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Defective Neutrophil Function in Workers Occupationally Exposed to Lead

M. L. S. Queiroz[1], M. Almeida[2], M. I. Gallão[2] and N. F. Höehr[3]

[1]Department of Pharmacology, [2]Haemocenter, and [3]Department of Clinical Pathology, Faculty of Medical Sciences, State University of Campinas, Campinas, CEP 13081, P.O. Box 6111, SP, Brazil

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Abstract: Chemotaxis and niroblue tetrazolium reduction were measured in peripheral blood neutrophils of workers occupationally exposed to lead. These two neutrophil functions were significantly reduced, as compared to controls, even in those workers with blood lead levels and urinary delta-aminolevulinic acid (ALA-U) concentrations below the currently accepted biological limit values of 60 μg/dl and 6 mg/l, respectively. The immunosuppressive effects of relatively low level lead absorption suggests that immune dysfunction may be a sensitive indicator of lead exposure.

Despite the well documented effects of lead on cellular and humoral immunity in experimental animals (Faith *et al.* 1979; Blakley & Archer 1981; Kowolenko *et al.* 1989), there have been few systematic studies of the influence of this metal in the human immune system. Immunosuppressive effects were observed in workers with toxic, high-level lead exposures (Jaremin 1983; Governa *et al.* 1988). Of particular interest, however, are those responses which may be elicited by "safe" levels of lead absorption, particularly since it has been demonstrated that relatively low-dose exposure may cause adverse effects which are not evident during a standard clinical examination (Landrigan 1989).

Since the ultimate actions of lead are exerted at the cellular level (Alessio & Foà 1983) any study evaluation of the adverse effects of this metal needs to take into account possible functional alterations to cells and tissues. Based on these assumptions and on previous clinical work in our laboratory (unpublished observations), we designed the present study to investigate the existence of alterations in the function of neutrophils isolated from workers exposed to lead acid batteries and who had lead absorption parameters ranging from "safe" to toxic levels.

Materials and Methods

Population. A total of 39 male workers who had been working for at least six months in storage-battery plants were studied. These plants smelt, refine and recast lead, mostly from automobile batteries. The mean exposure period to lead was 4 years (range: 0.5–20). The mean age of the workers was 33.9 ± 12.1 years (range: 18–56). Blood lead levels ranged from 14.8–91.4 μg/dl and the distribution among the workers was as follows: <30 μg/dl: 7 workers; 30–40 μg/dl: 4 workers; 40–50 μg/dl: 4 workers; 50–60 μg/dl: 7 workers; 60–70 μg/dl: 12 workers; >70 μg/dl 5 workers. The workers were chosen by random selection, with the exception that they had to have been employed for at least six months. Control subjects of comparable age and race with no history of lead exposure were chosen from blood donors arriving at the University hospital blood bank.

Each worker was examined in a standard fashion by a physician. A complete occupational history was noted and included the occurrence in the preceding six months of symptoms possibly related to lead exposure as well as observations on past episodes of lead poisoning and of chelation therapy and incidence of infections. A similar protocol was applied to the control individuals.

Urine and venous blood samples from each worker were collected in lead-free vacutainers for a laboratory check of lead absorption parameters and for the study of neutrophil chemotaxis and nitroblue tetrazolium reduction activity. Sampling was always performed between 8:00–9:00 a.m., when the subjects had been fasting for at least 12 hr. To avoid errors arising from inaccurate collection of 24 hr urine samples, use was made of spot samples voided during the period of sampling.

Isolation of leucocytes. Five ml of venous blood were collected into 20 U/ml heparin and the plasma separated after centrifugation at $500 \times g$ for 10 min. at room temperature. The cell sediment was resuspended in medium TC 199, mixed with 1 ml of 6% dextran (molecular weight 153,000 Sigma), and allowed to stand at 37° for 25 min. Leucocyte rich supernatant was then collected and washed three times with medium TC 199. The cells were resuspended in 1 ml of TC 199 and the final concentrations adjusted to 2×10^6 cells/ml.

