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Lead inhibits oxidative metabolism of macrophages exposed to macrophage-activating factor

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The present experiments were designed to evaluate the effect of lead on the capacity of macrophages to respond to activating signals by increased respiratory-burst activity. When mouse peritoneal macrophages were exposed for 24 h to macrophage-activating factor (MAF) and/or bacterial lipopolysaccharide in the presence of lead acetate, a marked inhibition of their oxidative metabolism was observed. The hexose-monophosphate-shunt (HMPS) activity and the release of oxygen derivatives upon triggering by phorbol myristate acetate (PMA) were impaired. Treatment with the metal for 1 h led, however, to stimulation rather than inhibition of the PMA-triggered superoxide production, suggesting that the metal interfered with neither the triggering steps nor the activity of the NADPH oxidase. Moreover, the lead-induced inhibition of macrophage oxidative metabolism did not result from blockade of enzymes of the HMPS pathway. Glucose-6-phosphate dehydrogenase in macrophage extracts, as well as CO_2 production from glucose, remained unaffected by the presence of lead, and extracts of lead-treated macrophages were as active as extracts from control cells in those two assays. Lead appeared to interfere with an early event in the MAF-induced activation process. In addition, lead decreased the uptake of 2-deoxyglucose by macrophages, suggesting that the metal might inhibit trans-membrane glucose-transport systems, a phenomenon that might explain in part the metabolic inhibition observed in lead-treated cells.

INTRODUCTION

Exposure of macrophages to heavy metals *in vitro* is known to affect several functions of these cells, such as phagocytosis, motility, response to migration-inhibitory factor, and antigen presentation [1–6]. Particularly striking is a decreased capacity to kill intracellular pathogens [1,7]. It is known that the microbicidal activity of macrophages depends, at least in part, on the production of highly reactive metabolites of oxygen [8,9], the synthesis of which is triggered during the phagocytic process. Similarly, upon treatment with macrophage-activating factor (MAF), macrophages acquire an enhanced capacity to convert oxygen into such toxic derivatives, correlating with increased microbicidal and tumoricidal capacities [10–15]. In view of the reported impairment by heavy metals of macrophage functional activities involved in microbial killing, it was decided to determine whether these substances would similarly interfere with macrophage stimulation by MAF and other co-activating agents. It is shown below that lead prevents the development of an oxidative response in macrophages exposed to lymphokines and to lipopolysaccharide (LPS), with a resulting loss in the capacity to generate oxygen metabolites.

MATERIALS AND METHODS

Animals

CBA/T6, C3H/OUJ, BALB/c and C57BL/6 mice (between 8 and 18 weeks old) were obtained from the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland.

Macrophage cultures and stimulation

Starch-elicited cells were obtained by peritoneal lavage of mice injected intraperitoneally 3 days earlier with hydrolysed starch [16]. Bone-marrow-derived macrophages were prepared as described in ref. [17]. Briefly, cells aspirated from the femurs of 2-month-old mice were grown in Dulbecco's medium (Seromed, Munich, Germany) supplemented with 20% (v/v) horse serum (Gibco A.G., Basel, Switzerland) and 30% (v/v) L-cell-conditioned medium for 10 days. Cells suspended in Dulbecco's medium supplemented with 10% (v/v) fetal-bovine serum (FBS; Seromed) were distributed as follows: for cytochrome *c* and NBT reduction tests and for protein determinations, in 96-well flat-bottom micro-culture plates (3596; Costar, Cambridge, MA, U.S.A.; 10^5–1.5×10^5 peritoneal cells/well); for H_2O_2 production, in 96-well U-bottom micro-culture plates (3799; Costar; 3×10^4–5×10^4 bone-marrow cells/well). After 24 h at 37 °C, the cells were washed twice with warm Hanks' balanced salt solution (HBSS; Seromed) and re-incubated at 37 °C with either a MAF-containing supernatant from concanavalin A-stimulated spleen cells from C57BL/6 or CBA/T6 mice [18], or control medium, in the presence of LPS (10 ng/ml, from *Escherichia coli* 055:B5; Difco Laboratories, Detroit, MI, U.S.A.). Lead(II) acetate (0–100 μM) or sodium acetate (0–100 μM) (Merck, Darmstadt, Germany) was added

Abbreviations used: FBS, fetal-bovine serum; G6PD, glucose-6-phosphate dehydrogenase; HBSS, Hanks' balanced salt solution; HMPS, hexose monophosphate shunt; IAA, iodoacetamide; LPS, lipopolysaccharide; MAF, macrophage-activating factor; NBT, Nitro Blue Tetrazolium; PBS, phosphate-buffered saline; 6PGD, 6-phosphogluconate dehydrogenase; PMA, phorbol myristate acetate.

