Minimal immunological effects on workers with prolonged low exposure to inorganic mercury

L Soleo, A Vacca, L Vimercati, S Bruno, M Di Loreto, C Zocchetti, R Di Stefano, G Candidio, G Lasorsa, G Franco, V Foa

Abstract

Objectives—This study was carried out to investigate possible immunological changes in workers with prolonged low exposure to inorganic mercury in a fluorescent light bulb factory.

Methods—29 immunological variables were examined in 34 workers with prolonged low level exposure to inorganic mercury (exposed workers) and 35 unexposed workers as the controls. The selected indicator of mercury exposure was concentration of mercury in the urine (U-Hg), which declined progressively from 36.0 µg/l in 1978 to 6.0 µg/l in the study year 1994.

Results—None of the exposed workers had ever shown signs of either acute or chronic inorganic mercury toxicity or had shown any form of hypersensitivity. The only changes found in the exposed workers, compared with the controls, were a reduction of the cells that express cluster differentiation (CD25, T activation antigen (Tac antigen)) and concentrations of tumour necrosis factor-α (TNF-α) in serum. However, the decrease of cells that express CD25 was unrelated to occupational exposure and was, in all likelihood, a chance finding. Conversely, the decline in serum TNF-α was closely associated with occupational exposure. However, no dose-response relation was found between U-Hg and TNF-α concentrations; nor were TNF-α concentrations affected by cumulative occupational exposure to inorganic mercury in over 20 years.

Conclusions—Tentatively, we suggest that reduced serum TNF-α concentrations might be indicative of an in vivo functional defect of the monocyte macrophage system in this particular group of workers even though they were clinically asymptomatic.

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Keywords: inorganic mercury; TNF-α; CD25

Most of the mercury used in industry occurs as inorganic mercury. Occupational (chloralkali plants, production of lamps and batteries, gold mining, dentistry) and environmental (dental amalgams, food) exposures to inorganic mercury are known to be frequent events. Both mercury and its salts induce abnormal responses in animal and human immune systems.1 2 In rats and mice the responses are controlled by the histocompatibility-2 (H-2) system, homologous to the human leukocyte associated (HLA) antigen system.3 4 In brown Norway rats, the most susceptible strain, administration of a non-toxic dose (50 µg/100 g body weight) of mercuric chloride causes cluster differentiation (CD) CD4+ T cell, and B cell proliferation, and an increase in immunoglobulin (Ig) concentrations, mainly IgE, together with production of several autoantibodies, of which those towards the glomerular basement membrane cause membranous glomerulonephritis.5

In Lewis rats, a non-toxic dose causes a significant increase in CD8+ T cells within the secondary lymphoid organs, but does not induce systemic autoimmunity and glomerulonephritis.6

Some susceptible mice strains show immunological alterations overlapping those of susceptible rats; by contrast, the resistant mice rarely develop these alterations.7 8 The effects of inorganic mercury on the human immune system have attracted little attention. Contact dermatitis has been found in subjects as a consequence of occupational and environmental exposure.9-12 Circulating anti-laminin antibodies were found in workers exposed to mercury vapours, but these findings were not confirmed in other studies.13 14

An immune nephrotic syndrome mediated by antibody complexes followed treatment with skin lightening creams and other medications containing mercury.15 16 Increased serum IgA and IgM concentrations and lower IgG concentration were found by Bencko et al17 but not by Langworth et al18 in workers occupationally exposed to inorganic mercury. Likewise, Ellingsen et al19 found no changes in Ig, autoimmune bodies, and complement components in chloralkali workers previously exposed to mercury vapours.

Variables of cell mediated immunity in subjects occupationally exposed to inorganic mercury20 21 or in those exposed to metallic mercury vapour from amalgam fillings22 were investigated in a few studies. Only Mosczynski and Slowinski23 showed an increase in CD4, and mainly in CD8 lymphocytes, with a resulting low CD4/CD8 ratio essentially related to the duration of exposure. Langworth et al20 studied in vitro production of interleukin-1 (IL-1) and TNF-α, without finding any alterations.