Chemotaxis. Neutrophil chemotaxis was measured by the leading front method in modified Boyden migration chambers using millipore membranes with a pore diameter of 3 μm (Akenzua & Amenghene 1981). Chemotaxic factor was prepared by adding 0.1 ml of endotoxin solution (2.5 mg/ml of lipopolysaccharide B from *Escherichia coli* 0127:B8 Sigma in phosphate buffered saline, pH 7.5) to 0.5 ml of autologous plasma and 4.4 ml of medium TC 199. To evaluate non-stimulated migration the chemotactic factor was replaced by medium TC 199. The chambers were kept at 37° for 30 min. in a moist atmosphere and the membranes then washed, stained with Harris' haematoxylin, cleared in isopropyl alcohol and xylene, and mounted on a microscope slide with Canada balsam. To measure the distances migrated by polymorphs the membrane was examined on a microscope fitted with a micrometer on the fine adjustment. Using an oil immersion objective at a $100\times$ magnification we focussed on the top of the membrane and then through the membrane until only three cells were in view. The reported values are the mean of five different fields. Results were expressed in micrometres as the difference between the migration of neutrophils with and without endotoxin.

Nitroblue tetrazolium test. A modified stimulated nitroblue tetrazolium test was performed as described previously (Falcão *et al.* 1982). Briefly, 0.1 ml of peripheral blood collected in EDTA was mixed with an equal volume of nitroblue tetrazolium solution (Sigma, 0.1%

nitroblue tetrazolium in PBS) plus 0.05 ml of endotoxin solution (1 mg/ml of lipopolysaccharide B from *Escherichia coli* 0127:B8 Sigma in PBS). This mixture was incubated at 37° for 15 min. and then centrifuged at 400 × g for 3 min. and the supernatant discarded. The cells were gently resuspended in a small volume of foetal calf serum and the smear made with special care to avoid damage to the white cells. The smears were air dried and stained with Leishman stain. Using a 100 × oil immersion objective, 200 neutrophils were counted and only those with large blue deposits in the cytoplasm were scored as positive.

Analytical methods. Lead concentration in blood was measured by atomic absorption spectrophotometry (Zeiss, FMD4) according to the direct chelation-extraction method (Hessel 1968). The accuracy and precision of this method were determined by analysing standardised bovine blood obtained from Koulson Laboratories Inc. The results were 43 ± 7 μg/dl (c.f. the control value of 45 ± 7 μg/dl) for five samples. The lead concentration in the specimen was determined by comparing its absorbance with that of a standard curve prepared from reference samples. In the range from 20–100 μg/100 ml, the relative error was of the order of 10% with a standard deviation of approximately 8 μg/100 ml.

Urinary delta-aminolevulinic acid (ALA-U) concentrations were measured by UV spectroscopy at 555 nm (Beckman model 14) following extraction into ethyl acetoacetate and reaction with Erlich reagent (Tomokumi & Ogata 1972).

Statistics. Statistical comparison of the results from exposed and non-exposed individuals was performed using the Mann Whitney U-Test. To ascertain the effect of the duration of exposure to lead on the immune response, one-way analysis of variance and the Duncan test were used.

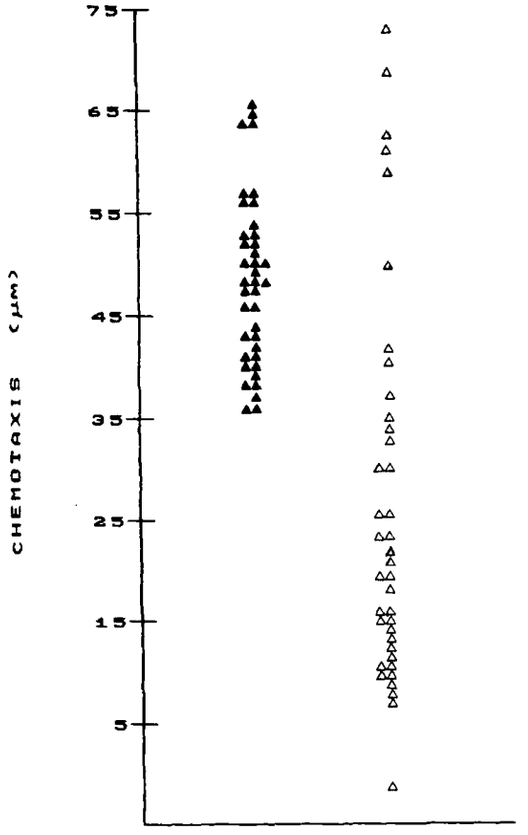

Fig. 1. Neutrophil chemotaxis of normal subjects (▲) or lead-exposed workers (△). Data are reported as neutrophil mobility in micrometres (n = 39). P < 0.001 (Mann-Whitney U-test).

