



RAPID COMMUNICATION

Distribution of secretory inhibitor of platelet microbicidal protein among anaerobic bacteria isolated from stool of children with diarrhea

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Supported by The Russian Foundation of Basic Research and Government of Orenburg region, No. 07-04-97624 and No. 08-04-99105

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Received: January 31, 2008 Revised: July 29, 2008

Accepted: August 5, 2008

Published online: September 21, 2008

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Ivanov IB, Gritsenko VA. Distribution of secretory inhibitor of platelet microbicidal protein among anaerobic bacteria isolated from stool of children with diarrhea. *World J Gastroenterol* 2008; 14(35): 5428-5431 Available from: URL: <http://www.wjgnet.com/1007-9327/14/5428.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.5428>

Abstract

AIM: To study the secretory inhibitor of platelet microbicidal protein (SIPMP) phenotypes of faecal anaerobic isolates from patients with diarrhea.

METHODS: Faecal isolates of anaerobic bacteria (*B. fragilis*, $n = 42$; *B. longum*, $n = 70$; *A. israelii*, $n = 21$; *E. lentum*, $n = 12$) from children with diarrhea were tested. SIPMP production was tested by inhibition of platelet microbicidal protein (PMP) bioactivity against *B. subtilis* and was expressed as percentage of inhibition of PMP bactericidal activity.

RESULTS: Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of $13.8\% \pm 0.7\%$, $14.7\% \pm 1.8\%$, $3.9\% \pm 0.9\%$ ($P < 0.05$) and $26.8\% \pm 7.5\%$ ($P < 0.05$) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis*, respectively.

CONCLUSION: Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in the intestine, as well as for future improvement in the prevention and therapy of anaerobe-associated infections.

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Key words: Platelet microbicidal protein; Secretory inhibitor; Anaerobic bacteria; Intestine

INTRODUCTION

Anaerobic microorganisms are important constituents of human intestinal microbiota^[1]. Enzymes produced by these bacteria provide nutrients for growth, participate in the pathogenesis of infections involving these bacteria, etc. Infections caused by anaerobic bacteria are increasingly being recognized as a major problem in clinical medicine^[2,3]. The commensal anaerobic bacterial flora of the colon may undergo changes during diarrhea, owing to colonization of the intestine by pathogens and to rapid intestinal transit^[4]. As it is difficult to establish exactly the significance of various anaerobic microorganisms in the pathogenesis of infections, it is imperative to delineate both microbial and host factors that contribute to its development. Identifying such a factor(s) produced by anaerobes is important for understanding and possibly modulating interactions between these bacteria and the host.

The intestinal mucosa forms a primary barrier providing both barrier function and immediate effective recognition of bacterial products invading the mucosa. This is of great importance for the prevention of permanent and chronic inflammation as a reaction to the commensal intestinal flora and the multitude of antigens present in the intestinal lumen^[5].

The major role of endogenous cationic antimicrobial peptides in preventing the onset of infection has been emphasized recently^[6,7]. In mammals, these peptides have evolved to have a central function in the host defense properties of granulocytic leukocytes, mucosal surfaces, skin and other epithelia^[7]. Antibacterial protection of intestinal mucosa is provided in part by Paneth cell-derived antibacterial peptides^[8-10]. Such peptides have also been found by several authors in human platelets

and are designated platelet microbicidal proteins (PMPs)^[11,12]. These peptides are secreted at sites of infection and exert microbicidal activity against many pathogens^[11]. Bukharin *et al* showed that an enhanced level of PMP in coprofiltrates of patients is associated with *Salmonella* gastroenteritis^[13]. However, we suspect that successful pathogens (especially anaerobes) would have involved mechanisms to resist or degrade the inhibitory and microbicidal activities presented by the host. For example, in a recent publication^[14], we reported the detection of an extracellular staphylococcal product, designated secretory inhibitor of platelet microbicidal protein (SIPMP), which causes local inhibition of the bactericidal action of PMP in the fluid phase. We also demonstrated that SIPMP represents a hitherto unrecognized determinant of staphylococcal pathogenicity and SIPMP production is associated with a prostatitis source.

At the same time, it is surprising that no extracellular product of anaerobic microorganisms with remarkable anti-PMP potential has been described. Thus, in this communication we report on *in vitro* detection of SIPMP phenotypes of faecal anaerobic isolates from patients with diarrhea.

