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Effects of heavy metals on the viability of A549 cells

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Heavy metals, such as Cd and Pb, are present in the particulate matter (PM) suspended in ambient air. Once internalized, they often accumulate in the human body and cannot be metabolized for long periods. Most previous studies on Cd and Pb metabolism used high concentrations of these heavy metals. In this study, we analysed the effects of low concentrations of Cd or Pb (similar to their concentrations in the PM) on the viability of lung epithelial cells and the secretion of pro-inflammatory factors, IL-6 and IL-8. A549 cells were cultured in the presence of Cd and Pb at 1 μg/L and 1 mg/L for 0, 4, 12, and 24 h and analysed using WST-8 and lactate dehydrogenase (LDH) assays and ELISA. Our results revealed that Cd and Pb induced comparable cytotoxicity and secretion of pro-inflammatory cytokines, IL-6 and IL-8, in a time-dependent manner, even at low concentrations. Altogether, our results suggest that heavy metals present in PM can cause chronic long-term effects on human lungs.

Keywords: Cytokine, Lactate dehydrogenase, Particulate matter

Heavy metals, such as cadmium (Cd), barium (Ba), hydrargyrum (Hg), and lead (Pb), are present as particulate matter (PM) in the air and can reportedly cause PM-induced biological damage to cells, animals, and humans[1]. Therefore, although heavy metals account for only a small proportion of PM mass, their potential toxicity should not be neglected[2].

Among these metals, Pb, a widespread occupational and environmental xenobiotic, is hazardous to humans and various ecosystems[3].It is a free metal in various compounds and enters the human body primarily by ingesting Pb-contaminated food or inhaling Pb-contaminated fumes or air[4]. Although it occurs naturally in the environment, it is also emitted during smelting and mining and from industries producing plastics and alloy batteries.

Furthermore, tobacco, a carcinogen, is a source of Cd, and direct or indirect exposure to tobacco is the largest source of Cd exposure in the human body[5]. Smoking is one of the most significant risk factors for the development of cardiovascular diseases. Smokers exposed to PM have a higher risk of arrhythmia, heart failure, cardiac arrest, and hypertensive disorders than non-smokers under similar environmental conditions[6].

Exposure to $PM_{2.5}$ and PM_{10} is directly associated with ventricular ectopy in smokers, suggesting that these individuals are more vulnerable to the arrhythmia-inducing effects of PM, although further studies are required to discriminate between acute and chronic effects of PM[7].

Cell proliferation and viability are essential parameters in the study of life sciences. Cytotoxicity and cell viability can be measured by various cellular functions, such as mitochondrial function and cell membrane permeability. Water-soluble tetrazolium salt (WST) and lactate hydrogenase (LDH) colorimetric methods are widely used. The demand for sensitive, reliable, fast, and simple techniques to analyse cell proliferation and viability has (nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) (WST-8), WST-8 is relatively stable[8].

The LDH leakage on the LDH activity in the extracellular medium is a reliable, fast, and simple method to evaluate cytotoxicity[9] and was used to determine HepG2 cell viability following exposure to $CdCl_2$[10]. The release of intracellular LDH into the culture medium indicates cell membrane damage leading to irreversible cell death[11].

Therefore, in this study, we evaluated the toxic effects of Pb- and Cd-containing fine dust (PM_{10}) on lung cells. We selected two concentrations of Cd and

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Abbreviations: Cd, Cadmium; Pb, lead; PM, particulate matter; IL-6, Interleukin-6; IL-8, Interleukin-8; LDH, lactose dehydrogenase

Pb to reflect the levels in water and PM and observed their cytotoxic effect on lung cells within 24 h of exposure.

Materials and Methods

Chemicals

Cd standard solution-1000 ppm (Cat. No. 07993-1B) and Pb standard solution-1000 ppm (Cat. No. 24239-2B) were obtained from Kanto Chemical (Tokyo, Japan). Thus, our study provides novel insights into the pathogenic effects of Cd and Pb exposure on cell toxicity.

