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Genotoxicity of Hydrochlorothiazide in Cultured Human Lymphocytes. I. Evaluation of Chromosome Delay and Chromosome Breakage

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Hypertension is often treated with diuretics, like hydrochlorothiazide (HCTZ). Previous results on the in vitro genotoxicity of HCTZ are equivocal. In the present study, we have evaluated the genotoxicity of HCTZ in cultured human lymphocytes using the Cytokinesis Blocked Micronucleus (CBMN) assay. In addition, micronucleus (MN) induction was analyzed by Fluorescence In Situ Hybridization (FISH) with an α-satellite DNA centromeric probe to distinguish between clastogenic and aneugenic effects. Lymphocyte cultures from 32 healthy adults were exposed to 5 and 40 μg/ml HCTZ. Age, gender, and smoking were evaluated as factors affecting the MN analysis. We found that HCTZ increased MN frequencies. FISH analysis revealed that HCTZ exerts its genotoxicity more strongly at the 40 μg/ml concentration, and principally through chromosome delay (aneugenicity). Multiregression analysis of our results confirmed the known effect of age and gender on MN induction in human lymphocytes. Smoking was also a confounding factor for MN induction, especially for centromere-negative MN frequencies. Under the experimental conditions used, only age had a clear positive effect on the response of lymphocytes to HCTZ. These data indicate that HCTZ produces micronuclei in cultured human lymphocytes by a mechanism that involves chromosome delay and to a lesser extent through chromosome breakage. Environ. Mol. Mutagen. 47:169–178, 2006.

Key words: hydrochlorothiazide; micronuclei; FISH; chromosome delay

INTRODUCTION

Hypertension is the most common cardiovascular disease, affecting about 25% of the adult U.S. population. A specific cause is identified for only 10–15% of the cases of hypertension. Diuretics lower blood pressure by depleting the body of sodium and reducing blood volume. Diuretic thiazides, which are commonly used for the initial treatment of high blood pressure, act by inhibiting the reabsorption of NaCl in the distal tubule of the kidney [Beaumont et al., 1988]. The thiazide, hydrochlorothiazide (HCTZ), has been used as a diuretic and antihypertensive agent since 1957 [Reynolds, 1989], and is used to reduce edema associated with heart failure, and for special indications such as Ménière's disease [IARC, 1990] and nephrogenic diabetes insipidus [Kirchlechner et al., 1999].

Data on the in vitro genotoxicity of HCTZ are equivocal. The first published report indicated that HCTZ (Esidrex) induced a high frequency (96%) of non-disjunction in diploid strains of *Aspergillus nidulans* [Bignami et al., 1974]. In a study of the mutagenicity of amine drugs and their products, reaction of HCTZ with nitrite in acetic acid solution resulted in products that were mutagenic in one of four strains of *Salmonella typhimurium* (TA98), with or without metabolic activation [Andrews et al., 1984]. By itself, HCTZ did not induce reversion in *Escherichia coli* strain Hs30R argF(Am); however, irradiation of the strain with near-ultraviolet light in the presence of HCTZ gave positive results [Fujita, 1985]. HCTZ was positive in the mouse lymphoma L5178Y $Tk^{+/-}$ forward mutation assay in the absence of exogenous metabolic activation [Myhr et al., 1990], while it was reported as questionable in the Salmonella reversion assay [Mortelmans et al., 1986]. In a reevaluation of the Salmonella data, HCTZ was characterized as nonmutagenic [Zeiger, 1990]. HCTZ was reported to induce sister chromatid exchanges, but not chromosome aberrations, in CHO cells

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[Anderson et al., 1990]. Up to 500 μg/ml of HCTZ also did not increase the frequency of chromosome aberrations in Chinese hamster lung cells, both with and without metabolic activation, but polyploidy was observed 48 hr after treatment [Ishidate et al., 1988; EMEA, 1999].

In contrast to the occasional positive responses using in vitro assays, with or without metabolic activation, in vivo studies on the genotoxicity of HCTZ and its metabolites have been uniformly negative. HCTZ, administered either by injection or in the feed, did not induce sex-linked recessive lethal mutations in *Drosophila melanogaster* [Valencia et al., 1985]. HCTZ was also negative for micronucleus and sister chromatid exchange induction in Chinese hamsters, negative in the mouse spermatogonia assay, and negative in the mouse dominant lethal assay [EMEA, 1999]. In a recent review of the genotoxicity of marketed pharmaceuticals, HCTZ was characterized as negative for bacterial mutation, negative for in vitro and in vivo cytogenetics assays, and positive for in vitro sister chromatid exchange and for the mouse lymphoma mutation assay. In addition, HCTZ was judged as negative for rat carcinogenicity and equivocal for mouse carcinogenicity [Snyder and Green, 2001].

In the present study, we have further evaluated the in vitro genotoxicity of HCTZ using the Cytokinesis Blocked Micronucleus (CBMN) assay in cultured human lymphocytes. First described by Fenech and Morley [1985a], the CBMN assay has become established as a method for assessing genotoxic damage in human biomonitoring studies and in human cells in vitro. The assay employs cytochalasin B (Cyt-B) to inhibit cytokinesis, and micronuclei (MN) are scored in binucleated cells. MN are genetic material that has not been integrated into the daughter nuclei and they are produced during nuclear division either from acentric chromosome fragments or delayed whole chromosomes. The two mechanisms by which MN originate, chromosome breakage (resulting in clastogenicity) and chromosome delay (resulting in aneuploidy), can be discriminated using Fluorescence In Situ Hybridization (FISH) with centromeric probes [Norppa and Falck, 2003].

We evaluated HCTZ-induced binucleated micronucleated cell (BNMN) frequency in lymphocytes from 32 male and female donors of different ages. To assess the aneugenic and clastogenic activity of HCTZ, FISH experiments using a pancentromeric probe were carried out in a subset of cultures from 20 healthy donors. We also conducted experiments by treating the lymphocytes with HCTZ, both before and after phytohemagglutinin (PHA) stimulation, so that the effects of the different centromere topology in cycling and resting lymphocytes could be evaluated [Weimer et al., 1992; Solovei et al., 2004]. Since HCTZ does not undergo significant metabolism [EMEA, 1999], our experiments were conducted without exogenous metabolic activation.

