Hemoglobin adducts, urinary metabolites and health effects in 2,4,6-trinitrotoluene exposed workers

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2,4,6-Trinitrotoluene (TNT) is an important occupational and environmental pollutant. In TNT exposed humans, the notable toxic manifestations have included aplastic anemia, toxic hepatitis, cataract, hepatomegaly and liver cancer. Therefore, we developed methods to biomonitor workers exposed to TNT. The workers were employed in a typical ammunition factory in China. The controls were recruited from the same factory. We determined hemoglobin (Hb) adducts and urine metabolites of TNT, Hb-adducts of TNT, 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrotoluene (2ADNT), and the urine metabolites of TNT, 4ADNT and 2ADNT were found in all the workers and in a few controls. 4ADNT was the main product. Although the levels of 2ADNT correlated well with 4ADNT, 2ADNT was not found in all the samples. Therefore, 4ADNT was the best marker of exposure for Hb-adducts and urine metabolites. The levels of the urine metabolites and Hb-adducts were related to the health status of the workers. The Hb-adduct 4ADNT was statistically significantly associated with risk of hepatomegaly, splenomegaly and cataract. The odds ratio (OR) for cataract, splenomegaly and hepatomegaly were 6.4 [95% confidence interval (CI) = 1.4-29.6], 9.6 (1.1-85.3) and 7.6 (1.3-43.7), respectively. No correlation was found between urine metabolites and health effects. These results were tested for confounding factors such as age, worker years, smoker status, smoke years, cigarettes per day and hepatitis B status using stepwise forward logistic regression analysis. In the case of splenomegaly, hepatitis B status is a confounder. In the case of cataract, age is a confounder. The Hb-adduct, 4ADNT, is a good biomarker of exposure and biomarker of biological effect.

Abbreviations: 2ADNT, 2-amino-4,6-dinitrotoluene; 4ADNT, 4-amino-2,6-dinitrotoluene; 4ABP, 4-aminobiphenyl; ECG, electrocardiogram; EI-MS, electron ionization-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; Hb, hemoglobin; NCI, negative chemical ionization; PFPA, pentafluoropropionic anhydride; SGPT, serum glutamicpyruvic transaminase; TNT, 2,4,6-trinitrotoluene.

Introduction

2,4,6-Trinitrotoluene (TNT) is an important environmental and occupational pollutant (1-6). In TNT exposed humans the notable toxic manifestations have included; aplastic anemia, toxic hepatitis, cataracts, and hepatomegaly. Similar conditions have been observed in animals. In China, chronic occupational exposure to TNT caused mainly hepatomegaly and cataract. The incidence of hepatomegaly was 41% and that of cataract was 79% in the TNT workers with prolonged exposure, whereas anemia was found rarely (7). A recent retrospective study, on male workers exposed to TNT over 1 year from eight Chinese military factories, from 1970 to 1995 demonstrated a higher relative risk for malignant tumors, especially liver cancer (8). The morbidity of total malignant tumor in male TNT exposed workers was markedly higher than that of controls, and the relative risk was 2.3. Liver cancer morbidity was 31.9% of the total malignant tumor, and its mortality was 3.97 times of the controls. A preliminary study of a German population living near the sites of two World War II munitions plants, indicated an association between increased rates of some types of leukemia and living in a town near TNT waste from these plants (9). The study showed increased relative risk of acute myelogenous leukemia for adult males and females living near the former explosives plants when compared with adults in a neighboring county. The relative risk was particularly high for individuals >65 years of age. However, the study case numbers were very small.

In mammalian systems, the principal metabolites of TNT are 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT); smaller amounts of other metabolites are formed (4,10,11). In humans mainly 2ADNT and 4ADNT were found in urine (4,12-14). Two studies showed that the urine metabolite levels were too high compared with expected levels calculated from the air measurements (12,13). It has been deduced from these data, that absorption through the skin must be the main pathway of exposure. Therefore, biological monitoring of workers is preferable as the method of assessing exposure rather than environmental monitoring. The metabolites of urine usually indicate only recent exposures up to 48 h post exposure (15). Hemoglobin (Hb) adducts are an indicator of exposure over the last 4 months, assuming that the adduct is stable and that the lifetime of the erythrocytes is not affected. The mechanism of adduct formation between aromatic amines or nitroarenes with Hb involves the reaction of the metabolite—nitrosoarene—with cysteine residues to form a sulfonic acid amide (16-20). Evidence for such reaction in vitro has been obtained by Ringe et al. (18). Sulfonic acid amide adducts are readily hydrolyzed under mild conditions, yielding the parent amine. Hb adducts are dosimeters for the internal and possibly of the target dose leading to toxic effects. Covalent Hb, plasma protein and protein adducts in various tissues were found in rats dosed with radiolabeled TNT.
and d5-4ADNT were synthesized in our laboratory as described below. At 90°C, the reaction mixture was cooled to room temperature. Cold D2O was added and extracted with benzene (2 × 3 ml). The collected organic phases were washed with 2 ml of saturated NaHCO3 and then with saturated NaCl solution (2 × 3 ml). The organic phase was dried through a pasteur pipet filled with anhydrous Na2SO4 and evaporated under reduced pressure. Slightly yellow crystals of d5-TNT were obtained (170 mg, 77% yield). Electron ionization-mass spectrometry (EI-MS) (70 eV), m/z (%): 215 (9), 214 (100), 213 (24), 196 (15), 184 (11), 168 (11), 152 (13), 138 (14), 94 (48), 93 (17), 92 (14), 66 (46), 64 (12), 54 (20). Negative chemical ionization-mass spectrometry (NCI-MS): 232 (100), 231 (8), 230 (4), 214 (19), 202 (28).

