Production of IL-10, TNF and IL-12 by Peripheral Blood Mononuclear Cells in Mexican Workers Exposed to a Mixture of Benzene—Toluene—Xylene

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Background and Aims. Occupational exposure to low-level benzene and the joint action of toluene—xylene probably cause effects on circulating monocytes immune response. We undertook this study to determine relationship between occupational exposure to benzene—toluene—xylene mixture (BTX) and IL-10, TNF and IL-12 production by peripheral blood mononuclear cells.

Methods. Exposure was estimated in 54 workers from a paint company in Mexico City through BTX accumulated potential dose (BTX-APD). Two exposure groups were formed: high and low BTX-APD established with a cutoff point at ≥1.0 of BTX-APD, as a function of the geometric mean of the estimator’s value distribution and the higher agreement between BTX-APD ≥1.0 and the areas referred as using (or not) organic solvents in the work process. IL-10, TNF and IL-12 concentrations were measured with ELISA. Through multiple linear regression models, the production of each of the proposed cytokines and of the whole set was assessed.

Results. Workers with high BTX-APD showed a significant reduction in TNF production (β = −1,196.0 pg/mL; p = 0.01); a reduction for IL-10 (β = −520.3; p = 0.13) and IL-12 (β = −843.3; p = 0.09) was also observed, although without statistical significance.

Conclusions. TNF production assessed in workers with a high BTX-APD is lower than in those with a low BTX-APD, but not in IL-10 and IL-12 production. © 2012 IMSS. Published by Elsevier Inc.

Key Words: Benzene—Toluene—Xylene, Occupational exposure, Immune response, Cytokines.

Introduction

It is important to highlight that, in a work environment, the mixture of benzene with toluene and xylene (BTX) is practically unavoidable (1−8). It is assumed that the two latter substances may interact and determine the toxicity of the benzene fraction by sharing the toxicokinetic mechanism (9,10), in addition to the fact that the joint action of toluene—xylene in itself probably causes effects on circulating lymphocytes and monocytes (11).

Epidemiological studies aimed at analyzing the functional integrity of the immune response of workers exposed to these substances in the last decade are contradictory, and

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the way in which the occupational exposure was determined is open to question (2,7,12,13).

The effects on the innate immune response may be assessed using different parameters, among which stands out cytokine production. These include interleukin-10 (IL-10), tumor necrosis factor (TNF) and interleukin-12 (IL-12) and are a set of soluble components that make up an important part of the human arsenal against viruses, bacteria and/or harmful external material (14). This set of cytokines shares the ability to be produced by monocytes that are activated by lipopolysaccharide (LPS) stimulation, whereas both TNF and IL-12 may be inhibited by IL-10 (15–17).

This study evaluates the relationship between exposure to a mixture of BTX and the ability of peripheral mononuclear cells (PBMCs) from a sample of workers at a company that manufactures paint in Mexico City to produce IL-10, TNF and IL-12.

Materials and Methods

Study Design

A cross-sectional study was conducted with 60 workers at a company that manufactures paint in Mexico City. The company had a total of 234 workers from 16 work areas. The group of workers consisted of both males and females with at least 4 consecutive months working at the location in their respective work areas, chosen depending on the feasibility of performing immunological studies and on the possibility of temporarily removing workers from the production line.

Information on age, name and the area to which workers were assigned and time worked was obtained through oral questionnaire, as well as any history of cigarette smoking and/or routine alcohol consumption; workers’ weight and height were recorded in order to calculate their body mass index (BMI) (18).

Exposure was estimated by means of personal sampling that collected benzene, toluene and xylene vapors that each worker in the study came into contact during an 8-h work shift in their respective workplace and during their working life. A blood sample was taken to obtain PBMCs, which were then stimulated with LPS.

