Correlation between the Urine Profile of 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Metabolites and N^7-Methylguanine in Urothelial Carcinoma Patients

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Abstract

A major carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is present in cigarette smoke and its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), is used as an exposure biomarker for environmental tobacco smoke (ETS). This metabolite (NNAL) can be either detoxified into glucuronidated NNAL (NNAL-Gluc) or activated into an unstable reactive metabolite that methylates DNA along with formation of 4-hydroxy-4-(3-pyridyl)-butyric acid [hydroxy acid (HA)]. Therefore, the carcinogenic risk associated with ETS exposure is greatly modulated by individual variations in metabolic activation and detoxification capabilities. In this study, we defined the urinary HA/total NNAL [HA/total NNAL] ratio as the activation index and NNAL-Gluc/free NNAL [(total NNAL-free NNAL)/free NNAL] ratio as the detoxification index of NNK. The major methylated DNA adduct N^7-methylguanine (N^7-MeG), considered as the carcinogenic biomarker for cigarette smoking, was excreted in urine. The objective of this study was to investigate the effects of these metabolic indexes of NNK on N^7-MeG urinary excretion in a population of urothelial carcinoma patients. Urinary levels of total NNAL (free NNAL plus NNAL-Gluc), free NNAL, HA, and N^7-MeG were positively correlated with smoking. Furthermore, activation index and detoxification index correlated positively and negatively with N^7-MeG levels, respectively. Our results suggest that these metabolic indices may represent the phenotype of individual metabolism capability and modulate the carcinogenic risk of ETS exposure. (Cancer Epidemiol Biomarkers Prev 2008;17(12):3390–5)

Introduction

Cigarette smoking is a well-established risk factor for the cancers of the lung, oral cavity, pharynx, and urinary bladder (1). Exposure to environmental tobacco smoke (ETS) is also considered as hazardous for nonsmokers (2). Recently, an epidemiologic study showed that cigarette smoking interacted with other environmental factors and enhanced carcinogenic risks (3). Thus, it is of importance to establish biomarkers to facilitate exposure and cancer risk assessments related to tobacco smoking. Appropriate biomarkers will also allow a better understanding of the interaction mechanisms between environmental factors and help in the development of preventive strategies for reducing cancer risks in humans.

Tobacco smoke is a complex mixture of ~4,500 chemicals. Nicotine is the most abundant component (4) and its urinary metabolite, cotinine, is readily detectable; thus, it is used as a exposure biomarker related to tobacco smoking (5-8). However, neither nicotine nor cotinine is a useful cancer-related biomarker for tobacco smoking because their relationship with carcinogenesis is obscure and several foods also contain small amounts of nicotine (9, 10). In this study, we set out to seek more specific cancer-related biomarkers as part of our effort to understand relationship between carcinogenesis and tobacco smoking.

A tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is formed by nitrosation of nicotine or of its related minor alkaloid, pseudo-oxynicotine, found only in tobacco products (11). NNK induces lung adenoma in animals (12) and is classified as a human carcinogen (13). Metabolic activation of NNK is required to turn it into carcinogenic metabolites. The general scheme of NNK metabolism is outlined in Fig. 1. NNK is rapidly metabolized to its carbonyl reduction product, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). The ultimate (or reactive) metabolites converted from NNAL methylate DNA and form DNA adducts, such as N^7-methylguanine (N^7-MeG) and O^6-methylguanine. Alternatively, NNAL can be detoxified by forming glucuronidated NNAL (NNAL-Gluc), which is readily excreted in the urine. N^7-MeG is by far the most abundant DNA adduct generated from metabolically activated NNK (14).

Urinary N^7-MeG has been used as an exposure biomarker related to tobacco smoking, as it is readily removed from methylated DNA by a DNA repair system

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and excreted into the urine (15). Indeed, urinary excretion of N^7-MeG was higher among smokers than nonsmokers (16). However, N^7-MeG is not specific for tobacco smoking exposure and is more likely to be a biomarker for cancer development after exposure. In comparison, urinary NNAL and NNAL-Gluc are much better biomarkers of NNK uptake in smokers and in people exposed to ETS (17). NNK is found only in tobacco products and seldom in the diet or the general environment unless contaminated by tobacco smoke. Furthermore, because metabolic activation of NNK is critical for its carcinogenic potential, different profiles of NNK metabolites may reflect a variation of cancer risks in different individuals. Therefore, it is of interest to elucidate the relationship between urinary N^7-MeG and the profile of NNK metabolites in humans.

