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Effects of intestinal bacteria-derived *p*-cresyl sulfate on Th1-type immune response in vivo and in vitro

Takahiro Shiba *, Koji Kawakami, Takashi Sasaki, Ikuyo Makino, Ikuo Kato, Toshihide Kobayashi, Kazumi Uchida, Kimiyuki Kaneko

Safety Research Department, Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi-Shi, Tokyo 186-8650, Japan

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ABSTRACT

Protein fermentation by intestinal bacteria generates various compounds that are not synthesized by their hosts. An example is *p*-cresol, which is produced from tyrosine. Patients with chronic kidney disease (CKD) accumulate high concentrations of intestinal bacteria-derived *p*-cresyl sulfate (pCS), which is the major metabolite of *p*-cresol, in their blood, and this accumulation contributes to certain CKD-associated disorders. Immune dysfunction is a CKD-associated disorder that frequently contributes to infectious diseases among CKD patients. Although some studies imply pCS as an etiological factor, the relation between pCS and immune systems is poorly understood. In the present study, we investigated the immunological effects of pCS derived from intestinal bacteria in mice. For this purpose, we fed mice a tyrosine-rich diet that causes the accumulation of pCS in their blood. The mice were shown to exhibit decreased Th1-driven 2, 4-dinitrofluorobenzene-induced contact hypersensitivity response. The concentration of pCS in blood was negatively correlated with the degree of the contact hypersensitivity response. In contrast, the T cell-dependent antibody response was not influenced by the accumulated pCS. We also examined the in vitro cytokine responses by T cells in the presence of pCS. The production of IFN-γ was suppressed by pCS. Further, pCS decreased the percentage of IFN-γ-producing Th1 cells. Our results suggest that intestinal bacteria-derived pCS suppressesTh1-type cellular immune responses.

Introduction

Protein fermentation by intestinal bacteria generates various compounds that are not synthesized by their hosts. An example is *p*-cresol, a product of the metabolism of tyrosine or phenylalanine (Evenepoel et al., 2009). The bacteria-derived *p*-cresol is absorbed in the intestine, conjugated primarily within the intestinal mucosa or liver, circulated in the blood, and finally excreted in the urine by the kidney (Evenepoel et al., 2009). In the human blood, *p*-cresol is circulated mainly as *p*-cresyl sulfate (pCS), a sulfate conjugate of *p*-cresol, and partially as *p*-cresyl glucuronide. The amount of nutrients, intestinal transit times, and composition of microbiota influence *p*-cresol production. This compound, known as uremic retention solute, accumulates in the blood of patients with chronic kidney disease (CKD) (Evenepoel et al., 2009) and interacts negatively with biological functions. Some clinical studies showed that the accumulation of pCS in the blood is associated with mortality (Liabeuf et al., 2010). pCS are not sufficiently removed by hemodialysis because of their high protein-binding properties (Martinez et al., 2005; Pham et al., 2008).

In patients with CKD, infectious diseases resulting from immune dysfunction are one of the main causes of death (Wang et al., 2011). Among uremic patients, immune dysfunction may be caused by some uremic toxins (Hauser et al., 2008). De Smet et al., (2003) showed that serum concentrations of free *p*-cresol were higher in hemodialysis patients hospitalized for infectious diseases. Several in vitro studies support this clinical observation. *p*-Cresol was shown to depress whole blood respiratory burst reactivity and inhibit cytokine-induced endothelial adhesion molecule expression and endothelium/monocyte adhesion (Dou et al., 2002; Faure et al., 2006; Vanholder et al., 1995). Further, we previously reported that *p*-cresol inhibits IL-12 production by murine macrophages stimulated with a bacterial immunostimulant (Kawakami et al., 2009). These in vitro studies suggest that *p*-cresol has immunosuppressive effects. However, few in vitro studies have focused on the effects of pCS, which is the major metabolite of *p*-cresol, on the immune function (Schepers et al., 2007; Viaene et al., 2012). Moreover, an in vivo immunological study of pCS remains to be conducted.

