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Chemico-Biological Interactions 153–154 (2005) 111–115
Chemico-Biological Interactions
www.elsevier.com/locate/chembioint

Lymphocyte toxicity and T cell receptor excision circles in workers exposed to benzene

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Available online 19 April 2005

Abstract

We have previously reported that benzene decreases peripheral white blood cell and platelet counts and specifically lowers subsets of several blood cell types, including $CD4^+$-T cells, B cells, NK cells, and granulocytes. Diminished thymus function has been implicated as a mechanism for $CD4^+$-T cell loss in other conditions such as AIDS by assays of T cell receptor excision circles (TRECs), a marker of naïve T cells that have recently emigrated from the thymus. To evaluate alteration of thymic function as a mechanism for benzene's effects on $CD4^+$-T cell counts, we measured total TREC levels in 45 benzene-exposed workers and 45 unexposed controls. There was no significant difference in TREC levels per 10^6 peripheral blood leukocytes in the benzene-exposed workers compared to the controls. Although our study does not rule out counterbalancing alterations of TREC levels in specific T cell subsets, benzene's lymphotoxicity does not appear to be mediated through diminished thymus function.
Published by Elsevier Ireland Ltd.

Keywords: T cell receptor excision circle (TREC); Thymic function; Benzene exposure; Occupational hazard; T cell subsets; Hematotoxicity; China; Organic vapor; Peripheral blood leukocytes

1. Introduction

Benzene is an established cause of leukemia, aplastic anemia and myelodysplastic syndromes, and may cause non-Hodgkin lymphoma [1–4], but its mecha-

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0009-2797/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.cbi.2005.03.015

nism of toxicity is uncertain. We previously reported that granulocytes, total lymphocytes, B cells, NK cells, and platelets decreased with increasing benzene exposure [5]. Among lymphocyte subsets, $CD4^+$-T cell counts decreased with increasing benzene exposure, whereas $CD8^+$-T cell counts were unaffected [5]. Because T cells derived from the bone marrow undergo a process of maturation in the thymus gland, we hypothesized that the decline in $CD4^+$-T cells in the benzene-exposed group could be partially due to effects of this chemical on thymic function.

Recently, it has been proposed that thymic function can be measured by T cell receptor (TCR) excision circles (TRECs) [6]. During the process of TCR rearrangement, episomal DNA circles are excised during the recombinational process. These fragments, termed TRECs, have been used as a quantitative marker of recent thymic emigrants, because they do not replicate and are gradually diluted out by T cell expansion in the periphery. The frequency of TREC in the peripheral blood gradually declines with age, despite relatively constant adult T cell numbers, consistent with the gradual age-dependent decline in thymic function. Patients infected with human immunodeficiency virus (HIV) have lower TREC levels, suggesting their decreased levels of $CD4^+$-T cells are due to a reduced thymic output [7]. To test the hypothesis that benzene exposure reduces thymic output, we measured TREC levels in peripheral blood leukocytes (PBL) from benzene-exposed workers and non-exposed controls.

2. Methods

We carried out a cross-sectional study of 250 benzene-exposed workers in two shoe-manufacturing factories, and 140 unexposed controls from three clothes-manufacturing factories in the same region of Tianjin, China [5,8]. In brief, controls were frequency matched by sex and age to exposed workers. The study was approved by Institutional Review Boards at the US National Cancer Institute and the Chinese Academy of Preventive Medicine. A written informed consent was obtained from each subject. Blood samples were collected from 88 workers in June 2000 and the remaining workers in May and June 2001, and 28 subjects were studied in both years [5].

Individual benzene and toluene exposure was monitored by wearing an organic vapor passive monitor badge as previously described [5,8]. Personal full shift air monitoring was measured every 1–2 months over a 16-month period in the shoe manufacture factory with lower benzene exposure, five times in the shoe manufacture factory that had higher benzene exposures, and three times in control factories. No benzene was detected in the control factories. Post-shift urine samples were collected during the week before phlebotomy and analyzed for benzene by gas chromatography–mass spectrometry.

Subjects were given a detailed questionnaire requesting information on lifetime occupational and environmental exposure history, medical history, and tobacco smoking status. A brief physical exam was carried out. Subjects provided a peripheral blood sample. Blood samples were delivered to the lab within 6 h after collection. The CBC and differential were analyzed by a Beckman-Coulter® T540 blood counter and the major lymphocyte subsets were analyzed by a Becton Dickinson FACSCalibur™ flow cytometer (software: SimulSET v3.1).