Department of Internal and Occupational Medicine L Soleo L Vimercati G Candidio G Lasorsa

Department of Biomedical Sciences and Human Oncology University of Bari Medical School, Bari, Italy A Vacca S Bruno R Di Stefano G Franco

Section of Immunohematology, Pediatric Hospital “Giovanni XXIII”, Bari M Di Loreto

Clinica Del Lavoro “Luigi Devoto”, Milan, Italy C Zocchetti V Foa

Correspondence to: Professor Leonardo Soleo, Dipartimento Di Medicina Interna E Del Lavoro, Universita’ Di Bari, Policlinico, Piazza G Cesare 11, I-70124 Bari, Italy.

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The aim of this study was to investigate the effects of prolonged low exposure to inorganic mercury on a wide range of immunological variables.

Methods

RECRUITMENT OF SUBJECTS

Thirty four male workers exposed to inorganic mercury for more than 20 years (mean (SD) 21.5 (1.2)) were recruited in 1994 from a fluorescent light bulb factory (exposed workers). They had never shown any symptoms of chronic toxicity from inorganic mercury, although this was not a criterion for selection.

Thirty five male workers who were not occupationally exposed to inorganic mercury were recruited in the same year to be the control group. These men were active in the chemical industry and in power plants without exposure to either solvents or metals. They were randomly selected from a group of workers participating in a surveillance programme that covered all the main industries in the area. The two groups were age matched (±3 years) (table 1).

Besides giving their job history, all workers were requested to fill in a questionnaire on the possible causes of non-occupational exposure to mercury: fish consumption as a possible source of exposure to organic and inorganic mercury and number of teeth filled with amalgam, which is made up of about 50% inorganic mercury. Alcohol intake and cigarette smoking habits were also considered as environmental factors. None of the differences between the two groups for the investigated variables was found to be significant (table 1). Both groups underwent a thorough medical examination. The investigating physician checked for the absence of immunological and immunmediated diseases, acute infections, and use of drugs that acted upon the immune system.

The study was approved by the local ethics committee and informed consent was obtained from all workers.

EXPOSURE TO INORGANIC MERCURY

The level of exposure to inorganic mercury was monitored by taking measurements of mercury concentrations in the working environment and of U-Hg excretion (table 2). Environmental surveys carried out with static samplers at different work places in the assembly manufacturing lines over 17 years, from 1978 to 1994, showed that average environmental concentration was always below the threshold limit value (TLV-TWA), or 0.05 mg/m³, allowed in the working environment by the American Conference of Governmental Industrial Hygienists (ACGIH). Before the study year 1994, and only separate values above the TLV-TWA were recorded sporadically at one workplace: 0.13, 0.085, and 0.055 mg/m³ in 1978, 1980, and 1983, respectively. Also, average environmental concentration gradually decreased over the years due to technical improvements in the production process by which the input of inorganic mercury into the working environment was curtailed.

Owing to the fact that the organisation of labour caused a continuous transfer of workers to other workplaces at the same plant, each having a different load of mercury, exposure of individual workers could not be assessed by environmental monitoring. It was, therefore, decided to refer to U-Hg as the appropriate indicator of exposure. The U-Hg was measured with the cold vapour atomic absorption technique (Spectrometer model 460 and MHS-1 idrie generator, Perkin-Elmer). Twenty four hour urine samples were collected by each subject every six months, at the end of a working week between Thursdays and Fridays (urine collecting time also included working hours at the factory) over the 17 years 1978–94. Average annual values were calculated for the whole group. Along with progressive abatement of environmental exposure to inorganic mercury, there was a progressive reduction of U-Hg values over the years until the lowest values were recorded in 1994 (table 2).