T cells play a critical role in the adaptive immune response. Naïve $CD4^+$ T cells differentiate into several subsets including Th1, Th2, and Th17 and regulate adaptive immune responses by producing cytokines such as IFN-γ, IL-4, and IL-17, respectively (Zhu and Paul, 2010). Regulatory T (Treg) cells expressing Foxp3 can also be differentiated from

Abbreviations: APC, allophycocyanin; CHS, contact hypersensitivity; CKD, chronic kidney disease; DNFB, 2, 4-dinitrofluorobenzene; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; pCS, *p*-cresyl sulfate; PE, phycoerythrin; PMA, phorbol 12-myristate 13-acetate; Treg cell, regulatory T cell.

* Corresponding author. Fax: +81 42 577 3020.
E-mail address: takahiro-shiba@yakult.co.jp (T. Shiba).

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naïve $CD4^+$ T cells and suppress immune responses. $CD8^+$ T cells differentiate into subsets including Tc1, Tc2, and Tc17, and are critical for host defense against intracellular bacteria and viruses (Sad et al., 1995; Yen et al., 2009). The ratio of Th1/Th2 cells in blood is reduced in patients with uremia (Alvarez-Lara et al., 2004), and the numbers of naïve and central memory $CD4^+$ and $CD8^+$ T cells are significantly reduced in patients with CKD (Yoon et al., 2006). Taken together, these studies suggest that uremic retention solutes influence the adaptive immune responses; however, the precise mechanisms remain to be elucidated.

Here we attempted to gain a better understanding of the immunological effects of pCS derived from intestinal bacteria, particularly on the T cell-mediated adaptive immune response. We established an in vivo mouse model in which pCS was accumulated in blood. The mice were used to evaluate the effects of pCS on cellular and humoral immune responses by 2, 4-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity (CHS) and keyhole limpet hemocyanin (KLH)-specific antibody response, respectively. Further, we conducted an in vitro study to examine the effects of pCS and *p*-cresol on cytokine production by $CD4^+$ and $CD8^+$ T cell subsets.

Materials and methods

Animals. Female BALB/c mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan), CLEA Japan Inc. (Tokyo, Japan), and Japan SLC Inc. (Shizuoka, Japan) and held for 1 week before use. They were housed at 20 °C–26 °C with 40%–60% humidity under artificial lighting conditions with a 12-h light/dark cycle. The mice had ad libitum access to the diets and water. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Yakult Central Institute for Microbiological Research. The animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Yakult Central Institute.

Diets. For in vivo experiments, an AIN-93G diet or AIN-93G diet containing 5% tyrosine (tyrosine-rich diet) was prepared in our laboratory. The composition of these diets is shown in Table 1. For in vitro experiments, mice were fed a normal commercial diet (MF, Oriental Yeast Co., Tokyo, Japan).

Reagents. pCS was obtained from Tokyo Chemical Industry (Tokyo, Japan). *p*-Cresol was purchased from Nacalai Tesque (Kyoto, Japan); DNFB, acetone, KLH, Tween20, phorbol 12-myristate 13-acetate (PMA), and ionomycin were from Sigma-Aldrich (St Louis, MO); and tyrosine, phenol, and olive oil were from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin fraction V was purchased from Roche (Basel, Switzerland); Hanks' balanced salt solution and phosphate-buffered saline (PBS) were from Nissui Pharmaceuticals (Tokyo, Japan); and horseradish peroxidase (HRP)-conjugated anti-mouse IgM monoclonal antibody (mAb) was from Southern Biotechnology Associates (Birmingham, AL). KLH-specific IgM mAb (clone C48-6), purified anti-mouse CD3ε mAb (clone 145-2C11), and BD GolgiPlug (brefeldin A) were purchased from BD Biosciences (San Jose, CA). PerCP-Cy5.5 anti-mouse CD3ε mAb (clone 145-2C11), fluorescein isothiocyanate (FITC) anti-mouse CD4 mAb (clone GK1.5), FITC anti-mouse CD8α mAb (clone 53-6.7), phycoerythrin (PE) anti-mouse IL-4 mAb (clone 11B11), allophycocyanin (APC) anti-mouse IFN-γ mAb (clone XMG1.2), PE anti-mouse CD25 mAb (clone 3C7), Alexa Fluor 647 anti-mouse/rat/human FOXP3 mAb (clone 150D), fixation buffer, permeabilization wash buffer, and a Foxp3 Fix/Perm buffer set were from BioLegend (San Diego, CA). RPMI1640 medium (Life Technologies, Carlsbad, CA) was supplemented with 10% fetal bovine serum, 5 mM 2-mercaptoethanol, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B, all from Life Technologies.

