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Bifidobacterial Lipoglycan as a New Cause for False-Positive Platelia Aspergillus Enzyme-Linked Immunosorbent Assay Reactivity

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We previously hypothesized that a lipoglycan of Bifidobacterium bifidum subsp. pennsylvaniaeum cross-reacts with the Platelia Aspergillus (PA) enzyme-linked immunosorbent assay (ELISA) based on the presence of galactofuranosyl epitopes in the cell wall (M. A. S. H. Mennink-Kersten, R. R. Klont, A. Warris, H. J. M. Op den Camp, and P. E. Verweij, Lancet 363:325–327, 2004). We tested this hypothesis by testing bacterial suspensions of different bifidobacterial species and other gram-positive and -negative bacteria with the PA ELISA, which is used to detect circulating galactomannan for the serodiagnosis of invasive aspergillosis. Furthermore, neonatal fecal samples were enumerated for bifidobacteria by fluorescence in situ hybridization (FISH) and tested for PA ELISA reactivity. All bifidobacteria, except B. infantis and B. adolescentis, showed reactivity 6- to 600-fold higher compared to the controls (i.e., Micrococcus luteus and Propionibacterium freudenreichii, which contain a cell wall lipomannan). Enterococcus lenta showed a 25-fold-higher reactivity. ELISA reactivity was clearly shown to be associated with bacterial lipoglycans containing a β-1,5-galactofuranosyl chain. All neonatal feces showed PA ELISA reactivity and associated numbers of bifidobacteria. Since high concentrations of bifidobacteria are present in the human gut, these bacteria or excreted lipoglycan may cause false serum PA ELISA reactivity in selected patient groups, especially neonates.

Invasive aspergillosis (IA) has become a leading cause of death among immunocompromised patients. A commercial sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia Aspergillus [PA] ELISA; Bio-Rad, Marnes-la-Coquette, France) that is widely used as a diagnostic tool in centers throughout the world detects a fungal antigen which is present in body fluids of patients with IA (5, 24, 44). The PA ELISA specifically detects circulating galactomannan (GM), a molecule present in body fluids of patients with IA (5, 24, 44). The PA ELISA cross-reacts with the Platelia Aspergillus (PA) enzyme-linked immunosorbent assay (ELISA) based on the presence of galactofuranosyl epitopes in the cell wall (M. A. S. H. Mennink-Kersten, R. R. Klont, A. Warris, H. J. M. Op den Camp, and P. E. Verweij, Lancet 363:325–327, 2004). We tested this hypothesis by testing bacterial suspensions of different bifidobacterial species and other gram-positive and -negative bacteria with the PA ELISA, which is used to detect circulating galactomannan for the serodiagnosis of invasive aspergillosis. Furthermore, neonatal fecal samples were enumerated for bifidobacteria by fluorescence in situ hybridization (FISH) and tested for PA ELISA reactivity. All bifidobacteria, except B. infantis and B. adolescentis, showed reactivity 6- to 600-fold higher compared to the controls (i.e., Micrococcus luteus and Propionibacterium freudenreichii, which contain a cell wall lipomannan). Enterococcus lenta showed a 25-fold-higher reactivity. ELISA reactivity was clearly shown to be associated with bacterial lipoglycans containing a β-1,5-galactofuranosyl chain. All neonatal feces showed PA ELISA reactivity and associated numbers of bifidobacteria. Since high concentrations of bifidobacteria are present in the human gut, these bacteria or excreted lipoglycan may cause false serum PA ELISA reactivity in selected patient groups, especially neonates.

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high-G+C subdivision of gram-positive bacteria, LTAs are functionally replaced by lipoglycans, which contain a linear or branched polysaccharide as a hydrophilic moiety; this polysaccharide may carry monogylycerophosphate side chains (Bifidobacterium species) or succinyl esters (lipomannan from Micrococcus, Mycobacterium, or Propionibacterium) (9). The macroamphiphiles are secreted even during normal growth both with and without their lipid anchor. Secretion is stimulated by exposure to β-lactam antibiotics (9, 12, 33, 35). In aqueous solutions, the acylated molecules form micellar aggregates (53).

