A Urinary Marker of Alcohol Intake[1]

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Abstract

Previously, one of us (B. K. T.) developed an assay that measures levels of free ethanol and ethanol conjugates in urine and showed that the mean levels of these ethanol markers in confirmed alcoholics were at least 20-fold higher than those levels in control subjects. In this study, we assessed the relationship of these biomarkers with self-reported levels of alcohol intake in a multiethnic sample of Los Angeles County residents who were male and over the age of 35 years (n = 128; 40 non-Hispanic whites, 46 blacks, 17 Chinese, and 25 Japanese). Regardless of race, the mean levels of free, bound, and total (free plus bound) ethanol were lowest in nondrinkers, intermediate in weekly drinkers, and highest in daily drinkers (P = 0.0001 in all three statistical tests of differences in the three biomarkers). Stepwise discriminant analysis showed that of the three potential biomarkers, total ethanol best discriminates between the three classes of drinkers (non, weekly, and daily), and that additional inclusion of either free or bound ethanol in the discriminant function had negligible effect. Overall, mean level of total ethanol was 2.2 times higher in weekly than in nondrinkers; daily drinkers, in turn, showed a 4.2-fold increase in mean total ethanol relative to weekly drinkers. However, there was no correlation between any of the three biomarkers and self-reported level (in grams of ethanol) of average consumption in either weekly or daily drinkers whose mean intake was about 13 and 42 g of ethanol/day, respectively. As the level of urinary free ethanol and ethanol conjugates showed extraordinary differences among racial groups for a given level of self-reported ethanol intake, the data suggest possible interracial differences in the *in vivo* elimination rate of ethanol; this latter finding needs to be confirmed in larger studies.

Introduction

Excessive intake of alcohol has been linked to the development of many serious diseases, including cancer of various sites (1–7). Given the possibility of underreporting, especially among heavy users, it is desirable to have available an objective marker of alcohol intake to complement the self-reported levels usually assessed in epidemiological investigations. A number of biological markers of ethanol intake and abuse have been proposed and most involve the collection of a blood specimen (8). Recently, Tang (9) developed an assay that measures levels of free ethanol and ethanol conjugates in urine and showed that the mean levels of these ethanol markers in confirmed alcoholics were at least 20-fold higher than those levels in control subjects. In this study, we assess the ability of this noninvasive procedure in identifying drinkers whose intake levels fall within the "normal range" of the general population.

Materials and Methods

Subjects. Detailed characteristics of our study subjects have been described elsewhere (10). Briefly, they were male residents of Los Angeles County who were over the age of 35 years and were either non-Hispanic white (white; n = 40), black (n = 46), or Asian (n = 42; 17 Chinese and 25 Japanese). By design, 67 (52%) subjects were lifelong nonsmokers; the remaining 61 subjects were current cigarette smokers of varying intensity.

Each subject was interviewed in-person by using a structured questionnaire, which asked about his drinking habits during the past 2 weeks. Subjects who did not consume any alcoholic beverages 2 weeks before the interview were classified as nondrinkers. The drinkers were asked whether they drank every day (classified as daily drinkers) or less frequently (classified as weekly drinkers). The average amounts of beer, wine, and hard liquor that a drinker consumed per day or per week also were recorded. All interviews were conducted no later than 5 p.m. A blood specimen was obtained from each subject immediately after the interview. In addition, the subject was given two packets of Nescafe instant coffee (about 70 mg of caffeine) to be drunk between 3 and 6 pm that same day. The subject collected an overnight urine sample (ending with the first morning void) into a 1-liter plastic bottle that was picked up and processed the same day. (This urine collection procedure was intended for acetylation phenotype determination, using caffeine as the test compound.) The urine specimens were acidified (20 mg of ascorbic acid/ml of urine) before storage at −20°C. Blind samples (identified only by code numbers) of serum and urine were subsequently sent on dry ice to the University of Toronto (Toronto, Ontario, Canada) for the analysis of free ethanol in serum and free ethanol and ethanol conjugates in urine, respectively.

