Lead pollution constitutes a major health problem that has been intensively debated. To reveal its effects on the immune response, the influence of lead on the \textit{in vitro} cytokine production of human peripheral mononuclear blood cells was investigated. Isolated cells were exposed to lead acetate or lead chloride for 24 h in the presence of either heat-killed \textit{Salmonella enteritidis} (hk-SE) or monoclonal antibodies (anti-CD3, anti-CD28, anti-CD40) as cell activators. Our results showed that while higher lead doses are toxic, lower ones evoke immunomodulatory effects. All tested lead doses significantly reduced cell vitality and/or proliferation and affected secretion of proinflammatory, T helper cell type (TH)1 cytokines. Expression of interferon (IFN)-\(\gamma\), interleukin (IL)-1\(\beta\), and tumor necrosis factor (TNF)-\(\alpha\) was reduced at lower lead doses in both models of cell stimulation. Although hk-SE failed to induce detectable IL-4, IL-6, and IL-10 secretion increased in the presence of lower lead doses. Thus, exposure to lower doses leads to suppression of the TH1 cytokine IFN-\(\gamma\) and the proinflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\). The elevated production of IL-4 and/or IL-10 can induce and maintain a TH2 immune response and might contribute to increased susceptibility to pathologic agents as well as the incidence of allergic hypersensitivity and/or TH2-dominated autoimmune diseases.

\textbf{Key Words:} allergy; autoimmune diseases; cytokine; immune response; lead salts; TH helper cells

\section*{INTRODUCTION}

Heavy metal exposure has long been known to be toxic to humans and animals, causing diseases and occasionally death (Yucesoy et al., 1997b). Many studies have addressed the toxic effects of heavy metals on specific organism(s) (Fischer and Skreb, 2001); other studies have documented the direct immunotoxicity caused by short-term exposure to heavy metals (Marth et al., 2001); still others observed genotoxic (Karayaka et al., 2005), apoptotic effects (de la Fuente et al., 2002), or even chronic effects resulting from long-term exposure (Koller and Roan, 1977). There is growing concern about the subtle health effects of lead, cadmium, and mercury. Lead has been used in the past as an additive in gasoline and eventually became widely dispersed throughout the environment. However, since the replacement of leaded gasoline with unleaded gasoline in many countries in the mid-1980s, lead exposure from that source has virtually disappeared (RAMP, 1996; Ransom et al., 1987). This has led to a welcome decrease in the median blood lead concentration (Rogan and Ware, 2003).

Indeed, the last decade was particularly fruitful in providing new information on the manifold toxic influences of lead (Lidsky and Schneider, 2003). However, reviewing these studies has revealed some inconsistency. For example, some publications reported that immunomodulatory effects could be found neither \textit{in vitro} nor \textit{in vivo}, even when very high doses were applied (Borella et al., 1990; Yucesoy et al., 1997a). Most investigators, however, have concluded that an effect exists. In \textit{in vitro} investigations employing human peripheral blood mononuclear cells (PBMC) or T cell clones (McCabe and Lawrence, 1991), as well as \textit{in vivo} studies using wild-type (Gupta et al., 2002; Heo et al., 1996; Kim and Lawrence, 2000; McCabe et al., 1999; Miller et al., 1998) or transgenic animals (Heo et al., 1998), have documented direct, although sometimes contradictory, effects of lead exposure on cytokine profiles or TH1 cell differentiation. The differentiation of naïve cell responses into TH1 or TH2 branches determines (1) resistance or susceptibility of hosts to infection, (2) development of allergic reactions, and (3) the degree of tissue damage that occurs in many autoimmune diseases (Abbas et al., 1996; Singh et al., 1999). The balance between allergy-promoting TH2 cells and infection-fighting...
**RESULTS**

**Cell Vitality**

Twenty-four-hour exposure to lead doses above 50.0 μg/ml exhibited strong cytotoxic effects (Fig. 1), but lower doses ranging from 15 pg/ml to 50 μg/ml significantly inhibited cell vitality and/or proliferation in a dose-dependent manner. These effects were independent of the kind of cell activators used or the metal salts tested. Similar results were obtained in both cases.

**Cytokine Release**

Changes in *in vitro* cytokine release by mAb or hk-SE-stimulated human PBMC after short-term exposure to one or both metal forms are represented as percentages of the accompanying controls (Figs. 2 and 3). The normal cytokine ranges of these controls, where cells are stimulated either by mAb or hk-SE, but not by lead, are represented in Table 1. A dose-dependent stimulation or inhibition of cytokine production resulted from exposure to lead.