The aim of our research was to test the in vitro reactivity of different bacteria including bifidobacterial species and other members of the gut microflora of humans with the PA ELISA. Any reactivity was correlated with the presence and structure of bacterial lipid macroamphiphiles based on review of the literature.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Reactivity with the PA ELISA was investigated in vitro by testing cell suspensions of a range of bacteria, including 11 bifidobacterial species and 15 other bacteria (gram-positive and -negative species), most known to be common inhabitants of the human gut (Table 1). For each bacterium the structure of the lipid macroamphiphile was documented based on review of the literature (7–9, 13, 16, 18, 30, 48). Bacteria were cultured anaerobically (80% N2, 10% CO2, 10% H2) on agar plates containing fastidious anaerobic agar (Lab M) or aerobically on Columbia agar (BBL), at 37°C. B. bifidum subsp. pennsylvaniaicum was also cultured on reinforced clostridial medium (RCM; Oxoid). Because this liquid medium showed ELISA reactivity, it was hydrolyzed with acid (pH 2 with HCl for 3 h at 100°C) and neutralized with NaOH at pH 6.8 before inoculation. Acid hydrolysis removes Galf chains that are present in the RCM, which originate from contaminated medium components like meat extracts.

Fecal sample collection and FISH analysis. Fecal samples from nine healthy neonates were obtained. Babies were fully breast-fed or formula fed and ranged in age from 3 days to 4.5 months. No baby had been on antibiotic treatment prior to fecal sampling. Fecal samples were stored at −80°C until shipment on dry ice to Groningen for fluorescence in situ hybridization (FISH) analysis. Samples were processed and FISH analysis was performed as described by Harmsen et al. (14). The bifidobacterial probe Bif164 (21), a 16S rRNA-based oligonucleotide probe, was used to enumerate total bifidobacteria in the fecal samples.

PA ELISA. Bacterial cells were scraped off the agar plates and suspended in 0.9% NaCl (wt/vol). Fresh fecal samples were suspended in saline to give a final concentration of 100 mg/ml (wet weight). The complete mixtures were serially diluted and used for detection of reactivity by the PA ELISA. The PA ELISA was performed according to the manufacturer’s instructions. However, the pre-treatment step intended to dissociate immune complexes was omitted, except for the R3, R4, and R5 calibration samples (serum spiked with GM) and the fecal samples. Briefly, 50 μl of a reaction mixture containing horseshadish peroxidase-conjugated anti-GM monoclonal antibody EA-A2 was added to each well of a microtitration plate coated with the same monoclonal antibody EB-A2, followed by addition of 50 μl of the bacterial cell suspension or pretreated sample. After 90 min of incubation at 37°C, the plates were washed five times with washing buffer before 200 μl of buffer containing tetramethylbenzidine solution was added. Then the plates were incubated for another 30 min in the dark at room temperature, followed by the addition of 100 μl of 1.5 N sulfuric acid to stop the reaction. The optical density (OD) was read at 450 and 620 nm. A test sample was considered positive when the OD at 450 nm was higher than the cutoff sample (i.e., 1.0 ng GM). Pretreatment of the R3, R4, and R5 calibration samples and the fecal samples was done by mixing 300 μl of each sample with 100 μl of treatment solution (4% EDTA), and the mixture was subsequently boiled for 5 min. After centrifugation (10,000 × g, 10 min), the supernatant was used for further testing.

All bacterial isolates were tested twice and in duplicate. In vitro reactivity was quantified as ELISA index (EI) correlated to the total protein content of the tested cell suspension. Total protein concentrations were determined with a protein assay kit (Bio-Rad, Richmond, CA), with bovine γ-globulin as a standard, after boiling the cells for 15 min in 1 M NaOH and neutralizing with 1 M HCl. The reactivity of Micrococcus luteus and Propionibacterium freudenreichii, which are known to have a lipomannan in the cell wall (9), served as negative controls. Furthermore, 1 ng/ml of purified GM (22) was added to M. luteus samples with different total protein contents. Fecal samples were tested twice, and in vitro reactivity was expressed as EI per gram of feces.

Cell count. Direct microscopic counts of tested bacterial cell samples were obtained by using duplicate smears of 0.01 ml of a 10-fold dilution spread over 1 cm2 of a glass slide. The smears were heat fixed and gently Gram stained. Ten fields were counted, and the counts were then correlated with the actual sample size.

Preparation and deacylation of cell extracts and cell pellet samples. After growth of B. bifidum subsp. pennsylvaniaicum on liquid RCM, samples were taken for PA ELISA. Part of the culture (1-ml samples) was centrifuged at 8,000 × g (10 min, room temperature) followed by filtration of the supernatant (0.2 μm) and washing with 0.9% NaCl. The clear supernatant was used as a cell-free culture fluid. The cell pellet was suspended in 200 μl 0.9% NaCl. The supernatant and suspended-pellet samples were serially diluted and used for detection of PA ELISA reactivity at 37°C. Fecal sample collection and FISH analysis. Fecal samples from nine healthy newborns were obtained. Babies were fully breast-fed or formula fed and ranged in age from 3 days to 4.5 months. No baby had been on antibiotic treatment prior to fecal sampling. Fecal samples were stored at −80°C until shipment on dry ice to Groningen for fluorescence in situ hybridization (FISH) analysis. Samples were processed and FISH analysis was performed as described by Harmsen et al. (14). The bifidobacterial probe Bif164 (21), a 16S rRNA-based oligonucleotide probe, was used to enumerate total bifidobacteria in the fecal samples.