Synthesis of d2-TNT. To 24, d4-dinitrotoluene (150 mg) (31), 96% D2SO4 (900 µl) and 100% HNO3 (500 µl) were added. After 1 h at 80°C and 2.5 h at 90°C, the reaction mixture was cooled to room temperature. Cold D2O was added and extracted with benzene (2 × 3 ml). The collected organic phases were washed with 2 ml of saturated NaHCO3 and then with saturated NaCl solution (2 × 3 ml). The organic phase was dried through a pasteur pipet filled with anhydrous Na2SO4 and evaporated under reduced pressure. Slightly yellow crystals of d5-TNT were obtained (170 mg, 77% yield). Electron ionization-mass spectrometry (EI-MS) (70 eV), m/z (%): 215 (9), 214 (100), 213 (24), 196 (15), 184 (11), 168 (11), 152 (13), 138 (14), 94 (48), 93 (17), 92 (14), 66 (46), 64 (12), 54 (20). Negative chemical ionization-mass spectrometry (NCI-MS): 232 (100), 231 (8), 230 (4), 214 (19), 202 (28).

Methods

Chemicals and reagents

2ADNT and 4ADNT were obtained from Promochem (Wesel, Germany). Dichloromethane (Pestanal) was obtained from Fluka (Buchs, Switzerland). pentafluorpropionionic anhydride (PFPA) was obtained from Supelco (Taufkirchen, Germany). diethylether (stabilized with EtOH), sodium hydroxide (No. 6498) and water (No. 15333) from Merck (Darmstadt, Germany). d3-4-Aminobiphenyl (d5-4ABP) (30), 2,4-d6-dinitrotoluene (31), d5-2ADNT and d4-4ADNT were synthesized in our laboratory as described below. 

For the present study, blood samples, work place description, air measurements and medical examinations were collected from Chinese workers and non-matching controls. The external dose, the urine metabolites and Hb-adducts were determined and compared with the health effects.

Isolation of Hb from blood and determination of Hb adducts

EDTA blood (1-2 ml) was centrifuged for 5 min at 2000 g. After removal of plasma, red blood cells were washed three times in equal volumes of 0.9% NaCl solution and lysed by the addition of 4 vols of water. The cell debris were removed by centrifugation. Hb was precipitated with ethanol from the lysed erythrocytes. The precipitate was washed with ethanol-water (8:2), ethanol, ethanol-diethyl ether (3:1) and diethyl ether. The dried Hb (50 mg) was dissolved in 0.1 M NaOH (3 ml) in new screw top glass tubes (16 mm × 100 mm) filled with teflon liner. The internal standard mixture of d5-2ADNT/d5-4ADNT (2 ng, 10 µl) in ethyl acetate was added to the Hb solution. After 1 h in a shaking bath at room temperature, dichloromethane (5 ml) was added. The mixture was vortex-mixed for 1 min, centrifuged for 5 min at 3000 g and frozen in liquid nitrogen. The thawed organic layer was transferred to a graduated tapered tube (98 mm × 15 mm) and concentrated down to 200 µl in a speed evaporator. The residue was then transferred to a microinsert (200 µl) for 12 mm × 32 mm autosampler vial and evaporated very carefully under a stream of nitrogen at 25°C. At the disappearance of the last drop, the stream of nitrogen was stopped. The residue was then taken up in 10 µl ethyl acetate containing 2 ng of the PFPA derivative of d5-4ABP.