Analysis of Free Ethanol in Urine/Serum. Detailed description of the GC-MS[3] method has been published (9). Briefly, 0.1 ml of sample was mixed with 10 μl (0.2 mM) of deuterated ethanol (CD_3CH_2OH, 98% deuterium; Isotope Labeling Corp.,

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[3] The abbreviations used are: GC-MS, gas chromatographic mass spectrometric; r, correlation coefficient; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

Table 1 Alcohol consumption status of study subjects

	Whites	Blacks	Asians	Total
Nondrinker	18[a] (45)[b]	21 (46)	24 (57)	63 (49)
Weekly drinker	10 (25)	14 (30)	13 (31)	37 (29)
1–19 g ethanol/day	6 (15)	14 (30)	11 (26)	31 (25)
20–39 g ethanol/day	3 (7)	0 (0)	0 (0)	3 (2)
40+ g ethanol/day	1 (3)	0 (0)	2 (5)	3 (2)
Daily drinker	12 (30)	11 (24)	5 (12)	28 (22)
1–19 g ethanol/day	2 (5)	1 (2)	0 (0)	3 (2)
20–39 g ethanol/day	8 (20)	2 (4)	4 (10)	14 (11)
40+ g ethanol/day	2 (5)	8 (18)	1 (2)	11 (9)
Total subjects	40 (100)	46 (100)	42 (100)	128 (100)
Mean daily intake of ethanol (g)[c]				
Weekly drinkers	22.5	7.0	12.0	12.9
Daily drinkers	30.8	57.5	33.8	41.8
All drinkers	27.0	29.2	18.0	25.4

[a] Data are number of subjects.
[b] Numbers in parentheses, percentages.
[c] Self-reported intake. For weekly drinkers, the total amount/week was divided by seven to obtain a daily intake equivalent.

Table 2 Geometric mean levels of urinary free, bound, and total ethanol by alcohol consumption status of study subjects

	Mean levels (nmole/mg creatinine)			
	Whites	Blacks	Asians	Total
Free ethanol				
Nondrinkers	6.3	2.8	5.5	4.5
Weekly drinkers	13.3	8.7	13.9	11.5
Daily drinkers	342.5	10.1	30.3	55.6
Bound ethanol				
Nondrinkers	24.9	15.0	23.8	20.7
Weekly drinkers	47.3	27.1	52.2	39.7
Daily drinkers	152.0	65.7	130.8	106.4
Total (free plus bound) ethanol				
Nondrinkers	32.0	18.2	29.6	25.7
Weekly drinkers	65.9	37.3	77.3	56.2
Daily drinkers	692.1	83.8	177.6	236.8

Whippany, NY) as internal standard. An aliquot of 0.1 μl of each sample was taken in a 10-μl syringe for GC-MS analysis. A capillary column (30 M × 0.25-mm internal diameter; J & W Scientific, Inc., Folsom, CA) was used. The GC conditions were: injector port at 200°C, column at 100°C, and helium at 4 ml/min. Under these conditions, the retention time of ethanol was 95 s. The MS was operated in the chemical ionization mode, and methane was used as the reagent gas (source pressure, 1.0 mm Hg). Protonated ethanol ion ($CH_3CH_2OH_2^+$) at m/z 47 and ethanol-d3 ($CD_3CH_2OH_2^+$) at m/z 50 were monitored by a DOS-based Vector/One control module and data acquisition system (Teknivent, Maryland Heights, MO). Peak height ratios of m/z 47 to m/z 50 were calculated, and the amounts of ethanol were estimated from the calibration curve. The recovery was quantitative and the coefficient of variation was <5%. Samples with ethanol concentrations >100 μM had to be diluted and reanalyzed.

Analysis of Ethanol Conjugates in Urine. An aliquot of 0.1 ml of urine was lyophilized in a glass screw cap vial to remove any free ethanol. Two nmol of ethanol-d3 as the internal standard and 0.1 M of 10 N sulphuric acid were added to the residue. The vial was then sealed with a cap. After reaction at 60°C for 1 h, an equivalent amount of 10 N sodium hydroxide was added for neutralization before GC-MS analysis for free ethanol as described above.

The chemical nature of the ethanol conjugates has not been characterized. However, because acid hydrolysis and hydrolysis by β-glucuronidases (type H-2, containing sulfatase activity; Sigma Chemical Co., St. Louis, MO) gave similar levels of ethanol, the conjugates were presumed to be ethanol glucuronate and/or sulfate conjugates.

