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Measurement of 7-methyl- and 7-(2-hydroxyethyl)-guanine DNA adducts in white blood cells of smokers and non-smokers

CHUNYAN ZHAO, RAJIV KUMAR AND KARI HEMMINKI*

Center for Nutrition and Toxicology, Karolinska Institute, NOVUM, 141 57 Huddinge, Sweden

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The goal of the present study was to measure the levels of 7-methylguanine and 7-(2-hydroxyethyl)guanine DNA adducts in human white blood cells in relation to smoking. DNA was isolated from samples of 11 smokers and eight non-smokers. The ^{32}P-postlabelled 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts were analysed by thin-layer chromatography (TLC) combined with a high pressure liquid chromatography (HPLC) assay. In smokers the mean 7-methylguanine and 7-(2-hydroxyethyl)guanine levels were 32·3 ± 7·1 and 6·6 ± 2·3 adducts per 10^8 nucleotides respectively. The corresponding values in non-smokers were 25·0 ± 7·0 and 3·7 ± 2·4 adducts per 10^8 nucleotides. There were significantly higher levels of 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts in WBC in smokers than in non-smokers ($p = 0{\cdot}041$; $p = 0{\cdot}018$), respectively. A positive correlation between 7-methylguanine and 7-(2-hydroxyethyl)guanine levels was observed.

Keywords: DNA adducts, HPLC, 7-(2-hydroxyethyl)guanine, 7-methylguanine.

Abbreviations: WBC, white blood cell; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; ETO, ethylene oxide; DMS, dimethylsulphate; NNK, 4-(methyl-nitrosoamino)-1-(3-pyridyl)-1-butanone.

Introduction

Tobacco smoke is considered one of the major causative factors for several types of cancers (IARC 1986). Many carcinogens present in tobacco smoke are capable of interacting with DNA to form adducts (Hemminki 1983). The presence of DNA adducts is a relevant indicator of individual human exposure to environmental genotoxicants (Shields and Harris 1991). Characterization of DNA adducts formed in the tissues of smokers can provide essential information for the identification of agents present in tobacco smoke that initiate cancer in humans (Phillips 1996).

Methylating and hydroxyethylating agents present in tobacco smoke include tobacco specific *N*-nitrosamines and ethene, a precursor of ethylene oxide (IARC 1986, 1994). These are potential human carcinogens, and smokers are estimated to be exposed to these compounds at biologically significant levels (Hecht and Hoffmann 1988, IARC 1994). These alkylating agents react mainly with the N-7 position of guanine leading to the formation of 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts (Hemminki 1983, Vogel *et al.* 1986, Walker *et al.* 1992). Minor reaction products are promutagenic lesions at the O^6 position of

* To whom correspondence should be addressed.

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guanine (Peterson and Hecht 1991, Walker *et al.* 1992), which result in G:C to A:T transitions during DNA synthesis (Eadie *et al.* 1984, Pegg 1984, Ludeke and Kleihues 1988). However, because of the high level of formation and relatively slow repair, 7-methylguanine adducts have been used in several studies as a marker of exposure to methylating agents (Bianchini and Wild 1994).

Several studies have demonstrated levels of 7-methylguanine DNA adducts in relation to tobacco smoking by the ^{32}P-postlabelling assay (Mustonen and Hemminki 1992, Mustonen *et al.* 1993, Szyfter *et al.* 1996), but similar studies for 7-(2-hydroxyethyl)guanine adducts have not been reported, even though 2-hydroxyethyl adducts in N-terminal valine of haemoglobin are characterized in smokers (Osterman-Golkar and Bond 1996). For DNA adducts methods have been limiting because the previously described assay (Mustonen and Hemminki 1992, Mustonen *et al.* 1993, Szyfter *et al.* 1996), relying on TLC, failed to separate 7-methylguanine, 7-(2-hydroxyethyl)guanine and probably also other simple alkylguanines, possibly confounding quantitation. Recently, the method of combined TLC and HPLC separation of ^{32}P-postlabelled 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts has been developed in this laboratory (Kumar and Hemminki 1996). In the present study we validate the technique further by measuring the 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts in human WBC DNA samples and for the first time investigate the levels of 7-(2-hydroxyethyl)guanine adducts in WBC in relation to smoking.

