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Original article

Staphylococcus epidermidis is involved in a mechanism for female reproduction in mice

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ABSTRACT

Both external and internal surfaces of organs (e.g., skin, mouth, gut, and intestine) are covered with bacteria, which often contribute to physiological events in host animals. Despite externally opened organs, the presence of bacteria in the mammalian female reproductive tract is uncertain. Here we assessed this problem using wild-type strains of mice, C57BL/6N and ICR. We first demonstrated that bacterial colonies were formed from the oviductal fluid in the C57BL/6N mice with birth experience ("parous"), but not in the mice without birth experience ("non-parous"). Sequence analysis of 16S ribosomal RNA (rRNA) revealed that *Staphylococcus epidermidis* existed in the oviductal fluid of the parous mice, confirmed by immunohistochemical analysis. Furthermore, extinction of bacterial population with intraperitoneal injection of antibiotics, penicillin G and streptomycin, disturbed the regularly implanted pattern of embryos in ICR mice. Our results indicate that symbiotic *S. epidermidis* plays a role in interaction between embryo and uterus upon implantation in mice.

1. Introduction

Bacteria are inhabited on the external surface and in the lumen of organs in host organisms [1,2]. Based on cooperative relations between bacterium and host organism, the bacteria are provided with a living place, a supply of food, and a constant condition of temperature and humidity from host animals [3,4]. They are often harmless, but also compete with pathogenic bacteria, and protect against diseases in host organisms [5]. The host and the bacteria both gain benefits from this peaceful relation, termed mutualism, a type of symbiosis [6]. As a mutualistic case in humans, the intestine is known to harbor complex societies of bacteria in the lumen [7]. The intestinal bacteria provide us with vitamin B and vitamin K, and help us digest foods [7]. And also, microbial symbiosis factors, such as polysaccharide A (PSA), prevent intestinal inflammatory diseases [8].

In a vagina as a gate of the female reproductive tract, an anerobic lactobacillus, *Doderleïn bacilli*, forms biofilm, a complex aggregation of microorganisms on the mucosa in humans [9]. Its symbiosis has a beneficial effect by inhibiting growth, adhesion or spread of other microorganisms [10]. In the vagina, this bacterium is also known to continually convert glycogen into lactic acid, and prevents pathogens from entering inside the vagina by keeping pH in the cavity acidic [9]. On the other hand, administration of antibiotics and spermicides, a contraceptive substance that stop sperm from moving, to women disturbs such a beneficial relation between host and bacterium [11]. Since *D. bacilli* plays a role in suppressing the pathogen entry, the maintenance of its symbiosis is an important issue for pregnant ability of women.

In mammals, the semen enters to the female reproductive tract along with ejaculated sperm [12]. The semen is known to include immunosuppressive materials potent for protecting ejaculated sperm from attack of female immune system, and weaken the female defensive ability against bacterial entry [13], implying that bacteria may enter the uterus and the oviduct along with the sperm entry. However, symbiotic bacteria have not ever found to enter the female reproductive tract beyond the vagina in mammals, and even more serve in reproductive events of females. Whether the female

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reproductive tract needs the symbiotic relationship between bacterium and reproductive tract is still unclear in mammals including humans. In the present study, we focused on the microbial environment as one of factors regulating the litter size in mice.

2. Materials and methods

2.1. Antibodies and chemicals

A mouse monoclonal antibody (mAb) and a rabbit biotinylated polyclonal antibody (polyAb) against whole soluble and structural antigens of *Staphylococcus epidermidis* and *Staphylococcus aureus* were purchased from Pierce Biotechnology Inc. and abcam Plc., respectively. For immunohistochemical analysis, Alexa647-conjugated IgG and Alexa488-conjugated streptavidin (Life Technologies Corp.) were used. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (WAKO Pure Chemical Industries, Ltd).

2.2. Animals

C57BL/6N and ICR mice (8–12 weeks old) were purchased from Japan SLC Inc., Shizuoka, Japan. Male 8- to 12-week-old B6C3F1 mice, a cross between female C57BL/6N and male C3H mice, were purchased from SLC Inc. (Shizuoka, Japan).

Mice were housed in specific pathogen-free controlled conditions. Food and water were available *ad libitum*. The procedures for performing animal experiments were in accordance with principles and guidelines of the *Care and Use of Laboratory Animals at National Institute for Child Health and Development*.

