Oral Lead Exposure Induces Dysbacteriosis in Rats

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Laboratory of Cellular Biophysics, Aachen University of Applied Sciences, Germany—Objectives: Lead's (Pb(II)) possible role in intestinal pathologies of microbial etiology remains mostly unknown. The aim of this study was to examine the effects of lead on the gut microbial community and its interactions with rat intestinal epithelium. Methods: The lead-induced changes in different intestinal microbial groups (lactose-positive lac(+) and -negative lac(–) E. coli strains, lactobacilli and yeasts) were followed separately by the colony-forming unit (CFU) method. Samples were taken from outbred white rats subjected to different exposure schedules. Additionally, the impact of different lead doses on microbial adhesion to cultured intestinal cells (IEC-6) was investigated. Finally, the lead accumulation and distribution were measured by means of atomic absorption spectrometry. Results: For the first time it was shown that oral lead exposure causes drastic changes in the gut microbial community. Proportional to the lead dose received, the relative number of lactose-negative E. coli cells increased dramatically (up to 1,000-fold) in comparison to the other microbial groups during 2 wk of exposure. Considering the number of microbes in the intestine, such a shift in intestinal microflora (dysbacteriosis) is very significant. Adhesion studies showed certain stimulating effects of lead on E. coli attachment to rat intestinal epithelium as compared to Lactobacillus attachment. Conclusions: The mechanisms providing the apparent competitive success of the lac(–) group are unclear but could be related to changes in surface interactions between microbial and host cells. This study may provide important clues for understanding the pathological effects of metal dietary toxins in human beings.

Key words: Dysbacteriosis, IEC-6, Intestinal microflora, Lead toxicity, Rat

Soluble lead, Pb (II), is one of the oldest known and most studied occupational and environmental toxins. Decades of use of lead-based paints and leaded gasoline have resulted in widespread contamination. Although lead has been later eliminated from household use, its compounds are still widely used in industrial processes and products. Pb mainly affects the nervous system, blood and blood-forming organs, kidneys and gastrointestinal tract. There is no apparent threshold for lead toxicity, suggesting that several different pathological mechanisms are involved. The problem of lead presence in drinking water and in airborne particles remains acute in many countries. Despite intensive study, there is still vigorous debate about the toxic effects of lead, mainly concerning low-level exposure in the general population owing to environmental pollution by lead alone and in combination with other compounds.

Total Pb dietary intake for an adult may vary from 20 to 150 µg per day in most countries with a daily dose of 0.2–2 mg regarded as safe. The main routes of lead entrance into the human body (food, water, dust) involve the intestinal system. Young children are especially susceptible to lead poisoning because they have 4–5 times higher intestinal absorption. Lead absorption is significantly influenced by the dietary intakes of iron, calcium, phosphorus, pectin etc., and has been reviewed extensively. The most easily accessible forms of lead are acetate, chloride and tetraethyl, whereas in the intestine, lead is mainly bound to bile acids (cholic and chenodeoxycholic) that contribute to lead transportation (translocation) in the intestinal epithelium.

Unabsorbed lead is excreted with feces. The fact that intestinal epithelium and intestinal microflora are usually subject to the highest Pb
concentrations in the body implies the importance of the detailed study of lead impact in this particular context. The human gastrointestinal tract displays an enormous variety of both aerobic and anaerobic microbial species. Some 400–500 species belonging mainly to the *Escherichia*, *Bacteroides*, *Bifidobacterium*, *Lactobacillus* genera have been identified so far. While the contribution of the indigenous gut microbial community is widely recognized, only recently has there been evidence pointing to indigenous flora in disease. Changes in microbe-host relationships and following abnormal expansion of gut microbial flora into the small bowel or small intestinal bacterial overgrowth are known causes of acute pancreatitis, bacterial gastroenteritis, irritable bowel syndrome, and many other diseases[20]. F.Savino has reported that changes in gut microflora could play an important role in the pathogenesis of infantile colic, a common problem in the first months of life[20].

