Pharmacological Reports 68 (2016) 44–50

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

Oral treatment with enrofloxacin early in life promotes Th2-mediated immune response in mice

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ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 29 June 2015
Accepted 1 July 2015
Available online 17 July 2015

Keywords:
Antibiotic
Natural gut flora
Allergy
Cytokines
IgE

ABSTRACT

Background: Th2 lymphocytes play a crucial role in the development of allergy. These pathologies are caused by coordinated production of the cytokines IL-4, IL-5 and IL-13 that regulate the activity of eosinophils, basophils and B cells. According to the 'hygiene hypothesis', the reduced exposure to microorganisms favors allergy occurrence. The advances in medicine in the field of infection therapy promoted an increasing application of antibiotics which, apart from eliminating pathogens, also partially eliminate the microbiota.

Methods: Epicutaneous (EC) immunization with ovalbumin (OVA) followed by OVA challenge was used to study the influence of partial gut flora depletion by oral treatment with enrofloxacin on type-2 immune response.

Results: Current work describes the influence of enrofloxacin application on anti-OVA antibody production and cytokine synthesis in young and adult mice. Immune response in adult mice is less sensitive to modification of natural gut flora. We observed that enrofloxacin treatment of adult mice leads to significant decrease of anti-OVA IgG2a production while synthesis of anti-OVA IgE was not changed. The production of type-1 (IFN-γ), type-2 (IL-4, IL-5, IL-10, IL-13) and Th17-associated (IL-17A) cytokines was inhibited. On the other hand, treatment of young mice with enrofloxacin significantly upregulates the production of anti-OVA IgE and inhibits the secretion of anti-OVA IgG2a antibodies. Additionally, treatment with enrofloxacin early in life prior to OVA immunization results in increased production of type-2 (IL-4, IL-10 and IL-13) cytokines.

Conclusion: Our results clearly indicate that the immune system is more vulnerable to decreased bacterial exposure early in life that may promote development of allergy.

Introduction

Allergic disorders develop as a consequence of immune responses to normally innocuous environmental antigens and now afflict roughly 25% of people in the developed countries [1]. These diseases include asthma, food allergy, atopic dermatitis, rhinoconjunctivitis, sisnusitis, urticaria, anaphylaxis, and insect and drug allergy, all of which can occur either alone or in combination [2]. It is believed that Th2 lymphocytes play a crucial role in this type of diseases. The phenotype of Th2 cell is characterized by the coordinated production of the cytokines IL-4, IL-5 and IL-13 which, in conjunction with other inflammatory mediators, direct the activation and tissue accumulation of eosinophils, basophils and B cells switching to pathogenic IgE [3].

Along with the development of our civilization and the increasing awareness of hygiene, there has been a permanent rise in the prevalence of autoimmune and allergic diseases. The observation collected in human is also directly reflected in animal studies. Hence, for example in non-obese diabetic mice (NOD), a change in the environment that reduces the number of microorganisms increases

Abbreviations: ALNC, axillary lymph node cells; CFU, colony forming unit; CIA, collagen-induced arthritis; EC, epicutaneous; FCS, fecal calf serum; IFN-γ, interferon gamma; Ig, immunoglobulin; IL, interleukin; NOD, non-obese diabetic mice; OVA, ovalbumin; PBS, phosphate-buffered saline; T1D, type 1 diabetes; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; TMB, 3,3′,5,5′-tetramethylbenzidine.

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http://dx.doi.org/10.1016/j.pharep.2015.07.002

the prevalence of type 1 diabetes (T1D), whereas the increase in the number of microorganisms in the surroundings decreases this prevalence [4]. Similar observations were made in an animal model of asthma which reflects the conditions observed in asthma patients [5].

Moreover, the advances in medicine in the field of infection therapy promoted an increasing application of antibiotics which, apart from eliminating pathogens, also partially eliminate the natural human bacterial flora. Studies conducted over many years have shown that the bacteria living in the alimentary tract have an essential role in the processes of food digestion, maintaining homeostasis, modulating lipid metabolism, promoting angiogenesis, supporting the immunity to infections, and maintaining the immune cell homeostasis [6].

The studies carried out in recent years have confirmed a strong connection between the natural gut flora and immune response. This interaction may differ in various experimental models. A strong inhibition of inflammatory reaction was observed, for instance, in the animal model of multiple sclerosis in mice treated orally with an antibiotic [7]. Similar observations were made in an animal model imitating rheumatoid arthritis [8]. Our previous study employing broad-spectrum antibiotic enrofloxacin showed that oral application of this therapeutic prior to the initiation of collagen-induced arthritis (CIA) aggravates disease symptoms in DBA1 mice [9].

In the current work, we determined the influence of partial gut flora depletion with broad spectrum antibiotic enrofloxacin on type-2 immune response in mice.

Materials and methods

Mice

6–8 weeks old female BALB/c mice were from the breeding unit of the Department of Medical Biology, Jagiellonian University College of Medicine. Mice were kept under pathogen-free conditions in individual cages using Aero-Mouse IVC Green Line system (Tecniplast S.p.A., Buguggiate, Italy) and fed autoclaved food and water *ad libitum*. All experiments were conducted to the guidelines of Jagiellonian University College of Medicine. All experiments were repeated 2–3 times, and a representative experiment is shown in the figures.