BTX Measurement

The measurement of benzene, toluene and xylene present in air contaminated with these solvents was taken from air collected at the breathing height of each of the workers included in the study by means of sampling pumps operating at flows below 500 mL/min and captured in coconut-shell activated carbon tubes that were 6 mm in diameter \times 70 mm long, with 50/100 mg sections of adsorbent material. Pumps were calibrated both before and after sampling using a primary calibrator. Base time for sampling was an 8-h shift covered by four or five samples collected continuously while calculating the pump’s operating flow and sampling time. For BTX separation, a Thermoquest Trace GC 2000 gas chromatographer (CE Instruments, Milan, Italy) with flame ionization detector and capillary columns was applied.

A first approach to air concentrations of benzene, toluene and xylene vapors in TWA (time-weighted average in 8-h work shift) by the group of workers that perform their job in areas that use or not use these substances in the production process at the company under study are shown in Table 2.

Personal Exposure Estimation

The dose of potential exposure to a mixture of BTX vapors through an inhalation route was estimated for each of the 60 workers, which represents the cumulative summation of vapors of each of the components of such a mixture ($\sum D_{APD}$). Each $D_{APD}$ is the cumulative addition of partial exposure concentrations during one work shift (in mg/m³), multiplied by the volume of inspired air consistent with the nature of the work performed, categorized as light (3.5 L/min), moderate (5.0 L/min) or heavy (5.7 L/min) expressed in m³ (19,20).

Finally, the potential-daily-dose-to-BTX estimator for an 8-h work shift was weighted according to the worker’s weight and time worked at the company so that the potential cumulative dose = cumulative mg of BTX mixture/kg of worker’s body weight/work shift for the number of months worked at the company, henceforth indicated as BTX-APD.

Collection of PBMCs

After informed consent, 10 mL of whole blood was extracted from the vein in the elbow crease into Vacutainer (Becton Dickinson, Franklin Lakes, NJ) tubes with heparin. Under a standard laminar flow hood, the samples were placed in 50 mL Corning (One Riverfront Plaza, NY) tubes and diluted 1:2 with Hanks’ balanced salt solution (Gibco, Carlsbad, CA), at a ratio of 2:1. Diluted blood was layered using a Ficoll-Hypaque (Sigma, Minneapolis, MN) solution at a ratio of 2:1 (blood: F-H). This suspension was centrifuged at 800 g for 30 min at room temperature in a free rotor; the PBMC cell pellet was collected using a Pasteur pipette and washed twice using 10 volumes of Hanks, centrifuged at 250 g for 10 min. Six mL of RPMI culture medium was resuspended with 5% fetal bovine serum; viable cells were quantified by means of Trypan blue staining. Finally, cells were counted and adjusted using AIM-V (Gibco) medium at 1 x 10^6/mL. One mL of the suspension was placed in each well of a 24-well (Corning) plate and the LPS (Sigma) stimulated cells at a concentration of 20 μg/mL.
Quantification of Cytokines Using ELISA Assay

To conduct ELISA assays for each of the cytokines proposed in the study, 1 x 10^6 PBMC/1 mL were placed in a 24-well plate. Twenty μL of LPS was added to three wells at a concentration of 20 μg/mL; another three were not activated (negative controls). After incubating at 37°C for 12 h, the supernatants of each well were harvested and centrifuged at 1,500 g for 5 min in a microcentrifuge. Centrifuged supernatants were stored at −70°C until required. Cytokine quantification was performed in 96-well microplates following the instructions of the commercial kit (BD OptEIA ELISA); 100 μL of the capture antibody diluted 1:250 were applied briefly to each well, incubated at +4°C for 12 h; the well content was removed and three washing procedures were carried out in each well with 300 μL of solution. Into the last wash, the microplate was dried with absorbent paper to remove the residues of the wash itself. Microplates were blocked by adding 200 μL of diluting solution to each well and incubating at room temperature for 1 h to, once again, perform the wash procedure mentioned above.