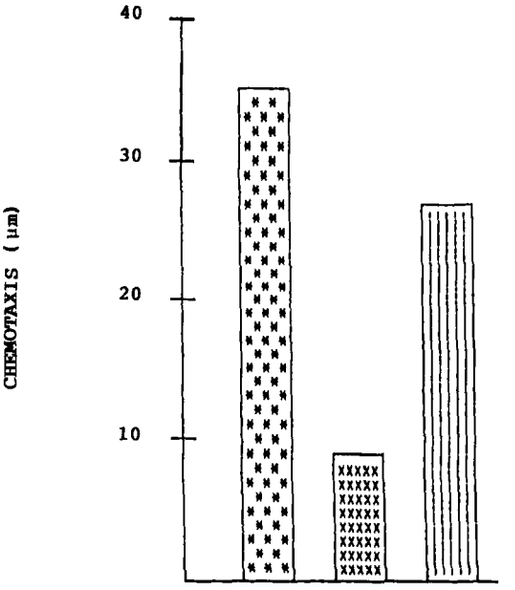

Fig. 2. Neutrophil chemotaxis of normal subjects (**) (n = 39) and lead-exposed workers with blood lead levels below (× × ×) (n = 17) and above (|||) (n = 22) the biological limit value of 60 μg/dl blood. P < 0.001 (Mann-Whitney U-test). Results are presented as medians.

Results

To monitor exposure we used blood lead levels as a measure of internal dose and delta-aminolevulinic acid in urine (ALA-U) as an indicator of effect. Twenty-two (56.4%) out of the 39 workers studied presented blood lead levels within the range of currently accepted biological limit values (40–60 μg/dl of blood). The average blood lead value for these 22 workers was 41.1 ± 12.65 μg/dl. ALA-U concentrations were below the currently accepted biological limit of 6 mg/l of urine in 16 (66.6%) of these 22 workers. None of the controls (n = 39) had blood lead levels above 10 μg/dl.

The individual values for neutrophil chemotaxis in the 39 workers and the 39 controls are plotted in fig. 1. A significant (P < 0.001 Mann Whitney U-test) impairment in neutrophil migration was observed in the exposed workers. Moreover, when polymorphonuclear migration results from only those workers presenting blood lead (fig. 2) or ALA-U (fig. 3) concentrations below the biological limit values were considered, a marked impairment as compared to non-exposed individuals was still observed. Similar results were obtained with the group of workers presenting blood lead or ALA-U concentrations above the biological limit values (fig. 2 and 3).

Fig. 4 compares the individual percentage of nitroblue tetrazolium-positive neutrophils in the exposed and non-exposed groups. A statistically significant impairment (P < 0.001 – Mann Whitney U-test) was observed in the exposed group. As with chemotaxis, the mean percentage

of nitroblue tetrazolium-positive neutrophils from workers with blood lead (20.8 ± 14.5) (fig. 5) and ALA-U (9.9 ± 7.9) (fig. 6) concentrations below the biological limits was significantly reduced as compared to non-exposed individuals (49.4 ± 11.5).

In relation to the duration of exposure, we observed a more pronounced impairment in the percent of nitroblue tetrazolium reduction in workers exposed for a period of up to 1 year when compared to workers exposed for longer periods. A reduction in chemotaxis, on the other hand, was not dependent on the length of exposure (table 1).

Discussion

It has been well demonstrated that lead suppresses the immune response in animals and that this suppression often occurs at subclinical dosages (Koller 1980). However, information about the effects of lead in the human immune response is still very limited.

In workers exposed to lead, a decrease in phytohemaglutinin-induced blast lymphocyte transformation (Jaremin 1983) and impaired chemotactic activity of polymorphonuclear leukocytes (Governa *et al.* 1988) have been observed. Such immunosuppressive effects, however, were investigated following toxic, high-level lead exposures. In another study, (Ewers *et al.* 1982), low serum complement C3, IgM, IgG and IgA levels were found in lead-exposed workers. These authors observed a negative correlation between blood lead levels and complement C3 and IgG as well as a linear correlation between IgA and blood lead levels. On the other hand, Kimber *et al.* (1986) observed no changes in immunoglobulin levels, phytohemaglutinin induced blast lymphocyte transformation and natural killer cell function in individuals exposed to low levels of lead.

