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Effect of Subacute Exposure to Lead and Estrogen on Immature Pre-Weaning Rat Leukocytes

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Lead is an environmental pollutant known to cause damage to human health, affecting specially the central nervous system, reproductive organs, the immune system and kidney.

From the perspective of reproduction, lead affects both men and women. Reported effects in women include infertility, miscarriage, pre-eclampsia, pregnancy hypertension and premature delivery (Winder 1993). In experimental animals, lead affects female reproductive organs through different mechanisms. The heavy metal may interact at the enzyme level (Wiebe et al. 1988). It may interfere with the action of reproductive hormones at the target organ, modifying the activity of estrogen receptors in the pregnant uterus (Wide & Wide 1980) and inhibiting responses where estrogens play a role (Wide 1980). Lead may induce imprinting mechanism (Csaba et al. 1986; Tchernitchin & Tchernitchin 1992), causing persistent changes in uterine estrogen receptors (Wiebe & Barr 1988) and ovary LH receptors (Wiebe et al 1988) following perinatal exposure. Finally, it may interfere at the level of hypothalamus-pituitary, decreasing pituitary response to growth hormone releasing factor (Camoratto et al. 1993), affecting levels of FSH and LH (McGivern et al. 1991) and increasing blood levels of glucocorticoids (Vyskocil et al. 1991), which modify the action of estrogens in the uterus (Tchernitchin et al. 1975).

Exposure to lead also affects the immune system, depressing the immunoregulatory capacity (Keller et al. 1990). This may be explained through various mechanisms. (a) Lead decreases plasma levels (Mahaffey et al. 1982) and action (Long & Rosen 1994) of 1,25 dihydroxyvitamin D_3, which stimulates macrophage differentiation and plays a role in monocytes and activated lymphocytes due to the presence, in these cells, of vitamin D receptors (Thomasset 1994). (b) Lead induces a stress reaction (Nation et al. 1987), that is characterized by the synthesis of specific stress proteins (Shelton et al. 1986), an alteration of the hormonal response to stress (Chowdhury et al. 1986; Agrawal & Chansouria 1989) and an increase in plasma levels of glucocorticoids (Vyskocil et al. 1991); the latter is known to inhibit several immune mechanisms (Cupps & Fauci 1982; Tchernitchin et al. 1990). (c) Lead induced alteration at the hypothalamus-pituitary level may also interfere with immune responses.

The role of blood leukocytes in reproductive functions and in immune processes lead us to suspect that lead may affect these functions, changing the proportions, levels and activity

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of the different leukocytes. These changes may occur due to increase in glucocorticoid levels (Vyskocil et al. 1991) or alteration in leukocyte glucocorticoid or sex steroid receptors and post-receptor events, as described in other target organs (Wide & Wide 1980).

In fact, the role of leukocytes in immune functions is well known. Lymphocytes possess glucocorticoid and sex steroid receptors, their ligands were suggested to play a role in immune regulation (Cupps & Fauci 1982; Danel et al. 1983). Eosinophils were also implicated in glucocorticoid-induced immune regulation in lymphoid organs (Tchernitchin et al. 1989, 1990). In addition to the uterine eosinophils (Tchernitchin et al. 1989), endometrial intraepithelial lymphocytes (Kamat & Isaacson 1987) were implicated in the inhibition of the immune response to implantation.

