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The effect of penicillin administration in early life on murine gut microbiota and blood lymphocyte subsets

Jaroslaw Daniluk [a, *], Urszula Daniluk [b], Malgorzata Rusak [c], Milena Dabrowska [c], Joanna Reszec [d], Magdalena Garbowicz [e], Kinga Huminska [e], Andrzej Dabrowski [a]

[a] Department of Gastroenterology and Internal Medicine, Medical University of Bialystok ul, M. Sklodowskiej-Curie 24a, 15-276 Bialystok, Poland
[b] Department of Pediatrics, Gastroenterology and Allergology, Medical University of Bialystok ul, J. Waszyngtona 17, 15-274 Bialystok, Poland
[c] Department of Haematological Diagnostics, Medical University of Bialystok ul, J. Waszyngtona 15A, 15-269 Bialystok, Poland
[d] Department of Medical Pathomorphology, Medical University of Bialystok ul, J. Waszyngtona 13, 15-269 Bialystok, Poland
[e] Genomic Laboratory, DNA Research Center ul, Mickiewicza 31, 60-385 Poznan, Poland

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ABSTRACT

Background and aim: Antibiotics have many beneficial effects but their uncontrolled use may lead to increased risk of serious diseases in the future. Our hypothesis is that an early antibiotic exposition may affect immune system by altering gut microbiota. Therefore, the aim of the study was to determine the effect of penicillin treatment on gut microorganisms and immune system of mice.
Methods: 21-days old C57BL6/J/cmdb male mice were treated with low-dose of penicillin (study group) or water only (control group) for 4 weeks. Tissue and stool samples for histology or microbiome assessment and peripheral blood for CBC and flow cytometry evaluation were collected.
Results: We found high variability in microbiota composition at different taxonomic levels between littermate mice kept in the same conditions, independently of treatment regimen. Interestingly, low-dose of penicillin caused significant increase of *Parabacteroides goldsteinii* in stool and in colon tissue in comparison to control group (9.5% vs. 4.9%, $p = 0.008$ and 10.7% vs. 6.1%, $p = 0.008$, respectively). Moreover, mice treated with penicillin demonstrated significantly elevated percentage of B cells (median 10.5% vs 8.0%, $p = 0.01$) and decrease in the percentage of total $CD4^+$ cell (median 75.4% vs 82.5%, $p = 0.0039$) with subsequent changes among subsets - increased percentage of regulatory T cells (Treg), T helper 1 (Th1) and T helper 2 (Th2) cells.
Conclusion: Our study showed significant effect of penicillin on B and T cells in peripheral blood of young mice. This effect may be mediated through changes in gut microbiota represented by the expansion of *Parabacteroides goldsteinii.*

1. Introduction

Discovery of penicillin in 1928 by Sir Alexander Fleming, saved many lives and revolutionized treatment of bacterial infections. Antibiotics have also found application in agriculture, due to their effect on weight gain (up to 15%) in farm animals [1]. Nowadays easy access to the antibiotics led to their overuse in humans and caused development of bacterial resistance to the treatment.

Despite of their benefit in infection treatment, antibiotics have profound influence on the structure and function of gut microbes. Our knowledge about human microbiome has dramatically increased over the last decade due to development of novel sequence-based molecular tests, like 16s rRNA-based sequencing, which became the gold standard technique in microbiome identification [2]. Commensal microorganisms prevent colonization of the gut by pathogens. They are involved in the process of appropriate development and maturation of innate and acquired immune system during the childhood, and maintaining homeostasis in the adults [3,4]. The fetal gastrointestinal tract is sterile but immediately after the birth it is colonized by many different microorganisms [5]. Recently, it has been reported that any perturbations in microorganisms composition caused by antibiotics or changes in the diet habits during the first two years of live, may predispose to the development of chronic diseases like obesity,

* Corresponding author.
E-mail addresses: danilukj@poczta.onet.pl, jaroslaw.daniluk@umb.edu.pl (J. Daniluk).

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type 1 diabetes, rheumatoid arthritis, coeliac disease, liver diseases and inflammatory bowel disease (IBD) [6–8]. It has been also suggested that antibiotic exposure during the first year of life increases the risk of asthma development [9]. Jernberg et al. reported that even a short, 1-week long course of antibiotic regimen causes significant and prolonged (up to 2 years) changes in microbiota composition [10]. Therefore, the aim of our study was to determine the effect of early antibiotic (penicillin) exposition on gut microorganisms and immune system of mice.

2. Material and methods

2.1. Animals

C57BL6/J/cmdb male mice (Medical University of Bialystok; Bialystok, Poland) were maintained in specific pathogen-free (SPF) conditions. All *in vivo* experiments were performed according to EU Directive 2010/63/EU and approved by the Local Committee for Experiments with the Use of Laboratory Animals, Bialystok, Poland. 21-days old mice, just after the weaning, were randomly divided into two groups: study group – animals receiving penicillin dissolved in drinking water (n = 10) and control group – mice receiving drinking water only (n = 10), *ad libitum* for 28 days. Dose of the antibiotic, 1 μg of penicillin/1 g of body weight, was determined on the basis of previous literature data and assumption that daily consumption of water is 15 ml per 100 g of mice [11,12]. Fresh containers with water and water with antibiotic were changed three times a week. Mice were housed 5 per cage and they were allowed to eat standard chow diet *ad libitum*. Consumption of water or water with penicillin was determined on daily basis for each cage. Body weight of each mouse was measured every fourth day.