As a means of adjusting for the varying concentration of urine among study subjects, levels of free ethanol and ethanol conjugates were expressed relative to that of urinary creatinine.

Statistical Analysis. We computed total ethanol intake from reported intake of amounts of beer, wine, and spirits by using the following conversion factors: 1 can (12 oz.) of beer contains 12.96 g, 1 glass (3.5 oz.) of wine contains 10.10 g, and 1 jigger (1.5 oz.) of spirits contains 14.03 g of ethanol (11). The distributions of all serum and urinary biomarkers of ethanol in our study populations were markedly skewed; therefore, formal statistical testings were performed on logarithmically transformed values of the biomarkers and geometric (as opposed to arithmetic) mean values are presented. The ANOVA method was used to compare ethanol biomarkers across racial and drinking categories simultaneously and to examine possible fluctuation in urinary ethanol biomarkers by day of the week on which the sample was collected (each day of the week separately or weekend days *versus* weekdays). The discriminant analysis method was used to assess which of the potential biomarkers of alcohol exposure offered the best discrimination between non-, weekly, and daily drinkers (12). All *P* values quoted are two sided.

Results

Sixty-three (49%) of the 128 study subjects were nondrinkers. Of the 65 subjects who drank alcoholic beverages during the previous 2 weeks, 28 of them drank on a daily basis. Fewer Asian subjects were drinkers relative to whites and blacks, and the Asian drinkers, on average, were more moderate in their