Identification and quantification by GC-MS

The analyses were performed on a Hewlett Packard chromatograph (HP 5890II) equipped with an autosampler (HP 7673) and interfaced to a mass spectrometer (HP 5970A). The amines were analyzed by splitless injection onto a fused silica capillary column (Rtx-5MS, I.D. 0.25 mm; length 30 m, 0.5 µm film thickness: Restek corporation, Bellefonte, PA) with a 0.25 mm × 1 mm methyl-silyl retention gap (Supelco). In all cases the initial oven temperature, the injector temperature and the transfer line temperature were set at 50, 220 and 220°C, respectively. The oven temperature was increased at a rate of 50°C/min to 200°C, held for 1.8 min and then heated at 30°C/min to 300°C. Helium was used as carrier gas with a flow rate of 1.5 ml/min. The elution order of the amines is represented in Figure 1. For negative chemical ionization (NCI), with methane as the reagent gas, the source pressure was typically 160 Pa, the electron energy was 160 eV, the emission current was 300 µA and the source temperature was 200°C. For the identification and quantification of 2ADNT, 4ADNT, d5-2ADNT, d5-4ADNT the molecular ions 197, 198, 201 and 202 were monitored. The molecular ion in the PCI mode the detection limits of the standard compounds are in the femtogram range. The determination limit from the present experiments was found to be 40 pg for 2ADNT and 4ADNT per analysis of 50 mg Hb. The samples were quantified against calibration curves obtained from Hb (50 mg) spiked with a constant amount of the surrogate internal standard d5-2ADNT/ d5-4ADNT and with different levels of 2ADNT and 4ADNT (0.2, 1, 5 and 10 ng).
Urine sample (100 µl) and 10 µl of internal standard (2.5 ng/µl of d5-2ADNT and d5-4ADNT) were pipetted into screw capped glass tubes (25 mm × 11 mm) with Teflon liners. The samples were vortex mixed for 30 s. The samples were extracted with hexane (250 µl), by vortex mixing for 1 min. The hexane-phase was transferred to a microinsert (200 µl capacity) and analyzed by GC–MS.

Urine analysis: β-glucuronidase treatment
Urine sample (100 µl), β-glucuronidase buffer (100 µl, 0.4 M NaOAc, pH 4.5) and 10 µl of internal standard (2.5 ng/µl of d5-2ADNT and d5-4ADNT) were pipetted into screw capped glass tubes (25 mm × 11 mm) with Teflon liners. The samples were vortex mixed for 30 s then 10 µl β-glucuronidase (1000 U from H.pomaria Typ HP-2) was added and the samples were incubated in a shaking bath overnight at 37°C. After cooling to room temperature, the samples were extracted with hexane (250 µl), by vortex mixing for 1 min. Following centrifugation, at 3000 g for 10 min, the samples were frozen in liquid nitrogen, thawed at room temperature to aid phase separation. The hexane-phase was transferred to a microinsert (200 µl capacity).

Quantitation of urine metabolites
The same instrumental conditions were used as described above for the Hb-adduct analyses. Calibration curves were obtained from urine treated in the same way as the in vivo samples and spiked with the internal standard and increasing amounts of 2ADNT/4ADNT: 0, 0.01, 0.1, 0.5, 1, 2 µg.

Statistical analyses
Statistical analyses were performed with the program SPSS 10.0, Sigma Stat 2.0 and Sigma Plot 3.0. The results of the questionnaire and of the medical examination were not known to the scientists performing the Hb-adduct and urine analyses. All results were disclosed at the end of the analyses. All tests of statistical significance were two sided. The distribution of the metabolic levels and of the Hb-adduct levels were markedly skewed; therefore, the data were transformed logarithmically. The logarithmically transformed data showed normal distribution only for the exposed worker group according to the one sample Kolmogorov–Smirnov test. Inclusion of the control group yielded a non-normal distribution. Therefore, parametric and non-parametric tests were performed on the data. Health effects were compared with the Hb-adduct and urine metabolite levels using the Mann–Whitney test and logistic regression analysis. In the first step univariate logistic regression analysis was used to compare disease with the log-transformed Hb-adduct.

Results
The biological samples were worked up following a standard method from our laboratory (25,30,31). The samples were hydrolyzed with sodium hydroxide according to methods for the hydrolysis of sulfanilamide adducts of arylamines (25,20 and literature cited therein). For the determination of Hb-adducts a method used previously was applied. However, instead of 3,5-dinitroaniline the deuterated d5-2ADNT and d5-4ADNT were used as internal standards. The samples were analyzed by GC–MS in the NCI mode. Chromatograms of an Hb extract from an exposed worker is presented in Figure 1.

The Hb adducts of 4ADNT and 2ADNT were found in 100 and 81% of the exposed workers (78 of 78 for 4ADNT and 63 of 78 for 2ADNT), 4ADNT and 2ADNT were found in 16 and 8% of the factory controls (4 in 25 for 4ADNT, 2 in 25 for 2ADNT), respectively. The mean and median levels in the exposed workers were significantly higher, as determined by ANOVA and Mann–Whitney test, respectively (Figure 2). The median levels of 4ADNT (59 ng/g Hb) were ~24 times higher than the levels of 2ADNT. Therefore, 4ADNT was found in most workers but not 2ADNT. However, the levels of 4ADNT and 2ADNT correlate well (r = 0.81, Table I).