As shown in Figure 2, after exposure to lead acetate, the release of the proinflammatory cytokines TNF-α by mAbs-stimulated PBMC was significantly reduced at lead doses above 1.5 ng/ml, IL-1β above 5.0 ng/ml, and IL-6 from 150.0 ng/ml to 15.0 ng/ml. The exception was the stimulation of IL-6 release at doses lower than 150.0 ng/ml. Furthermore, IFN-γ release was significantly reduced at all tested doses of lead.
from 1.5 ng to 5.0 mg/ml. In contrast, secretion of IL-10 and IL-4 was significantly increased at all doses below 150.0 µg/ml and 15.0 µg/ml, respectively. The production of all cytokines is drastically inhibited at lead doses above 150.0 µg/ml (Figs. 2 and 3). Estimating the ratio %IFN-γ/%IL-4 provided a picture of the Th1–Th2 balance. Because a balance between the two cell types is hypothesized to exist, a sample was obtained at all tested doses below 150.0 µg/ml (Fig. 2g); a value lower than unity for a given sample indicated a probable deviation toward the Th2 type. Similar results were obtained when comparing the ratios of %IFNγ and %IL-10 (Fig. 2h), which could also indicate a Th2 polarized immune response at all lead doses tested. With minor differences up to one dilution step, we obtained the same results by applying lead chloride (data not shown).

Cytokine profiles of hk-SE stimulated PBMC after 24 h of exposure to lead acetate and lead chloride were also identical. After exposure to lead chloride (Fig. 3), the release of TNF-α and IL-1β at all doses above 150.0 pg/ml and the release of IL-6 at doses above 1.5 ng/ml was significantly inhibited. Exceptions were the significant stimulation of TNF-α and IL-6 release at higher doses, in the range 0.5/ml to 150 µg/ml (Fig. 3a and 3c) and IL-6 at lower doses of 150 and 50 pg/ml. IFN-γ production by hk-SE-stimulated cells was reduced at all doses; this inhibition was significant at doses from 150 µg/ml to 150 ng/ml. Again, although hk-SE failed to produce detectable levels of IL-4 after exposure to lead chloride, IL-10 release was significantly stimulated at lower doses and inhibited at higher doses (Fig. 3e). Estimating the ratios %IFN-γ/%IL-10 revealed the polarization of the immune response toward IL-10 production; at low doses of 150.0 pg/ml and up to 15.0 ng/ml, a significant decrease in the ratios resulted (Fig. 3f).

**Intracellular Cytokines**

Intracellular cytokine staining was carried out to investigate the differentiation preference of T cells by lead. Of the total T lymphocytes, the proportions of IFN-γ–producing and IL-4–producing cells were estimated and expressed as percentages of the controls (Fig. 4). With few exceptions, a dose-dependent decrease in IFN-γ–producing CD3+ T cells was found after lead exposure. In contrast, the number of IL-4–producing T cells increased after exposure to non-toxic doses of lead (from 150.0 pg/ml to 15.0 ng/ml); it then decreased with the increase in lead toxicity (at doses higher than 15.0 µg/ml). Estimating the %IFN-γ/%IL-4 ratio could clearly represent the preferential proliferation of IL-4–producing T cells in comparison to IFN-γ–producing cells (Fig. 4c). A decrease in the ratio resulted at all tested doses, except at 15.0 ng/ml and 150.0 µg/ml, where about 90% of the tested samples showed a polarized Th2 response, reflected by the increase in the percentage of IL-4–producing T cells.

**DISCUSSION**

Heavy metals may influence cells by penetrating the interior of the cell through calcium channels and/or by reacting with surface structures of the cell, such as the cell receptors or associated ligands (Kerper and Hinkle, 1997; Marth et al., 2001). The hypothesis tested here is whether lead promotes Th2-dependent response over Th1 response, which may contribute to the increased incidence of atopy and allergic diseases in polluted areas.