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before, simultaneously with or after the activation medium; 16–24 h later, the cells were used for various metabolic studies.

Cell viability

The effect of lead acetate on the viability of macrophages was determined by the Trypan Blue exclusion test. Macrophages were cultured in 24-well Costar plates (0.6×10^6 cells/well) for 24 h, then washed; 0.4 ml of Trypan Blue [0.05% in Tris/Dulbecco buffer (135 mM-NaCl/5 mM-KCl/0.7 mM-Na_2HPO_4/25 mM-Tris, adjusted to pH 7.4 with 1 M-HCl)] was added to each well. Viability was determined by counting the number of unstained cells per 400–500 cells.

Preparation of cell extracts

(a) For HMPS activity. For this, 10^7 BALB/c peritoneal cells in 10%-FBS-supplemented Dulbecco's medium were incubated for 24 h in 90 mm-diam. plastic non-tissue-culture dishes (Sterilin, Teddington, Middx., U.K.) with or without 100 μM-lead acetate. The cells were then washed with HBSS and incubated with cold PBS (0.05 M-Na_2HPO_4/0.01 M-KH_2PO_4/0.1 M-NaCl, pH 7.4) for 20 min at 4 °C as previously described [12]. They were detached by gentle pipetting, centrifuged and resuspended at a concentration of 30×10^6/ml in PBS supplemented with 100 μM-phenylmethanesulphonyl fluoride (Sigma), 10 μg of soybean trypsin inhibitor (Sigma)/ml and the following proteinase inhibitors, each at 5 μg/ml: leupeptin, pepstatin and antipain (all purchased from Sigma). Macrophages were then sonicated for 15 s in ice by using a Branson model B-12 sonifier (Branson Sonic Power Co., Danbury, CT, U.S.A.) fitted with a micro-tip, with power on position 4. The cell extracts were then tested for HMPS activity as described below.

(b) For glucose-6-phosphate dehydrogenase (G6PD) activity. CBA/T6 peritoneal cells (20×10^6 per dish) were cultured overnight in 10%-FBS-supplemented Dulbecco's medium in 90 mm Sterilin Petri dishes as described above. The cells were then washed and re-incubated with Dulbecco's medium in the presence or absence of 50 μM-lead acetate or -sodium acetate at 37 °C. Then 24 h later, they were washed with HBSS without Phenol Red, and reincubated in a mixture containing 2.5 ml of HBSS, 1 ml of distilled water, 1 ml of Tris/HCl buffer (0.1 M-Tris/0.2 M-NaCl, adjusted to pH 7.5 with 1.0 M-HCl) and 0.5 ml of a saturated digitonin solution (Fluka A.G., Buchs, Switzerland) prepared by dissolving 1 g of digitonin in 100 ml of distilled water, shaking well and filtering off the precipitate [19]. After 60 min at 4 °C, the cells were scraped with a rubber policeman and the suspension was centrifuged in the cold (15 min at 3000 *g*). The sediment was discarded and the supernatant used for determination of G6PD activity.

Metabolic studies

Before measurement of oxygen-metabolite production by the cytochrome *c* and NBT reduction assays, the cells were washed twice with HBSS without Phenol Red.

(a) Cytochrome *c* reduction test. The method used was the semi-automated micro-assay of Pick & Mizel [20]. Washed cells were incubated with 200 μg of ferricytochrome *c* (from horse heart, type VI; Sigma) and 50 ng of phorbol myristate acetate (PMA) in 100 μl of HBSS. Wells used as blanks included 30 μg of superoxide dismutase (SOD; Sigma)/ml in the reaction medium. Cytochrome *c* reduction was measured at 550 nm in a micro-e.l.i.s.a. reader (Easy Reader EAR 340; Kontron Analytic, Zürich, Switzerland), with a 492 nm reference filter. For measuring the effect of lead on the triggering of the respiratory burst by PMA, the washed cells were treated for 1 h with lead acetate in HBSS, then tested for cytochrome *c* reduction in the continuous presence of the metal. Results were expressed as the difference in absorbance per mg of cell protein (ΔA/mg of protein) between cultures incubated in the absence (−SOD) and presence (+SOD) of SOD at each time point (t_i) relative to the time when measurements started (t_0), according to the following formula:

$$\Delta A/\text{mg of protein} = \frac{\Delta A_{t_i-t_0}(-\text{SOD}) - \Delta A_{t_i-t_0}(+\text{SOD})}{\text{mg of protein}}$$

