Phenotype analysis of lymphocytes of workers with chronic benzene poisoning

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Abstract

Lifetime exposure to benzene is associated to a variety of blood disorders, and except for the risk of cancer, almost nothing is known concerning health impairment in individuals who are no longer exposed. In Brazil, this exposure is one of the serious problems in workplaces, and many workers have been laid off their jobs due to this intoxication, particularly in the State of Bahia, the largest producer of benzene in Latin America, which is the area of this study. From a larger study to describe health effects and genetic polymorphisms among workers with chronic benzene poisoning (CBP), this previous specific investigation analyzes the association between CBP and the pattern of sub-populations of lymphocytes. The study was performed with a CBP group (n = 24) and a control group with other occupational diseases (n = 24), both were selected at the Workers Health Study Center in the State of Bahia, Brazil. Clinical and epidemiologic variables were collected from medical records and from a detailed questionnaire. The average age was similar in the two groups (51.1 and 50.7, respectively). Analyzing the mean proportions of the sub-populations of lymphocytes, statistically significant differences were found for T cytotoxic cells (TCD8) (27.9; 19.4; p = 0.002) and T helper memory cell (CD4CD45RO) (31.2; 37.0; p = 0.015), respectively, for the CBP group and control group. These results should be viewed with caution because of the small sample size, but they strengthen a previous impression that workers exposed to benzene have their immune system impaired, even in the long term, which may contribute to some disorders and carcinogenesis process. These workers must be strictly followed up in a medical surveillance program. Although this problem has been known for a long time, this is the first attempt to study these specific effects in Brazil.

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1. Introduction

In Brazil, exposure to benzene has been seen as one of the serious problems noted in workplaces [1]. The largest benzene production in Latin America, around 400,000 t/ year, occurs in the State of Bahia, more precisely in the Petrochemical Complex of Camaçan. At the beginning of the 1990s, many workers were laid off work in this region due to the presence of hematological alterations, most notably the reduction in the number of leukocytes/neutrophils in peripheral blood.

Although there are many forms of benzene exposure, such as industrial emissions through gasoline vapors, motor
exhaust fumes, cigarette smoke and water source contamina-
tion, occupational exposures are considered to be of a higher
level [2]. Evidence of the noxious effect of benzene dates
back to the end of the 20th century [3,4] and its carcinogenesis
on bone marrow is known [5–13]. In addition, this substance
is involved in a variety of blood abnormalities of which there
are many reports [14–17]. Accidentally, some studies with
the object of analyzing the relation between exposure to
benzene and leukemia have also shown an increase in the risk
of lymphoid neoplasms among workers exposed to benzene
[18]. In a review of 18 epidemiological studies about the
association between exposure to solvents and non-Hodgkin’s
lymphoma (NHL) found that out of the nine studies that char-
acterized exposure more precisely, only one was categorical
in demonstrating the non-existence of an association [19],
which may mean an important indication of the existence of
this association. It was concluded that considering the limita-
tions of each study and the leucemogenic action of benzene,
it is reasonable to classify it as one of the probable agents
involved in the etiology of NHL [20]. However, in spite of this
recognized carcinogenic action on the lympho-hematopoietic
system (classification in Group 1 of the International Agency
for Research on Cancer IARC: the patterns of exposed
populations (workers and the community in general)
becoming ill is still being discussed, and except for the risk
of cancer, little is known with regard to immunotoxicity
[21].

The effects of immunotoxicity induced by benzene are
probably a reflection of the toxicity to the bone marrow
[22], inducing depression and altering both the immune sys-
tem mediated by cells and the humoral with decrease in the
lympho-proliferative response of T and B cells and inhibi-
tion of the activity of T cytotoxic cells [23]. The increase in
the susceptibility to infections as a result of a depression of
the bone marrow may be the main cause of death related to
chronic exposure to benzene [24].

