observed in the black plus other group (consisting of 7 blacks, 1 American Indian, and one person of mixed racial background). Further studies with larger numbers of subjects are clearly needed to confirm this observation. Cigarette smoking and alcohol consumption are synergistic risk factors for cancer of the esophagus which is significantly more common in blacks than in whites in the U.S. (11,12). Acetaldehyde, a carcinogen derived from both cigarette smoke and alcohol consumption, is one possible etiologic agent in esophageal cancer. The higher impact of smoking cessation on acetaldehyde-DNA adducts in blacks, as observed in this study, could be related to these epidemiologic observations.

Many studies have investigated the relationship between polymorphisms in alcohol dehydrogenase (*ADH*) and aldehyde dehydrogenase (*ALDH*) genes and cancer, particularly of the head and neck (3,13,14). Polymorphisms in the *ADH* gene can affect the metabolism of ethanol to acetaldehyde while polymorphisms in the *ALDH* gene can affect the conversion of acetaldehyde to acetate. The main hypothesis underlying these studies is that fast ethanol metabolism combined with slow acetaldehyde metabolism leads to higher concentrations of acetaldehyde in drinkers. There is strong support for this in the literature with respect to the effect of *ALDH* polymorphisms on acetaldehyde accumulation in humans given an ethanol challenge, but not for *ADH* polymorphisms (15). Elevated amounts of acetaldehyde could result in higher levels of acetaldehyde-DNA adducts and thus higher risk for cancer. The analytical method described in this paper is suitable for determining the relationship of polymorphisms in *ADH* and *ALDH* to acetaldehyde DNA adducts and cancer of the head and neck. We plan to carry out such studies in the future.

The biological properties of adduct **1** in DNA are unknown. Although it is unstable at the nucleoside level, it is quite stable in DNA, with a half-life of approximately 24 h at 37 °C (16). Its instability at the nucleoside level has hindered synthetic approaches to specific incorporation of **1** in DNA and site specific mutagenesis studies. However, a number of investigations have been carried out on *N*²-ethyl-dGuo (**2**). Earlier studies indicating relatively low mutagenic potential of **2** were discussed previously (5). To summarize, depending on the DNA polymerase used, adduct **2** was either a block to DNA synthesis or was bypassed with relative accuracy. A recent report demonstrated that *N*²-ethyl-dGuo in DNA blocked translesion DNA synthesis in human cells, resulting in either failure of replication or frameshift deletion mutations (17).

In summary, we have developed a practical and quantitative method for analysis of *N*²-ethylidene-dGuo (**1**), the major acetaldehyde DNA adduct, in leukocyte DNA as its NaBH₃CN reduction product, *N*²-ethyl-dGuo (**2**). The method is applicable to low microgram amounts of human leukocyte DNA. We applied this method in a smoking cessation study and observed a significant 28% decrease in levels of adduct **1** after 4 weeks of abstinence from cigarette smoking. These results indicate that smoking contributes to leukocyte levels of this DNA adduct.

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Abbreviations

ADH

alcohol dehydrogenase gene

ALDH

aldehyde dehydrogenase gene

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Figure 1. Outline of analytical method for determination of N^2 -ethylidene-dGuo (1) in human leukocyte DNA, as N^2 -ethyl-dGuo (2).

Figure 2.

Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis of human leukocyte DNA by the method described in the text. A, Analysis of 340 μ g DNA containing 1000 fmol/ μ mol dGuo of adduct **2**; B, the same sample as in A, except that NaBH_3CN was omitted from the analysis; C, analysis of 0.8 μ g DNA containing 1000 fmol/ μ mol dGuo of adduct **2**. The signal corresponds to 0.4 fmol N^2 -ethyl-dGuo (S/N, 4).

Figure 3. Calibration curve for N^2 -ethyl-dGuo (**2**) (4 – 512 fmol) and $[^{15}\text{N}_5]N^2$ -ethyl-dGuo ($[^{15}\text{N}_5]\mathbf{2}$), 40 fmol; $R^2 = 1.0$. Each point is a single determination.