Materials and methods

White blood cells were collected from 19 male healthy volunteers: 11 smokers and eight non-smokers. Based on the questionnaire, both the smokers and the control persons were true smokers and non-smokers respectively and had no other exposure to methylating or hydroxyethylating agents. The average age of the smokers and non-smokers was 39·2 and 44·1 years respectively. The mean daily cigarette consumption by smokers was 20 cigarettes (one pack per day, as reported). All of the samples were coded for blinded analysis.

Ten ml blood was used for isolation of WBC DNA. WBC pellets were obtained by lysing red cells with 0·15 M NaCl, 10 mM Tris–HCl (pH 7·4), 0·1 % Triton X-100, followed by centrifugation. DNA was isolated from WBC pellet as described by Phillips *et al.* (1988). 7-Methyl- and 7-(2-hydroxyethyl)-deoxyguanosine-5′-monophosphate adducts were prepared by reaction of the unmodified nucleotide with dimethylsulphate (DMS) and ethylene oxide (ETO) respectively followed by purification on HLPC (Kumar *et al.* 1995). DNA standards for these two adducts were obtained by treating salmon testis DNA with ETO and DMS respectively and the level of 7-alkylguanine adducts was determined by depurination at neutral pH as described earlier (Kumar and Hemminki 1996).

The procedure for DNA digestion, adduct enrichment with anion exchange chromatography and ^{32}P-postlabelling of human DNA (10 μg) is described elsewhere (Mustonen and Hemminki 1992, Kumar *et al.* 1995). After postlabelling the samples were treated with nuclease P1 to remove the 3′-phosphate group. Analysis of 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts in DNA by TLC combined with an HPLC method was performed as described previously with minor modifications (Kumar and Hemminki 1996). In the present study the ^{32}P-postlabelled mixtures were applied to pre-washed 10×20 cm PEI TLC plates and developed with 0·1 M ammonium formate, pH 5·2 in the first dimension (D1) and 0·6 M ammonium formate, pH 5·2, mixed with 40 % *n*-propanol in the second dimension (D2). The areas of plates corresponding to 7-alkylguanine adducts, which contains 7-methylguanine and 7-(2-hydroxyethyl)guanine, were extracted with 10 mM ammonium formate, pH 5·3 by sonication. The ultimate analysis of 7-methylguanine and 7-(2-hydroxyethyl)guanine was based on reverse phase HPLC with on-line radioactivity and UV detectors. The retention times of the adducts were confirmed by analysing the aliquots of TLC extracts spiked with synthesized 7-methyl- and 7-(2-hydroxyethyl)-deoxyguanosine-5′-monophosphate adducts which were used as UV markers. The separation was performed using a gradient, started at 100 % 0·2 M ammonium formate buffer, pH 4·6 for 10 min, followed by a linear gradient over the next 10 min to 10 % methanol, which was maintained for 10 min. The methanol concentration was increased to 100 % in the next 10 min. 7-(2-Hydroxyethyl)guanine and 7-methylguanine adducts eluted separately at retention times of 7·1 and 8·5 min, respectively.

The *in vitro* modified DNA with DMS and ETO were labelled in parallel to each set of human DNA samples. The recovery of adducts from these *in vitro* samples were used to correct the levels of adducts in the human DNA samples. Individual 7-methylguanine and 7-(2-hydroxyethyl)guanine adduct determinations are based on at least three analyses.

Results

The levels of 7-alkylguanine adducts in the *in vitro* modified DNA with DMS and ETO were found to be 33·8 and 37·5 adducts per 10^6 nucleotides respectively. The total recovery of the 7-methylguanine and 7-(2-hydroxyethyl)guanine adduct was 21·0 ± 5·2 % (n = 5) and 9·0 ± 4·5 % (n = 5), respectively. The low recoveries were due to depurination and losses in sample work-up. It was thus necessary to include the standards in each assay in order to correct for the recoveries.