MATERIALS AND METHODS

Clinical isolates were obtained from diarrhea stool samples collected from April to December 2000 in Orenburg Regional Child Hospital. A total of 145 strains of anaerobic bacteria (*B. fragilis*, *n* = 42; *B. longum*, *n* = 70; *A. israelii*, *n* = 21; *E. lentum*, *n* = 12) were kindly provided by Natalia Elagina (Department of Dysbiosis, Institute of Cellular and Intracellular Symbiosis). Bacteria were isolated from children with diarrhea (ranging from 10 mo old to 6 year old) and identified at the Anaerobe Laboratory, Department of Dysbiosis, Institute of Cellular and Intracellular Symbiosis, Russian Academy of Sciences, Orenburg.

PMP was prepared and standardized as described previously^[12].

SIPMP production was performed by viable counting according to the recently proposed procedures^[14]. Strains were grown in brain heart infusion broth (BHI, Oxoid), supplemented with yeast extract (0.5%) under anaerobiosis conditions (90% N₂/10% CO₂), at 37°C, for 48 h and cell-free supernatants were obtained by centrifugation. Bacterial supernatants were sterilized by filtration *via* 0.45 µm pore-size membranes (Millipore). Each culture supernatant (0.6 mL) (an equal volume of BHI was loaded in the control tubes) was combined with 0.3 mL of PMP at 3.0 µg/mL and incubated at 37°C. After 1 h, 100 µL *B. subtilis* suspension at 10⁴ CFU/mL was added to each of the tubes. The tubes were incubated on a rotary shaker (300 r/min) at 37°C. After 1 h, aliquots of 200 µL were plated on blood agar plates. Colonies were counted after incubating overnight at 37°C and numbers of surviving microorganisms were calculated. The SIPMP production as expressed in percentage of inhibition of PMP bactericidal activity

Table 1 SIPMP production of fecal isolates of anaerobic bacteria *n* (%)

Organism	No. of SIPMP-producing strains (total/%) with different levels of SIPMP ¹			
	0	0.1-10.0	10.1-20.0	> 20
<i>B. longum</i> (<i>n</i> = 70)	14 (20)	0 (0)	56 (80)	0 (0)
<i>A. israelii</i> (<i>n</i> = 21)	3 (14.3)	0 (0)	18 (85.7)	0 (0)
<i>E. lentum</i> (<i>n</i> = 12)	6 (50)	6 (50)	0 (0)	0 (0)
<i>B. fragilis</i> (<i>n</i> = 42)	3 (7.14)	0 (0)	2 (4.76)	37 (88.1)

¹SIPMP was expressed in percentage of inhibition of PMP bactericidal activity.

and calculated by using the formula: % inhibition = (No. - Nk1) × 100/(Nk2 - Nk1), where No. was the number of surviving *B. subtilis* cells in the presence of bacterial supernatant and PMP, Nk1 was the number of surviving *B. subtilis* cells in the presence of PMP alone, and Nk2 was the number of surviving *B. subtilis* cells in BHI.

All of the experiments were carried out in triplicate and mean values and SEM were calculated. The differences between groups of microorganisms were assessed by using Student's *t*-test. *P* ≤ 0.05 was considered significant.

RESULTS

For exclusion the cooperative inhibitory effect of PMP and culture supernatants on *B. subtilis*, each culture supernatant was combined with *B. subtilis* suspension. After coincubation for 1 h, aliquots were plated on blood agar plates. Colonies were counted after incubating overnight at 37°C and numbers of surviving microorganisms were calculated. None of the supernatants tested inhibited growth of *B. subtilis* cells. The stability of SIPMP was tested by subjecting culture supernatants to boiling for 30 min. This treatment completely destroyed the biological activity of SIPMP. Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive (Table 1). The extracellular products of bacteria reduced the PMP-induced killing of *B. subtilis*. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of 13.8% ± 0.7%, 14.7% ± 1.8%, 3.9% ± 0.9% (*P* < 0.05) and 26.8% ± 7.5% (*P* < 0.05) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis* respectively.

DISCUSSION

At local sites of microbial infections, epithelial cells, platelets, neutrophils, or macrophages release large amounts of different bactericidal peptides^[7]. However, most infections are the result of contamination of host tissues with anaerobic flora from the gut^[15] despite the presence of multiple antibacterial peptides in intestinal cells and mucus^[7,16,17]. There is an urgent need to understand the virulence properties of anaerobic

organisms that may take part in their resistance to cationic antimicrobial peptides; identifying such a factor(s) would be helpful in devising effective treatment strategies.