We measured TREC in 45 exposed workers selected for relatively low ($n = 25$) or high ($n = 24$) levels of benzene exposure and 45 age and sex-matched unexposed controls. Within each stratum of benzene exposure, subjects were selected at random for inclusion in this analysis. Genomic DNA was extracted from buffy coat samples and used for real-time quantitative PCR using the 5′-nuclease (Taqman) assay on an ABI7700 system (Perkin-Elmer, Norwalk, CT), according to the protocol of Douek et al. [9] with some modifications. The primers 5′-CACATCCCTTTCAACCATGCT and 5′-GCCAGCTGCAGGGTTTAGTG were used with the probe FAM-5′-ACACCTCTGGTTTTTG-TAAAGGTGCCCACT-TAMRA (BioSource International Camarillo, CA). PCR reactions contained 0.5 μg PBL DNA, Platinum Quantitative PCR Mix-UDG (InVitrogen, Carlsbad, CA), 500 nM of each primer, 150 nM probe and Blue-636 reference dye (MegaBases, Evanston, IL). Conditions were one cycle at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. A standard curve was plotted [9] to determine TREC copies/10^6 cells. Samples from each subject were analyzed blindly in duplicate and the results averaged (intraclass correlation coefficient = 0.93). Samples from the study

subjects were tested in parallel wells for TREC and an endogenous gene control RNase P, a single-copy gene encoding the RNA moiety for the RNase P enzyme. RNase P was quantitated using the TaqMan® RNase P Detection Reagents Kit (Applied Biosystems, Foster City, CA). TRECs per 10^6 PBLs were calculated as two times the TREC frequency per 10^6 RNase P copies. Alternative analyses utilized TRECs/ml of blood, calculated as TRECs/10^6 PBLs times PBL count (in millions)/ml.

2.1. Statistical analysis

Initial data analysis was based on benzene exposures measured in the month prior to phlebotomy. Linear regression was used to test for differences between exposed subjects and controls and for trends in log-transformed hematologic outcomes and TRECs per 10^6 PBLs, adjusting for age, sex, current smoking status, current alcohol drinking, body mass index (BMI), and recent infections [5]. To take into account the likely correlation of repeated measurements, generalized estimating equations (GEE) analysis was used to adjust for the repeated measurements from a given subject [10]. Adjustment for potential confounders produced similar results to the presented results (data not shown). The selection of confounding factors was based on current literature concerning important hematotoxicity variables, our previous report on this population [5], and standard statistical procedures for variable selection.

3. Results

Age, sex, tobacco smoking, alcohol use, and frequency of recent infections were comparable for exposed subjects and controls (Table 1). The number of TRECs per 10^6 PBLs decreased significantly with increasing age (Fig. 1). However, TREC frequency per 10^6 PBLs (or per ml blood) was not significantly different between the benzene-exposed subjects compared with controls (Table 2). Furthermore, TREC frequency per 10^6 PBLs (or per ml blood) did not appreciably differ for workers with less than 10 ppm compared to those with heavier benzene exposure (data not shown). In addition, TREC levels were not significantly associated with benzene air levels in analyses for linear trend.

Table 1
Demographic characteristics and peripheral white blood cell counts of study subjects

	Controls ($n = 45$); N (%)	Exposed ($n = 45$); N (%)
Sex		
Male	14 (31)	13 (29)
Female	31 (69)	32 (71)
Current alcohol use		
Yes	14 (31)	13 (29)
No	31 (69)	32 (71)
Current smoking		
Yes	15 (33)	9 (20)
No	30 (67)	36 (80)
Recent infection		
Yes	3 (7)	5 (11)
No	42 (93)	40 (89)
Mean ± S.D.[a]		
Age	32.8 ± 8.0	34.1 ± 9.2
BMI	22.4 ± 2.8	22.7 ± 3.0

There are up to 94 observations on 90 unique subjects (45 controls and 45 benzene-exposed workers). For four subjects studied in both the first (2000) and second (2001) year of the study, data presented is from information collected in 2000.
[a] S.D.: standard deviation.

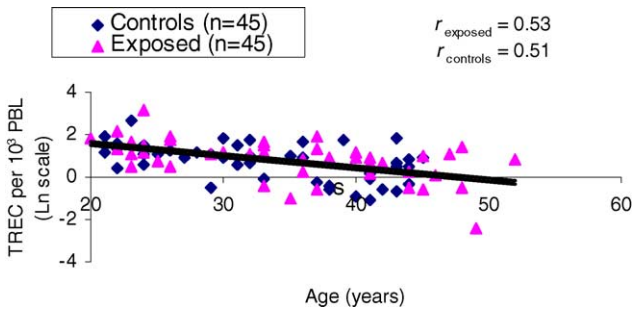

Fig. 1. Relation between TREC values (Ln 10 scale) and age.