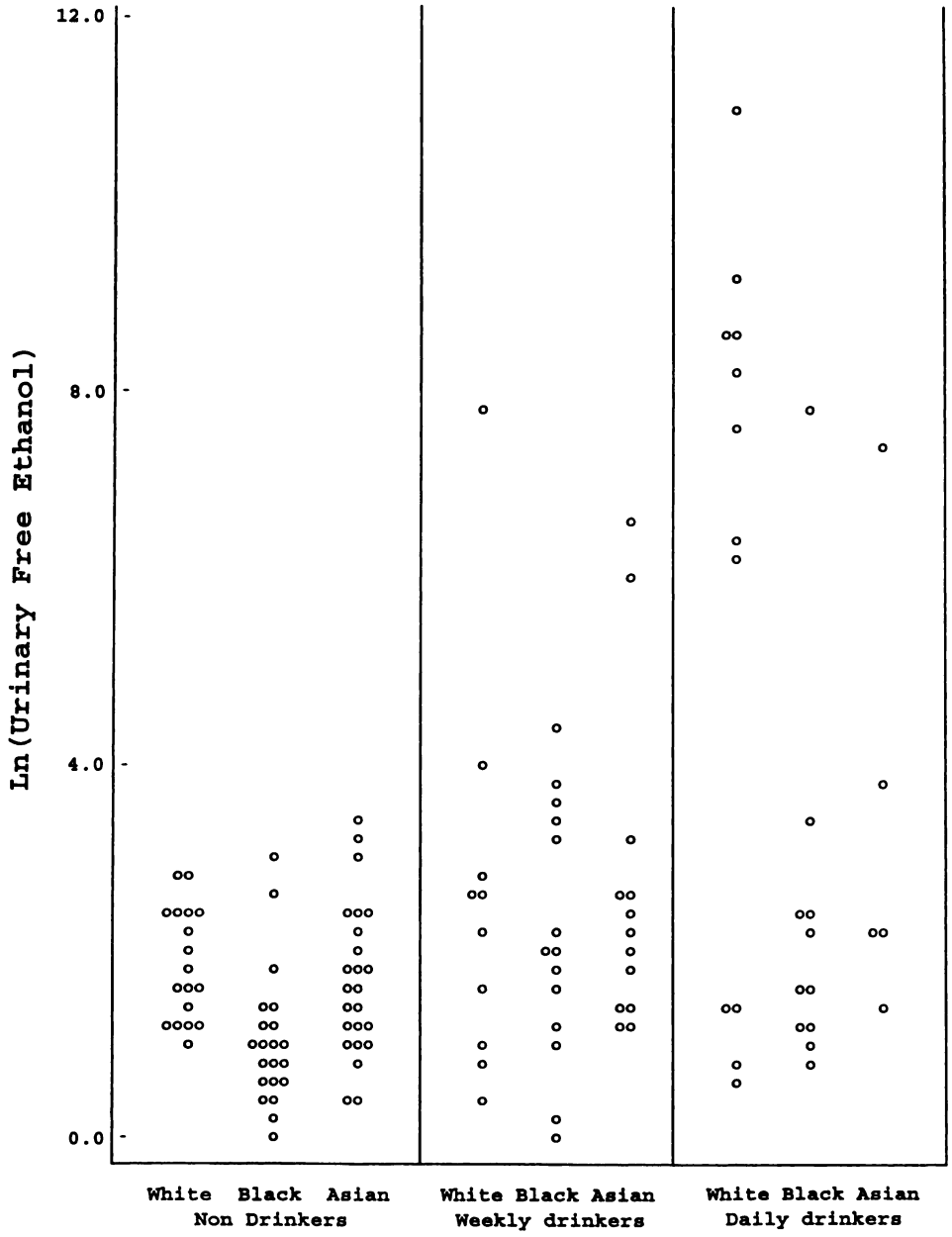

Fig. 1. Distribution of urinary free ethanol level (expressed in log units of nmol/mg of creatinine) among study subjects according to their race and alcohol consumption status.

intake relative to whites and blacks. Although a higher percentage of white relative to black drinkers drank alcohol on a daily basis, black daily drinkers drank more heavily than white daily drinkers, thus, resulting in similar levels of mean daily intake of ethanol between black and white drinkers overall (Table 1).

Table 2 presents the geometric mean levels of urinary free, bound, and total (free plus bound) ethanol (expressed in nmol/mg creatinine) by alcohol consumption status of study subjects. Regardless of race, the mean level of free ethanol was lowest in nondrinkers, intermediate in weekly drinkers, and highest in daily drinkers. This difference in mean levels of urinary free ethanol was highly significant ($P = 0.0001$). Overall, weekly drinkers had a mean level 2.6 times higher than that of nondrinkers, and the mean level in daily drinkers was 4.8-fold higher than that of weekly drinkers. However, there was no correlation between urinary level of free ethanol and self-reported level of average consumption among either weekly ($r = 0.14$; $P = 0.40$) or daily drinkers ($r = -0.14$; $P = 0.48$).

Race was a significant factor in determining level of urinary free ethanol ($P = 0.0002$). Although blacks who were daily drinkers, on average, reported heavier drinking habits than their white counterparts (57.5 *versus* 30.8 g of ethanol/day,