Our hypothesis is that the capability of individuals to metabolize NNK will modulate individual cancer risk on exposure to ETS. N^7-MeG is considered as a biomarker of tobacco smoking-associated cancer risk. Previously, we reported that cigarette smoking increased the risk of urothelial carcinoma (18). It has been shown that N^7-MeG levels were high in bladder cancer tissue and modulated by genotypes of a metabolic enzyme, glutathione *S*-transferase (19). In this study, we examined the association between urinary free NNAL and NNAL-Gluc, N^7-MeG, and the profile of NNK metabolites in urothelial carcinoma patients. The ultimate goal is to identify biomarkers that represent the phenotypes of metabolic capacity for NNK and related carcinogens.

Materials and Methods

Study Population. The study population consisted of 126 urothelial carcinoma patients including 15 current smokers, 42 ever smokers, and 69 never smokers from March 2004 to July 2007. All cases were diagnosed as urothelial carcinoma patients with pathologic confirmation. All urothelial carcinoma cases had not yet received any treatment before urine collection. The majority of study population lived in Taipei City and were recruited from the National Taiwan University Hospital.

Questionnaire Interview and Participant Specimen Collection. Standardized personal interviews based on structured questionnaire were carried out by well-trained personnel. Information collected included demographic and socioeconomic characteristics, general potential risk factors for malignancies such as lifestyle, alcohol consumption, cigarette smoking in quantified details, occupational history, and personal and family histories of disease. All patients provided informed consent before questionnaire interview and urine sample collection. Urine samples were stored at −20°C until further use for NNK and DNA adduct level analysis.

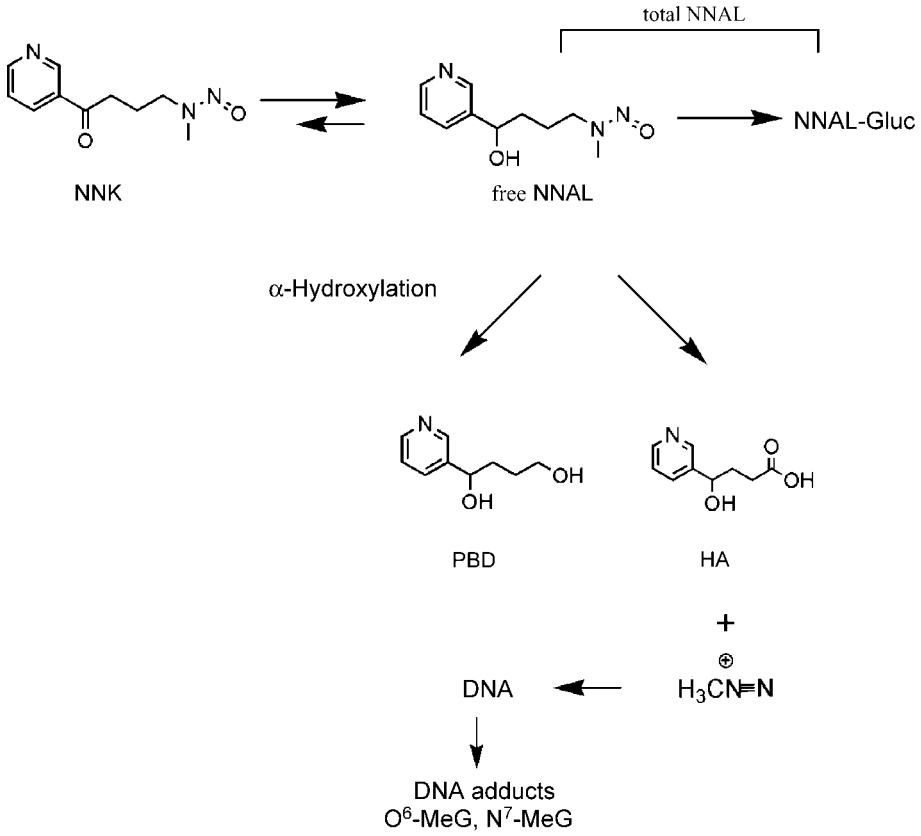

Figure 1. Overview of NNK metabolism showing structures of most urinary metabolites (for more details, see ref. 24). *PBD,* 1-(3-pyridyl)-1,4-butanediol; *O^6-MeG, O^6*-methylguanine.