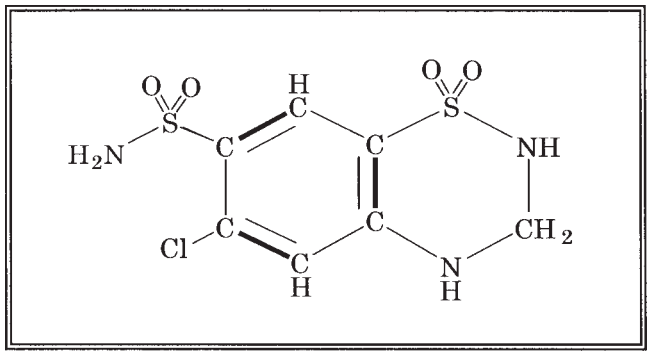

Fig. 1. Chemical structure of hydrochlorothiazide.

MATERIALS AND METHODS

Test Agent

Hydrochlorothiazide (HCTZ; 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1dioxide; commercially known as Aquarius, Apo-Hydro, Atenadon, Bremil, Cidrex, Dichlotride, Esidrex, Fluvin, Hydro-Diuril, Hypothiazide, Ivaugan, Oretic, Neoflumen, Ridaq, Thiuretic and others) also is found in numerous multi-ingredient preparations [IARC, 1990; Neutel et al., 1996]. The chemical structure of HCTZ is shown in Figure 1.

HCTZ was purchased from Sigma (H-4759; St. Louis, MO) and was dissolved in absolute ethanol and stored in the dark. The ethanol concentration in cultures was always less than 0.5%. For the definitive experiments the genotoxicity of HCTZ was evaluated at two final concentrations, 5 and 40 μg/ml.

Lymphocyte Culture and HCTZ Exposures

Donors

This study was approved by the Department of Biology of the University of Patras. After informed consent, 32 adults were recruited for this study. The subjects had no medications, X-ray exposure, or infection in the 6 months prior to the investigation. Donors were divided into groups according to their age and gender. The younger male donors were 23–24 years old (mean age 23.4 ± 0.18) and the younger females were 21–26 years old (mean age 23.6 ± 0.60). In the older age groups, males were 48–56 years old (mean age 52.4 ± 0.98) and females were 43–61 years old (mean age 51.9 ± 2.01). They also were divided in relation to their smoking habits. Less than 10 ml of blood were collected by venipuncture. None of the female subjects was pregnant at the time of blood collection. When necessary, a second blood sample was obtained from donors several weeks after the first collection.

Cycling (PHA-Stimulated) Cultures

Whole blood (0.5 ml) from each donor was added to 6.5 ml Hams nutrient mixture F-10 (Gibco-Invitrogen, Carlsbad, CA), 1.5 ml fetal calf serum (Gibco-Invitrogen), and 0.2 ml phytohemagglutinin (PHA; Gibco-Invitrogen). The culture medium was supplemented with penicillin and streptomycin (Gibco-Invitrogen) and L-glutamine (Sigma). Separate cultures were established from each donor for each experimental condition. The cultures were incubated at 37°C for 72 hr in a 5% CO_2 atmosphere with 95% humidity, with HCTZ exposure for the final 31 hr. After 41 hr of incubation, HCTZ was added into the culture medium at final concentrations of 5 and 40 μg/ml. After 44 hr of incubation, Cyt-B (Sigma) was added to the culture medium at a final concentration of 6 μg/ml. With this type of treatment schedule, the cells were exposed to HCTZ at G1/S/G2/M, in contrast to the experiments conducted with the resting

lymphocytes in which HCTZ treatment was performed in G0 phase. These are the typical protocols for testing MN induction by a chemical, as proposed by Fenech [2000].

Resting (Non-Stimulated) Cultures

Lymphocyte cultures were established as described for cycling lymphocytes, except that no PHA was added to the culture medium. At culture initiation, the cells were treated with HCTZ for a total of 6 hr. At the end of that time, the HCTZ was removed from the cultures by pelleting the lymphocytes and resuspending the cells in fresh medium containing PHA. Cyt-B was added into the culture medium 44-hr after PHA stimulation at the same concentration used for cycling cultures. The cells were harvested 78 hr after culture initiation.

Cytogenetic Procedures

Slide Preparation

Cells were harvested 72 hr after PHA stimulation. They were treated with a hypotonic solution of 1:1 Hams F-10:double-distilled H_2O for 2 min at 37°C. The cells were fixed immediately by treating them at least three times with a solution of 3:1 methanol:acetic acid (Merck, Darmstadt, Germany) at room temperature. Cells suspended in a small volume of the fixative were layered onto clean slides and stored at 4°C for in situ hybridization. To estimate MN frequency at least 1,000 binucleated cells were scored for each donor and for each treatment. MN frequency was determined for all donors.

FISH Analysis with Pancentromeric Probe

FISH analysis with the pancentromeric probe was performed using cell preparations from 20 of the adult donors from the cycling-lymphocyte experiments and from the six young donors used in the resting lymphocyte experiments. The slides were processed using the procedure of Bakou et al. [2002], with some modifications. Briefly, the slides were pretreated with a pepsin (Sigma) solution in 0.01 M HCl, (pH 3) for 5 min, dehydrated with a series of increasing concentrations of ethanol (Merck), and denatured in 70% formamide (Merck) at 70°C for 2 min. The slides were covered with a digoxigenin-conjugated α-satellite probe for all human centromeres (P5095:DG5; Oncor, Gaithersburg, MD) and hybridized overnight at 37°C in a humidified chamber. At the end of the hybridization, the slides were washed with 50% formamide in 2× SSC. Immunodetection of the probe was achieved using a monoclonal anti-digoxigenin antibody (D-8156; Sigma), anti-mouse Ig digoxigenin-conjugated secondary antibody (AQ 300D; Chemicon, Temecula, CA), and anti-digoxigenin-fluoroscein Fab fragments-FITC (1207741; Roche, Mannheim, Germany). Counterstaining was performed with a mixture of DAPI (4′,6-diamidino-2-phenylindole) and PI (propidium iodide) (Sigma), and slides were mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA).