(b) NBT reduction test. This was done as described by Pick *et al.* [21]. Washed cells were preincubated with either HBSS or 10 mM-iodoacetamide (IAA) (blanks) in the presence or absence of 100 μM-lead acetate for 60 min at 37 °C. The plates were then emptied and the wells supplemented with either 100 μl of HBSS containing 100 μg of NBT and 50 ng of PMA, or 100 μl of HBSS containing 100 μg of NBT and 10 mM-IAA (blanks) in the presence of the same concentration of lead as in the preincubation medium. After suitable incubation at 37 °C, NBT reduction was measured with a micro-e.l.i.s.a. reader (Easy Reader EAR 340) fitted with a 550 nm filter. Results were expressed as the difference in absorbance per mg of cell protein (ΔA/mg of protein) between cultures incubated in the absence (−IAA) and in the presence (+IAA) of IAA at each time point (t_i), according to the formula:

$$\Delta A/\text{mg of protein} = \frac{\Delta A_{t_i-t_0}(-\text{IAA}) - \Delta A_{t_i-t_0}(+\text{IAA})}{\text{mg of protein}}$$

(c) H_2O_2 production. This was measured by a semi-automated micro-assay based on the horseradish peroxidase-catalysed oxidation of fluorescent scopoletin by H_2O_2, as described by De La Harpe & Nathan [22].

(d) HMPS activity. (1) Whole cells. HMPS activity was determined as previously described [23] from the amount of $^{14}CO_2$ evolved by 5×10^5 macrophages from D-[1-^{14}C]glucose (0.3 μCi/ml final; sp. radioactivity 3.94 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.).

(2) Cell-free system. The reaction mixture (S. Wirth & J. Mauël, unpublished work) was prepared in 24-well Costar plates (no. 3524) and contained 2 mM-ATP (disodium salt, from horse muscle), 4.5 units of hexokinase (from baker's yeast, type V)/ml, 0.7 unit of G6PD (from *Torula* yeast, type XI)/ml, 0.7 unit of 6-phosphogluconate dehydrogenase (6PGD; from *Torula* yeast, type V)/ml (all purchased from Sigma Chemie, Munich, Germany), 0.05 mM-β-$NADP^+$ (disodium salt; Serva, Heidelberg, Germany), 8.5 mM-$MgCl_2$ and 30 mM-Methylene Blue, both purchased from Merck, Darmstadt, Germany, in PBS. D-[1-^{14}C]Glucose (0.3 μCi/well), with or without lead acetate (10, 30, 100 μM), was then added to the wells to a final volume of 300 μl,

and HMPS activity was determined after incubation for 90 min at room temperature.

(3) Cell extracts. Macrophage extracts obtained by sonication were diluted in PBS as indicated in the text and supplemented with various HMPS cofactors as described in Table 6. HMPS determinations were performed in 24-well Costar plates in 300 μl volumes at room temperature, with a 90 min incubation time.

(e) Determination of G6PD activity. Extracts from cells preincubated for 24 h with medium, lead acetate (50 μM) or sodium acetate (50 μM) were prepared as indicated above. G6PD activity was determined by a method previously described [19], which was adapted to a 96-well micro-titre plate assay. Cell extract (40 μl) was mixed with 130 μl of 1 mM-Tris/HCl buffer (pH 7.5) and 10 μl of 2 mM-lead acetate, 2 mM-sodium acetate or distilled water. After 15 min at 25 °C, 10 μl of 10 mM-$NADP^+$ solution was added to the wells, which were then re-incubated for 5 min at 25 °C; 10 μl of 13.33 mM-glucose 6-phosphate was then added to each well. Blanks included wells where $NADP^+$ was omitted. The A_{340} was then determined in a micro-e.l.i.s.a. reader (Easy Reader EAR 340), every 2 min for 20 min. Five replicates of each sample were tested in parallel.

Determination of cellular protein

Two different methods were used. (*a*) Measurements of cellular protein in macrophages assayed for cytochrome *c* and NBT reduction and for D-[1-^{14}C]-glucose oxidation were performed on separate micro-plate cultures by the Coomassie Blue dye-binding test [24]. The same assay was used to measure proteins in macrophage extracts: in this case, 50 μl samples of extracts were distributed into micro-titre wells, dried overnight at 37 °C and tested. Standards were prepared by adding 50 μl of increasing amounts of bovine serum albumin into micro-titre wells, which were air-dried overnight at 37 °C and tested as above. (*b*) Determination of protein in cultures assayed for H_2O_2 production was performed in the same micro-plate, by the method of Lowry *et al.* [25] adapted to a micro-assay by De La Harpe & Nathan [22].