2.3. Colony formation assay

For colony formation assay, the fluids were collected from the inner cavities of the female and male reproductive organs. One month after C57BL/6N female mice were mated with the male mice and produced their offspring, these female mice were superovulated. Female 8- to 12-week-old C57BL/6N mice were injected with 5 unites of pregnant mare's serum gonadotropin (PMSG; Merck4Biosciences, Darmstadt, Germany). Later, 5 units of human chorionic gonadotropin (hCG; Merck4Biosciences, Darmstadt) was injected. The oocytes were aseptically isolated from oviduct 14–16 h after hCG injection and gently dipped in 70% ethanol. Then, the oviductal fluids including oocyte–cumulus complexes (COCs) were collected from inner cavities of the oviducts. The oviductal fluids were placed in saline solution (Otsuka Pharmaceutical Co., Ltd.) without antibiotics, and spread on MRS agar plates (Kanto Chemical Co. Inc.), which were placed upside down and incubated at 37 °C for 16 h. Concomitantly, three kinds of male reproductive organs, epididymides, seminal vesicles, and preputial glands, were isolated aseptically from 8- to 12-weeks-old C57BL/6N mice. Similarly, after being collected, their inner fluids were seeded on MRS agar plates.

2.4. Polymerase chain reaction (PCR)

To identify the bacterial species, the colonies were picked up with toothpicks, grown in 10 ml liquid MRS medium without antibiotics, and the tubes were incubated overnight at 37 °C on a tube shaker (Thermo Scientific. Inc.). After they were centrifuged, its pellets were subjected to DNA extraction with MidiPrep kit (QIAGEN) and PCR analysis of microbial 16S rRNA genes. Since 16S rRNA gene contains hypervariable species-specific regions, the PCR analysis of this gene can provide signature sequences useful for bacterial identification [14]. The species-specificity of the 16S rRNA genes in microbial colonies was detected by PCR analysis using the following set of primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′, nucleotide no. 8–27 [GenBank Accession no. NR_041920]) and 1525R (5′-AAGGAGGTGATCCAGCC-3', nucleotide no. 1525–1541 [GenBank Accession no. NR_041920]), and this primer set yielded a band of 1499 bp. The amplified DNA fragments were inserted into pGEM-T Easy vector (Life Technologies Corp.), amplified, and sequenced with ABI3730 capillary sequencer (Life Technologies Corp.) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp.).

2.5. Gram's staining

For immunohistochemical analysis, female reproductive tract and male reproductive organs of C57BL/6N mice were stained with Gram's method, as described previously [15]. To avoid the bacterial contamination and grow, the female reproductive tract and male reproductive organs were fixed with 20% formalin solution, embedded in paraffin, sectioned, stained with Gram's method, and observed under a microscope (BX51; Olympus Corp.).

2.6. Immunohistochemical analysis

Bacteria identified in female reproductive tract and male reproductive organs of C57BL/6N mice were subjected to immunohistochemical analysis. Each tissue of female reproductive tract and male reproductive organs was aseptically collected from females and males, and investigated for the endogenous bacteria. The immunohistochemical analysis was performed using paraffin section of the tissues to find the habitats of two species, *S. epidermidis* and *S. aureus*.

2.7. In vitro fertilization (IVF)

Sperm were collected from the epididymis of 8- to 12-week-old B6C3F1 male mice and induced to capacitate by incubation of them in TYH medium for 90 min in an atmosphere of 5% CO_2 in air at 37 °C before insemination. The final concentration of sperm added to the oocytes was 1.5×10^5 sperm/ml. Oocytes were collected from oviducts of C57BL/6N female mice 14–16 h after injection with 5 units of human chorionic gonadotropin (hCG; Merk4Biosciences, Darmstadt, Germany) into pregnant mare's serum gonadotropin (PMSG; Merk4Biosciences, Darmstadt, Germany)-treated mice. Then, the oocytes were placed in a 30-μl drop of TYH medium covered with paraffin oil (Nacalai Tesque, Inc) equilibrated with 5% CO_2 in air at 37 °C. To investigate the physiological roles of bacteria in the female reproductive functions, IVF was then conducted in fertilization media including different concentrations of each antibiotic, penicillin G and streptomycin (0, 50, or 250 units/ml).

After being fertilized with sperm, the oocytes were transferred to a 30-μl drop of EmbryoMax KSOM medium (EMD Millipore Corp.) including each of antibiotics, penicillin G and streptomycin, KCl, and $MgSO_4 \cdot 7H_2O$, and continuously developed to the blastocyst stage. The rates of two-cell, morula, and blastocyst stages were determined under a differential interference microscope (BX51; Olympus Corp.).

2.8. Determination of litter size and number of implantation sites

Before mating, each of 8- to 12-week-old female and male ICR mice was intraperitoneally injected of 15 units of penicillin G (Sigma–Aldrich, Co. LLC.) and 68.4 units of streptomycin (Sigma–Aldrich, Co. LLC.) every day for 4 days. After treatment, the antibiotic-treated mice were mated with the untreated counterparts. To determine the litter size, the number of pups delivered