Urine samples were obtained from the same workers. For the determination of the metabolites of urine, a short and fast method was developed. Urine samples were analyzed with and without β-glucuronidase treatment. Urine was not collected from all workers. In the group of the exposed workers, 100% (71 of 71) and 97% (69 of 71) urine samples were positive for 4ADNT and 2ADNT, respectively, in the extracts of raw urine or in the extracts of enzyme treated urine. In the group of the control workers, 50% (4 of 8) of the urine samples were positive for 4ADNT and 2ADNT after both work-up

![Figure 1](https://example.com/figure1.png)

Figure 1. Chromatogram of an Hb extract from an exposed worker. Single ions were monitored in the NCI mode, with methane as the reagent gas (see Methods).

![Figure 2](https://example.com/figure2.png)

Figure 2. Box plot showing the median level of 4ADNT extracted from hydrolyzed Hb, from enzyme treated urine (U-gl) and from raw urine (U). Workers were grouped as exposed (E) and controls (C). The 10th, 25th, 75th and 90th percentiles have been presented as vertical boxes with error bars, and the outliers 5 and 95% have been highlighted as circles. The 95% outliers of E-U-gl-4ADNT is out of scale.
procedures. The levels of 4ADNT and 2ADNT in urine samples without enzyme treatment were much lower than after enzyme treatment. The median levels of 4ADNT and 2ADNT in enzyme treatment were much lower than in the exposed workers treated urine. The urine levels of 4ADNT and 2ADNT in enzyme treatment. The median levels of 4ADNT and 2ADNT ples without enzyme treatment were much lower than after procedures. The levels of 4ADNT and 2ADNT in urine samples without enzyme treatment were much lower than after enzyme treatment. The median levels of 4ADNT and 2ADNT were 4.5 and 2.4 times lower in raw urine than in enzyme treated urine. The urine levels of 4ADNT and 2ADNT in the control samples were much lower than in the exposed workers (Figure 2). The urine levels of 4ADNT in enzyme treated urine correlates with the levels of 4ADNT in raw urine (r = 0.77). The levels of 4ADNT and 2ADNT correlate for enzyme treated (r = 0.86) and raw urine (r = 0.85). The levels of urine metabolites and Hb-adducts do not correlate well (r = 0.34–0.55). Urine metabolites reflect the exposure of the last 48 h, Hb adducts reflect the exposure up to the last 120 days. Therefore, this moderate correlation between urine levels and Hb-adduct levels is not unexpected.

**Job related Hb-adduct and urine metabolite levels**

The workers were classified into five different categories: factory controls, mixers, loaders, grinders and packers. The Hb-adduct and the urine metabolite levels were compared between the different groups (Figure 3). The Hb-adduct levels, increase in the following order: factory controls < packers < mixers < loaders < grinders. The order is the same for the urine metabolite levels except for the packers. The median Hb-adduct levels are significantly different between all possible group pairs (n = 10) except for the group pairs: loaders versus grinders, and mixers versus loaders and packers. The median urine metabolite levels are significantly different between all possible group pairs (n = 10) except for the group pairs: loaders versus grinders; packers versus mixers, loaders and grinders. Thus, specific work tasks are critical in regards to the TNT body burden of the workers.

**Relationship between internal dose of TNT and health effects in Chinese workers**

A full medical examination was performed on each of the Chinese workers. Each worker was examined for the specific adverse health effects linked to exposure to TNT. Of particular interest was the investigation of dose–response relationships in the Chinese workers exposed to TNT. We were interested in defining whether biomarkers of recent exposure or chronic exposure correlated with the risk of suffering from one or more of the health conditions, symptomatic of toxic exposure to TNT. In the first analysis, the prevalence of cataract, splenomegaly, hepatomegaly, cardiovascular disease (ECG1 = sinus tachycardia, sinus bradycardia, ECG2 = arrhythmia, ECG3 = conduction), hepatitis B, cigarettes per day and smoke-years were compared between controls and exposed. The results are summarized in Table II. Except for cardiovascular effects the other diseases were more prevalent in the exposed workers. Smokers were more prevalent in the exposed group.