**Lead Reduces Cell Vitality and/or Proliferation**

One of the mechanisms by which lead and other heavy metals can affect the immune system, is through effects on cell vitality and proliferation. Our results demonstrate an inhibitory effect of lead on the vitality and/or proliferation of human PBMC. However, previous investigations showed conflicting
FIG. 2. The effect of lead (Pb) acetate on the mAbs-stimulated release of TNF-α (a), IL-1β (b), IL-6 (c), IFN-γ (d), IL-10 (e), and IL-4 (f) by human PBMC. Box-plots show the medians and the 10th, 25th, 75th, and 90th percentile ranges of 12 samples, each with three replicates. TH1:TH2 ratios were evaluated from %IFN-γ/%IL-4 (g) and %IFN-γ/%IL-10 (h). Symbols above each plot show whether, at each dose, cytokine release was significantly stimulated (#) or suppressed (*) compared with the controls ($p < 0.05$ in Wilcoxon test).
results. It was found in some studies that lead did not affect cell vitality or growth (Borella et al., 1990; Smith and Lawrence, 1988), whereas inhibitory or stimulatory effects of lead on immune competent cells were described in other reports. For example, a significant inhibitory effect of lead on rat spleno-cytes derived from rats exposed to 10 or 1000 µg/ml lead was demonstrated by Exon et al. (1985). Similarly, lead was found to enhance B cell proliferation (Lawrence, 1981) and T cell proliferation (Warner and Lawrence, 1986) in mice. Tellingly, lead augmented the proliferation of rat spleen cells at doses of 0.5 to 200 µg/ml, but it inhibited proliferation at doses above 200 µg/ml (Razani-Boroujerdi et al., 1999). These data provide a reason for the contradictory results: conflicting findings may be attributed to differences in lead doses or in the experimental models used. Our results confirm that exposure to lower as well as higher lead doses, namely, from 150 pg/ml up to 5 mg/ml, significantly inhibits cell vitality and/or proliferation. We propose that the capability of lead to impair cell vitality may
be the result of (1) the direct interaction between the lead ion and mAbs or hk-SE, and/or (2) interference with cell metabolism.

**Lead Exerts a Differential Effect on TH1-TH2 Balance**

The mechanisms by which lead affects the TH1–TH2 balance may include some or all of the following: (1) direct inhibition of TH1 cells by lead; (2) stimulation of TH2 cell activities, which induce cytokine release, which in turn inhibits TH1 cell activity; and (3) an indirect inhibition of TH1 cells mediated by impairment of antigen-presenting cells (APC). In light of previous investigations (e.g., McCabe et al., 1999; Shen et al., 2001), and as demonstrated here, it seems that the most rational approach to revealing the cellular interactions involved would be to measure cytokine profile changes. We found that short-term exposure of PBMC to low lead doses had immunomodulatory effects that might reflect skewness of the immune response toward the TH2 type.

In mAbs-activated cells, a significant increase in IL-4, IL-10, and IL-6 release occurred after exposure to lead acetate or lead chloride. Production of the TH1 cytokine IFN-γ and of the proinflammatory cytokines TNF-α and IL-1β was significantly inhibited. Slightly different results were obtained from hk-SE-stimulated cells. These included stimulation of TNF-α and IL-6 release at higher lead doses, the undetectable production of IL-4, and the relative increase in IFN-γ production, which is evident by comparing the IFN-γ/IL-10 ratios between the two models of cell stimulation. Therefore, we suggest that different lead immunomodulatory pathways become active according to the microenvironment of cell stimulation.

Evaluating the intracellular cytokines could also reveal the proliferation preference of TH2 cells at lower lead doses. Therefore, suppression of the IFN-γ release, together with the stimulation of IL-4 and/or IL-10 release indicated by ELISA, may be due to a reduction in the number of IFN-γ–producing cells and preferential proliferation of IL-4–producing and/or IL-10–producing cells (TH2 cells), or by changing cytokine production potency of both TH cell subsets.

Some previous investigations reported no influence of lead on the immune response (Blakley et al., 1982; Yucesoy et al., 1997a). Our results, however, are in agreement with other data.
documenting an inclination toward Th2 immune response following exposure to lead. In direct opposition to these data, Guo et al. (1996) and Krocova et al. (2000) found a preferential production of Th1 cytokines; TNF-α was produced by macrophages as well as by T cells. Our assertion that high lead doses stimulate TNF-α production by hk-SE-stimulated cells confirms the studies of Guo et al. (1996) and indicates a relative increase in the activity of hk-SE stimulated macrophages at high lead doses. The dissimilarity in reactivity of IFN-γ and TNF-α may result from the differing methods or cross-regulatory processes from other segments of the immune system that are compromised in our models. But, the finding that IL-10 inhibits cytokine production by Th1 cells (Mosmann and Moore, 1991) and the reciprocal regulation of Th1 and Th2 cells (Dubey et al., 1991; Manetti et al., 1993) rather confirms our results concerning the inhibition of IFN-γ and TNF-α levels and the preferential proliferation of Th2 in association with lower nontoxic doses of lead.