Some direct pathological effects of high doses of lead on the intestinal system have been mentioned in the literature and were attributed to dilatation of the gastric mucosal microcirculation accompanied by desquamation of surface mucus cells[22]. In contrast, the influence of sub-chronic intestinal lead exposure on the intestinal microbial community and its relationships with intestinal epithelium are still unestimated and poorly studied. This is also true for many dietary toxins, for which the study of the pathological effects is focused predominantly on host tissues and cells, but totally neglects the responses from commensal microbial flora.

The aim of the present study was to investigate the influence of lead at different doses on the structure of the rat intestinal microbial community in vivo and its relationships to intestinal epithelium in vitro. The understanding of the underlying role of altered commensal gut microbiota in dietary toxicity could lead to novel diagnostic and therapeutic strategies and would contribute to public health.

### Materials and Methods

#### Reagents

As a source of lead, soluble Pb salts (lead (II) acetate and lead (II) chloride) were used. The lead concentrations used in the experiments were calculated based on known lead toxicity data. In the case of oral administration, the 50% lethal dose LD₅₀ (rat) was assumed to be 4,665 mg/kg for lead acetate Pb(CH₃COO)₂ (trihydrate form). The toxicity data were obtained from accessible reference books[5, 13] and material safety data sheets (MSDS) provided by manufacturers[22]. All chemicals used were reagent grade and purchased from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

**Animal experiments**

White outbred adult male rats with a body mass of 180–220 g used for the experiments were obtained from the Biology Faculty’s vivarium of Al-Farabi Kazakh National University (Almaty). The experimental groups and the concentrations of lead were chosen to give maximum information from a reasonable number of experiments. The rats were separated into one control and 4 experimental groups, 3 rats in each group. All the animals were maintained on a standard laboratory diet (“Regal Rat” rat fodder), under the same standard conditions. The “ad libitum” feeding regimen was used. The first experimental group received 1/14 of oral LD₅₀ (corresponding to 333.21 mg/kg body weight) every day for 2 wk. The second, third and fourth experimental groups received 1/60 (77.75 mg/kg), 1/240 (19.44 mg/kg), and 1/960 (4.86 mg/kg), of LD₅₀, respectively. The lead acetate water solution (1 ml) was administered orally, once a day, using a plastic syringe with a catheter. The control group was administered 1 ml of sterile isotonic NaCl solution.

Before the actual experiments began, the fecal samples of all rats were collected for preliminary microbiological and chemical analyses. Further samples were taken every day during the whole period of lead administration (14 days). After 2 wk of lead exposure, the animals were euthanized by carbon dioxide inhalation[23]. The whole gastrointestinal tract from the lower esophagus to the colon was removed. Blood and organs (large and small intestine, stomach, kidneys and liver) were separately analyzed for lead content using atomic absorption spectrometry.

All animal procedures in this study were carried out by licensed investigators and in accordance with regulated procedures under the Animals (Scientific Procedures) Act (1986).

#### Microbiological analysis

A common criterion for bacterial viability is the ability to reproduce in a suitable nutrient medium (colony forming unit (CFU) counting.). For analysis of microbial flora present in excreta, samples of known mass containing different concentrations of Pb were collected individually from each rat (3 fecal samples from a single rat, 3 rats in a group) and transferred under sterile conditions into clean glass tubes. After that, the feces were thoroughly homogenized in sterile phosphate-buffered saline (PBS; pH 7.4, 1:9, v/v) and serial 10 × dilutions were made to obtain 10³, 10⁴ and 10⁵ times diluted working solutions. From every dilution, 100 µl were transferred into a Petri dish containing selective agar culture medium and spread out by a glass spatula. The media used were: Endo agar (for isolation and identification of *E.coli*), MRS agar (for *Lactobacillus sp.*) and Sabouraud agar (for yeasts). The media were purchased from LabM company (International Diagnostics Group, Lancashire, United Kingdom). The
Petri dishes containing MRS agar were placed into specialized exsiccators to create anaerobic conditions. After incubation at 37°C for 24–48 h, bacterial colonies were counted (using a “blind” method to avoid subjective bias in the results caused by experimenter’s intent) and additionally identified using light microscopy and corresponding microbiological staining. If the number of colonies ranged between 10 and 300, the data were included in the results. The successful counts of the colonies were further calculated as concentrations of colony-forming units per gram of fecal mass.