The health effects were compared with the adduct levels (Table III) using the Mann–Whitney test. In the first analysis, only the exposed workers were included in the calculations. The adduct levels in the exposed people with cataracts were significantly higher than in people without cataracts. The significance level for the difference of people with and without cataracts increased by multiplying the adduct levels with age or with the amount of years employed in the factory. Hepatomegaly was significantly present at higher adduct levels. For hepatitis B infected people, the adduct level difference was not significant. For workers without hepatitis B

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**Table I. Exposed TNT-workers: correlation of Hb adducts (Hb-2ADNT, Hb-4ADNT) with urine metabolites obtained from raw urine (U-4ADNT, U-2ADNT) and from β-glucuronidase treated urine (U-gl-4ADNT, U-gl-2ADNT)**

<table>
<thead>
<tr>
<th></th>
<th>Hb-4ADNT</th>
<th>Hb-2ADNT</th>
<th>U-4ADNT</th>
<th>U-2ADNT</th>
<th>U-gl-4ADNT</th>
<th>U-gl-2ADNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb-4ADNT</td>
<td>0.81**</td>
<td>0.41**</td>
<td>0.34b</td>
<td>0.55**</td>
<td>0.52**</td>
<td></td>
</tr>
<tr>
<td>Hb-2ADNT</td>
<td>0.44**</td>
<td>0.37b</td>
<td>0.52**</td>
<td>0.52**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-4ADNT</td>
<td>0.85**</td>
<td>0.77**</td>
<td>0.71**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-2ADNT</td>
<td>0.65**</td>
<td>0.78**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-gl-4ADNT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.86**</td>
<td></td>
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<tr>
<td>U-gl-2ADNT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.86**</td>
</tr>
</tbody>
</table>

Correlation coefficient r was determined from logarithmic transformed data (Pearson correlation). The statistical significance of each correlation was given by the two-sided P value.

*P < 0.001.

**P < 0.01.

**Fig. 3.** Box plot showing the median level of 4ADNT determined in Hb and enzyme treated urine from workers exposed to TNT. Workers were grouped according to their job description. The 10th, 25th, 75th and 90th percentiles have been presented as vertical boxes with error bars, and the outliers 5 and 95% have been highlighted as circles. The 95% outlier of the Hb-adduct from the loading workers is out of scale. Hb-4ADNT (U-gl-4ADNT) was determined in 25 (8) controls, 6 (5) packers, 35 (33) mixers, 26 (23) loaders and 10 (10) grinders.

**Table II. Comparison between the prevalence of adverse health effects and confounding factors in exposed and factory controls**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 25) (%)</th>
<th>Exposed (n = 78) (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cataract</td>
<td>26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.25</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>13&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.013</td>
</tr>
<tr>
<td>ECG1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28</td>
</tr>
<tr>
<td>ECG2&lt;sup&gt;h&lt;/sup&gt;</td>
<td>100&lt;sup&gt;i&lt;/sup&gt;</td>
<td>79&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.002</td>
</tr>
<tr>
<td>ECG3&lt;sup&gt;k&lt;/sup&gt;</td>
<td>24&lt;sup&gt;f&lt;/sup&gt;</td>
<td>17&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.45</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>29&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.34</td>
</tr>
<tr>
<td>Smoker</td>
<td>64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.033</td>
</tr>
</tbody>
</table>

The number of exposed workers where a symptom was recorded has been calculated as a percent of the total number of exposed workers analyzed. The number of factory control workers where a symptom was recorded has been calculated as a percent of the total number of factory control workers analyzed. The statistical difference of the prevalences between exposed and control workers was tested with the χ²-test (likelihood ratio).

Electrocardiogram (ECG): <sup>a</sup>sinus tachycardia, <sup>b</sup>sinus bradycardia, <sup>c</sup>arrhythmia, <sup>d</sup>abnormal conduction, <sup>e</sup>ECG1= -- tachycardia, ECG2 = arrhythmia, ECG3 = conduction. The data were available for: <sup>i</sup>n=23, <sup>j</sup>n=68, <sup>k</sup>n=72, <sup>f</sup>n=75, <sup>e</sup>n=65.
The odds of suffering from cataract were 6.4 times higher when such relationship was found for the urine metabolites. The suffering from cataract, hepatomegaly or splenomegaly. No between the Hb-cleavage product, 4ADNT and the risk of

(\text{Mann Whitney test}). In case of hepatomegaly, the workers were split in a category with hepatitis B and without hepatitis B. A significant difference for age and workyears was found for people with cataract. For workers with hepatitis B only splenomegaly is significantly prevalent (\chi^2-test, data not shown).