Determination of glucose incorporation

Peritoneal cells (10^5 cells/well) from starch-injected C3H/OUJ mice were incubated overnight at 37 °C in a 96-well micro-titre plate in FBS-supplemented Dulbecco's medium. Cells were then washed twice with HBSS and exposed to lead acetate (0–100 μM) at 37 °C. Cultures were then washed three times with HBSS and incubated for 1 h at 37 °C with 2-deoxy-D-[2,6-^{3}H]glucose (1 μCi/well; 36 Ci/mmol; The Radiochemical Centre) in glucose- and bicarbonate-free Eagle's medium in the presence of various concentrations of the metal. Cells were then washed extensively and lysed with 50 μl of 1 M-NaOH, and radioactivity was determined in the lysates.

RESULTS

Impairment by lead of the MAF-dependent stimulation of macrophage oxidative metabolism

CBA/T6 peritoneal macrophages were activated by incubation with MAF and LPS in the presence or absence of lead acetate (100 μM). Then 24 h later, cultures were tested for their capacity to generate superoxide after triggering with PMA, by using the cytochrome *c* reduction assay. Fig. 1 shows that the respiratory burst was stimulated in macrophages treated with MAF and/or LPS. The presence of lead acetate during activation

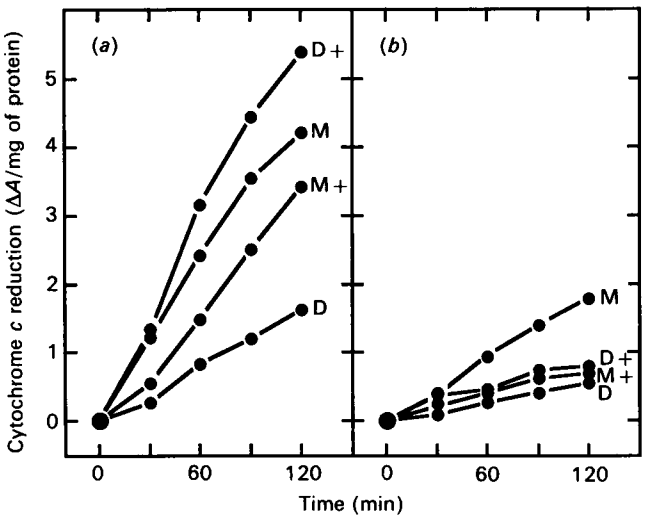

Fig. 1. Impairment of cytochrome *c* reduction by treatment of macrophages with lead acetate

CBA/T6 macrophages were incubated with a 1:16 dilution of MAF-rich (M) or control Dulbecco's medium (D) in the presence (+) or absence of added LPS (10 ng/ml) (*a*). In parallel cultures (*b*), lead acetate (100 μM) was added simultaneously with the MAF/LPS mixture or control medium. After 24 h, superoxide production was determined by the PMA-triggered cytochrome *c* reduction test. Results are expressed as ΔA/mg of protein, ±S.D., as measured at different time points. The data are representative of three experiments.

Table 1. Dose–response of the inhibitory effect of lead acetate on macrophage superoxide production

CBA/T6 macrophages were incubated with a 1:16 dilution of MAF-rich medium in the presence of added LPS (10 ng/ml). Increasing amounts of lead acetate or sodium acetate were also added to the cultures. After 24 h, superoxide production was determined by the PMA-triggered cytochrome *c* reduction assay. Results are expressed as ΔA/mg of cell protein ± S.D., after 120 min. Similar results were obtained in three other experiments.