**Adhesion assay**

The bacterial strains used in the adhesion experiments were *Lactobacillus plantarum* subsp. *plantarum* (ATCC 14917, serological group D), and *Escherichia coli* (ATCC 15223 serotype O13:H11, Lac⁻ (i⁺ z⁺ y⁻)-mutable). The microorganisms were cultured in culture media (MRS and Trypton Soy Broth, respectively) to reach the appropriate amount of biomass, washed 2 times with sterile phosphate-buffered saline (PBS; pH 7.4) and kept suspended in PBS until the experiments began.

As a model of intestinal epithelium, the normal rat epithelial cells IEC-6 from the small intestine were used. The IEC-6 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). They are adherent, transparent, epithelial cells growing as monolayers (polymorph of cell forms). They represent a non-continuous cell line with usually about 10–15 doublings and are able to synthesize fibronectin and collagen²⁵.

After thawing of the stock cells, the IEC-6 were cultured in 45% Dulbecco’s medium (DMEM) containing 4.5 g/l glucose with addition of 45% RPMI 1640, 10% fetal bovine serum and 0.1 U/ml insulin. To obtain the necessary amount of biomass, the confluent cultures were split from 1:2 to 1:4 about twice a week using trypsin/EDTA, seeded out at approx. 3–6 × 10⁵ cells/25 cm² and incubated at 37°C with 5% CO₂.

For adhesion studies, the confluent monolayer of cells (5th passage) was grown in 35 mm × 10 mm plastic chambers with Nunclon Surface (Nunc GmbH, Wiesbaden, Germany) in 2 ml of DMEM. Four-day post-confluent IEC-6 monolayers were washed twice with 1 ml of sterile DMEM (without phenol red indicator) before the adhesion assay.

The amount of attached bacteria depended on their initial concentration; therefore, the same final dilution of bacterial suspension was used for all experimental groups, allowing only the Pb concentration to vary between the groups. Since the lactic acid bacteria have a tendency to form chains and aggregates that make visual counting difficult, in this study the microplate method was adopted instead. After 24 h incubation of the monolayer with culture medium containing different concentrations of lead salts (both lead acetate and lead chloride were used), the cells were washed twice with 2 ml of pure DMEM. After that, 1 ml of the test bacteria suspensions at concentrations between 10⁴ and 10⁷ CFU/ml were added to 1 ml of complete culture medium. This suspension (2 ml) was added to each chamber and incubated for 1 hour at 37°C, in a 5% CO₂ atmosphere, with gentle rocking²⁶. After that, the medium with non-adhered bacterial cells was collected from each chamber into 3 microplate wells (200 μl each) and the cell concentration was measured at 490 nm using a microplate reader (Bio-Rad Co. USA). A lower optical density was interpreted as a lower concentration of bacterial cell, and thus, as a case of better adhesion.

For each concentration of lead, three plastic chambers were used. Control groups (each in triplicate) were created for adhesion experiments. In the first group, pure medium without microbial cell was added to the endothelial monolayer to check the contribution of the adhered cells alone to the photometric signal. In the second group, the microbial suspension was added into a plastic chamber without endothelial cells to take into account the unspecific adhesion of bacteria to plastic surface. Finally, the optical density of the native microbial suspension was measured to establish the initial reference point.

At the end of the adhesion experiments, after the samples had been taken for optical density measurements, the monolayers were washed twice with sterile PBS (pH 7.4), fixed with methanol, Giemsa stained, and examined microscopically²⁷. Additional visual counting of adhered cells was conducted in triplicate by two people and the number of adherent bacteria was counted on about 1,000 IEC-6 cells, in 50 randomly selected microscopic fields.