Cell culture

Human lung alveolar carcinoma epithelial cells, A549,were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in Dutch Modified (1×) RPMI 1640 medium (LM 011-07; Welgene, Gyeongsan-si, Republic of Korea) in a humidified incubator(VS-2180C;Vision Scientific, Daejeon-si, Korea) at 5% CO_2 and 37°C. Culture media were supplemented with 10% foetal bovine serum (FBS; Corning, Glendale, USA) andpenicillin-streptomycin (100×; CA005-010; GenDEPOT, Baker, USA)[12].

Cell exposure to standard Cd and Pb solutions

A549 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated in a humidified incubator (Vision Scientific) for 24 h at 37°C and 5% CO_2. Cells were treated with Cd or Pbat 10 µg/L and 10 mg/L for 0, 4, or 12 h.

Cell viability

A549 cells treated with different concentrations of Cd or Pb (1 µg/L and 1 mg/L) for 24 h and control cells incubated in culture medium were subjected to WST-8 cell viability assay. The effect of PM on cell viability was determined using the Quanti-MAX WST-8 Cell Viability Assay Kit (Biomax, Seoul, Republic of Korea). Briefly, 10 µL WST-8 was added to each well and incubated for 30 min at 37°C. Subsequently, the absorbance was measured at 450 nm using Flex Station 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, USA). RPMI 1640 medium without Cd or Pb was used as a negative control, and changes in cell viability were determined in triplicate[13].

LDH release

A549 cells were treated with different concentrations of Cd or Pb (1 µg/L and 1 mg/L), and the LDH release into the culture medium was determined using the Quanti-LDH PLUS Cytotoxicity Assay Kit (Biomax). The cytotoxicity of PM_{10} was determined using the Quanti-LDH PLUS Cytotoxicity Assay Kit, as mentioned earlier. Aliquots (100 µL) of the cell culture medium were collected from each well and placed on a fresh microtiter plate. LDH solution (100 µL) was added to each well, and plates were incubated for 30 min at 37°C[14]. Subsequently, the absorbance was measured at 490 nm using the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices). Each experiment was performed in quadruplicate. Cytotoxicity was expressed relative to the basal LDH release as determined using untreated control cells and the medium lacking FBS. The test was repeated three times, and the amount of LDH released by PM-exposed cells was expressed as a percentage of that released by non-exposed cells.

Cytokine assay using ELISA

A Human Premixed Multi-Analyte Kit from Bio-techne (Minneapolis, USA) was used to measure the levels of released cytokine in the cell culture supernatants from Cd-treated, Pb-treated, and control cells[15].All experiments were performed in triplicate to confirm data and curtail intra-group variation. Absorbance at 450 nm was determined using a microplate reader. Interleukin (IL)-6 and IL-8 levels were calculated using standard curves for each IL and expressed as pg/mL.

Fluorescence microscopy

Treated A549 cells were analysed with the MAX-View™ Live/Dead Cell Staining Kit (Biomax) according to the manufacturer's instructions. Fluorescence images were observed with a fluorescence microscope (ImageXpress Nano; Molecular Devices). Live cells (green fluorescence) were observed at 490nm, whereas dead cells (red fluorescence) were observed at 540nm.Images were processed using ImageXpress Nano Automated Imaging System using Cell Reporter Xpress software (Molecular Devices).

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) in Microsoft Excel 2019 (Build 14430.20306). The results are expressed as the mean ± standard error. Statistical significance was set at $P < 0.05$.

Results

We observed a time-dependent effect of Cd and Pb on cell viability.After 4, 12, and 24 h, Cd at 1µg/L reduced the cell viability to 91%, 86%, and 44%; Cd at

1mg/L reduced the cell viability to 78%, 67%, and 42%; Pb at1 μg/L reduced the cell viability to 99%, 82%, and 43%; and Pb at 1mg/L reduced the cell viability to 78%, 67%, and 44%, respectively,(Fig. 1A & B)(**P* <0.05).