Slide Scoring

The slides were stored in the dark at 4°C. Slides hybridized with the pancentromeric probe were analyzed with a Zeiss Axioskop epifluorescence microscope. The band pass filters used were of 546, 490, and 360 nm for green, blue, and ultraviolet light, respectively. At least 50 MN (56–110 for the young age group and 130–320 for the old age group) were analyzed for the presence (C^+MN) or the absence (C^-MN) of a centromere signal for each experimental point. C^+MN had the same staining intensity as the main nucleus. MN were characterized only in those cells whose nuclei contained clear pancentromeric signals. Standard criteria were used for the scoring of MN [Fenech, 2000; Kirsch-Volders et al., 2000]. BNMN frequency is the frequency of binucleated (BN) cells with MN in 1,000 BN cells and MN frequency is the frequency of MN in 1,000 BN cells. Two thousand cells were counted to estimate the Cytokinesis Block Proliferation Index (CBPI) [Surralles et al., 1995], which was calculated using the expression, $CBPI = M_1 + 2M_2 + 3(M_3 + M_4)/N$, where M_1, M_2, M_3, and M_4 correspond to the numbers of cells with one, two, three, and four nuclei, and N the total number of cells examined. The CBPI was used to estimate the cytotoxicity of the treatment, according to the formula proposed by the IWGT in vitro MN assay working group [Kirsch-Volders et al., 2003], ''Cytotoxicity $= 100 - 100[CBPI_T - 1/CBPI_C - 1]$''. Coded slides were analyzed by one microscopist. Cultures from young and old donors were established in parallel.

Statistical Analysis

Mean BNMN, MN, C^+MN, and C^-MN frequencies as well as CBPI, were analyzed by Students' t-test (SPSS 10.0; SPSS, Chicago, IL). One-way ANOVA was used to compare cell response to HCTZ between different groups using Tukeys' post hoc test. Multiple regression analysis was used to evaluate the influence of age, gender, smoking, and HCTZ treatment on MN frequencies and CBPI. The level of significance was taken as $P < 0.05$.

RESULTS

Cycling Lymphocytes

In a pilot experiment using the treatment protocol for cycling lymphocytes, lymphocytes from two young female donors were treated with 5, 10, 20, 40, and 60 μg/ml HCTZ; all HCTZ concentrations increased MN frequency (Appendix I). C^+MN frequency followed a linear-dose response ($P = 0.01$, $t_{reg} = 4.30$, $R^2 = 82.18\%$). HCTZ concentrations of 5 and 40 μg/ml were chosen as the low and high doses, respectively, used in subsequent analyses following the same experimental protocol.

Cytotoxicity and Micronucleus Frequency

At a concentration of 40 μg/ml, HCl slightly reduced the CBPI values for the 32 donor cultures (Table I). Calculating cytotoxicity as recommended by the IWGT in vitro MN assay working group [Kirsch-Volders et al., 2003] indicated that 40 μg/ml HCTZ reduced the overall CBPI by 12.05%, while the 5 μg/ml concentration reduced the CBPI by only 2.41%. There was no difference in cytotoxicity between the young and the old age group; 40 μg/ml HCTZ reduced the CBPI by 12.22% and 11.84% for the young and old groups, respectively, while 5 μg/ml reduced the CBPI by 3.33% and 2.63%.

The 5 μg/ml HCTZ concentration significantly increased the BNMN and MN frequency only for the young group [(7.37 ± 0.27)‰ to (8.69 ± 0.51)‰ and (7.67 ± 0.28)‰ to (9.08 ± 0.56)‰, respectively]. In contrast, the 40 μg/ml concentration increased the BNMN frequency for both the young and old groups [to (13.29 ± 0.53)‰ and (28.52 ± 2.41)‰, respectively] and the MN frequency in both the young and old groups [to (14.24 ± 0.56)‰ and (33.14 ± 2.77)‰, respectively]. Not stratifying the donors by age, HCTZ increased BNMN and MN frequencies only in cultures treated with 40 μg/ml [(12.58 ± 1.16‰) to (20.90 ± 1.83)‰ and (14.05 ± 1.41)‰ to (23.69 ± 2.19)‰, respectively].

TABLE I. Effect of Hydrochlorothiazide on Micronucleus Frequencies in Cultures of Cycling Human Lymphocytes