Table 1. Characteristics of the studied participants and biomarker levels in urine

Characteristic/biomarker	*n*	Mean (SE)	Range
Age (y)	126	64.23 (1.22)	25-102
Gender, *n* (%)			
Male	93 (73.81)		
Female	33 (26.19)		
Cigarette smoking, *n* (%)			
Never smokers	69 (54.76)		
Smokers/ever smokers	57 (45.24)		
Total NNAL (μg/g creatinine)	126	2.25 (0.48)	0.01-48.89
Free NNAL (μg/g creatinine)	126	0.74 (0.15)	0.005-11.30
HA (μg/g creatinine)	126	11.78 (3.24)	0.11-373.38
N^7-MeG (mg/g creatinine)	126	9.55 (1.48)	0.48-122.56

Analysis of NNK Metabolites. Urinary NNK metabolite concentrations were determined using the liquid chromatography-tandem mass spectrometry method (20, 21).

Analysis of Urinary N^7-MeG Level. A 50 μL aliquot of urine was diluted with 85% acetonitrile (450 μL) containing 0.1% formic acid followed by the addition of 3 ng/mL [$^2H_3$]-N^7-MeG as an internal standard for quantitation of N^7-MeG by liquid chromatography-tandem mass spectrometry method (21, 22). The [$^2H_3$]-N^7-MeG was synthesized based on published references with minor modifications at purity of >98% (22).

Statistical Analysis. Concentration of urinary metabolites was expressed as milligram creatinine to correct for variation in urine flow. Total NNAL concentration (ng/mg creatinine) was the sum of urinary free NNAL and NNAL-Gluc. The detoxification capability index was defined as the ratio between NNAL-Gluc and free NNAL levels. Activation index was defined as the ratio between 4-hydroxy-4-(3-pyridyl)-butyric acid [hydroxy acid (HA)] and free NNAL. All significant analyses of difference between NNK metabolites and DNA adducts were based on logarithmic transformed values. Pearson's correlation was used to assess the relationship between urinary NNK metabolites and DNA adducts levels. Simultaneously, we developed a simple linear regression to estimate the joint effects of various indices and DNA adducts on urothelial carcinoma risk adjusted for total NNAL. All data were analyzed using the SAS statistical package. $P < 0.05$ was considered significant.

Results

A total of 126 subjects [93 (73.81%) males and 33 (26.19%) females] with a mean age of 64.23 ± 1.22 years were recruited for the study. Their demographic characteristics, smoking status, and biomarkers data are summarized in Table 1. NNK metabolites, including free NNAL, total NNAL, HA, and N^7-MeG, were detectable in urine (Table 1). Among NNK metabolites, the HA levels were much higher than free NNAL and total NNAL. The relationship between biomarkers (NNK metabolites and N^7-MeG) and subject characteristics are presented in Table 2. Neither gender or age affected the levels of these biomarkers. We combined current smokers and ever smokers into one group. These biomarkers were not significantly different between smokers/ever smokers and never smokers.

Total NNAL, sum of free NNAL and NNAL-Gluc, is an accepted biomarker for NNK uptake (23). When free NNAL is not conjugated, it is metabolically activated to HA accompanied with N^7-MeG formation. NNAL-Gluc and HA represented the ultimate metabolites after detoxification and activation of NNK, respectively. N^7-MeG is an effective product of NNK activation. The urinary concentration of total NNAL were well correlated with those of free NNAL ($P < 0.0001$), HA ($P < 0.0001$), and N^7-MeG ($P < 0.0039$; Table 3). These results suggest that free NNAL, total NNAL, HA, and N^7-MeG are also good urinary biomarkers for ETS exposure in humans.