Cd and Pb exposure for up to 24 h increased LDH levels in the culture supernatant of treated cells in a time-dependent manner. Additionally, increased LDH release was observed in cells exposed to 1 mg/L Cd (Fig. 2A). The cytotoxic effects of Cd were more pronounced than those observed for Pb at the same concentration (Fig. 2B). Moreover, both Cd and Pb induced membrane damage. (**P* <0.05)

Furthermore, Cd and Pb exposure for 24 h enhanced IL-6 and IL-8 levels in A549 cells in a time-dependent manner. Exposure to Cd at 1 mg/L for 24 h yielded the IL-6 and IL-8 maximum values (Fig. 3). Fluorescent staining was performed to evaluate the cytotoxic effects of Cd and Pb on A549 cells, and the results revealed that exposure to 1 μg/L Cd or Pb did not cause notable cytotoxicity in A549 cells; however, treatment with 1 mg/L Cd or Pb triggered significant cell death. Cd caused significantly higher cell death than Pb (Fig. 4).

Discussion

In previous experiments, heavy metals contribute to PM-induced cytotoxicity (cellular oxidative stress and inflammatory response)[16], suggesting that heavy metal components are essential contributors to PM-induced cytotoxicity. Most studies were performed at moderate to high heavy metal doses; hence, the effects of low heavy metal exposure on cell viability and cytokine expression levels were thus far unknown. Therefore, in this study, we aimed to evaluate the cytotoxic effects of airborne heavy metal concentrations on human lung epithelial cells. We

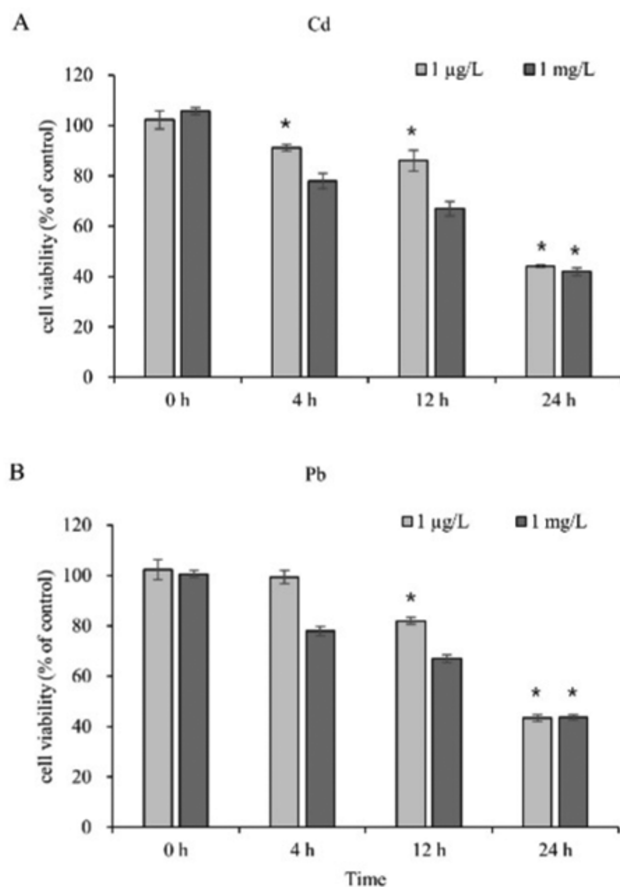

Fig. 1 — Effect of (A) cadmium (Cd); and (B) lead (Pb) standard solution extracts (1 μg/L and 1 mg/L) on A549 cell proliferation. The inhibitory effect on cell proliferation was determined by WST-8 assay and calculated by comparing the absorbance at 450 nm of the culture medium of heavy metal-exposed cells with that of the control group