Autoradiograms of PEI-TLC maps showing the 7-alkylguanine adducts from WBC of one smoker (A) and one non-smoker (B) are presented in figure 1. The 7-alkylguanine spot in which 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts co-migrated was well resolved from other adduct spots. The adduct spots were extracted from TLC plates and analysed on HPLC. Figure 2 shows representative chromatograms of HPLC separation of 7-methylguanine and 7-(2-hydroxyethyl)guanine adducts from WBC of one smoker (A) and non-smoker (B). The identities of these two adducts were established by their co-migration with the synthesized 7-methyl- and 7-(2-hydroxyethyl)-deoxyguanosine-5′-monophosphates standards used as UV markers.

The mean levels of 7-methylguanine and 7-(2-hydroxyethyl)guanine DNA adducts in WBC of smokers and non-smokers are presented in table 1. The mean 7-methylguanine adduct levels in WBC were 32·2 adducts per 10^8 nucleotides in smokers and 25·0 adducts per 10^8 nucleotides in non-smokers, respectively. The corresponding values for 7-(2-hydroxyethyl)guanine adducts were 6·6 and 3·7 adducts per 10^8 nucleotides, respectively. Statistical analysis showed that the adduct levels of both 7-methylguanine and 7-(2-hydroxyethyl)guanine in WBC in smokers were significantly higher than in non-smokers (p = 0·041; p = 0·018). Adduct levels of 7-methylguanine in WBC DNA were ~5–7 times higher than the levels of 7-(2-hydroxyethyl)guanine both in smokers and non-smokers. When 7-methylguanine and 7-(2-hydroxyethyl)guanine levels obtained from the same subjects were compared in the whole study group, a correlation (r = 0·60, $p < 0·05$) was observed. The correlation between 7-methylguanine or 7-(2-hydroxyethyl)guanine levels in WBC and subject age for the whole group was analysed and no correlation was found.

Discussion

Tobacco smoke contains many types of alkylating agents such as 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), a methylating agent, and ethene, which is metabolized to a hydroxyethylating agent ethylene oxide (IARC 1986, Sipes and Gandolfi 1991). These alkylating agents react mainly with the N-7 position of guanine (Hemminki 1983, Vogel *et al.* 1986, Walker *et al.* 1992). In recent years, 7-alkylguanine adducts have been detected with a number of techniques including mass spectrometry (Chang *et al.* 1986), immunoassay (Degan *et al.* 1988, Wild 1990, Bianchini *et al.* 1992), electrochemical detection (Park and Ames 1988), ^{32}P-postlabelling/HPLC (Shields *et al.* 1990, Mustonen *et al.* 1991

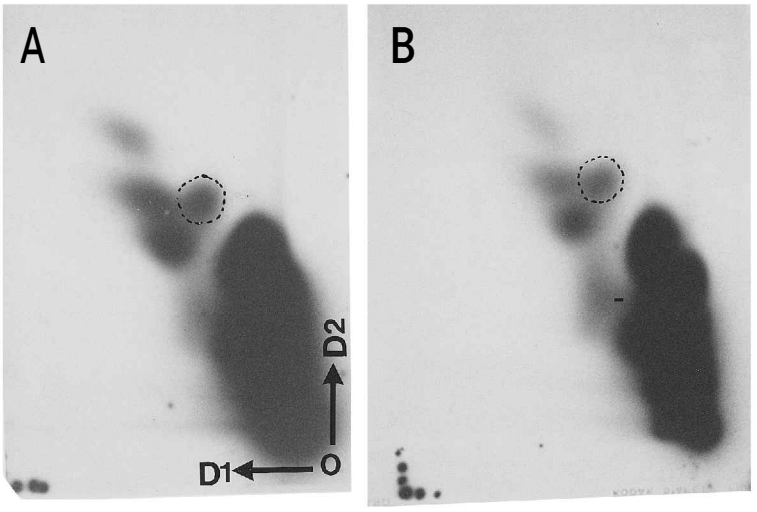

Figure 1. Autoradiograms of PEI-TLC maps of postlabelled WBC DNA samples from a smoker (A) and a non-smoker (B). Autoradiography was performed at –80 °C for 3 h. 7-Alkylguanine adducts are marked with circles. D1 and D2 are the directions of chromatography and 'O' is the origin.