2.2. Sample collection

After 28 days, mice were anesthetized and sacrificed. Blood samples were collected by cardiac puncture and divided into two parts - complete blood count (CBC) and flow cytometry analysis. The small and large intestine, pancreas, and liver were harvested aseptically and stored for further histological evaluation. For microbiota assessment samples were collected directly from tissue (distal part of small intestine and ascending colon), and from the stool (lumen of the caecum), and immediately snap-frozen and stored (−80 °C).

2.3. CBC and flow cytometry analysis of isolated cells

To determine CBC, 200 μl of peripheral blood was used and analyzed in the Department of Haematological Diagnostics, Medical University of Bialystok. Peripheral subpopulations of lymphocytes were assessed using flow cytometry. To prepare samples, approximately 200 μl of peripheral blood was treated with RBC lysis buffer (Sigma-Aldrich) for 10 min at room temperature, and the remaining cells were washed twice with cold PBS and centrifuged at 1200 rpm for 10 min. Cells were stained with the appropriate combinations of the following antibodies: FITC–anti-CD3e (145-2C11; BD Pharmigen), APC-anti-CD4 (MR4-5; BD Pharmigen), PE-anti-CD25 (3C7; BD Pharmigen), PE-Cy7-anti-CD127 (SB/199; BD Pharmigen), and Mouse T Lymphocyte Subset Antibody Cocktail with Isotype Control (BD Pharmigen) containing PE-Cy 7- anti-CD3e (145-2C11), PE-anti-CD4 (RM4-5), APC-anti- CD8a (53–6.7), Mouse B Lymphocyte Subset Antibody Cocktail with Isotype Control (BD Pharmigen) with PE-Cy 7- anti-CD45R/B220 (145-2C11); PE-anti-CD23 (RM4-5); APC-anti- sIgM (53–6.7). The Mouse Th1/Th2/Th17 Phenotyping Kit (PerCP-Cy5.5-anti-CD4 (RM4-5); PE-anti-IL-17A (TC11-18H10.1); FITC-anti-INF-GMA (XMG1.2); APC-anti-CD4 (11B11) BD Pharmigen) were used according to the manufacturer's instruction for the detection of CD4+IL-17+, CD4+IFNγ+, CD4+IL-4+ expression.

Flow cytometric data were acquired using a FACS Canto II cytometer with BD FACSDiva Software v6.1.3 (BD Biosciences) and analyzed with Worksheet software (BD).

2.4. Histology and immunohistochemistry

The organs were fixed with 10% PBS-buffered formalin for 24 h, embedded in paraffin, cut sagittally into 5-μm sections, stained with hematoxylin and eosin (H&E), and examined by light microscopy (Olympus BX45) for histological analysis. For each animal, ten fields at a magnification of × 100 were captured randomly from the four different parts of the intestine.

To determinate the lymphocytic infiltration we used antibodies against T cytotoxic cells, T helpers and against lymphocytes B. Following the deparaffinization and rehydration, epitope retrieval was carried out in the EnVision Flex Target Retrieval Solution (DAKO) at low pH. Endogenous peroxidases were blocked by incubating the sections in methanol and 3% hydrogen peroxidase for 40 min. Next, slides were incubated with proper anti-mouse antibodies against CD3, CD4, CD8 and CD20 in 1:100 dilutions for 1 h at room temperature. Visualization reagent EnVision (DAKO) was applied for 30 min and followed by DAB solution for 10 min. The slides were than counterstained with H&E and examined under the light microscope. The intensity of immunostaining was evaluated in random 10 fields under × 20 magnification. Appropriate positive and negative controls were performed.

2.5. Faecal microbiota analysis

The bacterial composition of small and large intestine, and stool samples of each group was carried out using 16S ribosomal RNA (rRNA) gene sequencing on the MiSeq apparatus (Illumina) via next-generation sequencing (NGS) technology. In order to analyze bacterial components, the V3-V4 hypervariable region of the 16S rRNA gene was firstly amplified from the genomic DNA extracted from fecal samples by using two primers: F (5′-CCTACGGGNGGCWGCAG-3′) and R (5′-GACTACHVGGGTATCTAATCC-3′) containing additional overhang adapter sequences, compatible with Illumina's indices. Incorporation of primers with indexes and adapters was followed by second PCR reactions. After each amplification step, the PCR products were purified by using AMPure XP beads (Beckman Coulter Genomic, CA, USA). DNA amplified fragments were normalized to 2 nM by Qubit® dsDNA HS kit (high sensitivity DNA; Life Technologies) and pooled prior to sequencing. 8pM libraries were loaded on the MiSeq platform (Illumina Inc., San Diego, CA, USA). Sequencing was performed with 300-nucleotide-long paired-end reads on the Illumina sequencer (MiSeq) according to the instructions of the manufacturer. The analysis output file generated for the 16S metagenomics workflow provided classification of reads for each sample. Obtained data were analyzed and interpreted using specialized software packages: Quantitative Insights into Microbial Ecology (QIIME), Greengenes, Metagenome Analyzer (MEGAN) I Ribosomal Database Project (RDP).

2.6. Data analysis

Data were analyzed using Statistica 10 software. Statistical significance was determined by U Mann-Whitney test; $p < 0.05$ was considered statistically significant.