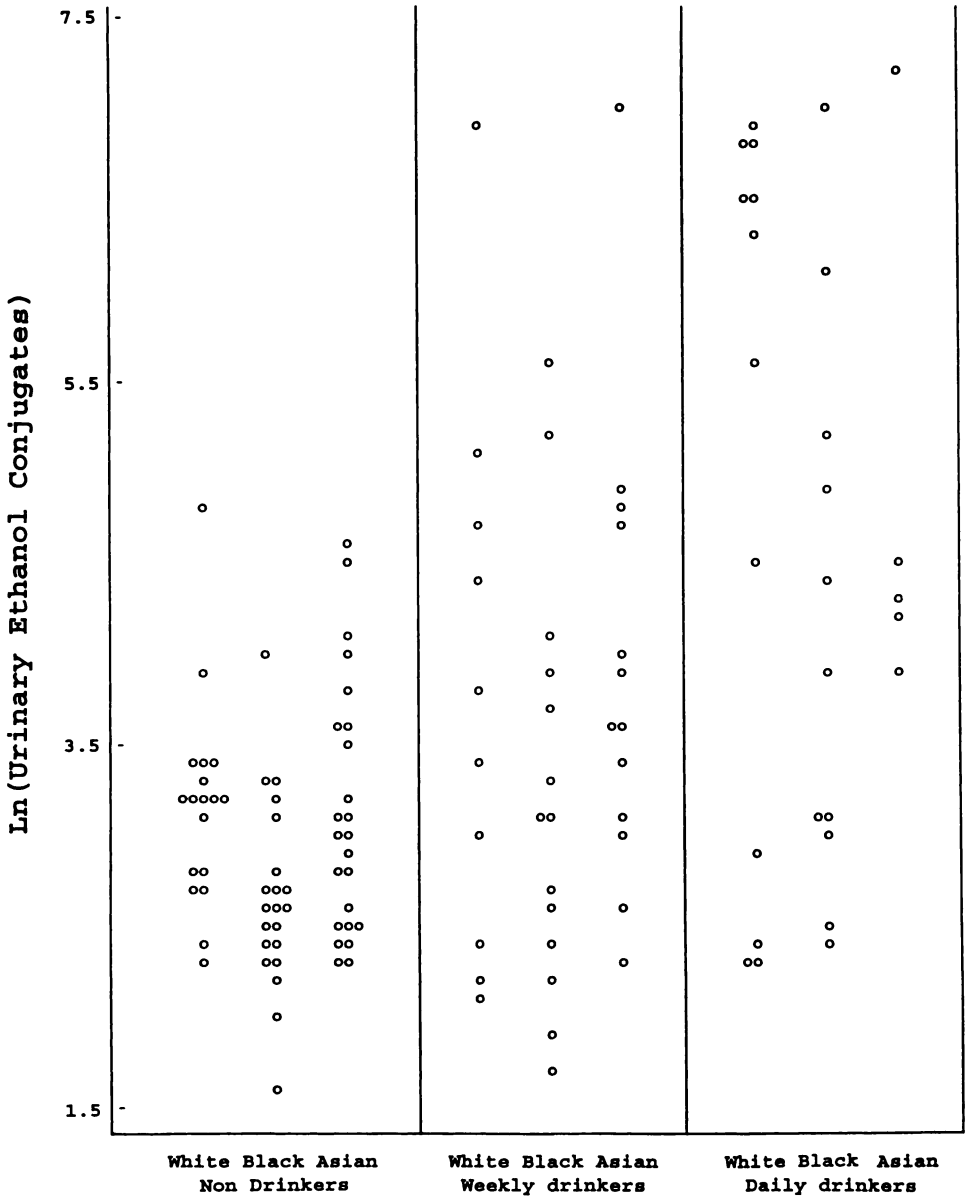

Fig. 2. Distribution of urinary ethanol conjugate level (expressed in log units of nmol/mg of creatinine) among study subjects according to their race and alcohol consumption status.

respectively), the mean level of urinary free ethanol was 34-fold higher in white than black daily drinkers (342.5 *versus* 10.1 nmol/mg of creatinine; Table 2). Fig. 1 shows the distribution of free ethanol values among all study subjects according to their race and drinking habits. The graph clearly shows an excess of white daily drinkers with very high free ethanol levels relative to black daily drinkers who actually reported higher amounts of consumption. Mean levels of free ethanol also were lower in black than white non- and weekly drinkers, although the differences were not as pronounced. We explored the possibility that the level of free ethanol varied according to which day of the week the urine sample was collected on (based on the assumption that weekend consumption might be greater than weekday consumption), and that black and white drinkers differed in their distributions of this potential confounder. We found no difference in levels of free ethanol by day of collection ($P = 0.35$).

For ethanol conjugates in urine, we again observed a significant difference in mean levels between the three categories of drinkers irrespective of race ($P = 0.0001$). Overall, weekly drinkers had a mean level 1.9 times higher than that in nondrinkers, and the mean level in daily drinkers was 2.7-fold higher than that in weekly drinkers. There was little correlation between urinary level of ethanol conjugates and self-reported level of average consumption within either weekly ($r = 0.27$; $P = 0.11$) or daily drinkers ($r = -0.03$; $P > 0.5$). Race was a