Except for workers with hepatitis B the median Hb-adduct levels were significantly higher in workers with hepatomegaly, cataract and splenomegaly (Mann-Whitney test). In case of hepatomegaly, the workers were split in a category with hepatitis B and without hepatitis B. A significant difference for age and workyears was found for people with cataract. For workers with hepatitis B only splenomegaly is significantly prevalent (\chi^2-test, data not shown).

\begin{table}[h]
\centering
\caption{Hb adduct levels of TNT (\textasciitilde 4ADNT), age and workyears of Chinese workers, compared with hepatomegaly, cataract and splenomegaly.}
\begin{tabular}{|c|c|c|c|c|}
\hline
& Hepatomegaly & Hepatomegaly & Hepatomegaly & Cataract & Splenomegaly \\
& hepatitis, yes & hepatitis, no & all & & \\
\hline
Exposed workers & & & & & \\
Hb-4ADNT & 54.6, 61.4, 0.88 & 54.5, 106.6, 0.009 & 54.5, 88.3, 0.02 & 49.6, 68.9, 0.021 & 55.6, 100.1, 0.023 \\
Age & 38.0, 39.9, 0.69 & 41.0, 41.9, 0.76 & 41.0, 41.9, 0.66 & 34.5, 43.1, \textless 0.001 & 41.0, 41.4, 0.56 \\
Workyears & 9.3, 9.9, 0.38 & 9.5, 10.8, 0.64 & 9.5, 10.6, 0.33 & 5.0, 11.0, \textless 0.001 & 9.0, 10.4, 0.48 \\
Hb-4ADNT+workyears & 402.4, 875.7, 0.34 & 468.4, 1401.8, 0.018 & 431.7, 1015.7, 0.011 & 211.3, 1007.3, \textless 0.001 & 418.1, 1006.3, 0.033 \\
Hb-4ADNT+age & 2101.2, 3022.1, 0.55 & 2016.9, 4005.7, 0.004 & 2031.9, 3721.9, 0.01 & 1733.1, 3031.3, \textless 0.001 & 2167.7, 3751.1, 0.022 \\
\hline
Exposed + controls workers & & & & & \\
Hb-4ADNT & 52.6, 47.5, 0.74 & 36.9, 83.3, 0.015 & 42.0, 75.3, 0.018 & 20.7, 63.3, \textless 0.001 & 42.8, 100.1, 0.002 \\
Age & 35.4, 35.0, 0.54 & 42.8, 41.9, 0.97 & 41.0, 41.0, 0.72 & 34.9, 43.4, \textless 0.001 & 41.0, 41.4, 0.55 \\
Workyears & 7.5, 9.0, 0.37 & 10.6, 11.0, 0.96 & 10.0, 10.7, 0.61 & 6.4, 11.5, 0.001 & 10.0, 10.4, 0.81 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Logistic regression analysis of Hb-adducts and disease in Chinese workers}
\begin{tabular}{|c|c|c|c|}
\hline
Hb-adduct: 4ADNT & Hepatomegaly OR\textsuperscript{a} (CI\textsuperscript{b}, \textit{P} \textsuperscript{c}) & Cataract OR (CI, \textit{P}) & Splenomegaly OR (CI, \textit{P}) \\
\hline
Exposed workers & & & \\
7.6 (1.3-43.7, 0.024) & 6.4 (1.4-29.6, 0.018) & 9.6 (1.1-85.3, 0.042) \\
Exposed + control workers & 2.1 (0.96-4.4, 0.063) & 2.9 (1.6-5.3, 0.001) & 11.1 (1.5-83.8, 0.019) \\
\hline
\textsuperscript{a}OR = odds ratio, \textsuperscript{b}CI = confidence interval, \textsuperscript{c}two sided significance level. The ORs were adjusted for: \textsuperscript{1}age, \textsuperscript{2}hepatitis B status and \textsuperscript{3}age and workyears. \\
\end{tabular}
\end{table}

infection, the Hb-adduct levels were higher in workers with hepatomegaly. In workers with cardiovascular defects, the adduct levels were not significantly different. In order to determine the confounding factors, the same analyses were performed with age, workyears, cigarettes per day and smoke years. Only for age and workyears was there a difference between people with disease. For workers with cataract, the median age and median workyears were significantly higher. For smoked cigarettes no such difference was seen. The same analyses were performed including the control group in the analyses. The results obtained were very similar (Table III). Thus Hb-adduct levels are a good indicator for the biological effect. The same statistical analyses were performed for the urine metabolites. All these analyses were statistically not significant \textit{P} > 0.35. Therefore, only Hb-adducts are a predictor for the health outcome.