Kato *et al.* 1993) and ^{32}P-postlabelling/TLC (Mustonen and Hemminki 1992, Mustonen *et al.* 1993, Szyfter *et al.* 1996). The most versatile and sensitive method is the ^{32}P-postlabelling method (Randerath *et al.* 1981). Studies analysing tobacco-related 7-methylguanine adducts in humans have used a number of tissues including blood, bronchus and larynx (Mustonen and Hemminki 1992, Mustonen *et al.* 1993, Szyfter *et al.* 1996). Higher adduct levels have been found in smokers as compared with non-smokers (Mustonen and Hemminki 1992, Mustonen *et al.* 1993, Szyfter *et al.* 1996). However, the analyses of 7-(2-hydroxyethyl)guanine adducts in human tissues are very limited (van Delft *et al.* 1994, Kumar and Hemminki 1996) and the effects of smoking have not been previously reported.

In this study we report 7-methylguanine and 7-(2-hydroxyethyl)guanine adduct levels in WBC of smokers and non-smokers by the recently developed TLC–HPLC assay. The 7-methylguanine adduct levels in smokers were found to range from 19·2 to 42·3/10^8 with a mean of 32·2/10^8, and in non-smokers from 17·8

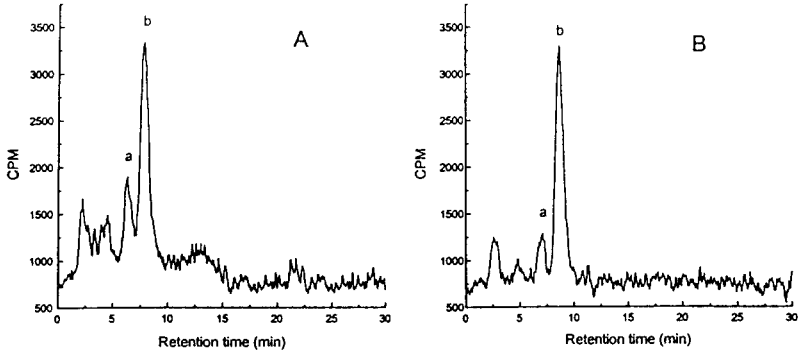

Figure 2. HPLC-radioactivity detector analysis of 7-(2-hydroxyethyl)guanine (peak 'a') and 7-methylguanine adducts (peak 'b') in WBC DNA from a smoker (A) and a non-smoker (B). The radioactive peaks were obtained from excision of corresponding TLC sp

Table 1. Individual 7-methylguanine and 7-(2-hydroxyethyl)guanine levels (adducts per 10^8 nucleotides; mean ± SD) in smokers' and non-smokers' white blood cells.

Subjects	Age	7-Methylguanine	7-(2-Hydroxyethyl)guanine
Smokers			
1	45	36·8 ± 5·2	5·9 ± 0·8
2	31	33·4 ± 3·9	9·7 ± 2·7
3	28	19·2 ± 4·5	2·8 ± 0·7
4	41	35·7 ± 2·9	7·6 ± 3·1
5	40	32·4 ± 6·7	7·5 ± 2·0
6	38	31·2 ± 3·3	8·9 ± 1·7
7	45	42·3 ± 3·8	4·6 ± 0·6
8	35	29·4 ± 5·7	3·7 ± 0·6
9	50	35·2 ± 6·0	9·0 ± 0·5
10	40	20·4 ± 4·8	5.3 ± 0·8
11	38	38·6 ± 3·0	7·3 ± 0·2
Mean ± SD		32·2 ± 7·1[a]	6·6 ± 2·3[b]
Non-smokers			
1	43	19·1 ± 2·6	2·4 ± 0·5
2	59	17·8 ± 4·2	2·2 ± 0·9
3	44	20·5 ± 3·1	2·4 ± 0·6
1	60	32·1 ± 1·8	8·1 ± 1·2
5	36	19·3 ± 3·0	3·3 ± 0·5
6	49	24·2 ± 5·2	2·2 ± 0·4
7	31	31·5 ± 2·6	7·1 ± 0·6
8	31	35·5 ± 5·1	2·1 ± 0·3
Mean ± SD		25·0 ± 7·0	3·7 ± 2·4

