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Stimulation of Human Innate Immune Defenses by Bacteria *in vitro*

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ABSTRACT

The microflora is essential for immune education and amplification of lymphoid effector cells, mainly at the mucosal level. It is well documented that the efficacy of the mucosal immune system can be significantly impaired in germ-free animals. In this study the bacteria used as antigens (mitogen) to the stimulation of human immune cells *in vitro*. Gram-positive *Streptococci spp* and gram-negative *Escherichia coli* was investigated. The stimulation of human peripheral blood mononuclear cells by the various bacterial strains induced a differential cytokine pattern. *Streptococci* and *E. coli* significantly ($P \leq 0.001$) induced gamma interferon (IFN-٢) and interleukin-12 (IL-12). All bacteria mediated the proliferation of human peripheral blood mononuclear cells. Proliferate activity of lymphocytes was observed when mitogens were added. The MTT is employed to assess the response of lymphocytes to a mitogen. Bacteria and PHA was effective in inducing the MTT index of lymphocytes. Therefore, PHA, gram-positive bacteria, and gram-negative bacteria showed a high significant ($P \leq 0.001$) increased percentage of MTT index (96.4, 88.2 and 74.7 %, respectively) as compared to control subjects (42.5%) with no treatment.

INTRODUCTION

The mixture of organisms regularly found at any anatomical site is referred to as the normal flora, except by researchers in the field who prefer the term "indigenous microbiota". The normal flora of humans consists of a few eucaryotic fungi and protists, but bacteria are the most numerous and obvious microbial components of the normal flora (1). Research suggests that the relationship between gut flora and humans is not merely commensal (a non-harmful coexistence), but rather is a mutually beneficial symbiotic relationship (2). Though people can survive without gut flora, the microorganisms perform a host of useful functions, such as fermenting unused energy substrates, training the immune system, preventing growth of harmful, pathogenic bacteria, regulating the development of the gut, producing vitamins for the host (such as biotin and vitamin K), and producing hormones to direct the host to store fats. However, in certain conditions, some species are thought to be capable of causing disease by producing infection or increasing cancer risk for the host (3, 4).

Gut flora have a continuous and dynamic effect on the host's gut and systemic immune systems. The bacteria are key in promoting the early development of the gut's mucosal immune system both in terms of its physical components and function and continue to play a role later in life in its operation. The bacteria stimulate the lymphoid tissue associated with the gut mucosa to produce antibodies to pathogens. The immune system recognizes and fights harmful bacteria, but leaves the helpful species alone, a tolerance developed in infancy (3, 4, 5, 6). As soon as an infant is born, bacteria begin colonizing its digestive tract. The first bacteria to settle in are able to affect the immune response, making it more favorable to their own survival and less so to competing species; thus the first bacteria to colonize the gut are important in determining the person's lifelong gut flora makeup (7, 8). However, there is a shift at the time of weaning from predominantly facultative aerobic species such as *Streptococci* and *Escherichia coli* to mostly obligate anaerobic species (8, 9, 10, 11).

Streptococci are members of the normal indigenous microflora. Many streptococcal species are non-pathogenic. Indeed, *Streptococci* are a necessary ingredient in Emmentaler ("Swiss") cheese. *Streptococci* are also part of the normal commensal flora of the mouth, skin, intestine, and upper respiratory tract of humans (10).

Recent findings have shown that gut bacteria play a role in the expression of Toll-like receptors (TLRs) in the intestines, molecules that help the host repair damage due to injury. TLRs cause parts of the immune system to repair injury caused for example by radiation (12, 13). TLRs are one of the two classes of pattern recognition receptors (PRR) that provide the intestine the ability to discriminate between the pathogenic and commensal bacteria. These PRRs identify the pathogens that have crossed the mucosal barriers and trigger a set of responses that take action against the pathogen which involve 3 main immunosensory cells; surface enterocytes, M cells and dendritic cells (13, 14, 15). The other classes of PRRs are known as the nucleotide-binding oligomerization domain/caspase recruitment domain isoforms (NOD/CARD) which are cytoplasmic proteins that recognize endogenous or microbial molecules or stress responses