N^7-MeG is both an exposure biomarker and an effective biomarker. The formation of N^7-MeG shall be modulated by metabolic activation and detoxification capabilities. The NNAL-Gluc/free NNAL ratio [(total NNAL-free NNAL)/free NNAL] and the HA/total NNAL ratios were defined as the detoxification index and activation index, respectively, and their relationship with urinary N^7-MeG levels was investigated (Table 4). N^7-MeG was negatively ($\beta = -0.17$; $P = 0.007$) correlated with the detoxification index [(total NNAL-free NNAL)/free NNAL] but positively ($\beta = 0.43$; $P < 0.0001$) correlated with the activation index (HA/total NNAL). After stratification with smoking status, the correlation between N^7-MeG and the detoxification index remained significant in smokers but not in never smokers. On the other hand, N^7-MeG correlated with activation index among both smokers and never smokers. These results

Table 2. Relationship between NNK metabolites, N^7-MeG, and characteristics

	Total NNAL (μg/g creatinine)	Free NNAL (μg/g creatinine)	HA (μg/g creatinine)	N^7-MeG (mg/g creatinine)
Stratification by gender				
Male	2.13 ± 0.59	0.84 ± 0.20	13.24 ± 4.37	10.13 ± 1.96
Female	2.58 ± 0.77	0.48 ± 0.10	7.66 ± 1.22	7.94 ± 1.26
P	0.68	0.12	0.22	0.35
Stratification by age (y)				
<58	2.04 ± 0.59	0.78 ± 0.27	7.16 ± 1.54	10.00 ± 2.69
58-75	2.97 ± 1.22	0.81 ± 0.30	12.85 ± 3.74	10.25 ± 3.00
≥75	1.65 ± 0.33	0.62 ± 0.19	16.52 ± 10.28	8.15 ± 1.42
P	0.53	0.88	0.50	0.83
Smoking status				
Never smokers	1.49 ± 0.28	0.48 ± 0.10	7.37 ± 0.99	7.57 ± 0.76
Smokers/ever smokers	3.16 ± 0.99	1.06 ± 0.31	17.10 ± 7.03	11.96 ± 3.13
P	0.11	0.08	0.18	0.18

Table 3. Correlation among biomarkers analyzed in this study

	Total NNAL (μg/g creatinine)	Free NNAL (μg/g creatinine)	HA (μg/g creatinine)	N^7-MeG (mg/g creatinine)
Total NNAL				
Pearson correlation coefficients	1.00			
P				
Free NNAL				
Pearson correlation coefficients	0.83	1.00		
P	<0.0001			
HA				
Pearson correlation coefficients	0.70	0.68	1.00	
P	<0.0001	<0.0001		
N^7-MeG				
Pearson correlation coefficients	0.26	0.38	0.47	1.00
P	0.0039	<0.0001	<0.0001	

suggest that individual variation in detoxification and activation capabilities might influence their carcinogenic effect (DNA methylation) of NNK in humans.

The frequency distribution of the detoxification index [(total NNAL-free NNAL)/free NNAL] was 5.0 ± 9.55 (n = 126; Fig. 2A). The detoxification index was >0.36 in ~10% of this population. The frequency distribution of the activation index [HA/free NNAL] was 10.2 ± 13.42 (n = 126) and was >1.5 in ~10% of this population (Fig. 2B).

Discussion

Cigarette smoking and exposure to ETS are important risk factors for many cancers (17). NNK is one of the major carcinogens in cigarette smoke (11, 13). However, carcinogenic effects of NNK are greatly modulated by its metabolic activation and detoxification. In humans, NNK from cigarette smoke is completely converted into metabolites after activation or detoxification, including free NNAL, NNAL-Gluc, and HA. N^7-MeG is one of the DNA adducts induced by NNK metabolites (12, 24). It is well known that CYP2A catalyzes the conversion of NNAL to HA (24) and UDP glucuronyl transferase catalyzes the conversion of free NNAL to NNAL-Gluc (total NNAL-free NNAL; ref. 25). Thus, the [HA/total NNAL] and [(total NNAL-free NNAL)/free NNAL] indexes may, respectively, represent individual variations in CYP2A and UDP glucuronyl transferase activities. In the present study, we showed that both NNK activation [HA/total NNAL] and detoxification [(total NNAL-free NNAL)/free NNAL] indices correlated with N^7-MeG levels in a human. It appears that N^7-MeG levels are modulated by CYP2A and UDP glucuronyl transferase activities. Thus, these indices could be used to investigate the role of individual variations in cigarette smoking-associated cancers in the future.

