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Time course of DNA adduct formation in peripheral blood granulocytes and lymphocytes after drinking alcohol

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Alcohol consumption is an established risk factor for cancers of the head and neck, colorectum, liver and female breast. Acetaldehyde, the primary metabolite of ethanol, is suspected to play a major role in alcohol-related carcinogenesis. Acetaldehyde binds to DNA resulting in formation of adducts. DNA adducts are involved in mutagenesis and carcinogenesis. N^2-Ethylidenedeoxyguanosine (N^2-ethylidene-dGuo) is the major adduct formed in this reaction. Studies have shown an association between alcohol drinking and levels of this DNA adduct, suggesting its potential use as a biomarker for studying alcohol-related carcinogenesis. However, there are no reports on the kinetics of formation and repair of N^2-ethylidene-dGuo after alcohol consumption. Therefore, we investigated levels of N^2-ethylidene-dGuo in DNA from human peripheral blood cells at several time points after consumption of increasing doses of alcohol. Ten healthy non-smokers were recruited and asked to abstain from alcohol consumption except for the study doses. The subjects were given measured doses of alcohol once a week for 3 weeks, targeting increasing blood alcohol levels. Blood was collected at several time points before and after each dose, DNA was isolated from granulocytes and lymphocytes and N^2-ethylidene-dGuo was quantified as its $NaBH_3CN$ reduction product N^2-ethyldeoxyguanosine by liquid chromatography–electrospray ionisation–tandem mass spectrometry. Significant increases in N^2-ethylidene-dGuo were observed after all doses and in both cell types. However, there was substantial intraindividual variability, indicating that there are other important sources of this adduct in peripheral blood DNA. Further studies are needed to better understand the origins of N^2-ethylidene-dGuo in blood cells, the exposures it reflects, and thus its potential use as a marker of alcohol's genotoxic effects.

Introduction

Acetaldehyde associated with alcohol consumption has been recently classified as 'carcinogenic to humans' by the International Agency for Research on Cancer (1). This classification is mainly based upon evidence indicating oesophagus and head and neck as principal sites of carcinogenicity of acetaldehyde metabolically formed from ethanol. Mechanistic evidence in humans deficient in aldehyde dehydrogenase, the primary enzyme responsible for acetaldehyde degradation, further supports the link between acetaldehyde and cancers of the upper aerodigestive tract (2).

Acetaldehyde reacts with nucleophilic sites of DNA bases forming adducts. DNA adducts are important in mutagenesis and carcinogenesis. The major DNA adduct produced in the reaction of acetaldehyde with DNA is N^2-ethylidenedeoxyguanosine (N^2-ethylidene-dGuo) (3). This adduct can be reduced *in vivo* to N^2-ethyldeoxyguanosine (N^2-ethyl-dGuo), although this is a minor pathway (4).

N^2-Ethyl-dGuo has been detected in the DNA of both ethanol-treated mice and human alcoholics (4,5). Aldehyde dehydrogenase–deficient drinkers with a high risk for esophageal cancer had relatively high levels of acetaldehyde-derived DNA adducts in their lymphocytes (2). We observed an increasing trend of N^2-ethylidene-dGuo levels in leukocyte DNA, measured as the $NaBH_3CN$ reduction product N^2-ethyl-dGuo, for increasing amounts of alcohol consumed per day (6). The effect of alcohol on adduct levels was observed only when including heavy drinkers in the analysis. Acetaldehyde-derived DNA adducts can also be formed after reaction of endogenously formed acetaldehyde with DNA (7). Collectively, these results indicate that N^2-ethylidene-dGuo or its reduction product N^2-ethyl-dGuo in leukocyte DNA could be used as biomarkers for understanding carcinogenesis by alcohol. However, there is no information in the literature on the kinetics of formation and persistence of acetaldehyde-DNA adducts after alcohol consumption.

Therefore, in this study, we quantified N^2-ethylidene-dGuo, as its reduction product N^2-ethyl-dGuo, in granulocyte and lymphocyte DNA at intervals after consumption of known amounts of alcohol. An understanding of the effects of alcohol consumption on levels of acetaldehyde–DNA adducts in blood cell DNA is critical for the further validation of N^2-ethylidene-dGuo as a biomarker for studies of alcohol carcinogenesis.