and forms oligomers that activate inflammatory caspases. This would result in the cleavage and activation of important inflammatory cytokines and/or activate NF-kB signaling pathway to induce the production of inflammatory molecules (14, 15).Bacteria can influence the phenomenon known as oral tolerance, in which the immune system is less sensitive to an antigen (including those produced by gut bacteria) once it has been ingested. This tolerance, mediated in part by the gastrointestinal immune system and in part by the liver, can reduce an overactive immune response like those found in allergies and auto-immune disease. Some species of gut flora, such as some of those in the Bacteroides genus, are able to change their surface receptors to mimic those of host cells in order to evade immune response. Bacteria with neutral and harmful effects on the host can also use these types of strategies. The host immune system has also adapted to this activity, preventing overgrowth of harmful species (16, 17, 18, 19).

MATERIALS AND METHODS

Bacteria. All bacteria used in this study isolated from healthy person, considered as non pathogen (normal flora) then identification in lab and cultivation on suitable media. All bacteria were harvested by centrifugation (3,000 × g, 15 min) at stationary growth phase (24 h). Bacteria were washed three times with phosphate-buffered saline (PBS) (pH 7.2; Gibco BRL) and subsequently diluted to final concentrations of 10^5 and 10^6 CFU/ml in RPMI 1640 (Gibco BRL) medium containing 20% native human AB serum (Sigma).

Isolation of human PBMC. Human PBMC (peripheral blood mononuclear cells) were purified from buffy coats (Blood) using Ficoll-Hypaque (1077; Pharmacia) gradient centrifugation. PBMC were harvested from the interface, washed five times with RPMI 1640, and diluted in RPMI 1640 containing 20% native human AB serum to a final concentration of 2×10^6/ml.

Stimulation of PBMC .Freshly isolated PBMC were seeded at 2×10^6/ml (2 ml) into six-well tissue culture plates and 2 ml of bacterial suspension was added to each well. For the stimulation, the final ratio between PBMC and bacteria was 1:1. For control treatment, culture medium alone was added to the PBMC suspension. To determine the cytokine expression by PBMC, the samples were incubated in the absence of antibiotics for 16 h at 37°C and 5% CO2. Subsequently, PBMC were collected, washed in cold PBS, and centrifuged and the cell pellet was lysed in guanidinium isothiocyanate denaturation solution. Cell culture supernatants were collected separately and kept at 20°C for cytokine analysis using the enzyme-linked immunosorbent assay (ELISA) technique.

ELISA. Cytokine concentration in cell culture supernatants (IFN-γ, and IL-12) was determined after 16 h of bacterial stimulation using ELISA (ImmunoKontact). Dose-response experiments performed for each cytokine indicated that maximal secretion was obtained with 10^6 CFU of bacteria/ml, corresponding to a ratio of 1:1 (bacteria to PBMC).

Proliferation assay.

Isolation of lymphocytes

By means of a density gradient centrifugation (21), the lymphocytes

were isolated from the whole blood. The following steps were followed:

The blood (5 ml) was centrifuged (1000 rpm) for 15 minutes.The plasma, Buffy coat and the most upper layer of erythrocytes were collected in a 5 ml centrifuge tube, and the cell-suspension was diluted with physiological saline up to 5 ml.The diluted cell suspension (2.5 ml) was layered on 2 ml of Ficoll-isopaque separation fluid (lymphoprep; specific gravity = 1.077).The tubes were centrifuged (2100 rpm) for 30 minutes in a cooled centrifuge. After centrifugation, the lymphocytes were visible as cloudy band between the plasma and lymphoprep layers. The band was collected in a 5 ml test tube, and the cells were suspended in washing medium (2 ml).The tube was centrifuged (2000 rpm) for 5 minutes (first wash), then the supernatant was discarded, and the cells were re-suspended in 3 ml washing medium. This step was repeated (second wash).A third wash was also done but at a lower speed (1000 rpm) and for 10 minutes. This step is very necessary, because it helps to maintain most of the platelets in the supernatant.The obtained cells were suspended in 1 ml Terasaki medium supplemented with 5 % heat inactivated (56ºC for 30 minutes) foetal calf serum.