**Results**

As shown in Fig. 1, even for the control group (left raw) some amount of lead is present in rat organs, mainly concentrated in the kidneys and large intestine, indicating background lead consumption via drinking water, air dust and fodder. The lowest dose of the lead used in these
experiments (1/960 LD\textsubscript{50}, which corresponds to 4.86 mg/kg), caused certain shifts in concentrations of lead in the organs but did not visibly influence its distribution. Further increases in the administered amount of lead resulted in corresponding increases of its detectable concentration in all the organs collected for measurement.

The collation of the data in Fig. 1 with the daily Pb amount measured in rat feces during a 2-wk period of time (Fig. 2 A,B) implies the constant increase of inter-intestinal Pb concentration even in the case of the lowest dose given. Higher Pb concentrations caused proportionally higher increases in the rate of Pb accumulation without any visible saturation dynamics.

The changes observed in the relative amount of lactose-positive and lactose-negative \textit{E.coli} cells as a reaction to elevated Pb concentration (Fig. 3) were most interesting. During the first 3–4 days of lead administration the concentration of both \textit{E.coli} lac(–) variants did not differ between the control and the experimental groups. However, the measurements made in the following 10–12 days showed intensifying differences between lac(+) and lac(–) \textit{E.coli} responses to Pb presence. While the lac(+) \textit{E. coli} variants were, as expected, inhibited by the presence of Pb in a concentration-dependent manner (Fig. 3 B), the lac(–) group appeared to use the weakening of competition caused by Pb to its advantage in the same concentration-dependent way. At the highest inter-intestinal Pb concentrations reached (10–15 mg/g feces, see Fig.2), the dominance of lac(–) forms was obvious (Figs. 3A and 4).

Figure 4 summarizes the changes induced in rat intestinal microflora after 2 wk of continuous Pb (II) intake according to the doses given. Though the absolute and relative amounts of different intestinal bacteria in the control group did not differ significantly after 2 wk from the initial values (the two sets of bars on the left), the smallest lead doses used in the experiment (4.86 mg/kg and 19.44 mg/kg, corresponding to 1/960 of LD\textsubscript{50} and 1/240 of LD\textsubscript{50}, respectively) resulted in distinct alterations in bacterial microflora (Fig. 4, the two bar sets in the middle).

To investigate possible reasons for such drastic changes in the intestinal microbial community caused even by low concentrations of lead, we performed a series of \textit{in vitro} experiments, using cultured rat intestinal cells, IEC-6, and homogenous bacterial suspensions. After being brought in contact with intestinal cell monolayer, some of the applied bacteria firmly adhered to the cell surface.

High concentrations of lead acetate (1–5 mmole per liter) showed in our studies distinct cytotoxic effects on IEC-6 cells after 24 h incubation and, therefore, were not used for adhesion studies. The dose of lead used for adhesion experiments was in the range 0.1 nM–0.5 \textmu M per chamber (2 ml), which corresponds to 50 nM–0.25 mM concentrations in the culture medium. The adhesion of the cells was measured spectrophotometrically which was additionally controlled by microscopic observation (Fig.5).
Fig. 2. A: Content of lead in rat feces measured during 2 wk of daily lead intake. B: Same data in logarithmic scale, to show the lead accumulation in feces during the 2-wk period at low concentrations. The concentration of lead is given as fractions of LD50, which corresponds to 4,665 µg of Pb acetate per kilogram of body mass.