[a] $p = 0·041$ between smokers and non-smokers.
[b] $p = 0·018$ between smokers and non-smokers.
Student's two-tailed *t*-test and ANOVA.

to $35·5/10^8$ with a mean of $25·0/10^8$. These levels are in very good agreement with the data established by Mustonen *et al.,* which showed 7-methylguanine levels in WBC DNA from 17 non-smokers of $25/10^8$ (Mustonen *et al.* 1991). Other authors have also reported similar values for 7-methylguanine (Mustonen and Hemminki 1992, Mustonen *et al.* 1993, Blömeke *et al.* 1996, Szyfter *et al.* 1996). Kato *et al.*, using combined two-step HPLC with ^{32}P-postlabelling assay, found levels of 7-methylguanine adducts in lung samples (range $14–54/10^8$) comparable to the present 7-methylguanine levels (Kato *et al.* 1993). In the present study, the 7-methylguanine level in smokers was significantly higher than in non-smokers. Increased 7-methylguanine adduct levels in smokers as compared with non-smokers have been reported in bronchial, blood cell and larynx DNA (Mustonen and Hemminki 1992, Mustonen *et al.* 1993, Szyfter *et al.* 1996). The data suggest that the smoking-related 7-methylguanine is likely to result from long-term exposures to tobacco-specific *N*-nitrosamines such as NNK and *N*-nitrosodimethylamine (NDMA) (Hoffmann *et al.* 1984).

The data presented here are the first demonstration of 7-(2-hydroxyethyl) guanine DNA adducts in WBC in relation to smoking. The levels of 7-(2-hydroxyethyl)guanine in smokers ($7·1/10^8$) were significantly higher than those in non-smokers ($3·7/10^8$). This is most likely related to the presence of ethene and ethylene oxide in tobacco smoke. Endogenous sources of ethene, including lipid peroxidation of unsaturated fats and metabolism of intestinal bacteria, might also contribute (Törnqvist *et al.* 1989). Our results on 7-(2-hydroxyethyl)guanine adducts obtained here are comparable to those reported by van D[illegible] *et al.* (1994) i[illegible]

WBC using immunochemical techniques. By contrast, in an earlier mass-spectrometric analysis the reported levels of 7-(2-hydroxyethyl)guanine adducts were some 50 times higher in subjects whose exposures were unspecified (Föst *et al.* 1989), probably due to technical problems, as discussed elsewhere (Eide *et al.* 1995).

The N-terminal valine adducts of ethylene oxide have been described in many studies (Törnqvist *et al.* 1992, Osterman-Golkar and Bond 1996), the background level in non-smokers being 20 pmol g^{-1} haemoglobin and the increment by 20 cigarettes per day by *ca* 10 times the background at steady-state. Interestingly the increment in white blood cell DNA was less than two-fold only, as shown here. In an animal study on alkenes, a dramatic difference was also observed in 2-hydroxyethyl adduct accumulation in lymphocyte DNA and haemoglobin (Eide *et al.* 1995). Exposure to 300 ppm of ethene for 12 h in 3 days increased haemoglobin adducts 1000 times over background but lymphocyte and liver DNA adducts only three-times (Eide *et al.* 1995). This may suggest that white blood cell and liver DNA is much more accessible to alkylation by endogenous ethylene oxide than haemoglobin is, which may have implications for biomonitoring.

A linear correlation between 7-methylguanine and 7-(2-hydroxyethyl)guanine levels was found in the present study. There was no direct relationship between these two adducts and subjects' age in the present study. One recent study also demonstrated that the levels of 7-alkylguanine adducts among individuals could not be explained by differences in age or gender (Blömeke *et al.* 1996). Interestingly, it was recently reported that individual 7-alkylguanine levels in larynx cells positively correlate with aromatic DNA adduct levels (Szyfer *et al.* 1996).

In conclusion, the TLC–HPLC method is sufficiently sensitive to be used for human DNA analysis of 7-(2-hydroxyethyl)guanine adducts. The adduct levels are relatively high and it will be of interest to trace the endogenous and exogenous sources of these adducts.

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