The type curve was prepared according to the cytokine to be quantified. To that end, 100 μL of the type curve and 100 μL of the sample were placed in the microplate wells and again incubated at room temperature for 2 h. The above-mentioned wash procedure was repeated five times, and 100 μL of the detection antibody was subsequently added together with streptavidin-horseadish peroxidase conjugate enzyme in the microplate’s wells. Once again, it was incubated for 1 h at room temperature, and then seven washes were carried out. Upon conclusion of the above, 100 μL of the substrate solution was added to each well, and the microplate was incubated at room temperature for 30 min in the dark. At the end of this incubation, 50 μL of STOP (phosphoric or sulfuric acid) solution was added and the absorbance read using an ELISA (EL808x, Biotek, Winooski, VT) reader at 450 nm. The corresponding calculation was performed using the absorbance data to obtain the concentration of each cytokine in picograms per mL (pg/mL).

S-phenylmercapturic Acid Measurement (S-PMA)

In order to identify with greater certainty the exposure of workers included to the benzene fraction of the BTX mixture, the S-phenylmercapturic acid elimination was used as an indicator of benzene exposure and measured in each of the workers included in the study (21). For this purpose, a urine sample was requested from the workers, preferably at the end of the week’s work; 5 mL was taken in special collection vials containing 50 μL of hydrochloric acid (7.5 mol) for preservation purposes. Once collected, the samples underwent ELISA testing and, from the ratio of nmol/L of S-PMA/mg/dL of creatinine, it was finally quantified in μmol of S-PMA/mol of creatinine. The correlation of the ELISA assay assessed and the gas chromatography/mass spectrometry reported by the same laboratory was 0.92 (21).

Statistical Analysis

The questionnaire results, cytokine concentration pre- and post-LPS stimulation, and concentrations of benzene, toluene and xylene, as well as the BTX mixture exposure estimator of the workers under study and S-PMA values were entered into a database using Stata v.10.0 software (StataCorp, LP, College Station, TX). Across the board, the database was explored in order to identify aberrant, extreme or missing values and to perform a descriptive analysis of included variables. Two exposure groups were established with a cutoff point at ≥1000 or 1.0 of BTX-APD as a function of the geometric mean of the estimator’s value distribution (999.6 of BTX-APD); the higher agreement between BTX-APD ≥1.0 and the areas referred to as using or not using organic solvents in the work process: kappa = 0.56 (moderate—substantial according to Landis and Koch, moderate—good according to Altman and almost good according to Fleiss) (22) and 77.8% of agreement.

Multiple linear regression models were used with the differential production of IL-10, TNF-α and IL-12 and the production set of the latter by PBMCs, pre- and post-LPS stimulation. In all these four models the respective interactions with cigarette smoking, alcohol consumption, and BMI with the aforementioned BTX-APD were included and tested. To assess these models, an F-test was performed and the assumptions of absence and multicollinearity (average VIF [i.e., variance inflation factor]), homoscedasticity (Breusch-Pagan/Cook-Weisberg) and residual value normal distribution (Shapiro–Wilks test) were explored.

Ethical Aspects

The project fulfilled requirements of the National Commission on Scientific Research of the Mexican Institute of Social Security, and was authorized in its entirety and registered under number 2005-785-175. The protocol was rated as being of minimal risk because it involved urine collection and blood extraction by venous tap in adults with an apparent good health status (23).