Expt. no.	[Lead acetate] (μM)	[Sodium acetate] (μM)	Cytochrome *c* reduction at 120 min
1	0	0	3.33±0.40
	5	–	2.29±0.25
	10	–	1.56±0.08
	50	–	0.75±0.20
	100	–	0.85±0.19
2	0	0	2.24±0.62
	50	–	0.53±0.15
	100	–	0.44±0.11
	–	50	2.11±0.45
	–	100	2.50±0.29

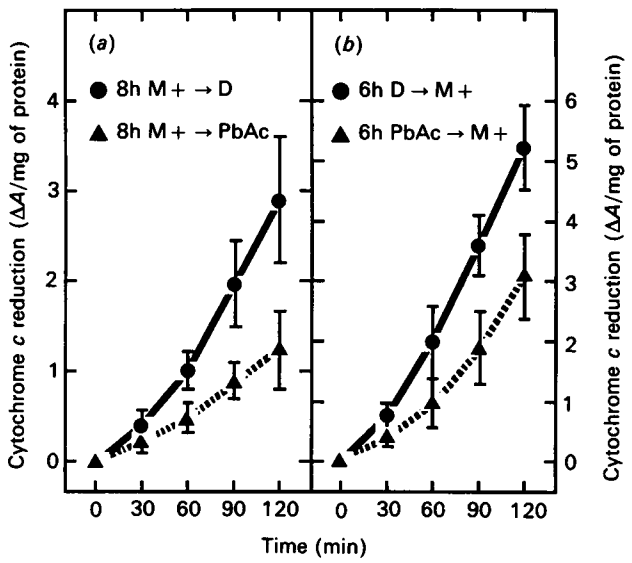

Fig. 2. Effect of the time of lead treatment relative to MAF-induced activation on macrophage respiratory burst

(*a*) CBA/T6 macrophages were pulsed for 8 h with a 1:16 dilution of MAF-rich medium in the presence of added LPS (M^+), then washed and re-incubated with Dulbecco's medium in the absence (D) or presence (PbAc) of 50 μM-lead acetate; 18 h later, cells were washed and superoxide production was determined by the PMA-triggered cytochrome *c* reduction assay. Data are means ± S.D. for four experiments. (*b*) CBA/T6 macrophages were incubated for 6 h with Dulbecco's medium in the absence (D) or in the presence (PbAc) of 100 μM-lead acetate, then washed and re-incubated with a 1:16 dilution of MAF-rich medium containing 10 ng of LPS/ml; 20 h later, cells were washed and tested as described above. Data are means ± S.D. for eight experiments.

Table 2. Effect of lead on H_2O_2 production by activated macrophages

C57BL/6 bone-marrow macrophages were incubated with a 1:128 dilution of MAF-rich medium containing 10 ng of LPS/ml in the presence of increasing concentrations of lead acetate or sodium acetate. After 24 h, H_2O_2 production was measured by the fluorescent scopoletin-oxidation assay. Results are expressed as arbitrary units of fluorescence change per μg of cellular protein, ± S.D., after 60 min. The data shown are representative of five experiments.

[Lead acetate] (μM)	[Sodium acetate] (μM)	Fluorescence change after 60 min
0	0	814 ± 133
10	–	450 ± 50
25	–	236 ± 85
50	–	137 ± 23
100	–	107 ± 18
–	10	714 ± 154
–	25	675 ± 157
–	50	716 ± 165
–	100	634 ± 142

Table 3. Effect of lead on the induction of HMPS activity in macrophages exposed to MAF and LPS

C57BL/6 bone-marrow (Expt. I) and Balb/c peritoneal (Expt. II) macrophages were exposed to a 1:64 dilution of MAF-rich medium containing 10 ng of LPS/ml in the presence of increasing concentrations of lead acetate; 24 h later, HMPS activity was measured by determining the amount of $^{14}CO_2$ evolved from [1-^{14}C]glucose. Results are means ± S.D. for four replicates; n.d., not determined.

[Lead acetate] (μM)	HMPS activity (c.p.m./μg of protein)	
	Expt. I	Expt. II
0	1519 ± 342	461 ± 62
10	1027 ± 373	n.d.
30	755 ± 46	371 ± 42
100	688 ± 217	261 ± 63

Table 4. Effect of lead acetate on the triggering by PMA of the macrophage respiratory burst

CBA/T6 macrophages were incubated with a 1:16 dilution of MAF-rich medium in the presence of 10 ng of LPS/ml; 24 h later, cultures were washed and re-incubated with Dulbecco's medium in the presence or absence of 50–100 μM-lead acetate for 1 h. Cultures were then tested for superoxide production by using the PMA-triggered cytochrome *c* or NBT reduction assays in the presence of the same concentrations of lead as in the preincubation medium. Results are expressed as ΔA/mg of protein, ± S.D.