Microculture Tetrazolium Test (MTT)

Principles

The MTT is employed to assess the response of lymphocytes to a mitogen. It measures the conversion of soluble 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (DMTB) to a purple DMTB formazan precipitate by the enzyme dehydrogenase that presents in the mitochondria of living cells. Based on this principle, the more active viable cells the more dehydrogenase is produced .

Method

The isolated lymphocytes were suspended in RPMI-1640 complete medium and adjusted to a cell count of 2 x 10^4 cell /ml. Then, 100 µl of cell suspension were dispensed in the well of 96-wells flat-bottom microtiter plates in triplicates, and each plate was incubated for 24 hours at 37ºC in a humidified 5% CO2 atmosphere. After incubation, 2×10^4 to 2×10^7 CFU of bacteria/ml, or 10 µl of a mitogen solution (PHA) were added, and the plate was further incubated for 24 hours at 37ºC in a humidified 5% CO2 atmosphere. In control wells, the bacteria or PHA was replaced with RPMI-1640 complete medium. Then, 20 µl of MTT working solution (5 mg/ml) were added to each culture well and the cultures were incubated for 4 hours at 37ºC in a humidified 5% CO2 atmosphere. After that, the culture medium was removed from wells and the converted dye was solubilized with 100 µl of acidic isopropanol (absolute isopropanol supplemented with 0.04N HCl). The absorbency of each well was measured with a microculture plate reader at 540 nm (22,23). The MTT index was calculated using the following formula:

$$\text{MTT Index (\%)} = \left(\frac{\text{Absorbency of Experimental Well}}{\text{Absorebency of Control Well}} \right) \times 100$$

RESULTS

Gram-positive and gram-negative nonpathogenic bacteria differentially induce cytokine expression in human PBMC. Freshly isolated PBMC were stimulated by nonpathogenic gram-positive *Streptococci spp* or gram-negative *E. coli* at a ratio of 1:1 (bacteria to PBMC) for 16 h in RPMI 1640-10% human AB serum. No bacterial overgrowth of the antibiotic-free cultures was detected at the given time point as controlled by the plating or nonacidification of the culture medium (phenol red indicator). In contrast, the E. coli-mediated IFN-γ induction was very low at the early time points and was completely abrogated at 16 h. The amount of IFN-γ secreted into cell culture supernatants was determined after 16 h of bacterial stimulation using ELISA techniques. The predominant induction of IFN-γ in human PBMC by the *Streptococci spp* was also confirmed at the secretory level, where significant amounts of IFN-γ (1130 pg/ml) by bacteria were detectable in cell culture supernatants compared to the untreated control (32 pg/ml). *E. coli*, although induced significant IFN-γ levels (350 pg/ml) compared to the amounts induced in to the untreated control but weaker than *Streptococci spp* (Fig. 1).

In contrast to IFN-γ, IL-12 was strongly induced by *Streptococci spp* bacterial treatments, with lower levels of induction for E. coli (Fig. 2).