In order to calculate the odds ratios (ORs), logistic regression analyses were performed with the data. The logistic regression was performed using the log-transformed data of Hb-cleavage products and urine metabolites. The obtained ORs are listed in Table IV. The OR indicates the odds of suffering for the various health effect of subjects with one log-unit more of each adduct compared with the odds of subjects with one log-unit less. The results presented in Table IV show that there is a significant dose-response relationship between the Hb-cleavage product, 4ADNT and the risk of suffering from cataract, hepatomegaly or splenomegaly. No such relationship was found for the urine metabolites. The odds of suffering from cataract were 6.4 times higher when the level of 4ADNT Hb-adducts increased by one log-unit (\textit{P} < 0.05). Similar ORs were observed with hepatomegaly (7.6) and splenomegaly (9.6). These results were tested for confounding factors like age, workyears, smoker status, smoke years, cigarettes per day and hepatitis B status using stepwise logistic regression analysis. In the case of splenomegaly, hepatitis B status is a confounder. In the case of cataract, the median age is a confounder. The hepatitis B adjusted OR for splenomegaly was 12.8 and the age adjusted OR for cataract was 25.6. All ORs have been listed in Table IV. These results inferred that quantitation of TNT Hb-adducts provided an effective biomarker of toxicity and could be used to estimate the risk associated with a particular exposure to TNT.

The clinical blood parameters of the exposed male workers were correlated with the Hb-adduct levels using the Spearman rank order methods. There was a significant correlation between the Hb-adduct levels and the Hb-concentration (\textit{r} = -0.51, \textit{P} < 0.001), hematocrit (\textit{r} = -0.25, \textit{P} < 0.05) and serum glutamic pyruvic transaminase (SGPT) levels (\textit{r} = -0.25, \textit{P} < 0.05). The other parameters did not correlate. For a further statistical analysis, the Hb-adduct levels of the exposed male workers were split in two categories: values below the median level and values above the median level. These two categories were compared with the clinical blood parameters using the Mann-Whitney test. Workers with a higher adduct level have a significantly lower hematocrit, red blood cell count level, Hb concentration and total blood protein levels. In addition, the SGPT levels and the neutrophil white blood cell count were significantly lower in workers with
higher Hb-adduct levels. The changes in the other parameters were not significant, although the bilirubin level, the albumin-globulin ratio and the lymphocyte blood cell count was higher in the group of workers with high adduct levels. The other parameters did not differ between the two Hb-adduct categories.

Discussion

Biomonitoring

For TNT, this is the first study combining different markers of exposure with biological effects. The external dose, skin exposure and air measurement was performed only on about one-third of the workers \((n = 35)\). The data and experiments are not included in this paper since the worker number is too small to observe significant correlations with the health effects. A Spearman rank analysis of the external dose with urine metabolites and/or Hb-adducts yielded correlation coefficients \(< 0.41\). For skin exposure the correlation was moderately better \((r < 0.54)\).

In all the other previous studies about workers exposed to TNT, urine metabolites or the Hb-adducts were measured separately. In German workers dismantling old munition, the median 4ADNT level found in raw urine was 0.23 \(\mu g/\text{ml}\) (51 workers) (32) and 0.71 \(\mu g/\text{ml}\) (9 workers) (14). We found similar 4ADNT levels (median = 0.21 \(\mu g/\text{ml}\)) in raw urine of exposed Chinese workers. Therefore, the same disease noticed in Chinese workers could appear in other populations also, if chronic exposure is given over the years. The Hb-adduct levels found in the present study were similar to an earlier study performed in a Chinese factory (25). In Germany, Hb-adducts were found in environmentally exposed people (33). Populations living on a formerly used industrial area for the production of TNT were compared with matched controls living in non-contaminated areas. The median levels found in people of the contaminated areas was 0.24 ng/g Hb 4ADNT. This is 262 times lower than the median levels found in Chinese workers. The maximum value found was up to 3.4 ng/g Hb. In control population, the values were similar, 0.08 ng/g Hb (median) and 1.9 ng/g Hb (maximum). Unfortunately, no experimental details were given for the determination of Hb-adducts. Therefore, the levels found are difficult to discuss and the method cannot be repeated.

Medical examination

The clinical blood parameters regarding the hematological effects, decrease of hematocrit and Hb concentration, corresponded to earlier findings (1). The other changes observed in the present study—hepatic, immunological and lymphoetheletic effects—have not been observed in earlier studies in humans, though observed in animal studies (1). In the present group of workers, the SGPT levels decreased significantly with increasing levels of Hb-adduct. The same was observed in mice, rats and dogs; in these animals, the SGPT levels decreased and the liver size increased (34). The serum glutamic-oxaloacetic transaminase remained the same in those animals. To monitor immunological and lymphoetheletic effects, white blood cell counts (lymphocytes, eosinophils and neutrophils) and spleen size was measured in the present group of Chinese workers. The amount of lymphocytes was higher in workers exposed to higher doses as observed earlier (1). The significant decrease in neutrophil white blood cell counts was not observed in earlier studies. Splenomegaly has never been reported in humans although reported in animals (1). Hepatomegaly was found both in animals and humans in the past. Cataract is a common disease among TNT workers.