Table 2
Benzene exposure, TREC concentration, and hematologic parameters for benzene exposed subjects and controls

	Controls ($n = 45$)	Exposed ($n = 49$)	P for exposed vs. controls[a]
Benzene air level (ppm)[b]	–	15.8 (17.9)	
Benzene urine (μg/l)[c]	0.3 (0.5)	452.6 (961.7)	
TREC/10^3 PBLs[d]	2.9 (2.5)	3.4 (3.6)	0.26
TREC/μl blood	6.3 (6.5)	6.7 (9.8)	0.64
WBC	6380 (1490)	5210 (1470)	<0.0001
Granulocytes	4060 (1330)	3140 (1160)	<0.0001
B cells	210 (80)	160 (90)	0.0006
CD3 T cells	1300 (400)	1240 (360)	0.46
Lymphocytes	2070 (580)	1860 (480)	0.054
$CD4^+$-T cells	730 (260)	600 (200)	0.006
$CD8^+$-T cells	530 (190)	570 (220)	0.31
$CD4^+/CD8^+$ ratio	1.48 (0.56)	1.11 (0.32)	0.0002
NK cells	570 (300)	460 (190)	0.068
Platelets	229,000 (58,000)	179,000 (44,000)	<0.0001

Values represent means (standard deviations). Data for 4 of the 49 exposed subjects were obtained from in both years (2000 and 2001) and are treated as independent observations. Results for peripheral white blood cell counts and blood cell subsets were previously reported [5].

[a] Adjusted for age, sex, BMI, alcohol drinking, and current smoking.

[b] Average of two measurements per subject collected during the month prior to phlebotomy.

[c] Urine data from 44 controls and 48 exposed subjects.

[d] PBL: peripheral blood leukocyte.

There was no effect of benzene air level on TREC levels in multivariable linear regression models adjusted for $CD4^+$ or $CD8^+$-T cell counts or $CD4^+/CD8^+$ ratio. As previously reported, total white blood cell, granulocyte, B cell, $CD4^+$-T cell, and platelet counts were significantly decreased in workers exposed to benzene compared to the controls (Table 2) [5].

4. Discussion

TRECs are episomal DNA generated during TCR rearrangement. As this circular DNA sequence does not replicate, it is primarily found in naïve T cells that have recently emigrated from the thymus. The TREC frequency in peripheral blood represents a balance of thymic output and peripheral blood cell division. Increased levels of TREC may reflect increases in thymic T cell production, whereas decreases in TREC frequency may represent declines in thymic output. In the present study, TRECs per 10^6 PBLs were inversely associated with age, consistent with previous reports [6,11]. Peripheral expansion of activated T cells, such as in response to infection or inflammation, can reduce TREC frequency (as the total number of T cells increases), but would not diminish total TREC/ml of blood.

Despite benzene's apparent toxicity to $CD4^+$-T cells, we did not detect an effect of benzene exposure on either the frequency of TREC/10^6 cells or the total level of TREC/ml of blood. The unaltered TREC levels may indicate that benzene affects neither thymic output nor T cell division in the periphery, although we cannot rule out counterbalancing increases or decreases in both of these parameters. This evidence, taken together with the wide range of other cell types decreased by benzene exposure, including B cells, NK cells, granulocytes and platelets, suggests that benzene's hematotoxicity (including lymphocyte toxicity) may be due to early events in the bone marrow during the development of all major blood cell lines.

Han et al. recently reported that the median value of TRECs within peripheral blood mononuclear cells was significantly higher in normal individuals than in benzene-exposed workers [12]. However, the results from that study were not adjusted for age, which is an important potential confounder. No information on the age distribution was available for either exposed workers or controls making their findings difficult to interpret.

A limitation of our study, however, is that a limited quantity of buffy coat DNA was available from each subject, because the amount of blood collected did not yield enough cells to separate T cell subsets. As a conse-

quence, the TREC levels we report are from $CD4^+$- and $CD8^+$-T cells combined. If TRECs decreased in one of these lymphocyte subsets and increased to an equivalent extent in the other, we would have not detected any change in our study. Nevertheless, adjustment for individual levels of $CD4^+$-T cells, $CD8^+$-T cells, or the $CD4^+/CD8^+$ ratio did not affect the results.

In summary, we previously reported that $CD4^+$-T cell counts decreased with benzene exposure in our cross-sectional study of healthy workers exposed to benzene and unexposed controls. However, in the present study, we found that total TREC levels were unassociated with benzene exposure. Assessment of TREC is needed in sorted $CD4^+$- and $CD8^+$-T cell fractions from subjects exposed to benzene to further explore the thymus as a potential locus of benzene-associated lymphotoxicity. Although, we could not rule out counterbalancing alterations of TREC levels in specific T cell subsets, our study suggesting that benzene's lymphotoxicity occurs prior to thymic maturation.

Acknowledgements

Supported by NIH grants RO1ES06721, P42ES-04705 and P30ES01896 (to MTS), P42ES05948, and P30ES10126 (to SMR). We thank the participants for taking part in this study.

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