Donor	Age/sex/ smoking	Untreated			5 μg/ml Hydrochlorothiazide			40 μg/ml Hydrochlorothiazide		
		CBPI	BNMN (‰)	MN (‰)	CBPI	BNMN (‰)	MN (‰)	CBPI	BNMN (‰)	MN (‰)
V.A.	O/M/+	1.69	19.87	23.14	1.70	23.96	25.89	1.61	34.71	40.59
K.G.	O/M/+	1.76	19.41	22.50	1.72	23.97	26.85	1.66	33.68	39.60
S.N.	O/M/+	1.69	20.59	24.94	1.66	23.94	27.36	1.61	36.06	41.15
C.V.	O/M/−	1.71	12.60	14.03	1.68	13.71	14.60	1.60	18.09	21.71
G.C.	O/M/−	1.82	19.07	21.65	1.80	19.87	23.30	1.68	33.85	37.64
G.T.	O/M/−	1.64	9.72	11.21	1.62	11.09	12.14	1.59	13.47	16.47
I.G.	O/M/−	1.82	10.44	11.74	1.81	10.57	11.82	1.79	15.77	19.59
S.C.	O/M/−	1.68	9.58	9.58	1.66	10.87	12.23	1.61	14.80	17.50
Old male mean ± SE		1.73 ± 0.02	15.16 ± 1.77	17.35 ± 2.22	1.71 ± 0.02	17.25 ± 2.23	19.27 ± 2.54	1.64 ± 0.02	25.05 ± 3.63	29.28 ± 4.01
K.S.	O/F/+	1.78	27.96	31.73	1.79	29.71	36.59	1.69	44.79	52.90
L.M.	O/F/−	1.89	17.62	20.82	1.85	18.96	22.13	1.74	29.32	36.56
N.M.	O/F/+	1.68	14.90	15.73	1.68	14.72	15.64	1.60	20.87	23.37
P.E.	O/F/−	1.90	21.33	24.81	1.87	21.58	23.50	1.78	34.32	40.18
S.S.	O/F/−	1.76	19.73	21.98	1.75	19.56	21.70	1.68	29.35	32.14
S.G.	O/F/−	1.88	27.15	31.80	1.85	25.87	29.63	1.75	40.62	45.81
C.E.	O/F/−	1.74	16.84	19.96	1.72	18.59	19.92	1.66	23.05	24.97
C.A.	O/F/+	1.74	17.94	21.43	1.70	22.72	25.56	1.63	33.52	40.14
Old female mean ± SE		1.80 ± 0.03	20.43 ± 1.69	23.53 ± 2.00	1.78 ± 0.03	21.46 ± 1.65	24.33 ± 2.26	1.69 ± 0.02	31.98 ± 2.87	37.01 ± 3.55
Old mean ± SE		1.76 ± 0.02	17.80 ± 1.36	20.44 ± 1.65	1.74 ± 0.02	19.35 ± 1.44	21.80 ± 1.77	$1.67^{a,b,c,d}$ ± 0.02	$28.52^{a,b,c,d}$ ± 2.41	$33.14^{a,b,c,d}$ ± 2.77
A.C.	Y/M/+	2.03	7.96	7.96	2.01	9.19	9.73	1.94	14.85	15.38
A.P.	Y/M/−	2.04	7.16	7.83	1.98	7.48	8.23	1.92	11.93	13.87
A.A.	Y/M/−	1.93	7.41	7.70	1.88	9.84	10.15	1.80	15.51	17.24
K.G.	Y/M/+	1.97	9.02	9.71	1.94	9.81	10.55	1.86	15.52	17.46
S.E.	Y/M/−	1.99	8.32	8.84	1.95	9.35	9.90	1.90	14.24	14.74
V.A.	Y/M/+	1.86	4.86	5.21	1.85	4.73	4.73	1.73	10.05	11.10
P.N.	Y/M/−	1.78	6.68	7.13	1.77	5.36	5.36	1.70	9.89	11.72
T.M.	Y/M/−	1.75	6.32	6.32	1.72	6.49	6.49	1.69	11.08	11.38
Young male mean ± SE		1.92 ± 0.04	7.22 ± 0.46	7.59 ± 0.49	1.89 ± 0.04	7.78 ± 0.73	8.14 ± 0.82	1.82 ± 0.04	12.88 ± 0.85	14.11 ± 0.89
K.M.	Y/F/+	1.61	8.62	8.62	1.62	8.80	8.80	1.55	11.48	11.48
K.A.	Y/F/−	2.02	7.60	7.60	1.98	12.30	13.29	1.85	15.50	15.50
B.K.	Y/F/+	1.79	7.54	7.54	1.77	7.86	8.65	1.71	12.34	13.06
N.C.	Y/F/+	1.93	7.75	7.75	1.92	10.98	10.98	1.79	14.50	14.50
P.N.	Y/F/+	1.98	7.49	7.91	1.95	9.97	10.38	1.86	15.10	16.26
P.P.	Y/F/−	1.97	5.89	6.23	1.94	8.63	9.25	1.84	11.51	12.23
P.M.	Y/F/−	1.76	6.66	7.32	1.78	7.58	7.58	1.68	13.03	14.40
S.K.	Y/F/−	1.98	8.69	9.04	1.93	10.68	11.19	1.85	16.16	17.58
Young female mean ± SE		1.88 ± 0.05	7.53 ± 0.33	7.75 ± 0.30	1.86 ± 0.04	9.60 ± 0.58	10.01 ± 0.64	1.77 ± 0.04	13.70 ± 0.65	14.38 ± 0.73
Young mean ± SE		1.90 ± 0.03	7.37 ± 0.27	7.67 ± 0.28	1.87 ± 0.03	8.69^{a} ± 0.51	9.08^{a} ± 0.56	$1.79^{a,b,c}$ ± 0.03	$13.29^{a,b,c,d}$ ± 0.53	$14.24^{a,b,c,d}$ ± 0.56
Total mean ± SE		1.83 ± 0.02	12.58 ± 1.16	14.05 ± 1.41	1.81 ± 0.02	14.02 ± 1.22	15.44 ± 1.46	$1.73^{a,b}$ ± 0.02	$20.90^{a,b}$ ± 1.83	$23.69^{a,b}$ ± 2.19

BNMN, Binucleated Micronucleated cells; CBPI, Cytokinesis Blocked Proliferation Index; MN, Micronuclei. F, Female; M, Male; O, Old; Y, young; +, smoker; −, nonsmoker.

[a] $P < 0.05$ in comparison with untreated cultures (Students' t-test).

[b] $P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (Student's t-test).

[c] $P < 0.05$ in comparison with untreated cultures (one-way ANOVA).

[d] $P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (one-way ANOVA).

Analysis of Micronucleus Induction by FISH

MN originating from chromosome breakage were discriminated from those formed through chromosome delay by analyzing for the presence of centromeres in MN using FISH (Table II).

