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Gut microbiota limits heavy metals burden caused by chronic oral exposure

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HIGHLIGHTS

- We model chronic ingestion of environmental lead and cadmium in axenic mice.
- We addressed the role of the microbiota in heavy-metal dissemination in organs.
- We delineate the direct impact of the non-absorbed heavy metals on gut homeostasis.
- We measure transport- and oxidative-gene expression in intestine.
- It enlightens risk assessment of heavy metals in intestinal disease's susceptibility.

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ABSTRACT

Environmental exposure to pollutants such as heavy metal(s) is responsible for various altered physiological functions which are detrimental for health. The gut microbiota is critical for intestinal homeostasis but its role on xenobiotic handling is not fully understood, especially when continuous sub-chronic exposure is addressed. We first confirmed the essential role of the intestinal microbiome to limit heavy metal body burden by using germ-free mice following 6-weeks oral exposure. Significant increases of cadmium and lead absorption and dissemination in blood and target organs were measured in germ-free mice when compared with conventional specific pathogen free (SPF) mice. Besides the "barrier" function of the luminal microbiota, this may involve specific host-genes such as metallothioneins, which are differentially expressed in the gastrointestinal tract of each group of mice. Considering genes relevant for divalent metal transporters and oxidative pathways, significant differences in basal gene expression were measured between control and germ-free mice. Moreover, the magnitude of induction of these genes upon stimulation by heavy metals varied greatly depending on the dose and type of metal as well as the microbial status of the animal. Collectively, these data illustrate the complex host-microbes interplay occurring with environmental pollutants inside the gut.

1. Introduction

Inorganic cadmium (Cd) and lead (Pb) ions are the most representative toxic non-essential elements which can contaminate food, water or air. In industrial areas, occupationally exposed workers as well as environmentally exposed populations can experience moderate to severe health perturbations due to the chronic ingestion of heavy metals. It has been shown that a significant fraction of inhaled Cd (60%) ends up in the gastrointestinal tract, as a result of mucociliary clearance and subsequent ingestion (Satarug et al., 2003). Following oral entry of Cd and Pb, the body burden of these metals has been clearly linked to various sorts of diseases based on various mechanisms, including those involved in oxidative stress and extended toxicity such as genotoxicity and carcinogenesis.

The impact of gut ecology on the absorption, distribution, metabolism and excretion of xenobiotics has received little attention (Nicholson et al., 2005). However, the gut microbiota is likely an important mediator of the bioavailability and toxicity of environmental pollutants including heavy metals. On the one hand, the

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microbiota by itself may interact with metals inside the gut, either by active uptake or by passive ad- or absorbtion (Morozzi et al., 1986; Halttunen et al., 2008). On the other hand, intestinal barrier integrity, as the first line to control the entry of ingested toxic metals, also depends on microbial-host interactions that involve epithelial junctions and physical impediments of the mucous layer. Finally, the gut microbiota and its metabolites will also impact on environmental parameters such as pH, oxidative balance, detoxification enzymes, and xenobiotic-metabolizing and transporting host proteins (Claus et al., 2011), all of which may highly influence the bioavailability of chemicals in the gut lumen. The microbial status furthermore affects hepatic and renal metabolism.

While Louis Pasteur considered that germ-free life was impossible (Pasteur, 1885), today we know that germ-free mice are available and can be grown as far as proper technical requirements associated with rearing are achieved; the concept of the gut microbiota as a "forgotten organ", essential for gut homeostasis, is also well accepted. Therefore, axenic mice, so called germ-free mice provide useful tools to study the interplay between the host and the gut inhabitants (Smith et al., 2007; Yi and Li, 2012). Germ-free animals are, however, physiologically different from conventional specific pathogen free (SPF) individuals and thus may show differences in the *in vivo* handling of xenobiotics (Ilett et al., 1990).

Recently, we reported on the dissemination to the intestine and to primary organs of continuous ingestion of low- and moderate environmentally relevant concentrations of Cd and Pb in mice (Breton et al., 2013). The aim of the current study was to address the role of the gut microbiota in the bioaccumulation and retention of Cd and Pb in primary organs following oral exposure. To examine these events, we used germ-free and SPF mice subjected to a chronic ingestion of various environmentally relevant doses of Cd and Pb. We also determined changes in gene expression of several intestinal markers, addressing aspects of transport- and oxidative stress functionality of the proximal (duodenum) and distal (colon) mucosa.

2. Materials and methods

2.1. Animals

Forty germ-free (GF) female mice, aged 6–7 weeks and with a C57BL/6J genetic background were bred in the animal facility at the Transgenose Institute (TAAM, National Center for Scientific Research, Orléans, France) while 40 age-matched C57BL/6J female specific pathogen free (SPF) mice were obtained from Charles River (Saint-Germain-sur-l'Arbresle, France). Both groups (GF and SPF, respectively) were kept in separate isolators in a controlled sterile environment (22 °C temperature, 12 h daylight cycle). Mice were housed in cages of $n = 5$ animals with free access to gamma irradiated food and water. Throughout the study, sterility of the germ-free isolator was checked weekly by analysis of fecal samples. All animal experiments were performed in the animal facility at the Transgenose Institute, following the guidelines of the Institut Pasteur de Lille Animal Study Board, which conforms to the Amsterdam Protocol on Animal Protection and Welfare, and the Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes, updated in the Council of Europe's Appendix A. The study has also been approved by the Ethic and Welfare Committee for Experiments on Animals of the region Nord-Pas-de-Calais, France (approval number 04/2011).