Materials and methods

Subjects

Ten healthy volunteers, 5 of whom were men, were recruited from students and employees of the University of Minnesota. They were all social drinkers who consumed alcoholic beverages regularly in moderation, defined as taking at least one drink a week and three drinks over a 4-h period at least one time in the month before the study. They were greater than 21 years old, non-smokers, in good mental and physical health and had no unstable medical conditions and no history of alcohol abuse based on a medical history questionnaire. The following exclusion criteria were used: Asian ethnicity, periodontal disease or other oral lesions that might affect drug absorption, chronic use of a drug that could interact with alcohol and insulin-dependent diabetes. For women, pregnancy and current breastfeeding were additional exclusion criteria. Subjects were asked to refrain from using any recreational drug and from

ingesting any ethanol-containing product other than the study dose for the 4-week duration of the study.

Study design

The study was approved by the University of Minnesota Human Research Protection Programs Institutional Review Board. Subjects were enrolled after signing a consent form and evaluation of the eligibility criteria. Medical and alcohol drinking history in the past 12 months and lifetime were obtained by questionnaire. This was an open-label study design in which each subject served as his/her own control. Subjects participated in an initial meeting in which they were provided with information about the study; a blood sample was collected at that time. Participants were asked to refrain from using any mouthwash containing alcohol and from drinking any alcohol other than that administered in the study, starting at the initial meeting. One week later, the subjects returned to the clinic for the first alcohol dose. The meeting was scheduled at 8 a.m. The subjects were asked to come to the clinic after having had a light breakfast (cereal, milk and coffee) consumed at least 90 min before the alcohol dose. Subjects provided a blood sample before drinking the alcohol dose. Vodka, 100 proof, was mixed with tonic water and 1 ml of Rose lime juice, all purchased at a local liquor store. The amount of the drink served to each subject was calculated based on body weight and gender (8) in order to reach a blood alcohol level of 0.03%. Subjects took one sip every 5 min over a 20-min period. Systemic alcohol concentration was measured with a breath alcohol analyser, 30 min after the last sip (9). Blood samples were collected 2, 4 and 6 h after completion of the dosing period. Subjects were asked to refrain from drinking and eating between completion of the alcohol dose and the 4-h sample collection. Between the 4- and 6-h samples, the subjects had a light meal. They returned to the clinic between 8 and 10 a.m, 1, 2 and 5 days after the dose, providing a blood sample at each time point. One week after the first alcohol dose, the subjects came back to the clinic for the second alcohol dose. The session started at 8 a.m. They provided a blood sample before drinking. The alcoholic beverage was prepared as in the first session, but with a target blood alcohol level of 0.05%. They took one sip every 5 min over a 30-min period. Samples were collected following the protocol described for the first dosing session. One week after the second alcohol dose, they came back to the clinic for the third and final alcohol dose. The session started at 8 a.m. They gave a blood sample before drinking. The alcoholic beverage was prepared as in the first dosing meeting, with a target blood alcohol level of 0.07%. They took one sip every 5 min over a 40-min period. Samples were collected following the protocol described for the first dosing session, with addition of one sample collected 7 days after the last dose.

Blood collection and cell isolation

Blood samples were collected by venipuncture. Separation of lymphocytes and granulocytes from whole blood was performed using a density-gradient protocol (miltenybiotec.com) with some modifications. Briefly, 10 ml of whole blood was diluted with 35 ml of 2 mM EDTA in phosphate-buffered saline and layered on top of 15 ml Histopaque (Sigma–Aldrich, St Louis, MO, USA). Samples were centrifuged at 400 g for 40 min. The layer of lymphocytes and then the layer of granulocytes were aspirated and transferred into two separate vials. DNA was isolated using the DNA purification from white blood cell protocol (Qiagen Corp., Valencia, CA, USA) with several modifications. Briefly, 3 ml of RBC solution were added to the cells. After 5-min incubation at room temperature, the samples were centrifuged at 2000 g for 10 min. Cell lysis solution (3 ml) was added to the cell pellet and the sample was incubated at room temperature overnight. A solution of RNase A (15 µl of 4 mg/ml) was added and the sample was incubated at room temperature for 2 h. Protein precipitation solution (1 ml) was added to the cell lysate and the mixture was centrifuged to remove proteins. DNA was precipitated from the supernatant by addition of 4 ml of isopropanol. After centrifugation, the DNA pellet was washed with 1 ml of 70% ethanol in H_2O and then 1 ml of 100% ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA). DNA was dried in a stream of N_2 and stored at −20°C until use.