Fig. 3. Dynamics of lac (−) (above) and lac (+) (below) E.coli in rat intestines in response to continual (2 wk) lead (II) intake. The number of colony-forming units (CFUs) was counted every day for the different concentrations of Pb administered and is shown respective to the dilution used. The number of CFU per gram corresponds to the number of colonies (y-axis) × 10^4 for both lac(+) and lac(−) E.coli. The concentration of lead is given as fractions of LD50, which corresponds to 4,665 mg/kg of rat body weight. *indicates the sets of data significantly (p<0.005) different from control.
It was found that for lactose-negative \textit{E.coli} cells, a broad interval of lead salt concentrations exists (2.5–250 µM), where the adhesion of the bacteria to intestinal cells was stimulated compared to the control. The maximum of \textit{E.coli} adsorption (approx. 104–108% relative to the control) was observed at –78 µM concentration (for both salts used) corresponding to the dose –0.1565 µmol per flask (Fig. 6). In Fig. 6B the points situated above the x-axis can be interpreted as decrease in attachment and those lying below as increase in attachment. In contrast to \textit{E.coli}, adhesion of \textit{Lactobacillus} cells was significantly inhibited by lead, showing some increase at only one concentration (–326 µM) which could possibly be attributable to electrostatic interactions. Similar effects were also found when lead (II) nitrate was used as a source of lead but the contribution of the nitric group cannot be
excluded in this case.

**Discussion**

A great amount of research effort has been devoted to the description of intestinal adsorption of metals but their effects on intestinal microflora have not drawn any serious attention. Previous studies have suggested that lead toxicity is the result of its effect on calcium metabolism, and channel dysfunction. Nevertheless, some mechanisms underlying lead toxicity at low concentrations are still unclear, maybe because of the absence of a systemic approach to understanding lead toxicity. Recent studies have revealed the intricate relationship between the vast population of microbes that live in our gut and the human host. Our experiments were designed, first, to investigate the effects of lead on the rat intestinal microbial community, and second, to study if the effect is related to microbial adhesion to rat intestinal epithelial cells.

The rat, as well as its intestinal cells, has been shown to be a good model for toxicological animal-human bioavailability correlations. Many aspects of concentration and distribution of lead in rat resemble those in the human body. Nevertheless, some interspecies variations in intestinal absorption (0.36 ± 0.07 for human beings and 0.23 ± 0.02 for rats) should be taken into account.

The results of our atomic absorption spectroscopy measurements of lead distribution in rat organs were in a good accordance with data published previously. They show in these experiments the lead distribution corresponded to typical profiles and was not influenced by unaccounted factors. Interestingly, the higher doses of Pb caused the accumulation of Pb mainly in the gastrointestinal system (small and large intestine and stomach) indicating the growing inability of intestinal epithelial transport mechanisms to absorb Pb.

The next step was to study the dynamics of the lead concentration in intestinal contents and feces during 2 wk of chronic exposure to evaluate the capacity of intestinal absorption processes. Risk assessments for toxicants in environmental media via oral exposure often rely on measurements of total concentration in a collected sample. However, the digestive system cannot mobilize all of a toxicant present in the binding matrix, and cannot absorb it with near 100% efficiency. Default values of 30% lead bioavailability are assumed to be a reasonable indicator for dose, but the recent studies by Yu and

**Fig. 6.** Comparison of *E. coli* (lac-) and *Lactobacillus plantarum* adhesion to rat intestinal cells according to lead acetate dose. A: Standard scale. B: The same data shown in logarithmic scale. The stimulation of adsorption of lactose-negative *E. coli* cells by Pb(II) is visible in data points below the x-axis.
coauthors suggest that higher values for gastric bioaccessibility of lead possibly need to be considered.

Our measurements revealed that the lead concentration in feces correlated well with the dose administered. Even in the case of small concentrations, the lead content gradually increased during the experiment, indicating a decreasing trend for lead absorption by gut epithelium, resulting in accumulation in the intestinal space. As a result, intestinal microorganisms were exposed to continuously increasing concentrations of lead. Taking into account that different microbial groups have different susceptibilities to lead, population shifts in intestinal flora were to be expected. Indeed, the CFU method showed clearly that the intake of lead (II) caused explosive growth of lactose-negative E. coli variants. In contrast, the yeasts, lactobacilli and lactose-positive coli bacteria were suppressed in proportion to lead concentration. Although particular health-related consequences of the shift in proportions of non-lactose fermenting relative to lactose-fermenting bacteria are not clear yet, rapid uncontrollable proliferation of lac(−) strains could be considered as an ecological imbalance causing a pathological state, referred to as dysbacteriosis, which affects many aspects of gastrointestinal system function.