Sample collection. Urine and feces were collected weekly. Blood was collected by cardiac puncture from anesthetized mice. Serum and plasma were obtained from blood using standard methods. Feces and cecal contents were collected, weighed, and mixed with 0.1 M phosphate buffer (pH 5.5). These samples were stored at −80 °C until use.

Sample preparation for HPLC. Sample (plasma, serum, urine, feces, or cecal contents) preparation for measurement of total *p*-cresol and phenol (conjugated and unconjugated) was performed as previously described (Kawakami et al., 2005). Briefly, samples were hydrolyzed by mixing with an equal volume of hydrochloric acid and heated at 100 °C for 60 min. The hydrolyzed samples were extracted using ethyl acetate and filtered through a 0.45-μm filter. The filtered samples were analyzed using HPLC as described in the next section. Sample preparation for determination of pCS and unconjugated *p*-cresol was performed as described with some modification (Martinez et al., 2005). Samples were deproteinized by mixing with 3 volumes of methanol. After centrifugation, supernatants were filtered through a 0.45-μm filter and analyzed using HPLC.

HPLC. HPLC was performed using a GL-7400 system (GL sciences, Tokyo Japan). Total *p*-cresol and phenol were analyzed as described previously (Kawakami et al., 2005). Briefly, a Shodex ODS pak F-411 column (4.6 × 150 mm, Showa Denko K.K., Tokyo, Japan) was used. The mobile phase consisting of 0.1% phosphoric acid (68%) and acetonitrile (32%) was delivered at a flow rate of 1.0 ml/min. *p*-Cresol and phenol were detected by means of a fluorescence monitor. The excitation/emission wavelengths were 260/305 nm, respectively. HPLC analysis for pCS and unconjugated *p*-cresol was performed using an Inertsil ODS-SP column (4.6 × 150 mm, GL sciences). The mobile phase consisted of 0.02 M phosphate buffer (pH 4.0) and methanol. The composition of the mobile phase was gradually changed from 80:20 to 62:38 at 12 to 30 min. The flow rate was 0.9 ml/min. pCS and unconjugated *p*-cresol were detected using a fluorescence monitor. The excitation/emission wavelengths were 214/306 nm, respectively.

DNFB-induced CHS. DNFB-induced CHS was performed as described previously with some modification (Riemann et al., 1996). Starting from day 0, 8-week-old mice were fed either the AIN-93G or tyrosine-rich diet. On day 10, 25 μl of a DNFB solution (0.3% in acetone:olive oil, 4:1) was epicutaneously applied to the shaved abdominal skin of anesthetized mice. On day 15, the ear thickness baseline (0 h) of each animal was determined using a dial thickness gauge (Mitsutoyo, Kanagawa, Japan). Mice were challenged with 10 μl of a DNFB solution (0.2% in acetone: olive oil) on the right auricle, and the ear thickness was measured after 24, 48, and 72 h. The left auricle was treated with 10 μl of acetone:olive oil alone as a control. Ear swelling at X h is defined as follows: [(thickness of the right ear at X h) − (thickness of the right ear at 0 h)] − [(thickness of the left ear at X h) − (thickness of the left ear at 0 h)]. After the 72-h

Table 1
Diet composition.

Ingredient	AIN-93G diet (%)	Tyrosine-rich diet (%)
Casein	20.00	20.00
L-Tyrosine	0.00	5.00
Cellulose powder	5.00	5.00
L-Cystine	0.30	0.30
Soybean oil	7.00	7.00
AIN-93G mineral mixture	3.50	3.50
AIN-93G vitamin mixture	1.00	1.00
Choline bitartrate	0.25	0.25
Sucrose	10.00	10.00
Cornstarch	52.95	47.95
Total	100	100

Numbers in the table indicate the percentage of each ingredient relative to the total diet weights.