Chemicals and enzymes

N^2-ethyl-dGuo and $[^{15}N_5]N^2$-ethyl-dGuo were prepared as described (10). Puregene DNA purification solutions were obtained from Qiagen. Calf thymus DNA was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Alkaline phosphatase (from calf intestine) was obtained from Roche Diagnostics Corporation (Indianapolis, IN, USA). All other chemicals and enzymes were purchased from Sigma–Aldrich.

DNA hydrolysis and sample enrichment

These were performed as previously reported (6). Briefly, for enzyme hydrolysis, DNA was dissolved in 400 µl of 10 mM Tris/5 mM $MgCl_2$ buffer containing $[^{15}N_5]N^2$-ethyl dGuo (50 fmol) and $NaBH_3CN$ (30 mg). After the pH was adjusted to 7 with 0.1 N HCl, the DNA was initially digested overnight at room temperature with 1300 units of DNase I (type II, from bovine pancreas). 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. It was then allowed to stand overnight at room temperature. The enzymes were removed by centrifugation using a centrifree ultrafiltration device (Molecular Weight cutoff of 30 000; Amicon, Beverly, MA, USA). A 10-µl aliquot of the hydrolysate was removed for dGuo analysis. The hydrolysate was desalted and purified using a solid-phase extraction cartridge [Strata-X 33 µm, 30 mg/1 ml (Phenomenex, Torrance, CA, USA)]. The 70% CH_3OH fraction was collected and concentrated to dryness, dissolved in 1 ml of H_2O and purified using a mixed mode, anion exchange reversed-phase extraction cartridge (Oasis MAX, 30 mg/cartridge; Waters). Adducts were eluted with 1 ml of 70% CH_3OH, and the solution was evaporated to dryness. The residue was dissolved in 20 µl of H_2O, and 8 µl aliquots were analysed by liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC-ESI-MS/MS). Samples from each dose for each subject, including baseline and time-points after consumption of the dose, were processed together as a set, resulting in three sets per subject. Buffer blanks containing internal standard were processed as above and analysed to check the MS instrument baseline and possible contamination. Calf thymus DNA (0.1 mg) with internal standard added as above was used as a positive control. Each set of samples was run together with one buffer blank and three positive controls.

High-performance liquid chromatography–ultraviolet spectrometric analysis

Quantitation of dGuo was performed using an Agilent 1100 capillary flow HPLC with a diode array UV detector set at 254 nm (Agilent Technologies, Palo Alto, CA, USA). A 4.6 mm × 25 cm Luna 5 µm C18 column (Phenomenex) was used with a gradient from 5 to 40% CH_3OH in H_2O over the course of 35 min at a flow rate of 10 µl/min.

LC-ESI-MS/MS analysis

This was carried out with an Agilent 1100 capillary flow HPLC (Agilent Technologies) equipped with a 250 mm × 0.5 mm 5-µm particle size Polar RP column (Phenomenex) and a Vantage (Thermoelectron, San Jose, CA, USA) triple quadrupole mass spectrometer. The solvent elution program was a 10 µl/min gradient from 5 to 40% CH_3OH in 35 min at 30°C. The ESI source was set in the positive ion mode as follows: voltage, 3.7 kV; current, 3 µA; 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 → m/z 180 ($[M + H]^+ \rightarrow [BH]^+$) for N^2-ethyl-dGuo and m/z 301 → m/z 185 for $[^{15}N_5]N^2$-ethyl-dGuo.

A calibration curve was constructed before each analysis using a standard solution of N^2-ethyl-dGuo and $[^{15}N_5]N^2$-ethyl-dGuo. A constant amount of $[^{15}N_5]N^2$-ethyl-dGuo (5 fmol) was mixed with differing amounts of N^2-ethyl-dGuo (0.1, 0.5, 4, 10 and 100 fmol) and analysed by LC-ESI-MS/MS-SRM.