In lymphocytes from young donors, treatment with 5 μg/ml HCTZ increased both the C^+ MN frequency (MN with at least one hybridization signal; aneugenic effect) from 3.23 ± 0.23 to 4.80 ± 0.34 and the C^- MN frequency (MN with no hybridization signal; clastogenic effect) from 5.12 ± 0.22 to 6.12 ± 0.17. The elevation was proportionately greater for aneugenic activity (1.5-fold increase) than clastogenic activity (1.2-fold increase). The high HCTZ concentration produced enhanced frequencies of C^+ MN in cells from both the young and old groups (to 9.64 ± 0.44 and to 25.96 ± 2.20, respectively), as well as C^- MN in lymphocytes from both the young and old groups (to 7.06 ± 0.50 and to 13.68 ± 1.08, respectively). In addition, the C^+ and C^- MN frequencies in cultures treated with 40 μg/ml HCTZ were significantly higher than the respective frequencies in cultures treated with 5 μg/ml HCTZ. Subdividing C^+ MN frequencies on the basis of the number of hybridization signals, 1 or ≥2, MN with one signal were the dominate category for cells from both young and old donors and for both exposure concentrations. The frequency of MN containing ≥2 hybridization signals, however, was significantly greater in cultures treated with 40 μg/ml HCTZ than in untreated cultures or cultures treated with 5 μg/ml.

Effect of Age, Gender, Smoking, and HCTZ Treatment

Multifactorial regression analysis was applied to the results described earlier using age, gender, smoking, and HCTZ treatment as independent variables (Model A-Table III).

Age and HCTZ treatment had significant effects on CBPI, while all the variables, including smoking, affected MN frequency. Age and HCTZ treatment were the major factors affecting C^+ and C^- MN frequencies, while gender affected the C^+ MN frequency to a greater extent than C^- MN frequency. Smoking was a significant variable for the C^- MN frequency, but not the C^+MN frequency.

A second model (Model B-Table IV) was used to evaluate the influence of the earlier mentioned independent variables on the ability of HCTZ to induce MN. Since the baseline MN level forms a considerable part of the MN observed in the treated cultures, we analyzed separately baseline MN frequency and induced MN frequency by subtracting, for each donor, the baseline MN frequency from the MN frequency of the treated cultures. Using this model, age was the major confounding factor for baseline and induced MN frequency, including both the frequencies of MN generated by chromosome delay and chromosome breakage. Gender marginally affected the baseline and did not influence induced MN frequency. However, gender was a significant confounding factor when C^+ and C^- MN frequencies were considered separately. Smoking was not a confounding factor either for baseline or induced MN level. If more subjects had been included in the study, the effects of gender and smoking on HCTZ-induced MN may have been more apparent.

Resting (Non-Stimulated) Lymphocytes

In these experiments, lymphocyte cultures were treated with both the low and high HCTZ concentrations without PHA stimulation. We established cultures from six donors of the young age group since the experiments on cycling lymphocytes indicated that cells from young donors had a higher sensitivity to HCTZ MN induction.

HCTZ cytotoxicity was slightly higher in cultures treated with 40 μg/ml (8.25%) than in those treated with 5 μg/ml (3.09%) (Table V). BNMN and MN frequencies were significantly elevated by both concentrations of HCTZ, from (8.63 ± 0.35)‰ and (8.85 ± 0.41)‰ for the untreated controls to (10.24 ± 0.29)‰ and (10.65 ± 0.31)‰ for the low concentration, and to (14.01 ± 0.86)‰ and (14.96 ± 0.87)‰ for the high concentration. MN frequencies induced at 40 μg/ml were significantly higher than those observed at 5 μg/ml.

Similar to what was observed in dividing lymphocytes, 40 μg/ml HCTZ increased both C^+ MN and C^- MN frequencies [(3.54 ± 0.29)‰ and (5.22 ± 0.41)‰ to (8.25 ± 0.76)‰ and (7.85 ± 0.73)‰]. Most of C^+ MN involved only one hybridization signal. There were no significant differences between the C^+ MN and C^- MN frequencies in cycling lymphocyte cultures (Table II) and in resting lymphocytes (Table VI).

DISCUSSION

In this study, we found that HCTZ increased MN frequencies in cultured human lymphocytes. This is the first report on the cytogenetic effects of HCTZ in human lymphocytes. Positive responses were generated with 40 μg/ml HCTZ in cells from both young and old donors, while 5 μg/ml HCTZ produced significant increases in MN frequencies only with lymphocytes from the young age group. This age effect may be due to cell kinetics, which affects MN formation. Since MN are expressed only in cells that pass through cell (nuclear) division, the proliferation rate of cells may affect MN frequencies [Fenech and Morley, 1985b]. In the present study, the CBPI of cells from older donors was lower than the CBPI for the young donor lymphocytes (Table I). This phenomenon has been noted in other studies [e.g., Pastor et al., 2001] and may have influenced the MN response produced by the lower concentration of HCTZ.

FISH analysis indicated that the MN formed after HCTZ treatment were generated by two different mechanisms, chromosome delay and chromosome breakage. At the high HCTZ

TABLE II. Analysis of Micronuclei Induced by Hydrochlorothiazide in Cycling Human Lymphocyte Cultures Using Fluorescence In Situ Hybridization with an α-Satellite Pancentromeric Probe