Two indicators of exposure to inorganic mercury were considered: U-Hg at the time of the immunological survey (current U-Hg), represented by the mean of the two U-Hg values in each worker in 1994, and a cumulative exposure indicator (cumulative U-Hg) obtained for each person by adding up the mean urinary mercury values for each year between 1978 and 1994. Cumulative U-Hg expresses the total amount of mercury to which each worker had been exposed during the years.
covered by the study. Current U-Hg and cumulative U-Hg (mean (SD) 259.1 (97.6), range 140.2–446.8) were found to be only partially correlated (r=0.30, P=0.08). The current U-Hg of exposed workers was significantly higher than that of the unexposed workers (table 2).

Furthermore, multiple regression was used to assess the role of occupational versus non-occupational exposure in assessing current U-Hg concentrations. It was found that occupational exposure to inorganic mercury is by far the most important factor affecting current U-Hg concentrations (table 3). A comparison between mean current U-Hg values from occupational exposure and from the presence of dental amalgams further highlights the importance of industrial exposure in assessment of current U-Hg concentrations (table 4).

IMMUNOLOGICAL VARIABLES

After medical examination, a peripheral venous blood sample was obtained from each subject and collected into sterile and heparinised Vacutainer test tubes for measurement of the various selected immunological variables, which was done immediately after sampling. The double blind approach was used in all cases.

BLOOD COUNTS

Leucocyte counts, differential counts, and other blood counts were measured with a cell counter (CELL.DIN 3000, Ciampolini, Milan, Italy).

LYMPHOCYTE POPULATIONS

These were assessed by flow cytometry, as described elsewhere. Briefly: 100 μl blood were incubated for 20 minutes at +4°C with 10 μl labelled fluorescein isothiocyanate (FITC) anti-CD3 (Leu-4), anti-CD4 (Leu-3), anti-CD8 (Leu-8), anti-CD19 (Leu-12), anti-CD20 (Leu-16), anti-CD25 (IL-2R) and anti-human leucocyte associated D receptor (HLA-DR) monoclonal antibodies, all from Becton Dickinson. Immediately after incubation, the cellular suspension was exposed to lysing solution G (Becton Dickinson) for 10 minutes at room temperature, washed twice with phosphate buffered saline (PBS), and was immediately (without being fixed) analysed with the FACScan flow cytometer (Becton Dickinson), equipped with Window Lysis 2 system and Consort 32 computer.

CD3 is expressed by all T cells and thus it is a pan-T marker; CD4 and CD8 mark T cells with helper inducer and cytotoxic suppressor functions, respectively; CD19 and CD20 are pan-B markers; CD25 is the chain (Tac antigen) of the IL-2 receptor and is expressed by activated B cells, natural killer cells, and T cells; HLA-DR is expressed by B cells and activated T cells. Isotype control antibodies consisted of murine indifferent FITC-labelled IgG (Becton Dickinson) which gave <3% background.

SERUM IgG, C3-C4, IMMUNE COMPLEXES, AND AUTOANTIBODIES

Serum IgG, complement fraction 3 (C3) and C4, immune complexes (ICs), and autoantibodies were measured by nephelometry (Nephelometer BN 100 Behring Institute, Scoppito, Italy) as described previously. Assays were carried out on IgG (SAS 15 NlGg), IgA (SAR 15 NlGa), IgM (SAT 15 NlGm), IgE (UXA 11 NA Latex), the C3 (SAP 15 NC3c), and C4 (SAO 15 NC4) complement fraction components, the immune complexes (IC, 11N Rea Latex CIC), and anti-IgG IgM (rheumatoid factor, WA 11 NA Latex); the nephelometer was calibrated with standard seroproteins (SAU 03 N) and quality control (SKE 07 N/T).