Statistical analysis

To determine whether the levels of N^2-ethyl-dGuo reached a peak, the ratio of the maximum level reached at each dose to the baseline level of N^2-ethyl-dGuo for that dose was calculated on the log scale and a 95% confidence interval (CI) for the ratio was formed. The estimate and confidence limits were then converted back to the original scale. The same method was used to determine whether the levels of N^2-ethyl-dGuo returned to baseline after reaching a peak, except that the ratio was formed using the baseline N^2-ethyl-dGuo level for the next alcohol dose or the final N^2-ethyl-dGuo level in the case of the third alcohol dose. To determine whether there was a dose–response, a repeated measures model was used, with the log of dose predicting the peak/baseline ratio, modelling the covariance matrix with dose as the repeated factor within subjects. To determine whether the granulocyte N^2-ethyl-dGuo peaks were greater than the lymphocyte N^2-ethyl-dGuo peaks, the differences between the peak/baseline ratios were calculated and 95% CIs for the differences were formed. The same method was used to determine whether the levels of N^2-ethyl-dGuo in granulocytes were higher than their levels in lymphocytes.

Results

The mean age of the subjects was 25 ± 3 (mean ± SD) and they were all Caucasian. All subjects participated in the introductory and three dosing meetings and provided the blood samples requested at all time points for a total of 230 samples. Demographics, amounts of ethanol administered, and systemic

alcohol levels reached after each dose are summarised in Table I. Women reached an average of 0.03 ± 0.02%, 0.05 ± 0.01% and 0.06 ± 0.01% blood alcohol after drinking an average of 19.4 ± 3.6, 28.0 ± 5.2 and 36.6 ± 6.8 g of alcohol in the three sessions. Men reached an average of 0.03 ± 0.01%, 0.05 ± 0.01% and 0.07 ± 0.01% after drinking 27.2 ±1.6, 39.3 ± 2.4 and 51.4 ± 3.1 g of alcohol in the three sessions.

Granulocytes and lymphocytes were isolated from each blood sample and DNA was extracted (mean ± SD, 94 ± 54 μg and 82 ± 32 μg, respectively), giving a total of 460 DNA samples. N^2-Ethylidene-dGuo (as N^2-ethyl-dGuo) was quantified in 423 samples treated with $NaBH_3CN$; the analysis failed in 37 samples due to poor recovery. The results are summarised in Tables II and III.

Levels of N^2-ethyl-dGuo increased in all subjects after most of the doses and the increase was up to 5-fold in DNA extracted from both lymphocytes and granulocytes. The results from granulocyte DNA (Table II) showed an increase in N^2-ethyl-dGuo levels in 8 out of 10 subjects after the first dose, in 8 out of 8 subjects after the second dose and in 9 out of 10 subjects after the third dose. N^2-Ethyl-dGuo increases were observed within 24 h in most cases, frequently starting 2–4 h

Table I. Study participants' demographics, grams of ethanol per dose and blood alcohol concentration (BAC %) attained after administration of each dose[a]

Subject	Gender	Age	Weight (kg)	Dose 1		Dose 2		Dose 3	
				Ethanol (g)	BAC[b]	Ethanol (g)	BAC	Ethanol (g)	BAC
1	F	30	59	16.1	0.02	23.2	0.04	30.4	0.04
2	F	23	65	17.7	0.02	25.6	0.05	33.4	0.06
3	F	23	64	17.6	0.03	25.4	0.05	33.3	0.05
4	F	26	92	25.2	0.04	36.4	0.05	47.6	0.07
5	F	28	74	20.2	0.05	29.2	0.06	38.2	0.07
	Mean ± SD	26 ± 3	71 ± 13	19.4 ± 3.6	0.03 ± 0.02	28.0 ± 5.2	0.05 ± 0.01	36.6 ± 6.8	0.06 ± 0.01
6	M	31	82	26.8	0.01	38.7	0.05	50.6	0.06
7	M	23	79	26.0	0.04	37.6	0.05	49.2	0.07
8	M	24	83	27.4	0.04	39.5	0.05	51.7	0.06
9	M	22	91	29.9	0.04	43.2	0.05	56.5	0.08
10	M	25	79	25.9	0.04	37.3	0.04	48.8	0.08
	Mean ± SD	25 ± 4	83 ± 5	27.2 ± 1.6	0.03 ± 0.01	39.3 ± 2.4	0.05 ± 0.01	51.4 ± 3.1	0.07 ± 0.01

[a]This table is also presented in a separate paper on oral cell DNA adducts in the same individuals (11).
[b]BAC, blood alcohol concentration: milligrams of alcohol in 100 ml blood expressed as %.