The roles of the different leukocyte cell-types in female reproductive physiology can be inferred from the presence of these cells in reproductive organs and their changes under the effect of hormonal status. Among these blood cells, the functions of the eosinophil leukocytes are best understood. These cells are involved in the mediation of a group of non-genomic responses to estrogen stimulation in the rat uterus (edema, increase in vascular permeability, release of histamine, increase in the production of nitric oxide, and possibly antiimmune protection of the blastocyst against its rejection) (Tchernitchin et al. 1985b, 1989, Suburo et al. 1995), independently from the cytosol-nuclear receptor-mediated mechanism of steroid hormone action that is implicated in the genomic responses to estrogen stimulation (Tchernitchin et al. 1985b). Eosinophils display two kind of estrogen receptors: the high affinity membrane receptor is a cell adhesion molecule involved in estrogen-induced uterine vascular endothelium recognition by the eosinophils (Tchernitchin et al. 1985b); the second, lower affinity, estrogen receptor is involved in eosinophil activation and degranulation within the uterine extravascular space (Tchernitchin et al. 1985b, 1989). The recognition of the uterus by the eosinophils, their migration through endothelial lining, their accumulation in the uterine stroma and subsequent activation and degranulation are required for the development of the eosinophil-mediated responses to estrogen (Tchernitchin et al. 1989, 1990). The finding that these responses develop even in the absence of estrogen, provided eosinophils have migrated to the uterus, suggests that agents released from degranulating eosinophils, and not estrogen itself, are involved in their development (see Tchemitchin et al. 1989 for a review). It was previously shown that agents interfering with the eosinophil estrogen receptors, with the number of eosinophils in the blood, causing or inhibiting the degranulation of these cells, selectively modify eosinophil-mediated responses to estrogen without alteration of cytosol-nuclear receptor-mediated genomic responses to estrogen (Tchernitchin et al. 1985b, 1989).

Taking into account the role of eosinophils in the action of estrogens in the uterus and in glucocorticoid immune regulation in lymphoid organs, and the role of lymphocytes in various immune mechanisms, the present study intends to further understand the mechanisms of lead-induced inteference with female reproductive and immune functions.

MATERIALS AND METHODS

Four groups of female rats (10 to 15 animals per group) from a Sprague-Dawley-derived colony bred at the vivarium of the Faculty of Medicine, University of Chile, were used in the present study. The animals received lead acetate (Merck, Darmstadt, Germany) (172 µg Pb^{++}/g

body wt s.c.) or saline physiological solution, every second day from the age of 14 days until the age of 20 days (total dose, 688 μg Pb^{++}/g body wt), and were treated with estradiol-17ß (Sigma Chemical Co., St. Louis, MO) (300 ng/g body wt s.c.) or its vehicle at the age of 21 days. This age is the most appropriate for the study of the effects of sex steroids on target organs, since estrogen and progesterone levels are extremely low and receptor levels and hormone responsiveness are already fully developed (Tchernitchin et al. 1985b). Table 1 summarizes the experimental conditions.

Table 1. Experimental conditions

Pretreatment (days 14, 16, 18 and 20)	Treatment (day 21; 24 h before blood collection)	
	Estradiol	Vehicle
Lead	L/E24	L/V24
Saline physiological solution	S/E24	S/V24

Blood samples were taken from the tail of each animal, under ether anesthesia, 24 h after treatment with estradiol or its vehicle. The blood was collected into tubes containing EDTA; two samples were obtained from each animal, one was used immediately after sample collection for blood cell quantification, the other one was kept at 4 °C for subsequent lead concentration determination.

Blood lead concentration was measured in saline or lead exposed animals without estrogen treatment, using atomic absorption spectroscopy with graphite furnace at the Chilean Institute of Public Health, Ministry of Health.

Eosinophil quantification and evaluation of eosinophil degranulation was performed according to the method of Tchernitchin et al. (1985a), as follows: Aliquots were taken from the tubes with EDTA immediately after blood sample collection and diluted 1:10 with freshly prepared eosin stain solution (1 mL of 1% eosin Y stock solution in 100% ethanol diluted in 10 mL of distilled water and 1 mL of acetone). Subsequently, an aliquot of blood-stain solution was transferred to a Neubauer chamber for eosinophil quantification and evaluation of their degranulation.

Blood leukocytes were quantified in a Neubauer camera diluting an aliquot of blood in Hayem B (0.5% of a saturated methylene blue solution (Merck, Darmstadt, Germany) in 3% acetic acid in distilled water) within 5 minutes of blood sample collection. Blood smears were fixed in methanol for 10 min and stained with May Grunwald-Giemsa (Merck, Darmstadt, Germany) for the quantification of blood leukocyte differential counts. Blood leukocyte absolute counts were calculated with the information obtained from blood smears and Neubauer camera counts.