Reagents

Ovalbumin (Grade V) and phosphate-buffered saline (PBS) were from Sigma–Aldrich (St. Louis, MO, USA). Enrofloxacin was purchased from Bayer Animal Healthcare GmbH (Leverkusen, Germany). DNA analysis was performed using sodium dodecyl sulfate (SDS), JumpStart™ Taq ReadyMix™ for qPCR, lysozyme, Tris–EDTA (Sigma–Aldrich, St. Louis, MO, USA), silica beads (BioSpec Products, Bartlesville, OK, USA) and proteinse K (Roche Diagnostics, Mannheim, Germany). RPMI 1640 was from Gibco (Grand Island, NY, USA), whereas fetal calf serum (FCS) was from PAA Laboratories (Pasching, Austria). The levels of OVA-specific IgE and IgG2a antibodies in sera were tested using reagents from mouse ELISA sets for IgE (BD Pharmingen, San Diego, CA, 555248) and for IgG2a (BD Pharmingen, San Diego, CA, 552576). The units of antibodies were calculated according to an internal standard. The concentration of IL-4, IL-5, IL-10 and IFN-γ in culture supernatants was measured with the use of mouse ELISA sets purchased from BD Pharmingen, San Diego, CA, USA. Additionally, the concentration of IL-17A was evaluated using a mouse ELISA Ready-SET-Go kit (eBioscience, San Diego, CA, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) (BD Biosciences, San Jose, CA, USA) reagent set was used as the substrate in our ELISAs (BD Pharmingen, San Diego, CA, USA). The level of IL-13 was measured by using a mouse IL-13 immunoassay (R&D Systems, Inc., Minneapolis, MN, USA).

Treatment with enrofloxacin

6–8 weeks old BALB/c mice received drinking water containing enrofloxacin (0.27 mg/ml) or water alone for 2 weeks prior to tests. When enrofloxacin treated animals were immunized with OVA, enrofloxacin application was continued for additional 7 days. To study the influence of early exposure to enrofloxacin on antibody production, nursing pups were exposed to enrofloxacin during breastfeeding. Antibiotic treatment of mothers began immediately after delivery and was continued for 2 weeks. After the weaning, 6 weeks old mice were additionally treated with enrofloxacin for 3 weeks prior to immunization with OVA, as described above.

Evaluation of gut flora depletion

Mice received drinking water containing enrofloxacin (0.27 mg/ml) or water alone for 3 weeks. Then, animals were sacrificed and colon content was collected and serial dilutions were cultured in general agar plates (blood agar, Bioshop Inc., Burlington, Canada) for 48 h or 72 h at 37 °C in aerobic and anaerobic conditions respectively. Total bacteria per gram of sample were calculated based on the Colony Forming Units (CFU) counted in each serial dilution [7,9]. Additionally, gut flora depletion was evaluated by quantitative PCR analysis of 16S rDNA genes by real-time RT-PCR. Briefly, stool pellets were collected and total DNA was extracted with *phenol*:*chloroform*:*isoamyl alcohol* (25:24:1) method. Quantification of rDNA was performed with universal primers (forward; 5′-TCCTACGGGAGGCAGCAGT-3′), (reverse; 5′-GGACTACCAGGGTATCTAATCCTGTT-3′) and probe (6-FAM)-5′-CGTATTACCGCGGCTGCTGGCAC-3′-(TAMRA) using CFX96 Touch (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Epicutaneous immunization with OVA

Epicutaneous immunization of mice was performed according to the method described by Spergel et al. [10] and Wang et al. [11] with our modifications. Briefly, immunization was performed by applying to the shaved skin of the mouse dorsum a 1 cm^2 gauze patch soaked with a solution containing 100 μg OVA in a volume of 100 μl PBS on day "0". Mice in control group were patched with PBS alone. The patches were secured by adhesive tape wrapped around the midsection. The patches were left in place from day "0" until day "+4" when they were replaced with fresh patches and kept to day "+7".

The refreshed patches were then replaced on day "+7" and kept until day "+11" and replaced once again with fresh patches that were kept until day "+14". On day "+14", the patches were removed and the mice were rested for 1 week. The animals were then EC challenged by patching with 100 μg OVA in sterile PBS on day "+21". Four days later (day "+25") patches were replaced by fresh ones and left until day "+28". After that, patches were removed, mice were bled and sera were used to test level of OVA-specific IgE and IgG2a antibodies. The scheme of immunization and challenge is described in Scheme 1.

Measurement of OVA-specific IgE and IgG2a antibodies

OVA-specific antibodies were measured according to the method described by Spergel et al. [10] and Wang et al. [11] with our modifications. Briefly, 96-well microtiter plates were coated with 50 μg/ml of OVA in 0.1 M $NaHCO_3$ at 4 °C overnight. The plates were washed with PBS–Tween 20 (0.05%), then blocked with