		Untreated				5 μg/ml Hydrochlorothiazide				40 μg/ml Hydrochlorothiazide			
Donor	Age/sex/ smoking	C^+ MN (‰)	C^- MN (‰)	C^{1+} MN (‰)	$C^{\geq 2+}$ MN (‰)	C^+ MN (‰)	C^- MN (‰)	C^{1+} MN (‰)	$C^{\geq 2+}$ MN (‰)	C^+ MN (‰)	C^- MN (‰)	C^{1+} MN (‰)	$C^{\geq 2+}$ MN (‰)
V.A.	O/M/+	9.48	13.27	7.58	1.90	13.00	15.00	11.00	2.00	22.33	20.47	17.67	4.65
K.G.	O/M/+	12.78	10.05	9.13	3.65	16.00	11.00	12.00	4.00	23.59	13.61	16.33	7.26
S.N.	O/M/+	11.74	13.12	8.98	2.76	13.33	12.67	10.00	3.33	24.73	17.39	19.23	5.50
C.V.	O/M/−	6.46	8.31	6.46	0	7.43	8.36	7.43	0	12.77	13.75	10.81	1.96
G.C.	O/M/−	11.43	10.71	9.29	2.14	10.42	9.68	8.93	1.49	22.16	15.34	17.05	5.11
C.A.	O/F/+	12.06	7.39	8.95	3.11	17.00	10.00	13.00	4.00	29.00	10.00	20.00	9.00
P.E.	O/F/−	17.44	8.72	10.26	7.18	17.00	7.00	11.00	6.00	31.03	9.01	19.02	12.01
S.G.	O/F/−	21.14	8.06	12.90	8.24	20.00	8.00	14.00	6.00	34.00	11.00	21.00	13.00
L.M.	O/F/−	11.79	8.42	10.11	1.68	13.00	7.80	11.27	1.73	23.14	12.85	17.99	5.14
K.S.	O/F/+	20.80	9.24	13.10	7.70	23.70	13.33	14.81	8.89	36.88	13.41	22.63	14.25
Old Mean ± SE		13.51 ± 1.51	9.73 ± 0.65	9.68 ± 0.66	3.84 ± 0.90	15.09 ± 1.49	10.28 ± 0.84	11.34 ± 0.71	3.74 ± 0.84	25.96[a,b,c,d] ± 2.20	13.68[a,b,c,d] ± 1.08	18.17[a,b,c,d] ± 1.01	7.79[a,b,c,d] ± 1.30
A.C.	Y/M/+	2.67	5.33	2.67	0	3.33	6.67	3.33	0	8.00	8.00	6.00	2.00
A.P.	Y/M/−	2.12	4.95	2.12	0	4.67	5.33	4.67	0	8.00	7.00	7.00	1.00
A.A.	Y/M/−	3.00	6.00	3.00	0	4.00	7.00	4.00	0	11.00	10.00	9.00	2.00
K.G.	Y/M/+	3.91	5.48	3.13	0.78	4.31	5.92	3.50	0.81	10.83	7.00	8.28	2.55
S.E.	Y/M/−	3.45	5.52	3.45	0	4.00	6.00	4.00	0	8.67	6.67	6.67	2.00
K.A.	Y/F/−	2.67	4.67	2.67	0	7.00	6.50	5.50	1.50	10.00	6.00	7.00	3.00
N.C.	Y/F/+	2.76	4.83	2.76	0	5.00	6.00	4.50	0.50	8.00	5.00	7.00	1.00
P.N.	Y/F/+	4.00	5.00	4.00	0	5.00	6.00	5.00	0	10.00	7.00	9.00	1.00
P.P.	Y/F/−	3.20	3.60	2.80	0.40	4.67	5.33	4.00	0.67	10.00	5.00	8.00	2.00
S.K.	Y/F/−	4.54	5.79	3.72	0.83	6.07	6.43	5.00	1.07	11.91	8.94	10.21	1.70
Young Mean ± SE		3.23 ± 0.23	5.12 ± 0.22	3.03 ± 0.18	0.20 ± 0.11	4.80[a,c] ± 0.34	6.12[a] ± 0.17	4.35[a,c] ± 0.22	0.45 ± 0.17	9.64[a,b,c,d] ± 0.44	7.06[a,c] ± 0.50	7.82[a,b,c,d] ± 0.41	1.82[a,b,c,d] ± 0.21
Total Mean ± SE		8.37 ± 1.39	7.42 ± 0.63	6.35 ± 0.83	2.02 ± 0.61	9.95 ± 1.39	8.20 ± 0.64	7.85 ± 0.88	2.10 ± 0.56	17.80[a,b] ± 2.17	10.37[a] ± 0.96	12.99[a,b] ± 1.30	4.81[a,b] ± 0.94

C^+ MN, Micronuclei with hybridization signal; C^- MN, Micronuclei with no hybridization signal; C^{1+} MN, Micronuclei with 1 hybridization signal; $C^{\geq 2+}$ MN, Micronuclei with 2 or more hybridization signals; F, Female; M, Male; O, Old; Y, Young; +, smoker; −, nonsmoker.

[a] $P < 0.05$ in comparison with untreated cultures (Students' t-test).

[b] $P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (Students' t-test).

[c] $P < 0.05$ in comparison with untreated cultures (one-way ANOVA).

[d] $P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (one-way ANOVA).

TABLE III. Multiple Regression Analysis of CBPI and Micronuclei Data (Model A)

	CBPI (significance)	MN (significance)	C^+ MN (significance)	C^- MN (significance)
Age	<0.0001	<0.0001	<0.0001	<0.0001
Gender	0.461	0.008	0.0001	0.0002
Smoking	0.043	0.008	0.267	0.014
Hydrochlorothiazide treatment	<0.0001	<0.0001	<0.0001	<0.0001
Models	$R^2 = 0.405$ ($P < 0.0001$)	$R^2 = 0.675$ ($P < 0.0001$)	$R^2 = 0.786$ ($P < 0.0001$)	$R^2 = 0.738$ ($P < 0.0001$)

CBPI, Cytokinesis Blocked Proliferation Index; MN, Micronuclei; C^+ MN, Micronuclei exhibiting hybridization signal; C^- MN, Micronuclei not exhibiting hybridization signal.

TABLE IV. Multiple Regression Analysis of Micronuclei Data (Model B)

	Variables	BNMN (significance)	MN (significance)	C^+ MN (significance)	C^- MN (significance)
Baseline MN	Age	<0.0001	<0.0001	<0.0001	<0.0001
	Gender	0.048	0.065	0.028	0.011
	Smoking	0.175	0.199	0.843	0.194
	Models	$R^2 = 0.688$ ($P < 0.0001$)	$R^2 = 0.687$ ($P < 0.0001$)	$R^2 = 0.751$ (P <0.0001)	$R^2 = 0.799$ ($P < 0.0001$)
HCTZ-induced MN	Age	0.0003	<0.0001	<0.0001	0.005
	Gender	0.434	0.624	0.042	0.040
	Smoking	0.072	0.062	0.178	0.981
	Models	$R^2 = 0.366$ ($P = 0.001$)	$R^2 = 0.421$ ($P = 0.0003$)	$R^2 = 0.721$ ($P < 0.0001$)	$R^2 = 0.396$ ($P = 0.011$)

BNMN, Binucleated Micronucleated cells; MN, Micronuclei; C^+ MN, Micronuclei exhibiting hybridization signal; C^- MN, Micronuclei not exhibiting hybridization signal.

concentration, chromosome delay (aneuploidy) appeared to be the stronger effect since MN frequencies associated with a hybridization signal, and thus presumably containing a whole chromosome, were increased ~2-fold in the old-age group and about 3-fold in the young-age group. In contrast, MN exhibiting no hybridization signal, and presumably generated by chromosome breakage, were increased only by about 1.4-fold in both age groups. In addition, the high dose of HCTZ produced a significantly higher C^+ MN frequency than the lower concentration of HCTZ, while the C^- MN frequencies produced by the two doses of HCTZ were not significantly different. Thus, HCTZ appears to have a relatively strong aneugenic activity.