The majority of data available in literature about CBP
and immunotoxicity was obtained from studies in animals
[15,25–35]. Data in humans are scarce and involves groups
of workers during the exposure [36–39]. Except for the risk
of cancer, little is known with respect to immunotoxicity [21]
and the alterations to health among individuals in whom occu-
pational exposure has ceased.

The purpose of this study is to describe and analyze the
phenoctical profile of lymphocytes, comparing the
leucogram values of two groups (individuals with and without
CBP). This study is a part of a broader research, in progress,
about laboratory assessment of immunocompetence, of geno-
toxic alterations and genetic polymorphism.

In spite of the various forms of toxicological assessment,
the evaluation of the immunological response is an important
parameter that could provide information about the immuno-
toxic mechanisms that may be responsible for a series of
reactions leading to pathogeneses. The findings in this study
will help to describe later even in individuals with a history
of CBP.

2. Material and method

2.1. Population and the study area

Within the scope of a wider project “Hematological,
Genotoxic, Immunological Alterations, Genetic Suscepti-
bility and Occupational Exposure to Benzene” in workers
registered in the Work Disease Outpatients (“Ambulatório
de Doenças do Trabalho (ADT)”) of the Worker Health
Study Center (“Centro de Estudos da Saúde do Trabalhador
(CESAT)”) in the period from 1988 to 1999, a hematol-
ogist selected 24 patients with a most probable diagnosis
of chronic intoxication by benzene based on the following
criteria: (1) occupational exposure to benzene; (2) num-
ber of leucocytes lower than 4000 and neutrophils lower
than 2000; (3) decreasing trend in the number of these
cells, as from the beginning of exposure. The controls were
selected from the same file and from where cases came from
(CESAT) diagnosed with other occupational diseases, at the
same time as the cases. The patients mainly originate from
the Metropolitan Region of Salvador, and work or worked
for companies, generally in the industrial sector of this
region.

2.2. Study design

This study is a part of a larger case control study. From the
definition of the two groups, those who have and those who
do not have CBP, a phenotype analysis was carried out of the
lymphocytes and comparisons were established between the
groups.

2.3. Data collection

2.3.1. Questionnaire

The individuals were asked to answer a question-
naire, structured specifically for the assessment of expo-
sure, personal data, determination of life-style factors like
smoking and alcohol and use of medications, among others.

2.3.2. Obtainment of peripheral blood samples

Blood was collected at fasting by venous puncture in the
antecubital region in a vacuum collection tube (Vacutainer®)
with tri-potassium EDTA.

2.3.3. Leucogram

For analyzing the blood sample, a hematological self-
analyzer Adivia 120-Bayer was used. Complete leucograms
were made and the total numbers of leucocytes, lympho-
cytes and segmented neutrophils were analyzed. For all
the reagents used to acquire and analyze the samples,
the manufacturer’s recommendations and guidance were
followed.

Morphological study of the samples in blood smears was
assessed by a hematologist of the group.
2.3.4. Preparation and analysis of peripheral blood lymphocyte populations and sub-populations by flow cytometry

EDTA was placed in a test tube and 2 μL of the following monoclonal antibodies conjugated with fluoro-chromes, in double and triple combinations were added: (1) CD3FITC/CD4PE (for lymphocytes TCD4+); (2) CD3FITC/CD8PE/CD45ROCY (for lymphocytes TCD4−); (3) CD4FITC/CD8PE (for lymphocytes TCD8); (4) CD56PE (for lymphocytes B and NK cells, respectively); (5) CD4FITC/CD8PE/CD25CY (for activated T lymphocytes); (6) CD3FITC/CD8PE/CD56PE (for lymphocytes B and NK cells); (7) CD3FITC/CD4PE (for lymphocytes TCD4+); (8) CD3FITC/CD8PE/CD45ROCY (for memory T lymphocytes). The monoclonal antibodies were obtained from Caltag Laboratories, Bayshore Blvd., Burlingame, CA, USA.