[Lead acetate] (μM)	Cytochrome *c* reduction at 120 min*	NBT reduction at 45 min†
0	3.03 ± 0.33	8.79 ± 1.96
50	4.00 ± 0.18	10.36 ± 1.71
100	4.79 ± 0.45	8.79 ± 1.66

* Representative of four experiments.
† Representative of three experiments.

strongly impaired the oxidative response (Fig. 1). Such inhibition was dose-dependent; more than 50% inhibition of the respiratory burst was observed in macrophages activated with MAF + LPS in the presence of 10 μM-lead acetate. This effect was not due to the acetate anion, since incubation with sodium acetate failed to decrease respiratory-burst activity (Table 1). The cell viability was, however, decreased by the presence of lead acetate at 100 μM (76.6 ± 5.3% viable cells in cultures treated for 24 h with 100 μM-lead, against 94.6 ± 4.9% in untreated cultures). At 50 μM-lead acetate, however, percentage cell viability was not significantly different from that of control cultures (87.2 ± 3.4 against 94.6 ± 4.9). Because of the possible toxicity of the metal, results were corrected for the amount of cell-associated protein present in each well at the end of incubation with lead.

The continuous presence of lead acetate during the activation process was not required to obtain inhibition

of respiratory-burst activity. Thus metabolic impairment was also observed when the metal was added as late as 8 h after exposure of macrophages to MAF and LPS (Fig. 2*a*). Under these conditions, however, inhibition of activation was incomplete, and a substantial capacity to generate superoxide was retained by the cells. Moreover, reversal of the lead effect was not immediate upon removal of the heavy metal, since a 6 h preincubation with lead acetate resulted in significant impairment of macrophage oxidative metabolism upon subsequent activation for 20 h in lead-free medium (Fig. 2*b*).

Inhibition of the lead-induced respiratory burst as measured by the cytochrome *c* reduction assay was confirmed by measurements of H_2O_2 release. The latter was indeed markedly decreased by the presence of the metal during MAF-induced activation (Table 2). A similar inhibition was observed by using three other assays, i.e. the NBT reduction test, measurements of chemiluminescence (results not shown), and HMPS activity (Table 3).

Table 5. Effect of lead acetate on macrophage G6PD activity

Macrophages were incubated for 24 h with Dulbecco's medium in the presence or absence of lead acetate (50 μM). Cells were then lysed as described in the Materials and methods section, and G6PD activity was determined in the cell extract directly or after a 15 min preincubation with lead acetate (100 μM). Controls with sodium acetate are included. Results are expressed as m-units/mg of extract protein, ±S.D., and are representative of three experiments.

Cell treatment	Extract treatment	G6PD activity (m-units/mg of protein)
Lead acetate	None	190±17
Sodium acetate	None	205±19
None	Lead acetate	209±23
None	Sodium acetate	237±31
None	None	244±34

Effect of lead acetate on triggering of the macrophage respiratory burst by PMA

Although the above experiments suggested that lead did interfere with the metabolic stimulation induced by MAF and LPS, they could not distinguish between an effect on the activation process itself, and inhibition of the triggering events induced by PMA. To investigate this point, the effect of the metal on the triggering step of the respiratory burst was examined. Macrophages were activated by MAF and LPS, then exposed to lead acetate for 1 h before triggering with PMA. Under these

Table 6. Effect of lead on cell-free HMPS activities

Data (±S.D.) are representative of two experiments.

(*a*) Effect of lead on HMPS activity of purified components and cell extracts

		10^{-3} × HMPS activity (c.p.m.)	
		Lead acetate (100 μM)	Without lead acetate
(a) Complete system*		16.0±0.5	16.1±0.8
(b) Complete system minus G6PD and 6PGD		n.d.	0.9±0.1
(c) As (b), +macrophage extracts† diluted:	1:4	170.7±8.8	184.2±12.9
	1:8	161.0±1.6	164.5±5.7

(*b*) HMPS activity in extracts of lead-treated macrophages

		10^{-3} × HMPS activity (c.p.m.)	
24 h incubation with 100 μM-lead acetate...		Yes	No
(d) Macrophage extract‡ diluted	1:4	41.1±3.1	43.1±5.7
	1:8	29.6±2.8	31.6±2.1
(e) Macrophage extract plus hexokinase§ diluted:	1:4	88.3±5.2	81.2±9.5
	1:8	n.d.	71.1±3.5
	1:16	45.2±9.2	56.9±2.4
	1:32	25.8±3.9	29.3±3.5
	1:64	16.7±1.9	16.6±0.7
	1:128	6.3±1.0	8.3±1.0
Control (no extract)		–	1.3±0.1

* The complete system (S. Wirth & J. Mauël, unpublished work) contained all enzymes, substrates and cofactors of the irreversible part of the HMPS (see the Materials and methods section), with 30 μM-Methylene Blue added as a stimulus.
† Prepared by sonication of macrophages at a concentration of 3×10^6 cells/ml in PBS (see the Materials and methods section).
‡ Tested in the presence of ATP (2 mM final concn.), $NADP^+$ (0.05 mM), $MgCl_2$ (8.5 mM) and Methylene Blue (3 μM).
§ Same reagents as in (d), with hexokinase (4.5 units/ml) added.