Table II. Levels of N^2-ethylidene-dGuo (fmol/μmol dGuo) in granulocyte DNA before and after each dose of alcohol

	Subject	1	2	3	4	5	6	7	8	9	10
Dose 1	Baseline	7446	8467	15 290	9894	7196	9865	9095	8210	8881	7371
	2 h	7621	9815	16 750	13 690	6811	11 540	5580	8224	13 390	5659
	4 h	10 270	9968	23 140	15 740	9825	9595	6806	7863	—	4637
	6 h	7399	6983	13 000	15 740	9453	9301	7464	6733	7189	3155
	24 h	7397	8292	3403	2104	2158	3576	7978	4930	6008	5931
	48 h	10 400	6869	13 900	16 290	25 160	14 610	7244	5169	11 050	6707
	120 h	7290	5521	12 600	14 240	14 260	12 910	13 580	5769	6660	5546
Dose 2	Baseline	7991	12 550	9898	6623	6773	—[a]	3877	2178	—	5476
	2 h	8618	13 620	8158	7550	5859	—	3252	3224	—	19 250
	4 h	6208	15 530	11 580	7919	7188	—	3918	1795	—	4781
	6 h	3379	10 650	10 400	3758	6984	—	2835	1887	—	3053
	24 h	8886	12 780	6590	7521	6946	—	4654	4613	—	1361
	48 h	8076	8168	10 270	4923	10 820	—	3998	3168	—	2063
	120 h	20 440	15 460	6256	9313	9394	—	3951	4501	—	653
Dose 3	Baseline	—[a]	8697	7893	4358	7833	5988	4555	8498	9354	520
	2 h	4662	21 200	4689	4948	5625	3405	3123	14 710	7515	—
	4 h	6591	17 500	3040	5065	4895	4036	10 790	7885	4282	1181
	6 h	4565	9053	2276	5109	4141	1808	6235	4819	7133	3391
	24 h	11 960	9644	11 230	4488	22 360	6224	1102	3968	1025	813
	48 h	4774	5629	7552	13 070	40 630	11 560	3751	3543	3430	—
	120 h	3316	761	10 500	—	14 670	12 300	795	789	887	723
	168 h	3589	862	12 170	5481	5535	4643	913	1693	1076	9276
	1 week before dose 1	12 520	9331	8883	8980	9045	9656	9655	7409	7225	7708

Levels of N^2-ethylidene-dGuo (as N^2-ethyl-dGuo) were measured in granulocyte DNA from each subject. The last row reports participants' adduct levels measured 1 week before the first dose was given. This is the point at which participants began to abstain from consumption of any alcoholic beverage other than the dose given for the study. 'Baseline' levels of N^2-ethyl-dGuo were also measured 1 h before each dose was given. The rows that follow report the levels of N^2-ethyl-dGuo at the various time points after consumption of the three increasing doses (from dose 1 to dose 3).
[a]Levels of N^2-ethyl-dGuo not quantified due to poor recovery.

after alcohol consumption. Similar results were observed for the lymphocyte DNA samples (Table III). The increase of N^2-ethyl-dGuo levels was seen in all subjects after the first dose, in 5 out of 8 subjects after the second dose and in 9 out of 10 subjects after the third dose. As in the granulocyte DNA samples, N^2-ethyl-dGuo increases were observed mostly within 24 h after alcohol consumption, mainly 2–4 h after the dose. Although in both granulocyte and lymphocyte DNA an increase was observed mostly 2–4 h after the dose, the peak levels of N^2-ethyl-dGuo were reached later in time.

Statistical analyses are summarised in Table IV. Significant peak/baseline ratios of N^2-ethyl-dGuo in granulocyte DNA were observed 36 h after the first dose, 43 h after the second dose and 32 h after the third dose. The increases were dose dependent ($P < 0.001$). After reaching a peak, levels of N^2-ethyl-dGuo returned to baseline after all doses. However, this observation was only statistically significant after the first and third doses. Baseline levels of N^2-ethyl-dGuo in granulocyte DNA significantly decreased throughout the duration of the study ($P = 0.021$).