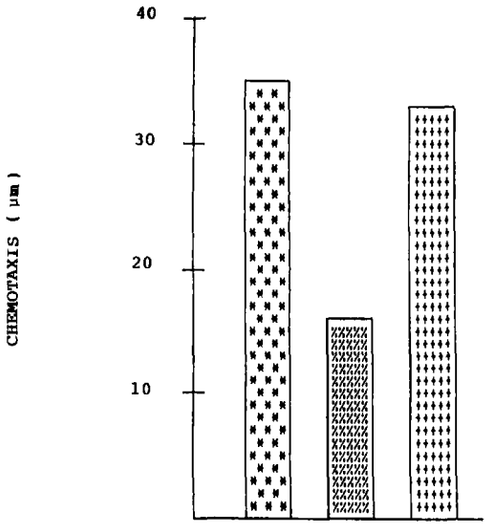

Fig. 3. Neutrophil chemotaxis of normal subjects (**) (n = 39) and lead exposed workers with urinary aminolevulinic acid concentration below (%%%) (n = 16) and above (+++) (n = 9) the biological limit value of 6 mg/l blood. $P < 0.001$ (Mann-Whitney U-test). Results are presented as medians.

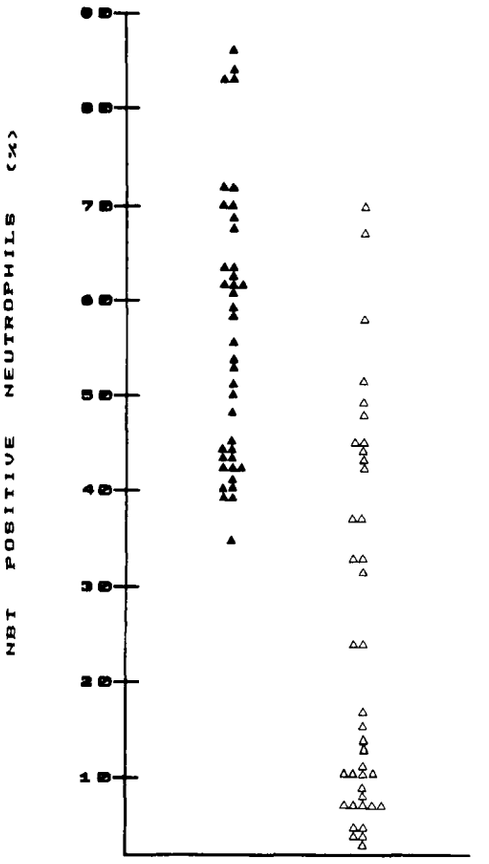

Fig. 4. The percentage of nitroblue tetrazolium positive neutrophils from normal individuals (▲) (n = 39) or lead-exposed workers (△) (n = 39). $P < 0.001$ (Mann-Whitney U-test).

In our study, a marked impairment of polymorphonuclear chemotaxis and nitroblue tetrazolium-dye reduction were observed in lead-exposed workers presenting lead absorption parameters ranging from "safe" to toxic levels. Of the 39 workers studied, 22 presented blood lead levels below the biological limit values. In 18 of these 22 individuals, blood lead concentrations had not exceeded 60 µg/dl within at least 6 months prior to the study. These results suggest that "safe" levels of lead exposure may lead to immunosuppression.

Our observation of impaired chemotaxis suggests that this deficiency may have been due to an intrinsic cell defect rather than to a plasma inhibitor, since the cells were washed prior to use. Impairment of chemotaxis may be explained by the reported changes in cell membrane fluidity in lead-acid battery workers (Valentino *et al.* 1982) and by the *in vitro* findings of lead interference with microtubule assembly (Roderer & Doenges 1983). It has been demonstrated that leukocytes with a deficit in microtubule assembly possess a decreased capacity for locomotion *in vitro* and *in vivo* (Allan & Wilkinson 1978; Becker 1990).

Nitroblue tetrazolium-dye reduction can occur in neutro-

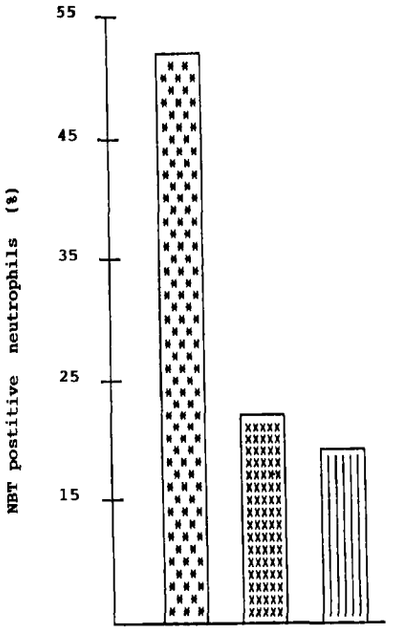

Fig. 5. The percentage of nitroblue tetrazolium (NBT) positive neutrophils from normal individuals (**) (n=39) and lead-exposed workers with blood lead levels below (× × ×) (n=21) and above (|||) (n=18) the biological limit value of 60 μg/dl blood. $P<0.001$ (Mann-Whitney U-test). Results are presented as medians.

