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Immunization with intestinal microbiota-derived *Staphylococcus aureus* and *Escherichia coli* reduces bacteria-specific recolonization of the intestinal tract

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ABSTRACT

A wide array of microorganisms colonizes distinctive anatomical regions of animals, being the intestine the one that harbors the most abundant and complex microbiota. Phylogenetic analyses indicate that it is composed mainly of bacteria, and that Bacterioidetes and Firmicutes are the most represented phyla (> 90% of the total eubacteria) in mice and humans. Intestinal microbiota plays an important role in host physiology, contributing to digestion, epithelial cells metabolism, stimulation of intestinal immune responses, and protection against intestinal pathogens. Changes in its composition may affect intestinal homeostasis, a condition known as dysbiosis, which may lead to non-specific inflammation and disease. The aim of this work was to analyze the effect that a bacteria-specific systemic immune response would have on the intestinal re-colonization by that particular bacterium.

Bacteria were isolated and identified from the feces of Balb/c mice, bacterial cell-free extracts were used to immunize the same mice from which bacteria came from. Concurrently with immunization, mice were subjected to a previously described antibiotic-based protocol to eliminate most of their intestinal bacteria.

Serum IgG and feces IgA, specific for the immunizing bacteria were determined. After antibiotic treatment was suspended, specific bacteria were orally administered, in an attempt to specifically re-colonize the intestine. Results showed that parenteral immunization with gut-derived bacteria elicited the production of both anti-bacterial IgG and IgA, and that immunization reduces bacteria specific recolonization of the gut. These findings support the idea that the systemic immune response may, at least in part, determine the bacterial composition of the gut.

1. Introduction

The intestinal tract must keep potentially pathogenic microorganisms at bay and, at the same time, it must provide a permissive microenvironment for commensal microorganisms [1–3]. As a result, each anatomical region within the intestine has a well-defined microbial consortium or "microbiota" [4–8].

Phylogenetic analyses indicate that the intestinal microbiota is very diverse, composed mostly of bacteria [3,4,7,9]. About 10^{14} bacteria reside in the human intestinal tract, outnumbering the whole body eukaryotic cells [4,5,7,10–13]. The number of bacterial species within the intestine is estimated to be greater than 1000 [3,7,9,14,15].

At birth, the phylogenetic diversity of intestinal microbiota is relatively low and it gradually increases over time [8,9,15–17]. Although new-born babies are exposed to a great variety of microorganisms, only a limited number of species are capable to permanently colonize specific tissues [10,17]. Colonization of the intestinal mucosa reaches its maximal density between 24 and 72 h after birth [15,17].

Some colonizing bacteria are transitory while others are still to be found later in life [16,18,19].

As the intestine grows, new ecological niches develop, contributing to microbiota diversity [8]. Therefore, early in life, the microbiota is different from that found at older ages; moreover, inter-individual differences in microbiota composition diminish as complexity increases with age [15,16]. Around 3 years after birth, the intestinal microbiota has developed into a "mature" stage, characterized by its stability and a whole new set of functions [15,16,20], such as favoring digestion, by the degradation of un-digestive polysaccharides [15,21], and epithelial

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cell metabolism. In addition, the intestinal microbiota stimulates cell proliferation and intestinal immune responses and confers protection against intestinal pathogens [10,22,23].

Intestinal microbiota, either directly or indirectly, helps in the suppression of pathogens by preventing their colonization, stimulating the development of gut associated lymphoid tissue, and enhancing local immune responses through the production of short chain fatty acids (SCFAs), vitamins, and bactericidal proteins [15,21,22,24,25].

Defined bacterial species and their products may have a role in the overall structure and function of secondary lymphoid organs, intestinal angiogenesis, and oral tolerance [15,22,26–28].

Given the role of the intestinal microbiota in the homeostasis and well-being of organisms, it is of the uppermost importance to know how its composition is shaped.

Bacterial colonization of the intestine depends on multiple factors, such as host genetics, environment, diet, the use of antibiotics, and even cultural practices and life styles [8,15,29,30].

Although there is substantial evidence that the intestinal microbiota contributes to the development of intestinal immune responses [31,32], a possible role for the immune system in the shaping of intestinal microbiota composition has been studied less. Palm et al. have considered that since the host and the microbiota are in constant communication, reciprocal interactions between these two entities shape both host immunity and microbial ecology [33].

We tested the hypothesis that the absence of a systemic immune response against intestinal bacteria is due to the lack of systemic bacterial antigen stimulation and, on the other hand, that parenteral immunization with intestinal bacterial antigens readily elicits a humoral immune response capable of interfering with bacteria specific intestinal re-colonization. Our results seem to support this hypothesis.

2. Materials and methods

2.1. Mice

Male Balb/c mice (Envigo, Mexico) of 4–6 weeks of age were maintained in a microventilation rack (Ecoflo; Allentown, USA) under controlled temperature and humidity, and 12 h dark–12 h light cycles, and were provided with sterilized food and drinking water ad libitum. All animal procedures were carried out according to the Institutional guidelines for the use of animals.