Significant peak/baseline ratios of N^2-ethyl-dGuo in lymphocyte DNA were observed 40 h after the first dose, 31 h after the second dose and 29 h after the third dose. The increases were significant after all three doses, but no dose-dependent

Table III. Levels of N^2-ethylidene-dGuo (fmol/ μmol dGuo) in lymphocyte DNA before and after each dose of alcohol

	Subject	1	2	3	4	5	6	7	8	9	10
Dose 1	Baseline	8741	11 770	9858	12 400	1006	10 970	4923	3765	6518	5094
	2 h	11 310	8069	10 180	13 300	8870	10 440	4136	5200	7648	3991
	4 h	11 480	15 100	8702	11 820	7061	8479	5620	6206	4444	5397
	6 h	7783	6318	10 080	12 240	7575	9796	6248	3573	6385	6022
	24 h	13 550	5970	19 070	11 720	9227	15 910	5801	4356	5925	5200
	48 h	5762	7454	16 320	13 660	6394	12 510	5274	5696	13 900	3955
	120 h	4795	5128	10 970	13 760	7866	7389	3904	5513	4250	6231
Dose 2	Baseline	9576	13 110	16 960	13 650	9716	—[a]	2663	1902	—	1909
	2 h	10 700	10 380	14 810	8383	10 590	—	2157	2473	—	1688
	4 h	6787	6463	16 220	11 300	19 800	—	2203	1730	—	4728
	6 h	9750	8363	15 790	11 250	9410	—	1748	2507	—	3815
	24 h	5684	8569	11 800	10 270	7724	—	3080	3321	—	3782
	48 h	3008	2001	4605	6239	3506	—	2887	1458	—	2196
	120 h	8602	11 610	6072	4628	4377	—	3237	2411	—	2102
Dose 3	Baseline	—[a]	8063	7259	2939	5971	6164	4384	2801	932	4809
	2 h	7219	40 190	5145	4963	3986	4279	9334	10670	4576	—
	4 h	6811	2850	2909	2936	2226	5026	5802	3380	—	5864
	6 h	5600	8478	2966	4005	4595	1342	3609	2079	1798	11 660
	24 h	8144	4905	5198	4512	8579	7615	5079	1780	1254	804
	48 h	6539	2282	5598	4271	13 430	17 940	2645	2945	2698	—
	120 h	13 400	776	4537	5588	7126	1911	3071	909	660	4231
	168 h	4960	2965	9647	4708	1852	3510	1236	5389	3365	789
	1 week before dose 1	10 170	5431	8095	15 430	5878	9724	8192	5421	9582	4595

Levels of N^2-ethylidene-dGuo (as N^2-ethyl-dGuo) were measured in lymphocyte DNA from each subject. The last row reports participants' adduct levels measured 1 week before the first dose was given. This is the point at which participants began to abstain from consumption of any alcoholic beverage other than the dose given for the study. 'Baseline' levels of N^2-ethyl-dGuo were also measured 1 h before each dose was given. The rows that follow report the levels of N^2-ethyl-dGuo at the various time points after consumption of the three increasing doses (from dose 1 to dose 3).
[a]Levels of N^2-ethyl-dGuo not quantified due to poor recovery.

Table IV. Variation of peak/baseline ratio and peak/next baseline ratio after increasing doses of alcohol considering the peak reached after consumption of the alcohol dose

		Dose 1	Dose 2	Dose 3
Granulocytes	Peak/baseline[a], mean (95% CI)	1.5 (1.2, 2.0)	1.7 (1.2, 2.4)	2.7 (1.7, 4.2)
	Peak timing (h)[b]	36	43	32
	Peak/next baseline[c], mean (95% CI)	2.2 (1.3, 3.9)	2.1 (0.6, 7.2)	3.8 (1.2, 12.1)
Lymphocytes	Peak/baseline[a], mean (95% CI)	1.8 (1.0, 2.7)	1.6 (1.1, 2.5)	3.00 (2.2, 4.1)
	Peak timing (h)[b]	40	31	29
	Peak/next baseline[c], mean (95% CI)	1.6 (1.0, 2.5)	1.3 (0.5, 3.7)	4.5 (2.0, 10.5)
Granulocyte–lymphocyte	Difference[d]	0.8 (0.61, 1.13)	1.25 (0.8, 2.1)	1.1 (0.6, 2.1)

[a]The ratio between the average maximum N^2-ethyl-dGuo level reached after each dose of alcohol and the average baseline level for that dose was calculated and a 95% CI for the ratio was formed.
[b]The average time at which the maximum N^2-ethyl-dGuo was reached after each dose was calculated.
[c]To determine whether the levels of N^2-ethyl-dGuo returned to baseline after reaching a peak, the ratio between the average maximum N^2-ethyl-dGuo level reached after each alcohol dose and the average baseline DNA adduct level measured for the next alcohol dose was calculated. The final N^2-ethyl-dGuo level measured at 168 h was used as next baseline level for the third alcohol dose.
[d]To verify whether there was a difference between the levels N^2-ethyl-dGuo in granulocyte and lymphocyte DNA, we calculated the difference between the granulocyte and lymphocyte peak/baseline ratios and constructed a 95% CI for the difference.