Results

The final analysis of the three cytokines was drawn from data obtained from the samples of only 54 workers as the PBMCs of four of them proved to be either nonresponsive to LPS-stimulation, or resulted in an aberrant response; a further two were excluded due to the fact that no information could be gathered with respect to the total number of months worked at the company. Cytokine production by
The mean age of the 54 workers was 36.25 ± 7.2 years (range: 19–49 years). Among them, the mean length of time working at the company was 54 months (range: 6–300 months), and 29 workers (54%) are based, use toluene and xylene in the work process although the company reported that no benzene was used there. The last six areas where 25 workers worked were identified: resins, solvent-based paint, solvent-based paint packaging, finished-product store, emulsion paints, and maintenance, aging, drum storage, formula development, quality control, and the PBMCs taken into account for the final analysis was the difference between production pre- and post-LPS stimulation. This same analysis includes the sum of the differential production pre- and post-LPS stimulation by groups with low (IL-10: 803.2 pg/mL and IL-12: 848.7 pg/mL) and high BTX-APD (IL-10: 1,273.9 pg/mL and IL-12: 1,586.1 pg/mL) (all samples) expressed as the production of TNF (1,843.9 pg/mL) and post-LPS stimulation by groups with low (IL-10: 803.2 pg/mL and IL-12: 848.7 pg/mL) and high BTX-APD (IL-10: 1,273.9 pg/mL and IL-12: 1,586.1 pg/mL) respectively. There was no significant difference (p = 0.08 and 0.07, respectively) between groups with low BTX-APD (all samples) and high BTX-APD (all samples) and the production aggregate of the three cytokines (4,270.6 pg/mL and 2,377.1 pg/mL, respectively). The mean age of the 54 workers was 36.25 ± 7.2 years (range: 19–49 years). Among them, the mean length of time working at the company was 54 months (range: 6–300 months), and 29 workers (54%) are based, use toluene and xylene in the work process although the company reported that no benzene was used there. The last six areas where 25 workers worked were identified: resins, solvent-based paint, solvent-based paint packaging, finished-product store, emulsion paints, and maintenance, aging, drum storage, formula development, quality control, and the PBMCs taken into account for the final analysis was the difference between production pre- and post-LPS stimulation. This same analysis includes the sum of the differential production pre- and post-LPS stimulation by groups with low (IL-10: 803.2 pg/mL and IL-12: 848.7 pg/mL) and high BTX-APD (IL-10: 1,273.9 pg/mL and IL-12: 1,586.1 pg/mL) (all samples) expressed as the production of TNF (1,843.9 pg/mL) and post-LPS stimulation by groups with low (IL-10: 803.2 pg/mL and IL-12: 848.7 pg/mL) and high BTX-APD (IL-10: 1,273.9 pg/mL and IL-12: 1,586.1 pg/mL) respectively. There was no significant difference (p = 0.08 and 0.07, respectively) between groups with low BTX-APD (all samples) and high BTX-APD (all samples) and the production aggregate of the three cytokines (4,270.6 pg/mL and 2,377.1 pg/mL, respectively).
Results of the Statistical Modeling

Multiple linear regression models that included alcohol consumption, cigarette smoking, and BMI showed that the first of these diminishes the production of the three cytokines and their sum. However, the results for cigarette smoking were open to question, although smoking reduces average production of IL-10 and TNF, it increases that of IL-12 and the sum of the three cytokines. The influence of BMI in production change units for the three cytokines and their sum proved to be low. With the exception of the reduction in the average production of IL-12 where the contribution of the routine alcohol consumption turned out to be statistically significant ($p = 0.02$), the other adjustment variables did not show it. In workers exposed to a BTX-APD ≥1, a reduction was observed both in the average production of each of the cytokines proposed as well as in their total amount, although this was only significant in the case of TNF and in the sum of the three cytokines studied (Table 3). The determination coefficients of IL-10, TNF, IL-12 and of the sum of the three cytokines were 7, 14, 17 and 15%, respectively. In the same order, the results of the F-test for interaction significance were 0.87, 0.56, 0.81 and 0.86, respectively, and of the Breusch-Pagan/Cook-Weisberg: 0.04, 0.002, 0.00001 and 0.006; for the four models, the VIF (Variant Inflation Factor for multicollinearity) average was $<10.0$, and the Shapiro-Wilk's test was 0.38.

Discussion

In general, the study revealed that the PBMC production of the three cytokines assessed in workers subject to a high BTX-APD is lower than in low BTX-APD workers. Despite the fact that no statistical differences were seen in the reduced production of IL-10 and IL-12 between the established groups, this production was nonetheless lower in samples taken from workers that have been subject to a high BTX-APD.

Although it has been observed that the IL-10 may persist even in the absence of a stimulus—in this case, LPS (27)—and that there are elements in our study that point to the preservation of the production of IL-12, it would be possible for these to become compensatory mechanisms given the reduction of TNF, perhaps due to the fact that it is more susceptible to chronic exposure to BTX, a fact that needs to be explored more comprehensively in further studies (28).