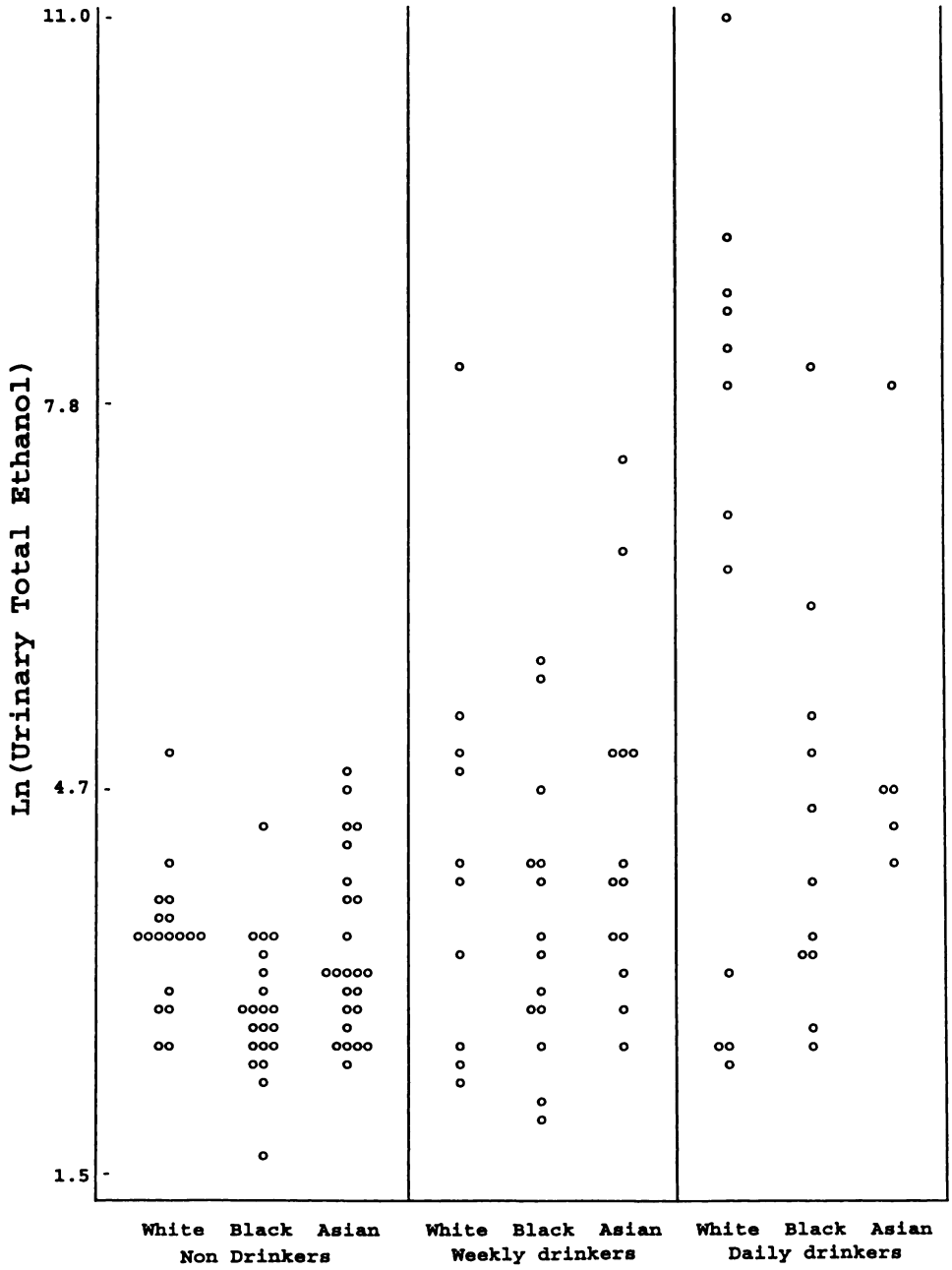

Fig. 3. Distribution of urinary total ethanol level (expressed in log units of nmol/mg of creatinine) among study subjects according to their race and alcohol consumption status.

significant factor in determining level of ethanol conjugates in urine ($P = 0.02$). Again, blacks had the lowest and whites had the highest mean level across all categories of drinking, although the differences were not as prominent as in free ethanol (Table 2). The distribution of urinary ethanol conjugate values among the 128 study subjects according to their race and drinking status is shown in Fig. 2. As in the case of urinary free ethanol, there was no relationship between level of urinary ethanol conjugates and the day of the week on which the sample was collected ($P = 0.45$).