An immunoblotting test (Delta Biologicals, Pomezia, Italy) was used to measure the anti-nuclear, anti-mitochondrial, anti-smooth muscle (F-391), anti-double-stranded DNA (F-158), anti-nuclear scleroderma 70 (Cl-70) antigen (I-470), and anti-ribonuclease protein Sm (I-410), sicca syndrome A, and B (I-450) autoantibodies.

SERUM CYTOKINES

The enzyme linked immunosorbent assay (ELISA) sandwich (Medenix Diagnostics SA, Fleurus, Belgium) was applied to measure concentrations of IL-1, IL-2, interferon-γ (IFN-γ), and TNF-α, as described elsewhere. Briefly: two monoclonal antibodies that recognise distinct epitopes of each cytokine were used. The cytokine in the serum samples or standard solutions bound to the first monoclonal antibody adsorbed to polystyrene microtiter wells. After overnight incubation at 4°C and PBS washings to remove the unbound cytokine, the wells received the second monoclonal antibody tagged with horseradish peroxidase, which completed the sandwich. A substrate solution was then added to the wells, and after one hour of incubation, their absorbance was measured at 490 nm with a Titertek ELISA reader. The concentrations of each cytokine were calculated on a calibration curve set up with the standard solutions.

T CELL PROLIFERATIVE ACTIVITY

This was measured in 18 exposed and 18 non-exposed randomly chosen workers as described...
Briefly, peripheral blood mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. After depleting adhering cells on plastic dishes, T cells were isolated by passing peripheral blood mononuclear cells through nylon wool columns and were then cultured in triplicate in U-bottomed 96 well plates (1x10⁵ cells/well). Their proliferative activity after phytohaemagglutinin (PHA) (Wellcome Diagnostics) stimulation (1 μg/ml) was assessed by ³H-thymidine uptake after four days.

STATISTICAL ANALYSIS

Frequency distribution and basic statistics (mean, median, range, SD, skewness, kurtosis) were computed for all quantitative variables, and log transformation was applied to those which showed some indication (mainly skewness) that they were not gaussian. Associations between categorical variables were assessed with the χ² test with appropriate degrees of freedom. Correlation and multiple regression analyses were used to study the effect of a set of predictors on the different quantitative response variables. Comparisons between mean values were made with analysis of covariance (ANCOVA) or analysis of variance (ANOVA) with and without control for some covariates (after log transformation no major violations of the assumptions for these analyses were found). Results of such analyses are reported in terms of the F test and its associated probability value. As well as parametric techniques, the Wilcoxon non-parametric test was applied to the comparison of some variables—for example, TNF-α—between exposed workers and controls. All the statistical analyses were performed with SAS (version 6.10).

Results

Blood counts and white blood cell subtypes were within the normal range for all the workers.

The circulating lymphocyte absolute value and the percentage of total T cells (CD3) and of their CD4 and CD8 subsets, the CD4/CD8 ratio, and concentrations of total B cells (CD19 and CD20), and HLA-DR cells of exposed workers and controls were comparable. Only the percentage of cells expressing CD25 was significantly lower in the exposed workers (table 5). There was no difference in T cell proliferative activities of the two groups (table 5).

Several serum variables, including IgG, C3-C4, IC, and autoantibody concentrations were similar in the exposed and in the control workers (table 6).

Seventy-eight workers and 39 controls were examined for cytokine concentrations. A two-way ANOVA (CD3 and CD8) or a Wilcoxon test (CD19 and CD20) was performed. There were no significant differences in any of the comparisons (table 7).

An attempt was made at evaluating a dose-response relation between current U-Hg and CD25 or TNF-α concentrations, irrespective of occupational or non-occupational exposure (figure). Notwithstanding the tendency of the concentrations of cells expressing CD25 to diminish with increasing U-Hg, the relation was not significant. Less definite, and still not significant, is the relation between U-Hg and TNF-α.

We thought it useful to consider both the effects of current and of cumulative U-Hg on concentrations of cells expressing CD25 and TNF-α on occupationally exposed workers. No associations of any kind were found with concentrations of cells expressing CD25.