On the other hand, the study involved a sample of relatively young workers and who could be assumed to have a well-developed immune system, influenced by genetic or environmental factors (29–31). In addition to considering the relatively long, strictly occupational exposure time to the mixture under study in the proposed high dose, the contribution of other factors needs to be included. These include the coincidence with cigarette smoking (32–37) as well as chronic alcohol consumption, which has been linked to diverse functions of the phagocytic cells, primarily the proinflammatory ones (38–42) and the combination with overweight or obesity that are involved in the production of proinflammatory adipocytokines. This includes a remarkable production of TNF, which has been shown to play a role in the activation of the inflammatory cascade, thus contributing to the development of obesity (43–46). However, our study did not reveal any significant involvement of such conditions.

It is likely that other additional exogenous factors exist in the work environment under study, especially because only the inhalation route was considered. Furthermore, in terms of endogenous factors, the individual conditions of the nutritional status must be taken into account, as well as the individuals’ metabolic capacity and the quality and variability of the immune response (47,48).

In conclusion, reduction in the production of the aforementioned group of cytokines prevails when this association is adjusted for alcohol consumption, cigarette smoking or overweight or obesity and, that under this analysis framework, the decline in the production of TNF is greater than that of IL-10 and IL-12 although limited to the stimulation of PBMCs with LPS. This without taking into account other mechanisms of the host’s immune response that could be preserved (49–51).

It must be kept in mind that all workers included in the study showed concentrations, albeit very low, of S-PMA. Even though the average reading was higher in workers assigned to areas reported as using BTX in the work process, the possibility that benzene is solely responsible for the generation of immunological effect is worth considering (1,8,52).

Essential to this study was the visit to the company, which proved to be a guiding tool to identify the work areas

Table 3. Regression coefficients ($b$) of multiple linear regression models for each of the three cytokines studied and the aggregate

<table>
<thead>
<tr>
<th></th>
<th>IL-10</th>
<th>TNF-α</th>
<th>IL-12</th>
<th>Three cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$b$ (pg/mL)</td>
<td>$p$</td>
<td>$b$ (pg/mL)</td>
<td>$p$</td>
</tr>
<tr>
<td>High BTX-APD</td>
<td>−520.3</td>
<td>0.13</td>
<td>−1,196.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Alcohol consumption+</td>
<td>−483.2</td>
<td>0.29</td>
<td>−751.8</td>
<td>0.24</td>
</tr>
<tr>
<td>Smoking+</td>
<td>−123.2</td>
<td>0.73</td>
<td>−131.4</td>
<td>0.79</td>
</tr>
<tr>
<td>BMI &gt; 25</td>
<td>−1.58</td>
<td>0.97</td>
<td>−68.6</td>
<td>0.34</td>
</tr>
</tbody>
</table>
in which the use of BTX solvents were reported and those in which presumably are not used in work process (53).

The proposed exposure estimator has several advantages over others referred in different studies (2,3,7,12,13, 54—57) because it was based on the accumulated exposure in the form of a potential dose and not on the TWA exposures. In principle this does not take into account any additive effect of the mixture or was not based on an analysis of each of its components independently. It was designed with the mixture of benzene with toluene and xylene that affects the cellular and soluble components analyzed in mind. It must be stressed that the estimator has been established as a simple mixture that needs to be studied in depth according to the National Occupational Research Agenda proposed by NIOSH (8).

Although it proved to be important to demonstrate the inhibition of the synthesis of some cytokines in workers exposed to a BTX mixture, it is necessary to consider using other tests to assess the inflammatory response (46,47,58—63). Notwithstanding, these findings warn of the need to carry out a more in-depth analysis of the exact immunotoxicology of the event at hand so that preventive practices may be developed accordingly within the industry—such as that subject to this study—and shed light on immunosuppression induced by occupational exposure to chemicals.

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