N^7-MeG is a major DNA adduct (70-90%) produced by methylation in biological systems (26), but it has little effect on DNA duplex structure (27). However, N^7-MeG is readily excised spontaneously or by methylpurine DNA *N*-glycosylase, converting it into mutagenic abasic sites (28). Because N^7-MeG is also formed by other endogenous methylation reactions, it is plausible that the N^7-MeG levels were ~100 times the levels of NNK metabolites in the urine. However, we still observed that the levels of N^7-MeG were well correlated with the levels of NNK metabolites and metabolism indices. Thus, N^7-MeG might be associated with the carcinogenic risk of ETS exposure. Recently, urinary N^7-MeG excretion was shown to be associated with lung cancer risk in smokers and subjects with null genotype of glutathione

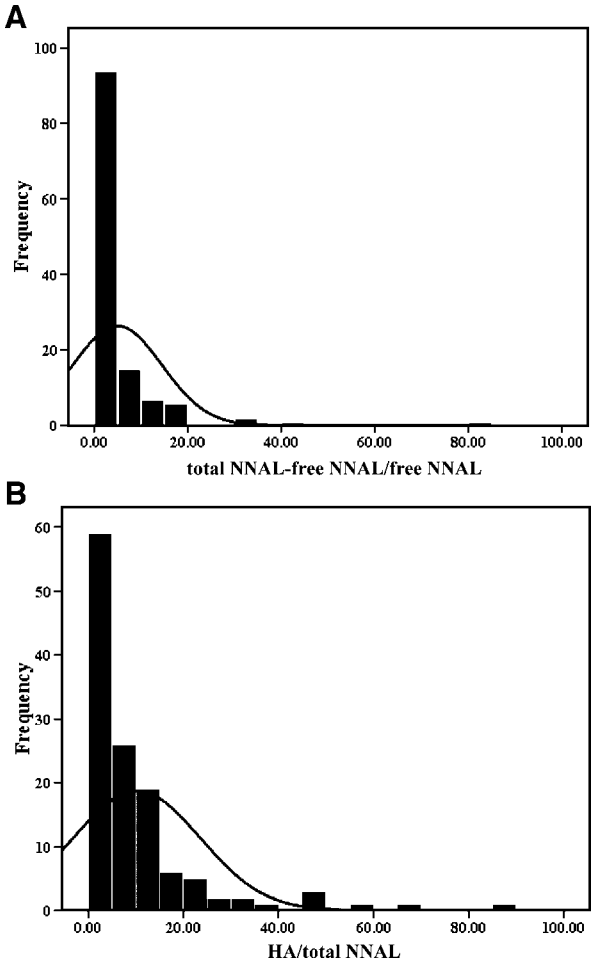

Figure 2. A, frequency distribution of total NNAL-free NNAL/free NNAL in human urine. **B,** frequency distribution of HA/total NNAL in human urine.

Table 4. Correlation between metabolic indices and N^7-MeG

Correlation with N^7-MeG	(Total NNAL-free NNAL)/free NNAL (n = 126)		HA/total NNAL (n = 126)	
	β	P	β	P
Total	−0.17	0.007	0.43	<0.0001
Smokers/ever smokers	−0.22	0.033	0.51	0.0003
Never smokers	−0.08	0.314	0.27	0.013

NOTE: Not adjusted for sex and age and adjusted for total NNAL, simple linear regression.

S-transferase M1 (29). The association between N^7-MeG and cancer risk may be modulated by individual variations in metabolism capability. In the future, we shall combine the metabolic indices of NNK and N^7-MeG for assessing cancer risks.