Table 7. Effect of lead acetate on glucose incorporation by macrophages

CBA/T6 macrophages were incubated for various lengths of time with increasing concentrations of lead acetate at 37 °C, then washed and exposed for 1 h to 2-deoxy-D-[2,6-^{3}H]glucose in glucose- and bicarbonate-free Eagle's medium in the continuous presence of the metal. Cells were then washed and lysed, and radioactivity was determined in the lysate. Data are means ± S.D. of three experiments.

[Lead acetate] (μM)	Duration of incubation with PbAc (h)...	2-Deoxyglucose incorporation (c.p.m./5 μg of protein)			
		1	4	8	24
0		1007 ± 209	685 ± 55	1886 ± 358	2529 ± 982
30		693 ± 83	826 ± 50	943 ± 160	1286 ± 116
100		1004 ± 30	700 ± 35	743 ± 89	770 ± 162

conditions, the metal failed to inhibit superoxide production as determined by the cytochrome *c* and NBT-reduction assays (Table 4). These results strongly suggest that lead-mediated respiratory-burst inhibition resulted from interference with events occurring during the activation process itself and not from direct blockade of the macrophage NADPH oxidase system.

Effect of lead acetate on the HMPS activity of macrophage extracts

The generation by phagocytes of oxygen metabolites such as superoxide is dependent on the activity of the HMPS pathway. However, lead has been reported to inhibit enzymes rich in thiol groups, including HMPS enzymes such as G6PD and 6GPD (see the Discussion section). Experiments were thus performed to determine whether the lead-induced metabolic inhibition of lymphokine-activated macrophages was due to a direct action of the metal on HMPS enzymes. To this end, macrophage extracts were prepared by sonication or by lysis with digitonin and tested for HMPS activity upon stimulation with Methylene Blue. The results can be summarized as follows. (1) Lead failed to inhibit G6PD activity of the extracts, whether the metal was added 24 h before, or after, cell lysis (Table 5). (2) When macrophage extracts were used to supplement a cell-free HMPS system (see the Materials and methods section) containing isolated enzymes (minus G6PD and 6PGD), cofactors and substrates of the irreversible part of the pathway, addition of lead failed to inhibit $^{14}CO_2$ production from D-[1-^{14}C]glucose (Table 6*a*). Interestingly, replacement of G6PD and 6PGD with the macrophage extract (line c) resulted in much higher HMPS activity, even when such extracts were diluted to contain the equivalent of approx. 0.4×10^6 macrophages/ml (1:8 dilution). (3) When macrophages were preincubated for 24 h with 100 μM-lead acetate, then extracted by sonication, the HMPS activity of the extract was not significantly different from that of control extracts (Table 6*b*, line d). Addition of hexokinase further stimulated the activity (line e). Significant HMPS activity could be detected down to a dilution of 1:128, containing the equivalent of approx. 25×10^3 macrophages/ml.

Effect of lead acetate on glucose incorporation by macrophages

The above experiments suggested that lead did not inhibit the macrophage respiratory burst by blocking HMPS activity. Experiments were thus performed to determine whether the metal might interfere with glucose uptake by the cells. Macrophages were incubated with lead acetate for various durations, then tested for glucose incorporation by measuring the uptake of 2-deoxyglucose, a non-metabolizable glucose analogue. Exposure to the metal indeed led within 8 h to a high inhibition of uptake of the labelled compound relative to controls run at each time point (Table 7). Interestingly, however, total glucose incorporation was not significantly different in macrophages exposed to 100 μM-lead acetate for 24 h from that in control macrophages tested after 1 h in culture.