Total (the sum of free and bound ethanol) ethanol in urine among the 128 study subjects was presented in Fig. 3. There was a close correlation between levels of free ethanol and ethanol conjugates in urine ($r = 0.88$; $P < 0.001$). Regardless of race, the mean levels of total ethanol were significantly different between non-, weekly, and daily drinkers (Table 2; $P = 0.0001$). The stepwise discriminant analysis method was used to compare the power of the three urinary biomarkers of alcohol exposure (free, bound, or total urinary ethanol) to discriminate between non-, weekly, and daily drinkers. Total

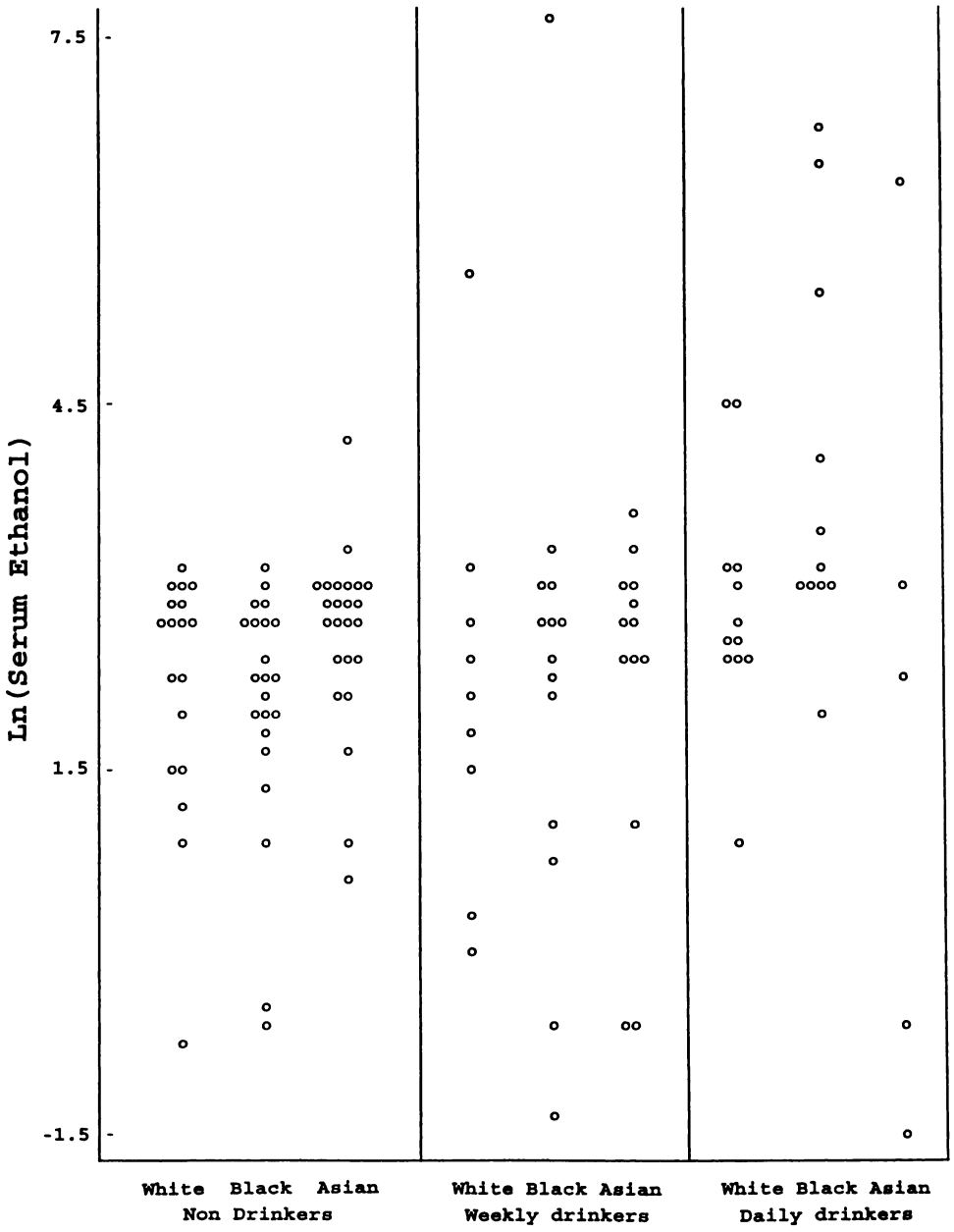

Fig. 4. Distribution of free ethanol level in serum (expressed in log units of μM) among study subjects according to their race and alcohol consumption status.

urinary ethanol was selected, and additional inclusion of either one of the remaining two biomarkers resulted in no additional improvement in discriminating among the three classes of drinkers.