All parameters, except lead blood levels, were expressed as percent of values in control animals receiving saline physiological solution (instead of lead) and vehicle of estradiol.

Statistics: Since multiple comparisons were performed between the four experimental conditions, data were subjected to the least significant difference (LSD) test. Following Bartlett's chi-squared test to ensure homogeneity of variances, the common variance was estimated from a one-way unbalanced analysis of variance (ANOVA), and no significant differences were declared unless ANOVA was significant.

RESULTS AND DISCUSSION

The geometric mean concentration of lead in the blood was 47.17 µg/100 mL in lead exposed animals without estrogen treatment, while it was 2.97 µg/100 mL in animals receiving saline physiological solution instead. The values in lead exposed animals are equivalent to those frequently found in occupational exposure in humans in several developing countries, such as Chile, where they are considered below the legal safety limits.

Table 2 shows the basal values of blood leukocytes in control animals, expressed as absolute values and differential counts.

Table 2. Blood leukocyte absolute and differential counts in controls

	Absolute count (cells/µL)	Differential count (%)
Lymphocytes	7728	78.1
Monocytes	102	1.0
Total eosinophils	58	0.6
Intact (non-degranulated) eos.	3	0.03 (5.7% of total eos.)
Total neutrophils	2008	20.3
Segmented neutrophils	1920	19.4
Band neutrophils	89	0.9
Total leukocytes	9895	100

Figure 1 shows the effect of exposure to lead and of estrogen treatment on differential counts. It can be observed that exposure to lead induces a 50% decrease in the percentage of eosinophils in the blood in both estrogen treated and untreated animals. Lead induces a disappearance of intact (non-degranulated) eosinophils in estrogen-treated rats and a tendency for a decrease ($0.05<p<0.1$) in rats without estrogen treatment. In lead-exposed estrogen-treated rats the percentage of lymphocytes is significantly smaller than that of estrogen treated without lead and those lead exposed without estrogen. An increase in total neutrophils and in segmented neutrophils is detected in lead-exposed estrogen-treated animals, that is significantly different from that in estrogen treated animals submitted to exposure to saline physiological solution instead of lead and that in lead exposed animals treated with vehicle instead of estrogen.

Figure 2 shows the effect of exposure to lead and of estrogen treatment on absolute leukocyte counts. Lead exposure induces an increase in blood leukocytes, that is observed in both estrogen treated and untreated animals. Lead exposure doubles total and segmented neutrophils in both estrogen treated and untreated rats, but causes a three-fold increase in band neutrophils in animals without estrogen treatment, but not in animals treated with estrogen; the inhibition by estrogen of this alteration induced by lead is statistically significant. The other change detected in absolute leukocyte values is a disappearance of non-degranulated eosinophils under the effect of lead; this change is statistically significant in estrogen-treated rats only, while a tendency for a decrease ($0.05<p<0.1$) is observed in lead-exposed animals without estrogen treatment.

The decrease in non-degranulated eosinophils under the effect of lead exposure suggests an increase in eosinophil degranulation in the blood. Studies are in progress to further investigate this effect using a morphometrical approach allowing the quantification of the degree of degranulation of each eosinophil. The present findings provide however the first