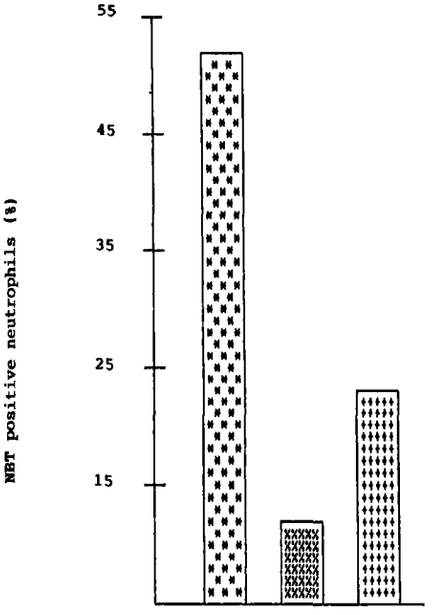

Fig. 6. The percentage of nitroblue tetrazolium (NBT) positive neutrophils from normal individuals (**) (n=39) and lead-exposed workers with urinary delta aminolevulinic acid concentrations below (%%%) (n=16) and above (+ + +) (n=9) the biological limit value of 6 mg/l blood. $P<0.001$ (Mann-Whitney U-test). Results are presented as medians.

phils after previous phagocytosis of the dye. We have found (unpublished observations) that the phagocytic capacity but not the lytic function, of neutrophils from lead-exposed workers is normal, thus indicating that the observed impairment in respiratory burst activity, as measured by nitroblue tetrazolium reduction, is not due to defects in polymorphonuclear phagocytosis. An explanation for this finding may be found in the metabolic changes occurring in leukocytes of workers exposed to lead. It has been demonstrated that the elevated blood ALA concentration in workers exposed to lead may act as an endogenous pro-oxidant (Hermes Lima *et al.* 1991). *In vitro*, ALA has been shown to yield reactive oxygen species and *in vivo* to cause a shift from aerobic to increased glycolitic metabolism due to impairment of mitocondrial functions (Pereira *et al.* 1992). According to Halliwell & Gutteridge (1989), the electron transport chain of mitocondria, together with that of the endoplasmic reticulum constitute the most important sources of superoxide *in vivo* in most aerobic cells. Therefore it is possible to assume that the decreased ability of the neutrophils of these workers to internally reduce nitroblue tetrazolium is attributed to lower intracellular superoxide production due to mitocondrial injury.

Although impairment of chemotaxis and nitroblue tetrazolium reduction were observed in the lead-exposed workers regardless of the length and level of exposure, the extent of impairment in nitroblue tetrazolium reduction, but not in chemotaxis, was significantly deeper in workers exposed to lead for periods up to one year, as compared to those exposed for longer periods (table 1). This inverse dose-response relationship between length of exposure and nitroblue tetrazolium-dye reduction function may be associated

Table 1.

The effect of duration of exposure on chemotaxis and nitroblue tetrazolium (NBT) reduction in neutrophils isolated from lead-exposed workers.

Duration of exposure	N	Chemotaxis (μm) Mean ± S.D.	Median	NBT reduction (%) Mean ± S.D.	Median
0	39	53.5 ± 17	50	49.3 ± 11	46
0.5–1 yr	13	21.0 ± 18	25	15.1 ± 11**	13
1.1 yr–5 yr	16	27.3 ± 20	25	30.8 ± 19*	24
> 5 yr	10	19.3 ± 20	8	22.8 ± 21*	22

* Significantly reduced as compared to controls.

** Significantly reduced as compared to control and the other exposed groups.

$P<0.05$ – one way analysis of variance followed by the Duncan test.

with proliferation of mitocondria which occurs as a result of endurance oxidative stress (Davies *et al.* 1982).

Increased incidence of infections in the lead exposed group was not found. This fact may be partly explained by the observation that chemotaxic defects as well as deficiency of some neutrophil enzymes involved in respiratory burst activity contribute little to the decreased resistance to infections (Boxer & Morganroth 1987).

Adverse effects that may occur at relatively low levels of exposure to lead are of particular interest in relation to preventive action, since these effects can be regarded as a critical piece of information when discussing, for example, exposure limits. Our results strengthen further the argument for the elimination of lead hazard in working places. Furthermore, our studies also indicate that polymorphonuclear may provide a sensitive functional indicator of heavy metal and other chemical contaminants present in the environment.

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