2.2. Animal contamination procedures and experimental setup

$CdCl_2$ and $PbCl_2$ were provided as powders by Sigma–Aldrich (St. Quentin–Fallavier, France). Cd and Pb doses are expressed as ppm (parts per million) equivalent to mg l^{-1}. Cd and Pb levels in control food and drinking water were negligible. Doses were determined according to the lowest observed adverse effect level (LOAEL) for chronic exposure in rodents (Lukacinova et al., 2011) and based on previous studies (Breton et al., 2013). Based on these data, distinct doses of either Cd or Pb (5, 20 and 100 ppm) were administered in drinking water, while control groups for both SPF and germ-free mice consist in standard uncontaminated drinking water. Mice were exposed to these concentrations for 8 weeks. At necropsy, whole heparinized blood was collected and heavy metal accumulation was measured on 50 μl samples. Spleen, liver and left kidney were isolated, and small intestine and colon were extensively flushed before resecting 3 cm duodenum and mid-colon pieces. Organs were precisely weighed and stored until processed for further metal content determination.

2.3. Metal determination in mice tissues

Two different digestion procedures were carried out: one for whole blood samples and another for mice tissue samples. 50 μl of whole blood were wet digested with 500 μl of nitric acid 3% at 65 °C during 1 h in a plastic digestion vessel on a block heater. For mice tissue digestion (liver, spleen and kidney), a portion was accurately weighed and digested on a block heater with 500 μl of nitric acid 3% at 65 °C during 12 h, and then 500 μl of hydrogen peroxide were added. After partial evaporation, samples were cooled down and diluted to 5 or 10 ml with ultrapure water.

Determinations of Cd and Pb concentrations in whole blood digested samples were measured by a quadrupole-based ICP mass spectrometer, Thermo Elemental X series II (Thermo Fisher Scientific, Bremen, Germany). Data are expressed in μg l^{-1}. Determinations of Cd and Pb, in mice tissue were carried out by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) Varian Vista AX (Varian, Victoria, Australia), cyclonic spray chamber, with an expansion concentric nebulizer. Data are expressed in μg kg^{-1}.

2.4. Gene expression analysis

Intestinal mice tissues (0.5 cm of the duodenum and colon) were processed with RNA stabilization solution (RNA-later, Ambion, Life technologies, France) and stored at −80 °C. Total RNA from ultra-turax homogenized samples was isolated using RNA spin columns from a commercial isolation kit (Macherey–Nagel, France). Reverse transcription and real-time PCR were performed with reaction kits (high capacity cDNA RT kit) and reagents (universal PCR master mix) from Applied Biosystems (Courtaboeuf, France) according to the manufacturer's instructions and according to published procedures. The PCRs were performed with a MX3005P Stratagene machine (Agilent Technologies, Massy, France). For the targeted genes, a custom gene expression assay (TaqMan, Applied Biosystems) was used with the commercially designed and validated primers as previously described (Breton et al., 2013). The housekeeping gene beta actin was run as an internal control. Five biological replicates were measured for each exposure condition. The recorded data were analyzed and expressed by using the $2^{\Delta\Delta}$Ct calculating method.

2.5. Statistics and data analysis

All statistical analyses were performed by comparing experimental groups to their respective controls. A nonparametric one-way analysis of variance, Mann–Whitney *U* test or a Student *t* test was used where appropriate. Data are presented as mean ± SEM. Differences were judged to be statistically significant when the *p* value was <0.05.

3. Results

3.1. General comments

While germ-free mice had a slightly higher (non-significant) food intake to compensate the lower metabolic rate of these animals, both groups showed similar daily drinking consumption, both for control untreated and metal-contaminated water. No significant difference was found for body weights at the end of the experiment. An oral gavage with FITC-dextran of 4 kDa was used as a marker of paracellular permeability for macromolecules. No differences in blood fluorescence were measured between SPF ($n = 5$) and germ-free mice ($n = 5$) after 4 h (data not shown). Thus, no overall intestinal barrier impairment can be assumed, confirming that the lack of gut microbiota is not responsible for a leaky gut. No external sign of apparent toxicity was observed after 6-weeks intake of Cd or Pb, and this for all experimental doses in SPF and germ-free mice.

3.2. Fecal excretion of cadmium and lead accumulation in SPF and germ-free animals

We weekly determined the average Cd and Pb content in representative (pooled) samples of fecal pellets. The amounts of each metal changed dose-dependently with increasing dose of the contaminant. However, fecal samples from germ-free mice consistently contained approximately 5–30 fold more cadmium and 7–40 fold more lead, respectively (Table 1), suggesting a massive