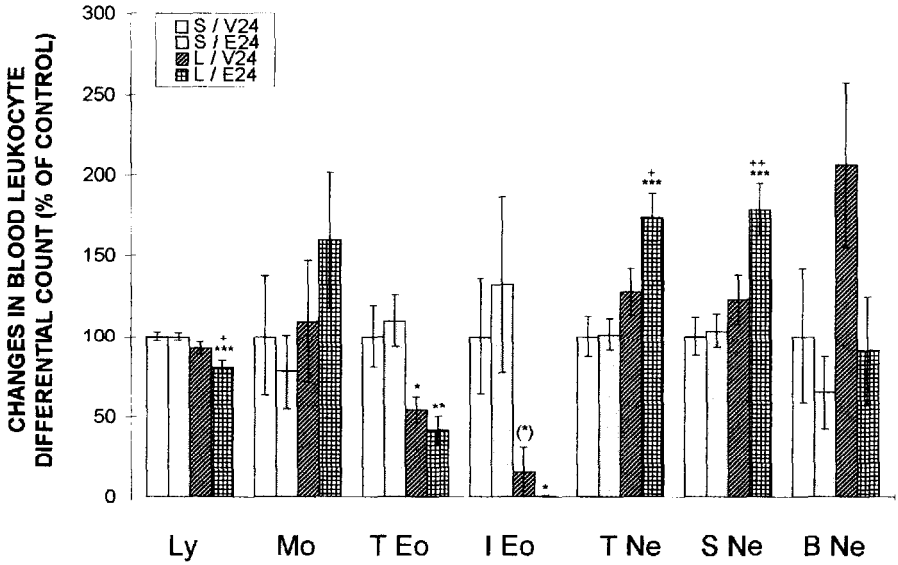

Figure 1. Effect of exposure to lead and/or estradiol-17ß treatment on blood leukocyte differential counts, and on the percent of intact (non-degranulated) eosinophils. Values were obtained 24 h after treatment with estrogen or its vehicle, and are expressed as % of the values in controls. Ly, lymphocytes; Mo, monocytes; T Eo, total eosinophils; I Eo, intact eosinophils; T Ne, total neutrophils; S Ne, segmented neutrophils; B Ne, band neutrophils. Total and intact eosinophils counted in Neubauer chamber, the remaining leukocytes, from blood smears. For analysis of significance, the Least Significant Difference (LSD) test was used, (*) $0.05<p<0.1$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to the homologous condition without lead; + $p<0.05$, ++ $p<0.01$, compared to the homologous condition without estradiol.

suggestion of a possible direct mechanism of interaction by lead with non-genomic estrogen action in the uterus. In fact, it was previously reported that eosinophils are involved in the mediation of several non-genomic parameters of estrogen stimulation in the uterus (Tchernitchin et al., 1989). Eosinophils migrate to the uterus under the effect of estrogen, and release several granular agents and enzymes that are involved in the development of non-genomic estrogenic responses. If the eosinophils loose their granule content through a process of degranulation, they may not be able to induce a full non-genomic response (Tchernitchin et al., 1985a). Current studies at our laboratory are intended to investigate the interference of non-genomic responses to estrogen by lead.

Eosinophils were proposed to play a role in glucocorticoid-induced immune regulation in the rat (Tchernitchin et al. 1990). In fact, they migrate to the spleen, thymus and lymph nodes under stimulation by glucocorticoid hormones (Sabag et al. 1978), where they release the eosinophil cationic protein (ECP) and eosinophil protein X (EPX) (Tchernitchin et al. 1990), known to inhibit the proliferation of T lymphocytes in a non-cytotoxic way (Peterson et al. 1986). Further work is necessary to explore the possibility that lead-induced eosinophil degranulation interferes with glucocorticoid-induced immune regulation, and to investigate whether these findings do apply to humans.