Other antihypertensive drugs have produced positive aneugenic responses in CBMN assays with human lymphocytes conducted in conjunction with FISH analysis. Télez et al. [2000] found that lymphocytes from patients treated with atenolol, a β-blocker antihypertensive drug, had a significantly increased frequency of MN containing whole chromosomes. This same research group [Télez et al., 2001] found that another antihypertensive drug, the calcium antagonist nimodipine, induced high frequencies of MN containing whole chromosomes when human lymphocytes were treated in vitro. C^+ MN frequencies were not elevated, however, in lymphocytes from patients treated with the drug in vivo, indicating that there may be metabolism of the drug in vivo to a less genotoxic derivative or that there may be an adaptive response to nimodipine in vivo.

Along with the assays conducted on cycling lymphocytes, we also evaluated the ability of HCTZ to induce MN when the treatments were conducted with resting lymphocyte cultures. Previous studies indicate that the centromere positioning in human cell interphase nuclei is related to the cell-cycle phase [Weimer et al., 1992; Solovei et al., 2004]. It also was suggested that the spatial organization of the centromeric region in the interphase nucleus might play a role in the cellular response to genotoxic agents [Renzi et al., 1996]. The two types of HCTZ genetic activity, chromosome delay and chromosome breakage, as measured by C^+ MN and C^- MN frequencies, were increased about 2.3-fold and 1.50-fold by the high HCTZ concentration, indicating that HCTZ seems to have a stronger aneugenic effect in agreement with the results from the cycling lymphocytes. Therefore, in CBMN assay it appears that the genotoxic activity of HCTZ is not affected by the schedule of treatment, before or after PHA stimulation, and probably not by the different centromere topology in cycling and resting lymphocytes.

Thus, we conclude that 40 μg/ml HCTZ is clearly genotoxic in human lymphocytes and this genotoxicity occurs principally through chromosome delay, with chromosome breakage occurring to a lesser extent. Chemicals with clastogenic properties preferentially produce MN containing acentric chromosome fragments, while those with aneugenic potential induce MN containing whole chromosomes. However, it has been noted previously that there are very few genotoxic agents that induce only C^+ MN or only C^- MN [Schuler et al., 1997]. Indeed, many clastogens [Vlachodimitropoulos et al., 1997; Andrianopoulos et al., 2000; Kouloumenta et al., 2005] and aneugens [Bakou et al., 2002] produce both types of MN. Myhr et al. [1990] reported that

TABLE V. Effect of Hydrochlorothiazide on Micronucleus Frequencies in Cultures of Resting Human Lymphocytes

		Untreated			5 μg/ml Hydrochlorothiazide			40 μg/ml Hydrochlorothiazide		
Donor	Age/sex/ smoking	CBPI	BNMN (‰)	MN (‰)	CBPI	BNMN (‰)	MN (‰)	CBPI	BNMN (‰)	MN (‰)
A.P.	Y/M/−	1.99	8.21	8.21	1.97	9.67	10.48	1.91	11.07	12.40
K.G.	Y/M/+	1.95	10.11	10.70	1.95	10.00	10.42	1.87	17.29	18.50
S.E.	Y/M/−	1.98	8.44	8.44	1.94	9.91	9.91	1.90	13.25	13.72
K.A.	Y/F/−	2.01	8.50	8.50	1.96	11.66	11.66	1.92	14.37	15.24
N.C.	Y/F/+	1.98	7.55	7.95	1.95	9.95	9.95	1.89	12.95	13.94
S.K.	Y/F/−	1.90	8.99	9.30	1.89	10.23	11.46	1.83	15.14	16.00
Mean ± SE		1.97 ± 0.02	8.63 ± 0.35	8.85 ± 0.41	1.94 ± 0.01	10.24[a] ± 0.29	10.65[a] ± 0.31	1.89[a,b,c,d] ± 0.01	14.01[a,b,c,d] ± 0.86	14.96[a,b,c,d] ± 0.87

BNMN, Binucleated Micronucleated cells; CBPI, Cytokinesis Blocked Proliferation Index; MN, Micronuclei; F, female; M, male; Y, young; +, smoker; −, non-smoker.
[a]$P < 0.05$ in comparison with untreated cultures (Students' t-test).
[b]$P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (Students' t-test).
[c]$P < 0.05$ in comparison with untreated cultures (one-way ANOVA).
[d]$P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (one-way ANOVA).