The incubation of the tubes was standardized for 30 min with the above-mentioned antibodies. The red cells were lysed with lysing solution (Beckman-Coulter). After being lysed, each sample was centrifuged twice, at 1000 rpm for 5 min. The cells were re-suspended in 300 μL of PBS.

The acquisition and analysis of immuno-marked cells was standardized for 10,000 events per sample in FACSort BD (US–BD Biosciences Clontech). Percentages were obtained standardized for 10,000 events per sample in FACSort BD. The red cells were included in this study, and no qualitative alterations were shown in the red cells, leucocytes or platelets.

Means of leucocyte counts and its sub-types between groups with CBP and control

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucocytes</td>
<td>3244</td>
<td>6668</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1400</td>
<td>3892</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1375</td>
<td>2091</td>
</tr>
<tr>
<td>Monocytes</td>
<td>254</td>
<td>344</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>296</td>
<td>309</td>
</tr>
</tbody>
</table>

The study population was composed of 48 individuals, 24 with CBP and 24 without CBP. In both groups, there were 22 men and two women. The mean age was 51.1 years in the CBP group, ranging from 36 to 66 years of age, and 50.7 years in the control group, ranging from 36 to 71 years of age (p = 0.866). In general, workers were employed in chemical companies and the mean time of exposure to benzene was 140 months, varying from 24 to 276 months. When this study was conducted, many workers had already been withdrawn from the exposure; the mean time was 112 months varying from 26 to 192 months.

No individual from either of the groups mentioned a history of disease related to hypersensitivity, immunodeficiency or self-immunity, or involvement of the immune system. Of the 48 individuals, only one belonging to the control group reported a recent history of diabetes mellitus.

4.3. Phenotype analysis of lymphocyte sub-populations

Table 3 describes the means of the absolute numbers of lymphocyte sub-populations of the groups with and without CBP. The comparison of the means between groups, however, was done with the value of the mean percentage of each group due to the heterogeneity of the groups in relation to the number of cells. Differences were found in the lymphocyte sub-population T CD3+CD8+ (27.9 ± 11.3 for the
group with CBP and 19.4 ± 5.9 for the control) and in the T memory helper cells (31.2 ± 9.4 for the group with CBP and 37.0 ± 5.9 for the control), data available in Table 4.

5. Discussion

The carcinogenic effect of benzene is recognized by the scientific community. However, studies about the immunotoxic action are rare, generally carried out in animals and are related to the term of exposure. In the literature, no papers analyzing the immunological pattern of individuals with occupational exposure to benzene after exposure had ceased were found.

In one of the rare studies carried out in Brazil [40], the authors evaluated the peripheral hematological condition of patients with CBP after 5 years of having been removed from exposure and showed that 48% of the patients had not normalized their blood cell counts. In the present study, the data demonstrated that leucopenia was maintained even after a period of approximately 11 years after exposure had ceased. This finding may suggest a long-term effect.

The half-life of exhaled benzene in humans varies depending on the benzene exposure concentration and duration. Exposure to 99 ppm for 1 h resulted in an initial phase half-life of 45 min, and exposure to 6.4 ppm for 8 h resulted in an initial phase half-life of 72 min, with a terminal phase half-life (from 10 to 100 h after exposure) of 23–31 h. Because of this short half-life, it is impossible to evaluate past exposure to benzene using biomarkers. In spite of this limitation, chronic health effects caused by an old exposure, such as low blood cell counting, immunological impairments, chromosome aberrations and cancer can be evaluated many years after exposure cessation.

With the delineation and execution of this stage, one is unable to understand the mechanism that leads to the increase in the population of monocytes in peripheral blood, although one recognizes the role of this important phagocyte cell that has the characteristic potential of an antigen-presenting cell. This cell may compensate the decrease of the other important phagocyte type, the neutrophil, in the individual’s defense.