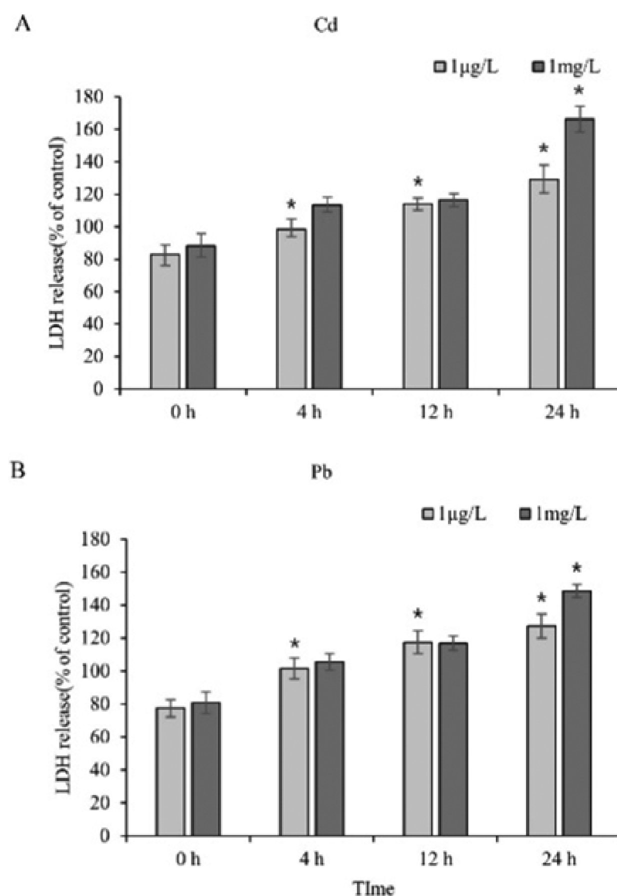

Fig. 2 — LDH released from A549 cells after exposure to (A) cadmium (Cd); and (B) lead (Pb) standard solution extracts (1 μg/L and 1 mg/L). LDH release was calculated spectrophotometrically using a culture medium of PM-exposed cells; non-PM-exposed cells were used as a control. **P* <0.05 compared with the control