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Table 5

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exposed (n=34)</th>
<th>Control (n=35)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes/mm³</td>
<td>2228 ± 370</td>
<td>2171 ± 335</td>
<td>0.45 0.50</td>
</tr>
<tr>
<td>CD3 (%)</td>
<td>60.0 ± 7.3</td>
<td>54.8 ± 10.1</td>
<td>3.07 0.09</td>
</tr>
<tr>
<td>CD4 (%)</td>
<td>45.2 ± 7.0</td>
<td>43.1 ± 7.4</td>
<td>0.59* 0.45</td>
</tr>
<tr>
<td>CD8 (%)</td>
<td>31.8 ± 9.0</td>
<td>30.9 ± 7.5</td>
<td>0.11 0.74</td>
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<tr>
<td>CD4/CD8</td>
<td>1.5 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>1.09* 0.03</td>
</tr>
<tr>
<td>CD19 (%)</td>
<td>10.9 ± 4.2</td>
<td>12.2 ± 3.6</td>
<td>1.97 0.18</td>
</tr>
<tr>
<td>CD20 (%)</td>
<td>11.8 ± 4.4</td>
<td>12.8 ± 3.3</td>
<td>1.01 0.32</td>
</tr>
<tr>
<td>HLA-DR (%)</td>
<td>3.2 ± 0.8</td>
<td>3.4 ± 0.6</td>
<td>0.35 0.55</td>
</tr>
<tr>
<td>CD25 (%)</td>
<td>1.5 ± 0.7</td>
<td>1.9 ± 0.8</td>
<td>5.48* 0.02</td>
</tr>
</tbody>
</table>

*Test performed on log transformed values.
†Was calculated as % H-thymidine uptake on 18 exposed and 18 control workers. Values from unstimulated cultures were 20.5 (3.2) and 25.4 (4.6), respectively.

Table 6

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exposed (n=34)</th>
<th>Control (n=35)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG (mg/dl)</td>
<td>1514.0 ± 272.3</td>
<td>1571.8 ± 452.4</td>
<td>0.41 0.52</td>
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<tr>
<td>IgA (mg/dl)</td>
<td>292.4 ± 101.9</td>
<td>335.2 ± 189.6</td>
<td>0.25* 0.62</td>
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<tr>
<td>IgM (mg/dl)</td>
<td>150.5 ± 73.2</td>
<td>193.5 ± 164.2</td>
<td>1.01* 0.32</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>79.0 ± 87.9</td>
<td>71.2 ± 60.1</td>
<td>0.01* 0.91</td>
</tr>
<tr>
<td>C3b (mg/dl)</td>
<td>92.7 ± 14.0</td>
<td>95.1 ± 31.2</td>
<td>0.01* 0.92</td>
</tr>
<tr>
<td>C4 (mg/dl)</td>
<td>35.0 ± 10.5</td>
<td>39.6 ± 28.5</td>
<td>0.11* 0.74</td>
</tr>
<tr>
<td>IC (gp/ml)</td>
<td>5.8 ± 12.2</td>
<td>2.5 ± 2.5</td>
<td>3.09* 0.08</td>
</tr>
</tbody>
</table>

*Test performed on log transformed values.
Peripheral blood cells expressing CD25 and concentrations of TNF-α in serum samples in comparison with current U-Hg in occupationally exposed and control workers.

Concentrations of TNF-α were associated with current U-Hg only (F=3.74, P=0.026), whereas no relation was found for cumulative U-Hg.