Immunotoxicity studies carried out in animals have shown a reduction in the functions of lymphocytes. These include: reduction in the T and B response to mitogens; in the production of IL-2 by T helper cells; in cytotoxic activity by T cytotoxic cells; production of antibodies by the B lymphocytes; and in the resistance of the macrophage to intracellular infections [41,29]. In studies in humans [36,37], there was evaluated the number of leucocytes in workers during the period of exposure, and found a reduction in the percentages and absolute numbers of total T lymphocytes, lymphocytes TCD4, TCD8, NK cells and an increase in the monocytes count. With these findings, the authors suggest that the depressive effect of benzene on the T cells may be a factor of the probable carcinogenic activity of benzene via the immune system.

The most recent immunophenotyping study using flow cytometry was done in Hungary by Biró and collaborators [39], but as with the other studies it was also carried out during the term of exposure. In this study, no decrease was found in the numbers relative to the lymphocyte sub-populations (CD3, CD4, CD8, CD19, CD25, CD45RO, CD56);
authors point out a super expression in the activation markers like CD4+CD25+, drawing attention to their importance for maintaining self-tolerance. In this study, there was a difference in the mean percentage of lymphocyte sub-populations for the cytotoxic T cells (CD3+CD8+) and T memory helper cells (CD4+CD45RO).

This study shows an increase in the values of lymphocytes TCD3+CD8−, although no alterations had been noted in the NK cell values. Probably, these lymphocytes interceded in a specific way in possible cell alterations to maintain the homeostasis of these individuals, which may, at least partly, justify the non-reporting of serious clinical conditions referred to by the patients.

On the other hand, the memory cells, fundamental for maintaining the individual’s health and balance, are responsible for the protection under secondary challenge by giving the defense system an increased response to the first stimulus with different qualitative and quantitative characteristics. Thus, this cellular phenotype gives and provides long-lasting immunity in different pathological processes. Little is known about the mechanisms involved in the “appearance” of long-life memory cells. Even less is known about the proportions and reference values of the peripheral blood of normal individuals or those involved in pathological processes. Only over the last few years has scientific information appeared, which has culminated in associating the individual’s clinical condition, as well as markers and consequently, the use of methodologies that enable these phenotypes in the blood and tissues of human beings to be characterized.

A reduction was found in the number of B lymphocytes during exposure [38]. However, the results of this study did not show differences between the groups with regard to the percentage of lymphocytes. These results should be analyzed with caution, so that one does not precipitately infer that the lymphocytes are not the target of immunotoxicity to benzene. A study was carried out by Sul and collaborators [42] showed damage to the DNA of B lymphocytes in workers exposed to low levels of benzene. The authors suggested the molecular assessment of the B lymphocytes for the bio-monitoring of human exposure to benzene at low levels.

No serious systemic dysfunctions were identified or were associated with immunodeficiency in the patients of the present study. It is known, however, that there is a well-documented correlation in man between qualitative and quantitative changes in the immune function and the appearance of clinical signs of immunodeficiencies. Many factors, including age and nutritional status, have considerable influence on the immunological competence and harm to the immune system. The characteristics of the group with CBP and control were similar in the present study, and there were no significant differences with regard to age and sex. Therefore, the reduction in the numbers of lymphocytes and sub-populations did not occur as a result of immune system aging, but probably because of exposure to benzene.

Some investigators demonstrated that the relative number of the lymphocyte sub-populations does not reflect their true size and are of limited value, and diagnosis of immunological alterations based on absolute numbers are more recommendable [43]. The absence of reference values for lymphocyte phenotypes in the healthy population reinforces the need for population studies.

Within the limitation of the studied sample, and respecting the characteristics of the present studies, the results observed enable one to draw the following conclusions: workers with a history of hematological alteration due to CBP maintained the leukopenia after a long period of occupational exposure having ceased; the patients in the CBP group presented immunological impairment with alterations in the relative numbers only of the LT cytotoxic and LT memory helper phenotypes; the analysis of the phenotype profile of the lymphocytes and their sub-populations may be useful for monitoring exposed patients.

These results may contribute towards the understanding of the action of benzene on the immune system. Further studies are required to check the cellular immune function.

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