2.2. Experimental design

At the beginning of the experiments, mice were kept together for four weeks to favor a more homogeneous intestinal microbiota. Feces were collected at the end of this conditioning period, for bacteria isolation, identification and antigen preparation. Mice were separated in 4 different experimental groups as follows: I) Untreated mice; II) mice treated with Incomplete Freunds Adjuvant (IFA) only, as a mock immunization control; III) mice treated with *S. aureus* cell-free extracts plus IFA (*S. aureus* CFx); and IV) mice treated with *E. coli* cell- free extracts plus IFA (*E. coli* CFx). Mice were parenterally immunized with cell-free extracts from feces-derived specific bacteria, while a protocol to reduce intestinal microbiota was applied [34]. Sera IgG and feces IgA specific for the bacteria used for immunization were assessed; mice were then re-colonized with the same bacterial species used for immunization and, finally, colony-forming units (CFUs) of fecal bacteria were determined.

2.3. Bacteria isolation and identification

Freshly collected feces from Balb/c mice that had been kept together in a single cage for four weeks (adaptation phase) were placed into sterile microtubes (Eppendorf, GE), feces were homogenized and plated on McConkey agar, brain hearth infusion (BHI), and trypticase soy agar (TSA) (MCD LAB, México). Individual colonies were selected based on morphology, and they were individually seeded in BHI medium until axenic cultures were obtained. Isolated bacterial strains were kept in minimal medium. Bacteria identification was carried out by using API Staph and API 20 E biochemical tests (BioMérieux, USA), following the manufacturer's instructions. Bacterial identity was confirmed by automatized sequencing of a gene fragment of bacterial 16 s ribosomal RNA, by using a GA3500 genetic analyzer (Applied Biosystems, USA). The specific primers used were: 5494F 5′- TGACTG ACTGAGTGCCAGCMGCCGCGG-3′ (forward) and 1494R 5′- TGACTG-ACTGAGGYTACCTTGTTACGACTT-5′ (reverse). PCR conditions were: initial denaturalization (10 min at 95 °C), followed by 30 cycles of denaturalization (1 min, 95 °C), aligning (1 min, 42 °C), and extension (90 s, 72 °C), respectively. Sequence analysis was performed through BLAST.

2.4. Reduction of intestinal microbiota in mice

To reduce the amount of intestinal bacteria, without compromising their well-being, Balb/c mice were treated according to a well described antimicrobial protocol by Reikvam et al. [34]. Briefly, ketoconazol (1 mg/kg) was intragastrically administered twice a day, for 2 days, followed by the intragastric administration of vancomycin (50 mg/kg), neomycin (100 mg/kg), and metronidazole (100 mg/kg), twice a day for 12 days. Ampicillin (1 g/L) (Sigma-Aldrich, USA) was supplied in the drinking water. Immediately after the microbiota-reducing protocol was over, mice feces were collected and the CFUs were determined by using a modified Miles et al. method [35]. Briefly, feces were transferred into sterile microtubes and weighted, 500 µL of PBS were added and then, serial dilutions in sterile PBS were carried out; 50 µL of each dilution were plated on BHI-containing Petri dishes. After 24 h incubation at 37 °C, CFUs were counted and the total number of bacteria in feces was calculated.

2.5. Antigen preparation and immunization

Bacteria that had initially been isolated from mice feces and identified for genus and species were grown in TSA medium, washed with PBS, and suspended in PBS to a final density of 1.5×10^8 bacteria/mL by using the McFarland standard. The bacterial suspension, kept at 4 °C, was sonicated in a Vibra Cell VC375 (Sonic & Materials, USA), by applying three bursts of 1-min each with 1-min rest intervals between sonication. Bacterial cell-free extracts were prepared by centrifugation at 3000 × *g* for 10 min. Bacterial cell supernatants were recovered and kept at − 70 °C until use. Mice were immunized three times, with 7 days intervals from each other, by subcutaneous injection of 100 µL of a 1:1 mixture of bacterial free-extract and incomplete Freunds adjuvant (IFA) (Sigma-Aldrich, US) in the base of the tail.

2.6. Intestinal bacteria re-colonization

One day after the anti-microbial scheme was completed, specific bacteria recolonization of the intestine started. Briefly, 300 µL of a bacterial suspension (9×10^8 bacteria/ml) of *Staphylococcus aureus* and *Escherichia coli* were orally administered with the aid of a 1-mL syringe and intragastric gavage. Bacteria administration was carried out daily for 7 days.

2.7. IgG and IgA assessment

Bacteria-specific IgG and IgA were assessed in mice sera and feces, respectively. Blood samples were obtained by mandibular vein puncture; after coagulation, blood samples were centrifuged at 3000 × *g* for 15 min. Sera were transferred into fresh microtubes and kept at −20 °C until use. Feces were processed by following the Grewal et al. protocol [36], feces supernatants were kept at −20 °C until use. Bacterial cell-