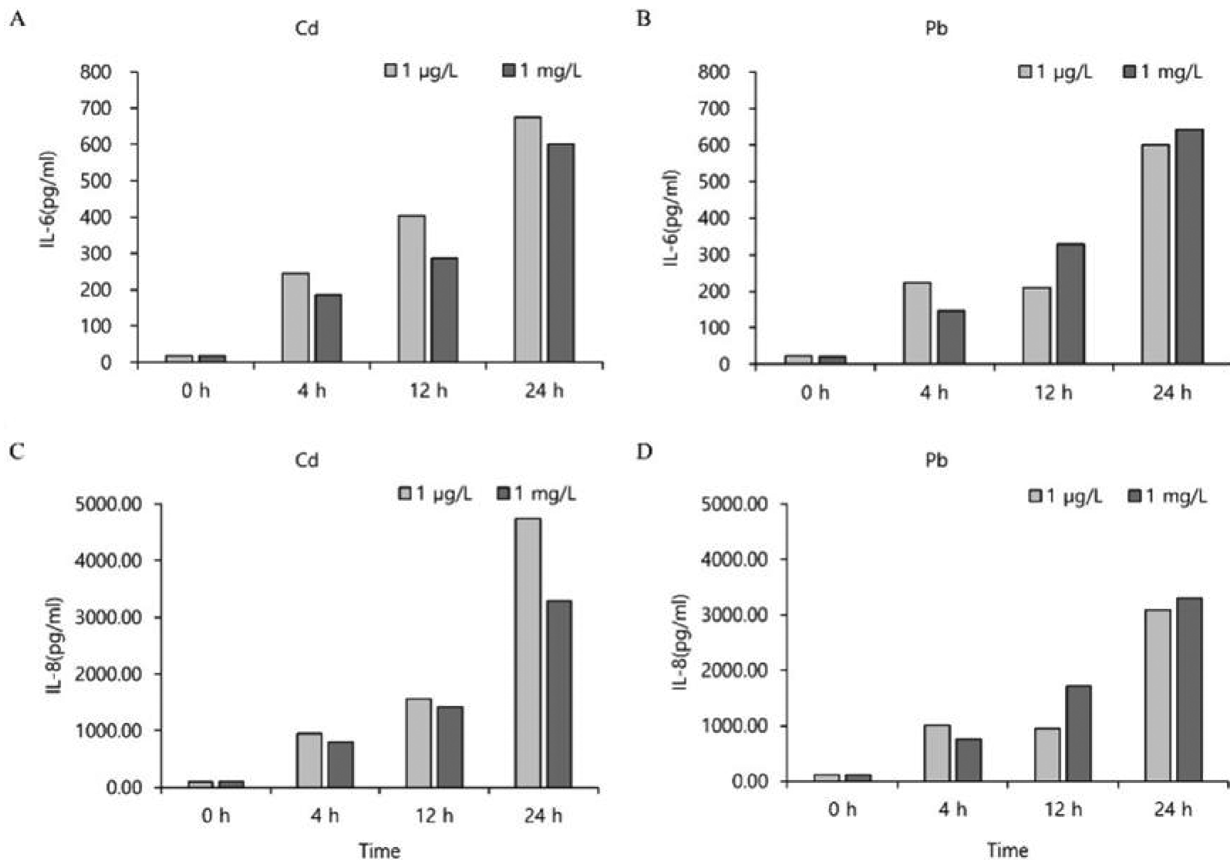

Fig. 3 — ELISA analysis of the expression of IL-6 and IL-8 induced by cadmium (Cd) and lead (Pb) standard solutions (1 μg/L and 1 mg/L) in A549 cells. (A & B) IL-6 exposed to Cd and Pb and (C & D) IL-8 exposed to Cd and Pb. The figure shows the means ± SD of three independent experiments within each treatment group

investigated the effects of low doses of Cd and Pb on lung cell viability using a WST assay.

The MTT assay is based on the ability of NAD(P)H-dependent cellular oxido reductases to reduce MTT to formazan crystals. However, it may not distinguish between cytostatic and cytotoxic agents. Therefore, to assess the cytotoxicity of Cd and Pb in A549 cells, we quantified LDH release, which is associated with loss of cell membrane integrity and subsequent cell death. The LDH release profile mirrored the results of the MTT assay. The leakage of the LDH enzyme from the cytosol into the culture medium following heavy metal exposure is a marker of membrane damage.

Smelter workers who were occupationally exposed to Pb and Cd had blood Cd levels as high as 4.27 μg/dL[17]. However, in our study, the dose used for cellular exposure measurements was extremely low (1 μg/L). Furthermore, by using the same concentration range for Cd and Pb, we could directly compare the toxicity of the two heavy metals and determine whether a common mechanism underlies the cytotoxicity observed at a given concentration.

As the main site of reactive oxygen species (ROS) generation, most mitochondria are prone to pathological changes associated with oxidative stress with lethal consequences, such as mitochondrial DNA (mDNA) damage, ultimately promoting apoptosis[18]. Upon mitochondrial dysfunction, endothelial cells secrete multiple pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α. The critical role of inflammatory responses in health and diseases has long been recognized. Pro-inflammatory responses are critical for developing various health effects associated with airborne PM[19]. Our findings suggest that Cd and Pb elicit pro-inflammatory effects in A549 cells in a time-dependent manner. Differences in the cytokine/chemokine profiles induced by the test compounds indicate that they may activate distinct immune cell populations. IL-6 and IL-8 are well-known cytokines associated with cancer progression. Airway inflammation leads to tissue damage, and inflammatory cytokines released by inflammatory cells and airway resident cells are thought to play a major role in airway inflammation[20]. In addition,