effect was observed ($P = 0.061$). After reaching a peak, levels of N^2-ethyl-dGuo decreased after all doses. However, the decrease was significant only after the first and third dose. A decreasing trend in baseline levels of N^2-ethyl-dGuo was not observed in lymphocyte DNA samples ($P = 0.261$). However, the levels measured in the samples taken at the end of the study (168 h after the third dose of alcohol) were significantly lower than the levels measured in the first samples taken at the beginning of the study ($P = 0.002$).

Levels of N^2-ethyl-dGuo showed considerable intra- and interindividual variation in the DNA of both cell types. Overall, the levels of N^2-ethyl-dGuo were similar in lymphocyte and granulocyte DNA, but no clear correspondence in the N^2-ethyl-dGuo trends was observed when comparing the levels in lymphocyte and granulocyte DNA within subjects.

Discussion

The results of this study demonstrate that alcohol consumption increases levels of the major acetaldehyde–DNA adduct N^2-ethylidene-dGuo, measured as N^2-ethyl-dGuo, in peripheral blood cells. In both granulocytes and lymphocytes, the effect was significant within 40 h, even after consumption of the lowest dose of alcohol tested, comparable to roughly one drink. The effect observed in granulocyte DNA was dose dependent. In lymphocyte DNA, the effect was significant at all doses although no dose dependence was observed. To our knowledge, this is the first study to investigate the effects of alcohol consumption on the time course of DNA adduct formation in blood cells from healthy volunteers. All previously published studies reporting effects of alcohol consumption on N^2-ethyl-dGuo levels were performed with heavy drinkers or alcoholics (5) and no time dependence of DNA adduct formation was reported. Very few previous studies have reported the time course of DNA adduct formation after exposure to any dietary or lifestyle-associated carcinogen (12,13). Investigation of the kinetics of formation of DNA adducts in humans after exposure to DNA-binding compounds has been limited mostly to studies testing clinical response to platinum-based therapies in cancer patients (14).

The rationale for this study originated in our recent observation of a dose–response effect of ethanol consumption on levels of N^2-ethylidene-dGuo in leukocyte DNA (6). In that study, some of the subjects were hospitalised and a decreasing trend in adduct levels was observed with increasing length of the subjects' stay in the hospital. This observation suggested the need to understand the kinetics of N^2-ethylidene-dGuo formation and removal. Furthermore, the large interindividual variation observed in that study suggested exploration of potential differences in exposure reflected by DNA from cells with differing lifespans or repair capacities. Another previous study reported higher levels of N^2-ethyl-dGuo in granulocyte DNA compared to lymphocyte DNA in alcoholic patients (5). Therefore, in this study, we investigated the role of alcohol consumption on DNA adduct formation in granulocytes and lymphocytes from healthy subjects who consumed increasing doses of ethanol in a controlled setting. We targeted blood alcohol levels below intoxication (considered to arise at a blood alcohol level of 0.08%) (8). The three doses selected for our study can roughly be described as 1, 2 or 3 drinks per subject. Potential exposure to acetaldehyde already present in the alcoholic beverage was reduced by selecting 100 proof vodka as the source of alcohol. Acetaldehyde content in vodka is low compared to that in other hard liquors (15). Since tobacco smoke can be a source of acetaldehyde, we restricted the recruitment to non-smokers. Food can interfere with alcohol absorption and can also be a source of acetaldehyde. Therefore, the study participants were asked to eat only a light breakfast at least 90 min before administration of the alcohol dose and they were not allowed to eat until 4 h after the dose. Finally, to reduce the potential influence of other sources of alcohol on the results, our subjects were asked to refrain from using any alcohol or any mouthwash containing alcohol for the duration of the study.