Table 3 presents the geometric mean levels of serum ethanol (expressed in μM) by alcohol consumption status of study subjects. The distribution of this variable among all subjects according to their race and drinking status is shown in Fig. 4. Contrary to findings on urinary ethanol levels, there was no evidence of an increase in serum ethanol between weekly and nondrinkers. There was no relationship between alcohol drinking status and serum alcohol level in Asians ($P = 0.49$), although there were noticeable increases in mean serum ethanol levels between daily and nondrinkers among both white and black subjects (1.9- and 6.5-fold, respectively). Mean serum alcohol level was 50% higher in Asian nondrinkers relative to black and white nondrinkers, but the difference was not statistically significant ($P = 0.12$). Although the collections of blood and urine specimens from the 128 study subjects were no more than 24-h apart and considerably less for most subjects, there was no correlation between serum ethanol and either free, bound, or total urinary ethanol ($r = 0.01$, 0.08, and 0.05, respectively).

Table 3 Geometric mean levels of serum ethanol by alcohol consumption status of study subjects

	Whites	Blacks	Asians	Total
Serum ethanol (μM)				
Nondrinkers	9.7	8.5	14.7	10.8
Weekly drinkers	9.4	10.5	9.6	9.9
Daily drinkers	18.6	55.7	7.3	24.2

Discussion

Previously, Tang (9) showed significant differences in free and bound ethanol in urine between confirmed alcoholics and subjects with "socially acceptable" levels of alcohol intake. The present study extends those earlier findings and demonstrates that the urinary biomarkers can discriminate additionally between heavier *versus* lighter users of alcohol within the range of community norms. Across the three races studied, daily drinkers consistently showed significantly higher mean levels of urinary total (free plus bound) ethanol relative to weekly drinkers whose levels, in turn, were significantly higher than nondrinkers. However, the urinary biomarkers could not further predict intake level within the categories of weekly or daily drinkers.

This study also indicates that blood ethanol level at a single, random time point is not a valid indicator of general self-reported drinking habits. This marker, unlike the urinary ethanol values, showed no elevation in mean value among weekly as opposed to never- drinkers. It also failed to discriminate among daily, weekly, or nondrinkers among Asian subjects.

Interestingly, we observed large differences in levels of urinary ethanol across white, black, and Asian men who reported daily alcohol intake. Among the 28 daily drinkers in the study (12 whites, 11 blacks, and 5 Asians), intake levels were highest in blacks, whereas whites and Asians drank comparable amounts (mean was 57.5, 30.8, and 33.8 g/day, respectively). However, 8 of 12 (67%) of white daily drinkers had free ethanol levels above 400 nmol/mg creatinine compared to 1 of 11 (9%) blacks and 1 of 5 (20%) Asian daily drinkers (Fig. 1). In contrast, blacks had the lowest mean levels of free, bound, and total ethanol across all categories of drinking, and these differences could not be explained by average daily consumption differences within these categories (Table 2).

It has been shown that there exists racial variation in allelic distributions of the alcohol metabolizing enzymes, ADH, ALDH, and CYP2E1 (13). For example, an atypical form of ADH (ADH_2), which differs substantially from the usual form in its kinetic properties, is more prevalent among Asians than it is among whites and blacks (13). Also, unlike whites and blacks, about one-half of Japanese and Chinese possess an inactive form of ALDH ($ALDH_2$ isozyme). In fact, this racial difference in ALDH polymorphism has been postulated as one explanation for the well-documented low rate of alcoholism in Asians because individuals possessing the $ALDH_2$ genotype are more sensitive to acute responses to alcohol and, thereby, would be discouraged from excessive intake (13). Recently, RFLPs (*Pst*I and *Rsa*I restriction sites) have been identified in the transcription regulation region of CYP2E1 and have been shown to be associated with the level of gene expression (14). The allelic frequencies of both *Pst*I and *Rsa*I differ markedly between Japanese and American blacks and whites; 24–27% of Japanese possess the rare alleles compared to 2–5% of American blacks and whites (14). There is some suggestion from our data that the *in vivo* elimination rate of ethanol is higher in whites relative to blacks and Asians, possibly as a consequence of genetic variation in alcohol metabolism among the races. However, we caution that the number of subjects is relatively small in the present study, and our results require confirmation in larger studies.

Acknowledgments

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