Cancer risk

In a recent study, higher incidences of cancer were found in TNT workers. How many additional cancer cases are expected for people exposed to the levels of TNT found in the present study? For a risk assessment, the daily dose of the Chinese workers has to be estimated. With the knowledge from animal data, it is possible to estimate the daily dose from the measured Hb-adduct levels. However, it is necessary to make the following assumptions: (i) The steady-state level of Hb-adducts is estimated with the following formula (35,36): steady-state adduct level = daily-adduct level \(\times 0.5\) \(\times\) lifetime of the erythrocytes. Thus, to calculate the single dose, the adduct level has to be divided by 60. (ii) Modified Hb has the same life span as unmodified Hb and the adducts are stable to repair mechanisms. (iii) The pharmacokinetics of the xenobiotic compound is comparable in rats and humans. For 4ABP the percentage of the dose bound to Hb in rats was very similar to the percentage of the dose bound to Hb of smokers (36), although two additional cysteine groups are present in the alpha-chain of the Hb of rats (37) but not in humans or mice. The Hb-adduct levels for five amines were 2–28 times lower in mice than in rats (38). Therefore, the estimation of the daily dose from Hb-adducts in rats could be an underestimation.

The absorbed dose per day was calculated from the Hb-adduct levels using the equation for the steady-state level of Hb-adducts. With the Hb-adducts, the daily dose was estimated assuming that the same amount of the dose binds in rats and in humans. In rats \(-0.09\%\) of the dose binds to Hb (39). The median adduct levels of the workers was 62.1 \((\text{mean} = 93.9 \text{ng/g Hb}, \text{95th percentile} = 226.3 \text{ng/g Hb})\). The daily dose for the median Hb-adduct level was estimated to be 14.1 \(\mu g/\text{kg/day}\). An excess lifetime cancer risk (ELCR) for exposure to TNT was estimated using the formula published by the US regulatory agencies (40,41) (http://risk. lsd.ornl.gov): \(\text{ELCR} = (\text{cancer slope factor}) \times \text{(human dose)}\).

Workers are not exposed 7 days a week and not all 52 weeks of the year to the work place contaminant. Since we deduced the external dose from the internal dose, days without exposure were included. Therefore, the dose was not corrected for the days without exposure. Since workers are not exposed for a lifetime of 70 years to the occupational pollutants, but only for 40 years of work life, the dose was corrected with the factor 40/70. The cancer slope factors for TNT is 0.03 (mg/kg/day)\(^{-1}\) (www.epa.gov/iris). Therefore, the excess cancer risk for lifetime exposure is 23 in 100 000 for the median adduct levels found in workers. For the 95th percentile adduct levels the estimated daily dose was 51.4 \(\mu g/\text{kg/day}\). This dose translates into an estimated lifetime cancer risk of 88 in 100 000. Thus, for workers exposed to this dose only an excess of 0.09\% more cancers is expected. However, based on an approximation using standard mortality rates, it was recently reported that TNT-exposed Chinese workers have a higher relative risk of 2.3 for all cancers (8). The average age of death was 51.7 years for the TNT-exposed workers and 54.1 years for the unexposed controls in this study. We calculated a cumulative lifetime risk of 7\% for this particular age group (15–64 years) in China using age-specific cancer mortality rates (42,43). Based on this
calculation, a relative risk of 2.3 would yield a 9.1% excess of cancers for the exposed workers in the earlier study (8). This estimate exceeds our estimate. There are several possible explanations for the discrepancy: (i) The dose estimated from Hb-adducts is too low if less TNT binds to Hb than in rats. (ii) The cancer slope factor provided by the Environmental Protection Agency is inaccurate and too low for TNT. (iii) The previous study from China does not differentiate between an ammunition factory, where only TNT is used, and other TNT factories where other intermediates are the more prevalent chemicals in the work environment. 2,4-Dinitrotoluene, 2,6-dinitrotoluene and 2-nitrotoluene are the major occupational pollutants in most TNT factories, and dinitrotoluenes have a cancer slope factor that is 23 times larger than the factor for TNT. We previously found a 1.5% excess of lifetime cancer in another TNT factory (44) using the same approximation methods that are described herein. This risk estimate is in the same order of magnitude as the estimate deduced from the other study (8).

In conclusion, this is one of the rare biomonitoring studies where a dose-relationship could be found between biological effects and biomarkers in people exposed to occupational pollutants. The findings of the present study provide a useful tool for future studies involving the risk assessment of people involved in the clean up of contaminated sites, for people living in contaminated areas (www.atsdr.cdc.gov) (6,45) and for workers of TNT factories.

Acknowledgements

We acknowledge the technical assistance of Renate Hartley and the financial support by the European Commission, ERB-IC-CT97-0221.

Conflict of Interest Statement: None declared.

References


Received December 21, 2004; revised March 17, 2005; accepted March 22, 2005