Although we observed increases in the levels of N^2-ethyl-dGuo in most subjects after each dose, as well as a dose–response effect of alcohol consumption on DNA adduct levels in granulocytes, there was, in spite of the study design features discussed above, considerable intraindividual variation in adduct levels and adduct peak times, even for the same subject in both lymphocyte and granulocyte DNA. Furthermore, a high background level was observed for all subjects recruited clearly indicating other major sources of N^2-ethylidene-dGuo in blood cell DNA than ethanol consumption or cigarette smoking, both of which were controlled in this study. Average levels of N^2-ethylidene-dGuo in the subjects analysed were ~7500 fmol/μmol Gua or 15 adducts per 10^7 nucleotides, which is comparable to the levels we previously reported in leukocyte DNA (6) but roughly 10-fold higher than the levels we found in human liver DNA (10) and the levels described in human lung DNA (16). These levels are in the high range of reported 'endogenous' DNA adducts, similar to reports of oxidative DNA adducts like 8-oxo-dGua that have been attributed to oxidative stress (17). These factors detract from the potential utility of this biomarker for studies in alcohol carcinogenesis and from formulating any conclusion on the biological significance of this DNA adduct, at least until additional origins of adduct levels and variation can be more clearly delineated. Acetaldehyde can be produced in the body through metabolism of threonine, alanine and deoxyribose phosphate (18). Alanine is synthesised in the body but is also present in a number of foods. Oxidative stress is another factor that could play a role in endogenous levels of acetaldehyde, possibly due to inhibition of aldehyde oxidation by products of oxidative stress (19) or to production of acetaldehyde by activated immune cells (20). Additionally, phagocytes oxidise α-amino acids to aldehydes and form acetaldehyde through myeloperoxidase mediation of alanine oxidation (21). Myeloperoxidase is found in high abundance in human neutrophils and circulating monocytes. This endogenous source of acetaldehyde could influence specifically its concentration in the blood, explaining the higher levels of N^2-ethyl-dGuo levels measured in whole blood cells compared to the levels reported in human lung and liver. In another part of this study, levels of N^2-ethyl-dGuo were quantified in oral cells obtained from the same individuals. The results, which will be described separately (11), showed lower background levels, lower variability and a clearer effect of alcohol consumption on peak DNA adduct levels. A dose-dependent increase was observed for all subjects after all doses and N^2-ethyl-dGuo levels reached a peak within 2–4 h after exposure. Concentrations of acetaldehyde in saliva after drinking alcohol are much higher than in blood, due in part to oral microflora metabolism of ethanol, likely contributing to the differing results (18).

Ethanol is absorbed from the intestines and metabolised in the liver. Blood alcohol concentration reaches a peak generally

between 30 and 90 min after a drink. Blood acetaldehyde levels after alcohol consumption follow a similar trend (18). However, acetaldehyde can bind to a wide variety of proteins such as albumin, cytochromes P450, low-density lipoproteins and haemoglobin. Some haemoglobin adducts degrade within 5 days from alcohol exposure (22). The release of acetaldehyde from the degradation of protein adducts could contribute to the increases in N^2-ethyl-dGuo which we observed at some of the longer intervals after alcohol exposure.

Levels of N^2-ethylidene-dGuo in granulocyte DNA decreased significantly during the course of the study. In lymphocyte DNA, this trend was not observed. However, N^2-ethylidene-dGuo levels in the first sample, taken at the beginning of the study, were significantly higher than in the last sample taken 168 h after the last dose. Subjects were asked to refrain from alcohol drinking for the duration of the study (other than the study doses). The decrease in the baseline levels of N^2-ethylidene-dGuo could be due to this abstinence from alcohol.

A limitation of this study was the relatively small number of participants, which was dictated mainly by the large number of DNA adduct analyses required to determine the time course of N^2-ethylidene-dGuo formation and removal. These mass spectrometry–based analyses, while highly sensitive and dependable, are nevertheless time consuming and expensive. The relatively small number of participants also prevented us from examining the influence of polymorphisms in alcohol and aldehyde dehydrogenase genes. The unexpected within-subject variability observed in this study underlines the need for further investigations on a larger number of study participants, possibly with dietary restrictions, and the need for a comparison with controls not exposed to ethanol. This would allow assessment of the normal variation in adduct levels over the time interval studied. The absence of this control group was another limitation in this study.

In conclusion, we present evidence that N^2-ethylidene-dGuo levels, measured as N^2-ethyl-dGuo in granulocyte and lymphocyte DNA, are influenced by ethanol metabolism to acetaldehyde even at the lowest alcohol dose tested. However, the significance of this increase is limited by variability observed both in the time to biomarker peak intensity and in the peak intensity itself, even within the same subject. These results underline the importance of investigating the kinetics of formation of DNA adducts proposed as biomarkers for human studies.

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