Discussion
In 1994, 34 workers occupationally exposed to inorganic mercury were tested for 29 immunological variables. Exposure had lasted for more than 20 years, although only at average environmental concentrations—as calculated between 1978 and 1994—that were always below the TLV-TWA standard recommended by the ACGIH (0.05 mg/m³). The biological indicator of exposure, U-Hg, was also low and decreased further, as environmental exposure declined progressively, from an average 36 μg/l in 1978 to 6 μg/l in 1994. The exposed workers had significantly higher concentrations of U-Hg than the controls. Evaluation of total exposure to inorganic mercury indicated that higher U-Hg excretion in the exposed workers was mostly due to occupational exposure. Compared with the control workers, the 34 exposed workers only showed significant decreases in the percentage of the peripheral lymphocytes that expressed CD25 and in concentrations of TNF-α in serum samples.

In the exposed workers, the decrease of cells expressing CD25 did not correlate with U-Hg (both current and cumulative) according to a dose-response relation. Cells expressing CD25 include activated B cells, T cells, and natural killer cells. Under normal conditions, they account for 1%-2% of total T cells as in the control workers. However, in the exposed workers, still lower values cannot be indicative of a reduced activation capability of these cell populations, as very few if any CD25 cells can be found in normal conditions. On the other hand, no functional data are provided by this work. Lastly, without a dose-response correlation and a significant association with U-Hg, this finding is not significant.

The TNF-α values too were lower in exposed workers, but no dose-response relation was found. However, when only the occupationally exposed workers were considered, it seemed that the decrease in TNF-α was associated with increases in current U-Hg, although not in cumulative U-Hg, which seems to suggest that the effect on TNF-α is only transient. Possibly, by moving the worker away from the source of hazard, or by decreasing environmental exposure to inorganic mercury, TNF-α can get up to values of control workers, similar to the findings for Ig, complement proteins, and autoantibodies. Tumour necrosis factor-α is a typical product of monocyte macrophages. Lower TNF-α concentration in serum could then be indicative of a subtle condition of in vivo functional deficit after occupational exposure to low dose inorganic mercury, although this has not been proved here. Also, Langworth et al were unable to prove a functional deficit with in vitro systems. Therefore, the exposure to inorganic Hg cannot imply the functional deficit of the monocyte macrophage cell population. Tumour necrosis factor-α modulates the function of certain immune cells: it enhances proliferation of T cells, T cell receptor expression, natural killer cell cytotoxicity, phagocytosis, and adherence to endothelial cells by neutrophils, proliferation of fibroblasts, and secretion of IL-1, IL-2, and granulocyte macrophage-colony stimulating factor (GM-CSF), which are all functions involved in the inflammatory and immune response, as well as in the antitumour defence. The decreased serum concentrations of TNF-α in exposed workers might be associated with a decline in one or more of these functions. However, this warrants a functional assessment on the different cell populations, which will be made in a further study. It has been shown in vitro by Steffensen et al that mercury chloride can injure the cell membranes of human T and B cells and monocytes; they suggested that the mechanism of action could either be the binding of mercury to membrane structures or the formation of precipitates. Such events could be
due to the well documented high affinity of mercury for cell thiol groups, as a decrease of such groups seems to play an important part in the inhibition of the cell activation processes. Research on rodents has allowed identification of a genetic predisposition as a necessary condition for the manifestation of abnormal immune response after administration of immunotoxic chemicals, such as mercury. It may well be that in humans also genetic factors play an important part in modulating the immune response to inorganic mercury; however, further studies are required before this point can be confirmed. Little is known about the possible existence in humans of a threshold dose of mercury capable of evoking an immune response. It has been found in susceptible rats that the lowest dose producing autoimmune effects was 50 μg/100 g body weight. This dose corresponds to 16 μg/kg body weight as ionic mercury, equivalent by extrapolation to about 1000 μg for a 70 kg adult human. Our study shows that occupational exposure to much lower environmental concentrations of inorganic mercury (0.002–0.024 mg/m³) than those given to hypersusceptible rats may produce a substantial decrease of serum TNF-α. In any case, the workers showed no clinical symptoms and no alteration of blood counts that could be correlated with such a finding. The meaning of this association and the possible effects upon the workers’ health need elucidation by further investigations.

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