The metabolic indices of activation [HA/total NNAL] and detoxification [(total NNAL-free NNAL)/free NNAL] were highly variable in this population. Theoretically, individuals with either high [HA/total NNAL] or low [(total NNAL-free NNAL)/free NNAL] are at higher risk of cigarette smoking-associated cancers. Indeed, [HA/total NNAL] was positively correlated but [(total NNAL-free NNAL)/free NNAL] negatively correlated with N^7-MeG in this population. We also noticed that majority of individuals were low in both [HA/total NNAL] and [(total NNAL-free NNAL)/free NNAL]. In other words, majority of individuals carry a low risk of [HA/total NNAL] index and high risk of [(total NNAL-free NNAL)/free NNAL] index. Because cancer incidence is relatively low in general population, majority of individuals are at low cancer risk. Therefore, the [HA/total NNAL], but not the [(total NNAL-free NNAL)/free NNAL], distribution was consistent with individual susceptibility to cigarette smoke-associated cancers. As mentioned in the previous paragraph, CYP2A converts NNAL to HA. Hepatic CYP2A6 and pulmonary CYP2A13 were reported to be involved in metabolic activation of NNK (24). Genetic polymorphism of CYP2A6 was not associated with cancer risks (30-32). Recently, we showed that arsenic increased hepatic CYP2A expression and activity, subsequently enhancing urinary HA, keto acid, and N^7-MeG levels in NNK-treated mice (21). We believe that some environmental factors or foods could also modulate metabolic activation or detoxification of NNK. By measuring the metabolic indices and corresponding genetic polymorphisms, we should be able to study the interaction between metabolism capability, environmental factors, and cigarette smoking on cancer risks in future.

Whereas the activation index was associated with N^7-MeG in both smokers/ever smokers and never smokers, the detoxification index was associated with N^7-MeG only in smokers/ever smokers but not in never smokers. The activation index (CYP2A activity) is relatively specific to NNK-associated N^7-MeG production. On the other hand, glucuronyl conjugation widely involves in metabolism of exogenous and endogenous chemicals. The detoxification activity (UDP glucuronyl transferase) may differently modulate other endogenous methylation reactions and detoxification of NNK. Therefore, the detoxification index was not associated with N^7-MeG in never smokers. It further implied that the activation index might modulate the cigarette smoke-associated cancer risk in both active and passive smokers.

HA is the most abundant NNK metabolite detected in urine and it is also produced during *N*′-nitrosonornicotine and nicotine metabolism (33-35). However, the formation of HA from *N*′-nitrosonornicotine and nicotine is not accompanied with N^7-MeG generation. It has been shown that 12% of urinary HA is generated from NNK, 31% from *N*′-nitrosonornicotine, 1% from keto acid, and only 0.1% from nicotine in rats (33). More recently, Stepanov et al. (36) showed that the levels of NNK-derived HA were much higher than the levels of total NNAL in smokers. It may explain why the HA level was higher than total NNAL and free NNAL levels in the present study. Furthermore, urinary HA levels correlated well with urinary total NNAL and N^7-MeG levels, suggesting that NNAL has significant contribution to HA formation in humans. Keto acids were also detectable, as low as 25% of HA, in urine of smokers (37). However, we failed to detect keto acids in our specimens perhaps due to lower sensitivity of our analytic conditions. The total NNAL and free NNAL levels measured in this study were similar to those reported by others (38, 39). Total NNAL has been used as an exposure biomarker for ETS. In our population, total NNAL and HA were detectable in all cases regardless of smoking status. NNAL and NNAL-Gluc was detectable 281 days after smoking cessation (35). In addition, it appears that never smokers were exposed to ETS via passive smoking. We believe that NNK metabolites could serve as better biomarkers for studying the carcinogenic potential of ETS in humans in future.

Exposure to chemical carcinogens, such as DNA alkylating agent (40) and cigarette smoke, has been associated with urothelial carcinoma risk. A variety of DNA adducts, including N^7-methyldeoxyguanosine, were detected in the urothelial carcinoma tumor tissues. In our study, urinary NNK metabolites, metabolism index, and N^7-MeG were well correlated in urothelial carcinoma patients. Previously, we observed an interaction between cigarette smoking and arsenic methylation profile in the risk of urothelial carcinoma (18). Therefore, these biomarkers could be used to investigate the mechanism of cigarette smoking-increased risk of urothelial carcinoma in future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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