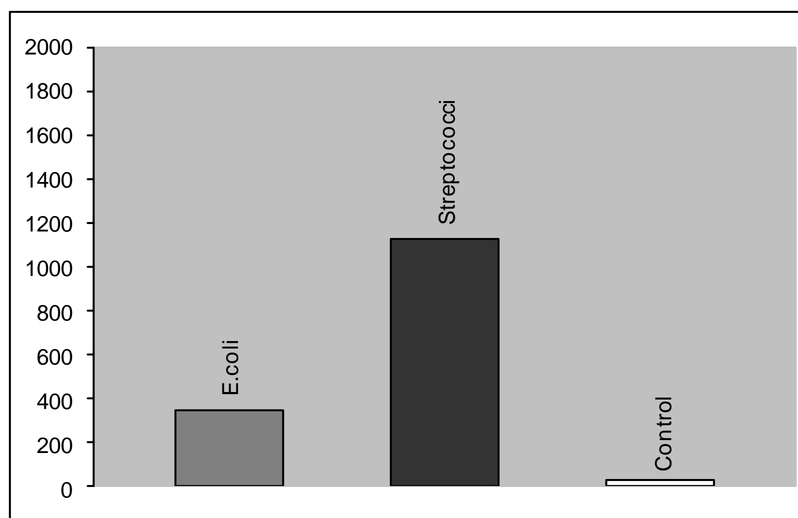

FIG. 1. Expression of IFN-γ by PBMC upon stimulation with bacteria. ELISA analyses was used to determine IFN-γ expression by PBMC (10^6/ml) upon stimulation with bacterial cells (10^6 CFU/ml) of *E. coli*, *Streptococci spp.*.Control (white bars) without stimulation.

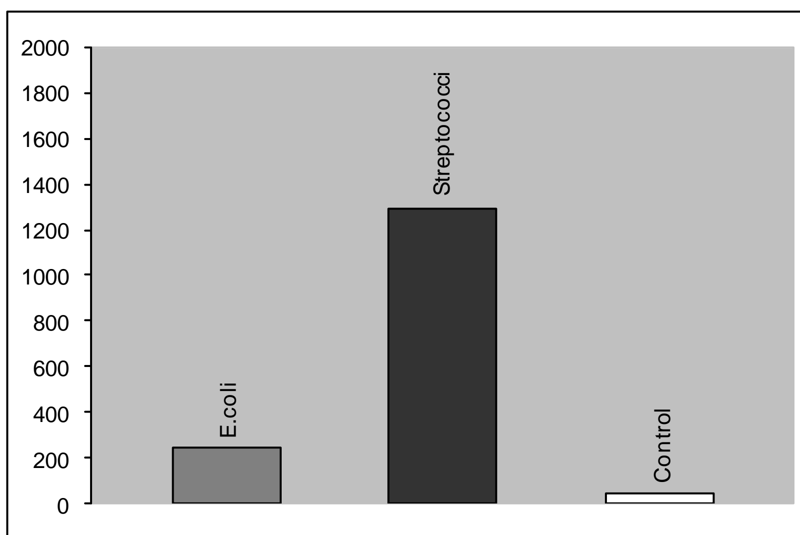

FIG. 2. Expression of IL-12 by PBMC upon stimulation with bacteria. ELISA analyses was used to determine IL-12 expression by PBMC (10^6/ml) upon stimulation with bacterial cells (10^6 CFU/ml) of *E. coli*, *Streptococci spp.* Control (white bars) withoutstimulation.

MTT (Microculture Tetrazolium Test) Index

Bacteria and PHA was effective in inducing the MTT index of lymphocytes. Therefore, PHA, gram-positive bacteria, and gram-negative bacteria showed a high significant ($P \leq 0.001$) increased percentage of MTT index (96.4, 74.7, and 88.2%, respectively) as compared to control subjects (42.5%) (Table 1).

Table 1: Microculture tetrazolium test (MTT) index (mean ± S.E.) of peripheral lymphocytes in PHA, gram-positive bacteria , gram-negative bacteria and controls.