Figure 4.

Effect of smoking cessation on levels of N^2 -ethyl-dGuo (2) in NaBH_3CN -treated leukocyte DNA. Baseline values were obtained at weeks 1 and 2 while the 25 subjects were still smoking. The 6 week value was obtained after 4 weeks of smoking cessation. Values are natural log transformed from data in Table 3. Boxes represent 25th – 75th percentile (interquartile range), outer lines represent the maximum observation below the upper fence (1.5 times the interquartile range above the 75th percentile) and the minimum observation above the lower fence (1.5 times the interquartile range below the 25th percentile). Dots represent outliers. The line in the box represents the median.

Table 1Analysis of calf thymus DNA to which N^2 -ethyl-dGuo was added

N^2 -ethyl-dGuo (fmol) ^{a,b}		CV (%)
Added	Detected ^c	
25	21.0 ± 4.16	20
50	47.0 ± 5.03	11
100	99.0 ± 5.13	5.2
200	193 ± 4.36	2.3
400	395 ± 2.89	0.73

^a Added to 0.3 mg calf thymus DNA^b N^2 -Ethyl-dGuo in NaBH₃CN-treated calf thymus DNA (130 fmol/0.3 mg) was subtracted from each value^c Mean ± S.D. (N = 3)

Table 2Investigation of artifact formation in the analysis of N^2 -ethyl-dGuo in human leukocyte DNA^a

Sample	Addition of NaBH ₃ CN	Adduct levels (fmol/μmol dGuo)	
		[¹³ C ₂]2	2
A	Enzyme hydrolysis only	816	588
B	All steps	41	464

^a [¹³C₂]CH₃CHO (0.57 mM) was added following cell lysis and samples were analyzed as described in Experimental Procedures.

Table 3

Effect of smoking cessation on levels of *N*²-ethylidene-dGuo (1), quantified as *N*²-ethyl-dGuo (2), in leukocyte DNA

Subject	Gender	Age	Race ^a	Total NNAL (pmol/mL)		<i>N</i> ² -Ethyl-dGuo (2) (fmol/μmol dGuo)			
				Baseline ^b	4 Weeks Cessation	Baseline 1	Baseline 2	Baseline Mean	4 Weeks Cessation
1	F	25	B	0.84	0.05	895	473	684	326
2	F	59	I	1.23	0.28	1030	1100	1063	440
3	F	30	W	1.98	0.22	1210	1470	1340	776
4	F	25	W	5.16	0.14	563	780	671	776
5	M	37	B	1.22	0.16	655	646	651	1150
6	F	50	W	0.24	0.04	863	547	705	751
7	M	38	W	0.90	0.09	366	667	516	260
8	M	34	W	0.33	0.09	135	172	154	111
9	F	40	B	1.65	0.10	542	427	484	311
10	M	33	B	3.32	0.16	5830	1430	3630	792
11	F	27	W	1.55	0.09	994	728	861	602
12	F	25	W	2.41	0.10	1700	2120	1910	1530
13	F	28	O	0.68	0.12	1860	1980	1920	1500
14	F	44	B	1.74	0.23	1310	1540	1430	367
15	F	53	W	0.75	0.06	1020	1570	1300	1280
16	F	44	W	1.31	0.10	1020	1490	1260	891
17	F	44	W	0.88	0.07	1620	871	1250	1350
18	M	55	W	0.36	0.11	975	832	904	480
19	F	45	B	1.32	0.09	7700	5760	6730	491
20	M	31	B	1.04	0.08	400	N.A.	N.A.	273
21	F	55	W	0.72	0.09	124	140	132	223
22	F	50	W	0.33	0.09	361	594	478	174
23	F	49	W	1.66	0.17	691	1140	916	1060
24	F	43	W	2.33	0.11	390	138	264	484
25	F	32	W	2.46	0.12	616	185	401	1230

^aB, black; W, white; I, American Indian; O, other^bOne baseline sample was analyzed