DISCUSSION

The present study was aimed at determining the effects of lead on the capacity of macrophages to develop a respiratory burst in response to activation by MAF and LPS. Treatment with the metal during activation resulted in marked impairment of the oxidative response. This was evidenced by decreased HMPS activity and decreased superoxide and H_2O_2 production. Metabolic inhibition by lead acetate was due to the metal rather than to the acetate anion, as shown by the absence of effect of sodium acetate in the various assays. Moreover, respiratory-burst inhibition was not an artifact due to cell death. Since lead at high concentration (0.1 mM) did affect to some extent cell viability after 24 h of incubation, the measured activities were corrected for the amount of cell protein in each experimental group at the end of incubation with lead. At lower concentrations, however, lead did not decrease macrophage viability, although it still inhibited the cellular metabolic response, indicating that the impaired oxidative burst was not the result of death of an active subpopulation of cells.

The above results with MAF-activated macrophages confirm the observations by Hilbertz *et al.* [3] on non-activated resident murine peritoneal cells. These authors showed that a 20 h incubation with 0.1 mM-lead suppressed lucigenin-enhanced chemiluminescence induced by both zymosan and PMA. As described in the present paper, whereas a 24 h exposure to lead markedly impaired the PMA-dependent oxidative response, a short incubation with the metal led to stimulation rather than inhibition of respiratory-burst activity as measured by the cytochrome *c* reduction test. Although the results of Castranova *et al.* [26] suggest that this technique is

inappropriate for measuring superoxide release in the presence of lead, owing to binding of the metal to the cytochrome *c* molecule, we failed to notice any interference with the assay. These results were further confirmed by the NBT reduction test. Our findings are again in agreement with those of Hilbertz *et al.* [3], who demonstrated that, within the first 1 h of exposure to lead, the PMA-triggered chemiluminescence of murine peritoneal macrophages was enhanced by this ion. When zymosan was used as a triggering agent, however, a short preincubation with lead was sufficient to impair the macrophage respiratory burst (results not shown), as also reported by others [2,26,27]. These observations are consistent with the hypothesis that inhibition by lead of macrophage oxidative metabolism is not due to inactivation of the membrane-bound NADPH oxidase thought to be responsible for superoxide production.

The different effects observed after a 1 h and a 24 h exposure to lead might be explained by differences in the site or mode of action of the metal. Increase of respiratory-burst activity after short treatment with lead might result from an effect of the ion on the cell membrane. Heavy metals modify the ionic configuration of the cell surface [5], which might promote the activation of specific surface receptors involved in triggering the respiratory burst. However, a longer incubation with lead might affect intracellular target molecules. Indeed, heavy metals have been shown to inhibit various thiol-dependent enzymes, to alter mitochondrial structures and to interact with DNA and RNA in multiple ways [28,29]. In addition, when added to cells together with MAF, the metal might inhibit the priming of the respiratory burst through direct interaction with the lymphokine, or through competition with the lymphokine for sites on the cell surface, as already speculated for migration-inhibitory factor [5]. The latter possibility appears unlikely, however, since no decrease in the number of bound lymphokine molecules could be detected on the surface membrane of lead-treated relative to untreated macrophages [30].

On the other hand, lead has a high affinity for thiol groups, leading to inhibition of thiol-dependent enzymes [28,29]. In particular, the metal has been shown to depress the activity of erythrocytic G6PD and 6PGD, thus inhibiting the HMPS pathway in these cells [31,32]. To determine whether the respiratory-burst decrease was due to an inhibition of enzymes of the HMPS pathway, we tested the effect of the metal on HMPS activities in several cell-free systems. At concentrations that depressed the MAF-dependent HMPS stimulation in macrophages, lead failed to inhibit the conversion of glucose *in vitro* by the HMPS enzymes, nor did it interfere with HMPS activity of fresh macrophage extracts, whether obtained from lead-treated or untreated cells. Moreover, when the specific activity of G6PD was measured in macrophage extracts, no inhibition of the enzyme by lead could be detected. These observations suggest that a direct effect of the metal on the enzymes responsible for HMPS activity is unlikely, and that respiratory-burst inhibition in lead-treated macrophages was not due to impairment of the HMPS enzymes.

Reactive intermediates of oxygen are formed by reduction of NADPH generated in the HMPS. Activity of the latter metabolic pathway is strictly dependent on the availability of glucose, and indeed, glucose deprivation strongly interferes with the microbicidal mechanisms of phagocytes [8]. We therefore tested the possible inhibitory effect of lead on glucose transport into the cells. Glucose uptake can be conveniently measured by incorporation of labelled 2-deoxyglucose, a glucose analogue that shares a common membrane transport system with glucose but is not metabolized [33–36]. Incubation with lead did decrease glucose incorporation in macrophages, which might explain inhibition of the oxidative metabolism reported in the present paper, and might also be partially responsible for depressed microbicidal action observed in MAF-treated macrophages [30].

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