One important aspect yet to be studied is to what extent the lac(−) strains differ from lac(+) strains. Sometimes E. coli strains can be lac(−) on first isolation and become lac(+) after subculture.

The gastrointestinal microflora is a complex ecological system, normally characterized by a flexible equilibrium. Bifidobacteria and lactobacilli are Gram-positive lactic acid-producing bacteria constituting a major part of the intestinal microflora in humans and other mammals. Successful probiotic bacteria are usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa. By doing that, probiotic bacteria might prevent the attachment of pathogens, such as coli-form bacteria and clostridia, and stimulate their removal from an infected intestinal tract. Administration of antimicrobial agents notoriously causes disturbances in the ecological balance of the gastrointestinal microflora with several unwanted effects such as colonization by potential pathogens, many of them belonging to lactose-negative coli bacteria. To our knowledge, our study is the first one to show a relation between dysbacteriosis and oral lead exposure.

The most important role of the commensal microflora, from the point of view of the host, is to act in colonization resistance against exogenous, potentially pathogenic, microorganisms. Thus, one of the causes of the observed lead-induced dysbacteriosis could be the disturbance in microbial adhesion to epithelia. To verify this hypothesis, a series of model in-vitro experiments can be performed. Recently, several laboratory models using human and rat intestinal cell lines have been developed to study the adhesion of probiotic lactic acid bacteria to epithelial cells (IECs) as a model for studying Pb transport as well as Pb accumulation. They found that Pb uptake by IECs depended on the extracellular Pb concentration. In our experiments, cultured intestinal cells IEC-6 were first exposed to lead, and then their ability to bind different bacteria was examined.

One of the principal difficulties in modeling intestinal environments lies in the uncertainty over mechanical stresses, and the topology and composition of the surfaces involved. The cultured cell model is a simple system that excludes many physiological variables such as peristalsis, blood flow, gastric emptying, etc., allowing the adsorption processes to be studied in isolation. Nevertheless, there are obvious limitations with cell culture techniques, including the lack of systemic control, short incubation times, etc. Thus, we realize that in these studies the adhesion process only partially mimics the in vivo interactions of bacterial and intestinal cells and that the data must be interpreted guardedly.

Our in vitro study showed clear differences in lead influence on adsorption profiles between Lac(−) (i+ z+ y−) E. coli strain (representing a pathological group) and Lactobacillus plantarum ATCC 14917 (known as a probiotic strain). In contrast to Lactobacillus, there was a broad range of lead concentrations where the adhesion of E. coli cells was not inhibited but rather stimulated as compared to the control. The particular molecular reasons for this phenomenon are unclear and could include both unspecific changes in electrostatic interactions and Pb-induced conformational rearrangements of surface proteins.

Conclusions

Several conclusions can be drawn. First, the oral exposure of human beings and animals to low Pb concentrations deserves more careful attention. Lead doses that were much lower than those causing visible harmful effects on cell culture or on the level of the whole body could induce significant changes in intestinal microflora. The presence of Pb somehow creates selective advantages for pathological lactose-negative coli forms and thus contributes to dysbacteriosis development. Second, the adhesion of bacteria to intestinal epithelium is a possible cause of lead-induced dysbacteriosis. Third, the results could be possibly extrapolated to human beings, opening the opportunity of understanding some mysterious cases of persistent dysbacteriosis as a result of latent Pb intoxication. This, in turn, would suggest probiotic treatment as a promising therapy for lead-exposed patients on the one hand and the possibility of Pb-scavenging therapy for some patients with dysbacteriosis on the other.
Regarding public health, monitoring and control over Pb intestinal levels would not only diminish the risk of lead specific pathologies, but would also be of major importance in maintaining the normal gut microflora.

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