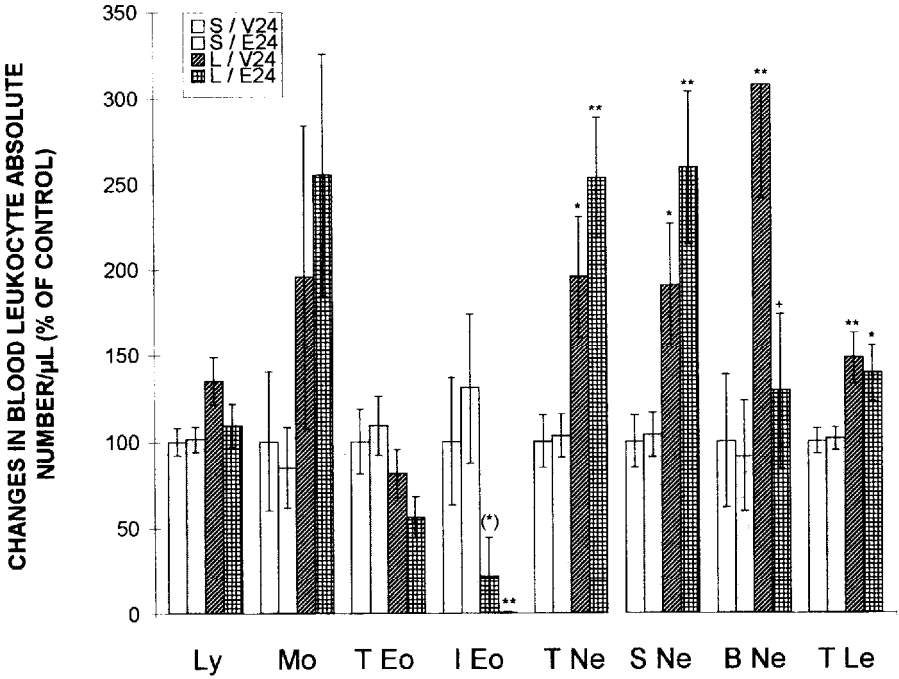

Figure 2. Effect of exposure to lead and/or estradiol-17ß treatment on blood leukocyte absolute counts, and on the number of intact (non-degranulated) eosinophils. Values were obtained 24 h after treatment with estrogen or its vehicle, and are expressed as % of the values in controls. Ly, lymphocytes; Mo, monocytes; T Eo, total eosinophils; I Eo, intact eosinophils; T Ne, total neutrophils; S Ne, segmented neutrophils; B Ne, band neutrophils; T Le, total leukocytes. Total and intact eosinophils and total leukocytes counted in Neubauer chamber, the remaining leukocytes, from blood smears. For analysis of significance, the LSD test was used. (*) $0.05<p<0.1$, * $p<0.05$, ** $p<0.01$, compared to the homologous condition without lead; + $p<0.05$, compared to the homologous condition without estradiol.

The present finding of an increase in mature neutrophils in the rat under the effect of lead adds further information to previous findings of decreases in neutrophil chemotactic activity (Queiroz et al. 1993) and phagocytic capacity (Baginski & Grube 1991) in humans, while neutrophil cytotoxicity, due to enhanced generation of toxic oxygen species, is increased despite the reduced phagocytic capacity (Baginski & Grube 1991). Although rats and humans display similar effects of lead toxicity on the immune systems (Lang et al. 1993), comparison between both species should be interpreted with caution since rodents display higher sensitivity to immunomodulation by lead than humans (Lang et al. 1993). At present, it is not possible to ascribe changes in neutrophils to lead toxicity, to a result of combined stress and physiological adaptation, or to a defense mechanism to compensate lead-induced immune impairment proposed by Keller et al. (1990). The increase in band neutrophils in animals without estrogen treatment only suggests that estrogen may accelerate neutrophil maturation in lead-exposed animals. The effect of lead on band neutrophils reveals an increased neutrophilopoiesis rather than release from intravascularly sequestered forms in lead-exposed animals. This may be caused by the increased corticosteroid levels in the

blood of adult female rats, but not males, following chronic exposure to lead (Vyskocil et al. 199 1), provided that a similar response occurs at the prepubertal age. Further studies are necessary to understand the mechanisms explaining the effects of lead on neutrophils as well as the implications of these changes in immune processes under lead exposure.

Present finding of lead exposure-induced decrease in blood lymphocyte differential count, but not in absolute counts, should be interpreted with caution. It most probably reflects a relative increase in neutrophils rather than a real lymphopenia. Studies are in progress to determine whether lead exposure causes any change on single lymphocyte subpopulations.

In summary, this study clearly demonstrates that prepubertal rat exposure to lead affects blood neutrophil and eosinophil leukocyte levels and induces eosinophil degranulation. Taking into account that similar blood lead levels in humans are considered in many developing countries below the legal safety limits in occupational exposure, present findings suggest a need for further studies to investigate if these changes occur in humans under similar blood lead levels and whether these alterations have a meaning to mechanisms of leukocyte receptors and homeostasis or play a role in lead induced impairment in immune and female reproductive functions in both humans and experimental animals.

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