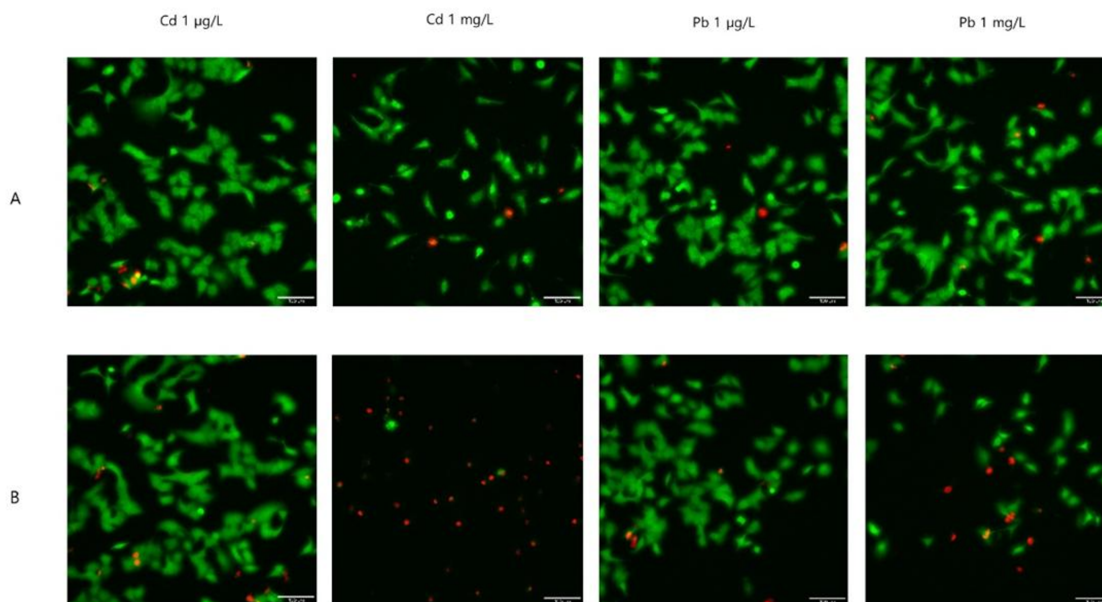

Fig. 4 — Cells were treated with 1 μg/L and 1 mg/L of cadmium (Cd) and lead (Pb) and subjected to Live/Dead cell staining. Panel (A)0 h after treatment. Panel (B) 24 h post-treatment. Scale bar 5200 lumens

inflammation contributes to many diseases, including tissue fibrosis and tumour formation[21]. Unlike previous studies wherein high Cd concentrations promoted the production of inflammatory factors[22], after 4 h of exposure, IL-6 and IL-8 levels in cells exposed to 1 mg/L Cd were lower than those observed in cells treated with 1 μg/L Cd at the same time-point. This was associated with cell viability, as cell death increased after 24 h of exposure to 1 mg/L Cd, as evidenced by fluorescence microscopy. However, Cd at high concentrations inhibited the inflammatory response, resulting in decreased immunity. Initially, 1 mg/L Pb exposure induced cell death, releasing IL-6 and IL-8 at concentrations lower than those observed for 1 μg/L Pb exposure. These findings were similar to those of Cd exposure. However, after 12 h, 1 mg/L Pb elicited a greater production of inflammatory factors than 1 μg/L Pb, thereby inducing a stronger inflammatory response. The effects of Cd on lung epithelial cells likely manifest as a long-term process. It has also been reported that human lung cells retain Cd resistance, abnormal expression of DNA repair genes, and reduced DNA repair capacity after Cd exposure[23]. Although Pb influenced cell viability during early exposure, cells gradually showed signs of recovery in the later stages. The cytotoxicity analysis also showed that the damage caused by Pb was milder thanthat caused by Cd, and the duration of these cytotoxic effects was shorter for Pb exposure. Altogether, within 24 h of exposure, the production of inflammatory factors induced by Cd and Pb at the same concentrations was time dependent.

Nonetheless, the effects of Cd and Pb might vary with different cell types. For instance, the stronger cytotoxic effects of Cd compared to Pb observed in this study do not apply to U87MG cells[24]. Exposure to Cd is associated with many diseases, including early atherosclerosis, bone lesions, and cardiovascular diseases[25].Furthermore, Pb can cause severe neurological damage, cross the blood-brain barrier, interfering with neurotransmission pathways, and reduce neuronal growth[26]. This seems to indicate that the effect of Cd on nerve cells is not as common as that of Pb and that the concentrations of Cd and Pb in PM affect the cellular activity and cytotoxicity of lung epithelial cells and nerve cells.Our focus for future studies is now on the effect of viral infection on cell viability, cytotoxicity, and cytokine production under the same Cd and Pb conditions.

Conclusion

In the present study, we found that exposure to low concentrations of Cd or Pb induced time-dependent changes in cell viability and expression of pro-inflammatory cytokines such as IL-6 and IL-8 in A549 cells. This finding suggests lung cells can absorb heavy metals even at low concentrations. Furthermore, although the responses were time-dependent, the initial reactions were not significantly different between 1 μg/L and 1 mg/L Cd and Pb concentrations. The range of long-term effects of

these pollutants should be further studied to elucidate the underlying mechanisms of action.

Conflict of interest

All authors declare no conflict of interest.

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