In the present work, we detected an extracellular bacterial product of anaerobic microorganisms with remarkable anti-PMP potential which, to our knowledge, has not been described before. We anticipate that SIPMP serves to protect invading bacteria by inducing local consumption of PMP in the fluid phase. The strategy underlying this process would be straightforward and effective. We believe that SIPMP represents a widespread and hitherto unrecognized determinant of bacterial pathogenicity. Similarly, in a study of distribution of streptococcal inhibitor of complement variants in pharyngitis and invasive isolates by Hoe *et al*^[18], 62% of group A streptococci from patients with pharyngitis produced this extracellular protein. Collectively, our study and the results of several studies^[19-21] suggest that the inactivation of components of innate immunity may be important for bacterial pathogens to induce and perpetuate infections of different localization by surviving or avoiding microbicidal proteins mediated clearance. Bacteria-derived proteases may contribute to mucosal surface destruction, and are likely to impair host defense by degrading antimicrobial peptides^[22]. It was confirmed by the fact that the lowest level of SIPMP production was observed with the non-protease producing species *E. lentum*. On the other hand, proteases of anaerobic microorganisms caused platelet aggregation with followed by release of a number of antibacterial proteins^[11,23].

In contrast to *B. fragilis*, normal microflora have low levels of SIPMP. Hypothetically, the constituents of normal flora must have basal levels of resistance to the antimicrobial host defense factors. It is possible that low levels of inactivation of PMP activity by normal organisms are sufficient to protect them from PMP-dependent killing, thus providing stability of intestinal microflora. We believe that SIPMP is the stable characteristic and the same strains express more SIPMP in case of infection. On the other hand, our results suggest that the normal microflora was replaced by other organisms with pronounced pathogenic properties in patients with persistent infection^[24].

At the same time, in the presence of infections, properties of normal microflora probably could change. The constituents of normal microflora, receiving signs of pathogenicity, are capable of causing diseases, as has been shown for lactobacilli and staphylococci^[24,25].

The predominantly anaerobic microbiota of the distal ileum and colon contain an extraordinarily complex variety of metabolically active bacteria that intimately interact with the host's epithelial cells and mucosal immune system^[26]. Crohn's disease, ulcerative colitis, and pouchitis are the result of continuous microbial antigenic stimulation of pathogenic immune responses as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing, or immunoregulation. Identification of these host and

microbial alterations in individual patients should lead to selective targeted interventions that correct underlying abnormalities and induce sustained and predictable therapeutic responses^[27]. New treatment strategies aim at neutralization of such pathogenic properties of microorganisms as pronounced resistance to the cationic antimicrobial peptides and/or ability to inhibit the antimicrobial host defense factors and thereby improve the quality of life in patients^[28-30].

Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in intestine, as well as for future improvement in the prevention of and therapy for anaerobe-associated infections. However, the exact mechanism of PMP inhibition in anaerobic bacteria remains to be determined, as does its molecular characteristics, occurrence and possible significance *in vivo*.

COMMENTS

Background

Anaerobic microorganisms are important constituents of human intestinal microbiota. Infections caused by anaerobic bacteria are increasingly being recognized as a major problem in clinical medicine. The commensal anaerobic bacterial flora of the colon may undergo changes during diarrhea, owing to colonization of the intestine by pathogens and to rapid intestinal transit. The major role of endogenous cationic antimicrobial peptides in preventing the onset of infection has been emphasized recently. Such peptides have been found in platelets and are designated platelet microbicidal proteins (PMPs). It is shown that an enhanced level of PMP in coprofiltrates of patients is associated with *Salmonella* gastroenteritis. Here we made an attempt to *in vitro* detection of secretory inhibitor of platelet microbicidal protein (SIPMP) phenotypes of faecal anaerobic isolates from patients with diarrhea.

Research frontiers

The article focuses on inhibition of PMP by extracellular bacterial products of faecal anaerobic microorganisms isolated from stool of children with diarrhea. Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of $13.8\% \pm 0.7\%$, $14.7\% \pm 1.8\%$, $3.9\% \pm 0.9\%$ ($P < 0.05$) and $26.8\% \pm 7.5\%$ ($P < 0.05$) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis*, respectively.

Innovations and breakthroughs

In the present work, the authors detected an extracellular bacterial product of anaerobic microorganisms with remarkable anti-PMP potential that has not been described before. SIPMP represents a widespread and hitherto unrecognized determinant of bacterial pathogenicity.

Applications

Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in intestine, as well as for future improvement in the prevention and therapy of anaerobe-associated infections.

Terminology

PMP is a group of small cationic peptides isolated from rabbit and human platelets after stimulation by acid or thrombin; the secretory inhibitor of PMP is an extracellular bacterial product with anti-PMP activity.

Peer review

In this manuscript, the authors reported the detection of SIPMP phenotypes of faecal anaerobic isolates from patients with diarrhea. The study was well performed and interesting.

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S- Editor Li DL L- Editor Li M E- Editor Yin DH