TABLE VI. Analysis of Micronuclei Induced by Hydrochlorothiazide in Resting Human Lymphocytes Cultures Using Fluorescence In Situ Hybridization with an α-Satellite Pancentromeric Probe

		Untreated				5 μg/ml Hydrochlorothiazide				40 μg/ml Hydrochlorothiazide			
Donor	Age/sex/ smoking	C^+ MN (‰)	C^- MN (‰)	C^{1+} MN (‰)	$C^{\geq 2+}$ MN (‰)	C^+ MN (‰)	C^- MN (‰)	C^{1+} MN (‰)	$C^{\geq 2+}$ MN (‰)	C^+ MN (‰)	C^- MN (‰)	C^{1+} MN (‰)	$C^{\geq 2+}$ MN (‰)
A.P.	Y/M/−	2.38	5.56	2.38	0	3.52	6.34	3.52	0	5.80	6.63	4.97	0.83
K.G.	Y/M/+	4.02	6.90	3.45	0.57	4.53	6.04	3.77	0.76	10.00	11.00	8.00	2.00
S.E.	Y/M/−	3.23	4.84	3.23	0	3.93	5.90	3.93	0	7.00	7.00	5.00	2.00
K.A.	Y/F/−	4.00	4.00	4.00	0	4.96	6.62	4.13	0.83	9.73	6.49	7.30	2.43
N.C.	Y/F/+	3.30	4.62	2.64	0.66	3.61	5.42	3.61	0	7.00	7.00	6.00	1.00
S.K.	Y/F/−	4.32	5.41	3.78	0.54	6.09	6.09	5.22	0.87	10.00	9.00	9.00	1.00
Mean ± SE		3.54 ± 0.29	5.22 ± 0.41	3.25 ± 0.26	0.29 ± 0.13	4.44 ± 0.40	6.07 ± 0.17	4.03 ± 0.25	0.41 ± 0.18	8.25[a,b,c,d] ± 0.76	7.85[a,b,c,] ± 0.73	6.71[a,b,c,d] ± 0.68	1.54[a,b,c,d] ± 0.28

C^+MN, Micronuclei with hybridization signal; C^-MN, Micronuclei with no hybridization signal; C^{1+} MN, Micronuclei with 1 hybridization signal; $C^{\geq 2+}$ MN, Micronuclei with 2 or more hybridization signals; F, female; M, male; Y, young; +, smoker; −, non-smoker.
[a]$P < 0.05$ in comparison with untreated cultures (Students' t test).
[b]$P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (Students' t test).
[c]$P < 0.05$ in comparison with untreated cultures (one-way ANOVA).
[d]$P < 0.05$ in comparison with 5 μg/ml Hydrochlorothiazide treated cultures (one-way ANOVA).

HCTZ induced forward mutations at the heterozygous *Tk* locus in L5178Y mouse lymphoma cells. Such mutations may occur by more than one mechanism, including somatic recombination, chromosome loss, and chromosome missegregation, or through the action of agents on alternate sites in the cell, such as microtubules [Henry et al., 1998]. Our findings indicating that HCTZ affects chromosome segregation by causing chromosome delay are consistent with the first report on the genotoxicity of HCTZ, which showed that the drug caused mitotic recombination and chromosome non-disjunction in *Aspergillus nidulans* [Bignami et al., 1974]. Experiments that are now in progress in our laboratory (data not yet published) indicate that HCTZ causes chromosome non-disjunction in human lymphocytes cultured from the same donors involved in the present study and that HCTZ also affects microtubule organization in a mouse cell line.

The multiregression analysis of our results confirmed the known effect of age and gender on MN induction in human lymphocytes [Fenech and Morley, 1985b; Migliore et al., 1991; Fenech et al., 1994]. In the Human Micronucleus Project [Fenech et al., 1999; Bonassi et al., 2001], in which the effect of age was evaluated in subjects of 40 years of age and over vs. younger subjects, an age effect was evident in all but two of the databases examined. The Human Micronucleus Project also found that gender was one of the most important variables affecting MN frequency, with frequencies in females being greater than those in males [Fenech et al., 1999; Bonassi et al., 2001]. In our study, Students' t-test analysis indicated that older females had higher C^+ MN frequencies than males for all HCTZ treatment conditions ($P = 0.03$ for the control, $P = 0.03$ for the low HCTZ concentration, and $P = 0.02$ for the high HCTZ concentration). This difference may be due to the overrepresentation of the X chromosome in MN in older females [Hando et al., 1994; Zijno et al., 1996; Catalan et al., 1998; Bakou et al., 2002]. Smoking was another confounding factor for MN frequency in our study, and especially for C^- MN frequency. The Human Micronucleus Project found that smokers exhibit an increase in overall MN frequency, although when the interaction with occupational exposure was taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the MN assay in lymphocytes [Bonassi et al., 2003]. In addition, age and smoking seem to affect CBPI. Finally, using the present experimental design and with the number of donors included in this study, among the three variables that we evaluated (age, gender, and smoking), only age clearly affected the response of lymphocytes to HCTZ.

In conclusion, the results of our study can be summarized as follows:

- HCTZ treatment induces MN in cultured human lymphocytes
- FISH analysis indicated that 40 μg/ml HCTZ induces MN principally through chromosome delay, with a lesser frequency of MN induced through chromosome breakage
- Multiregression analysis of our results confirmed the known effect of age and gender on MN induction in human lymphocytes. Smoking was another confounding factor for MN frequency and especially for MN generated through chromosome breakage
- Under the experimental conditions used, age clearly affected the response of lymphocytes to HCTZ in a positive way.

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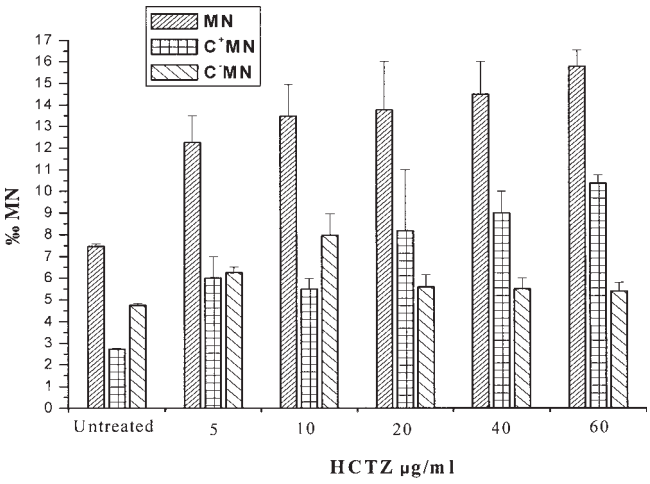
APPENDIX: PILOT EXPERIMENT DATA



MN, Micronuclei; C^+ MN, Micronuclei exhibiting hybridization signal; C^- MN, Micronuclei not exhibiting hybridization signal.