The Role of IL-4, -6, and -10 in Lead-Induced Immunomodulation

The preferential stimulation of Th2 immune response by lead is suggested here by the increase in IL-4, IL-6, and IL-10 production observed after exposure to lead. Indeed, many investigations have addressed IL-4 as the major determinant for differentiation of antigen-stimulated naive T cells into Th2 cells (Le Gros et al., 1990). The importance of IL-10 in the Th1–Th2 immune balance, however, has long been debated. Although IL-10 has been reported to be secreted by both CD4+ subsets (Yssel et al., 1992), the finding that IL-10 inhibits cytokine production by Th1 cells (Fiorentino et al., 1989; Mosmann and Moore, 1991) would support its role in inducing Th2 immune responses. Thus, it is reasonable here to suggest a skewed Th2 immune response after lead exposure, even in the absence of detectable levels of IL-4 in the hk-SE–stimulation model. Therefore, not all cytokines are indicative of impairment in Th1 cell balance and, in some instances, cytokine levels could be misleading. Mihara et al. (1991) found that IL-6 inhibited delayed-type hypersensitivity reactions that are known to be elicited by Th1 responses. Therefore, the elevated IL-6 levels may be a further indication of a Th2-polarized response, which suggests a heightened stress based on the absence of a priming level of IFN-γ.

In asthma, Th1 type reactions are deficient and Th2 reactions predominate. Th2 reactions are driven by IL-4, IL-5, IL-10, and IL-13 and result in an increase in IgE production. Indeed, significantly elevated levels of IgE have been found in lead-exposed workers, although no significant relationship between blood lead levels and serum IL-4 or IFN-γ levels was observed (Heo et al., 2004). The range of lower doses of lead used in our study is comparable to the blood lead levels found in workers occupationally exposed to lead—range: 0.07 to >0.8 μg/ml (Basaran and Undeger, 2000; Heo et al., 2004; Palus et al., 2003; Sata et al., 1998; Stollery et al., 1991; Truckenbrodt et al., 1984). Thus, the lower Th1/Th2 ratio we obtained confirms some previous reports of immunomodulation by lead and raises the possibility for the development of allergic and/or autoimmune diseases as suggested by Heo et al. (1996).

Lead Reduces the Proinflammatory Response

Exposure to lead resulted in a significant inhibition in the production of the proinflammatory cytokines TNF-α and IL-1β, which are mediators of the inflammatory response and which are released from different cell types (Schuerwegh et al., 2003). The importance of IL-1β, a primary mediator of immune response to injury and infection (Brough et al., 2003), and TNF-α in immune response stems largely from their ability to promote Th1 versus Th2 pathways by antigen presenting cells (APC; Cervi et al., 2004). These cytokines increase the efficiency with which an APC can bind and activate Th1 cells. It seems then, that lead exerts an inhibitory effect on APC, leading to the inhibition of TNF-α and IL-1β release and consequently to clonal proliferation.

Overall, we conclude that lead skews the immune response toward the Th1 pathway, leading to a weakened ability of the body to defend against various infections and an enhanced risk of allergic diseases. The use of two forms of the metal confirms the hypothesis that it is the action of the metal ion that is operative, regardless of the applied form. This was not the case when applying two models of immune stimulation; both revealed identical effects, but they demonstrated that lead may act through different pathways including different cytokine mediators. Identification of the intermediate molecules, as well as the mechanisms that establish such susceptibility or the initiation of the Th1–Th2 imbalance, may be an important goal for future experiments.

ACKNOWLEDGMENTS

We thank Katrin Bauer (IKIT, University of Leipzig, Germany) and Sylke Drubig (UFZ, Department of Environmental Immunology, Leipzig, Germany) for technical assistance and Dr. Sonya Faber (Coordination Centre for Clinical Trials, Leipzig, Germany) and Ms. Audrey Braun (University of Leipzig, Germany) for reviewing the manuscript. Ulrich Sack, as well as this work, has been funded by a grant from the Interdisziplinäres Zentrum für Klinische Forschung Leipzig (IZKF) of the Medical Faculty, University of Leipzig, Germany (project Z10). Nasr Y. A. Hemdan was funded by DAAD (Deutscher Akademischer Austausch Dienst, The German Academic Exchange Service).

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