Groups	Number	MTT index (%)			Probability ≤
		Mean ± S.E.	Minimum	Maximum	
Controls	50	42.5 ± 0.45	28.0	64.0	
PHA	50	96.4 ± 0.71	88.0	99.0	0.001
gram-positive bacteria	50	88.2 ± 1.10	77.0	91.0	0.001
gram-negative bacteria	50	74.7±1.61	62.0	87.0	0.001

DISCUSSION

The present study on the direct interaction of nonpathogenic bacteria with human PBMC is based on the assumption that bacteria and immunocompetent cells may physically interact in definite mucosal environments. The epithelial compartment, the lamina propria, and M-cell pockets are potential sites where commensal, nonpathogenic bacteria may encounter immunocompetent cells. It is well documented that M cells promote the interaction between luminal antigens, including bacteria, and immunocompetent cells (14, 15). The occurrence of limited bacterial translocation to the lamina propria in humans has also been reported (3, 4). Although PBMC are only partially representative of immunocompetent cells in intestinal mucosal compartments, phenotypical similarities with respect to the germ line-encoded receptors involved in the recognition of bacterial antigens on lymphocytes and macrophages, such as pattern recognition receptors (14), could constitute the link between both populations and thus may provide important indications of the functional aspects of the mucosal immune response to luminal bacteria.

We showed that gram-positive and gram-negative nonpathogenic bacteria induced different cytokine patterns in human PBMC. All bacteria induced the Th1-like cytokines IL-12 and IFN-ϒ were observed. Streptococci spp strongly induced IFN-ϒ and IL-12. In contrast, the gram-negative E. coli had little capacity to induce IFN-ϒ or IL-12 in human PBMC. These results are in agreement with reports by Miettinen et al. (20) comparing different nonpathogenic and pathogenic gram-positive bacteria with respect to the induction of cytokines in PBMC.

The optimal proliferative activity of lymphocytes was observed when mitogens were added. Bacteria and PHA was effective in inducing the MTT index of lymphocytes. Therefore, PHA, Gram-positive bacteria, and gram-negative bacteria showed a high significant ($P \leq 0.001$) increased percentage of MTT index (96.4, 88.2, and 74.7%, respectively) as compared to control subjects (42.5%).In this test bacteria showed high effect on immune cells when express mitogen properties.

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تحفيز ألمناعه ألذاتية للإنسان بواسطة البكتريا خارج جسم الكائن ألحي

منال بادي صالح التميمي

فرع الاحياء المجهرية الطبية - كلية الطب - جامعة ذي قار

الخلاصه

البكتريا غير ألممرضه المتعايشة مع الإنسان أساسيه لتعزيز ألمناعة ودعم الخلايا أللمفاويه.وقد لوحظ أن ألحيوانات ألخاليه من هذه البكتريا ألتعايشيه تكون مضعفه مناعيا. وفي هذه الدراسة تم استخدام هذه البكتريا كعوامل حيوية لتحفيز ألمناعة بواسطة تحفيز خلايا دم الإنسان الدفاعية أحادية الخلية بواسطة البكتريا غير ألممرضه الموجبة والسالبة لصبغة كرام (*Streptococci spp* and *Escherichia coli*).

قد لوحظ بشكل واضح ان السلالات البكتيريا المختلفة أدت إلى تحفيز إنتاج الحركيات الخلوية المناعية (السايتوكينات) IFNγ ,IL-12 بتراكيز مختلفة حيث إن بكتريا *Streptococci spp* حفزت إنتاج IFN-γ , IL-12 بشكل كبير ($P \leq 0.001$) مقارنة بالسيطرة ألغير معاملة .ومن جهة أخرى وجد إن *Escherichia coli* حفزت ألإنتاج وبشكل معنوي ($P \leq 0.001$) ولكن أقل من ألأولى.

ارتفاع ألنسبة المئوية للاستجابة الانقسامية للخلايا أللمفية (MTT index) ألمحفزة بالمشطر PHA وألبكتريا عند مقارنتها بالسيطرة.أظهرت ألنسبة ارتفاع معنوي ($P \leq 0.001$) لكل من الخلايا أللمفية ألمحفزة بالمشطر PHA (96.4%) وألبكتريا ألموجبة(88.2%) وألبكتريا ألسالبة (74.7%) مقارنة بالسيطرة(42.5%).