RESULTS

ELISA reactivities of different bacterial samples. B. bifidum subsp. pennsylvaniaicum bacterial suspensions showed reactivity when tested in the PA ELISA. Reactivity was tested with a range of sample dilutions and showed saturation kinetics (Fig. 1).
2). Reactivity was determined in the linear part of the graph and was expressed as EI per milligram of protein. Protein concentrations of the undiluted samples ranged from 0.1 to 0.5 mg/ml. Some cell samples reacted so strongly that even a 40-fold dilution gave a positive PA ELISA result (EI/H11022 1.0) as shown for Bifidobacterium lactis (Fig. 2). The reactivity of all tested bacteria is shown in Fig. 3. Most bifidobacteria (from human, animal, or food origin) showed cross-reactivity with the PA ELISA. Reactivities ranged from 6-fold (Bifidobacterium breve) to 600-fold (B. lactis) higher than the controls. Some of these species are known to contain a lipoglycan with more than 7 \( \beta-1,5 \)-linked Gal residues (B. bifidum, B. breve, and Bifidobacterium longum; Table 1). Bifidobacterium infantis and Bifidobacterium adolescentis, which do not seem to have \( \beta-1,5 \)-
linked Gal residues in their cell wall, showed low reactivity. Furthermore, other gram-positive bacteria containing LTA or lipoglycan showed low reactivity, including the negative controls containing a lipomannan. Gram-negative bacteria containing lipopolysaccharide (LPS) also reacted negatively. The only nonbifidobacterial species that showed PA ELISA reactivity was *Eubacterium lentum*. This species, which is one of the predominant microorganisms from the human intestine, has been reclassified as *Eggerthella lenta* (19). In contrast to other eubacteria, which belong to the low-G\(\text{C}\)/H\(\text{C}\) group, this bacterium has a high G\(\text{C}\) content and by definition a lipoglycan instead of an LTA (31).

**PA ELISA reactivity per gram of feces.** Table 2 shows a calculation of the EI per gram of neonatal feces for the most frequently found bifidobacterial species, i.e., *B. longum*, *B. bifidum*, and *B. breve*. Matsuki et al. (25) studied the distribution of bifidobacterial species in the intestinal tract with 16S rRNA gene-targeted species-specific primers and found about one to three species per neonate (25). The EI was corrected for the dilution factor. This reactivity was then correlated to the

![Cross-reactivity of different bacterial species and strains with the PA ELISA. Reactivity is quantified as EI correlated to the total protein content of the cell suspension tested. Black bars indicate the control samples. Total protein contents of cell samples ranged from 0.1 to 0.5 mg/ml. Control samples include 1 ng/ml GM (clinical cutoff value) added to *M. luteus* samples with different total protein contents.](http://jcm.asm.org/)

**TABLE 2.** Calculated and measured PA ELISA reactivity per gram of neonatal feces

<table>
<thead>
<tr>
<th>Tested sample</th>
<th>Occurrence (%)*a</th>
<th>EL \times dilution factor</th>
<th>No. of cells (10^7)*c</th>
<th>No. present in feces g(^{-1})</th>
<th>EI g feces(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. longum</em></td>
<td>37</td>
<td>80.7</td>
<td>1.0</td>
<td>10^9.8–10.1 &amp;</td>
<td>51,000–102,000</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>22</td>
<td>10.2</td>
<td>0.7</td>
<td>10^{10.3} &amp;</td>
<td>29,000</td>
</tr>
<tr>
<td><em>B. breve</em></td>
<td>70</td>
<td>4.8</td>
<td>3.8</td>
<td>10^{10.5–10.7} &amp;</td>
<td>4,000–6,000</td>
</tr>
<tr>
<td>Fecal samples from neonates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>5.6</td>
<td></td>
<td>1.40 \times 10^7d &amp;</td>
<td>1,120</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>72.0</td>
<td></td>
<td>1.90 \times 10^7d &amp;</td>
<td>14,400</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.0</td>
<td></td>
<td>2.50 \times 10^6d &amp;</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>11.5</td>
<td></td>
<td>1.20 \times 10^5d &amp;</td>
<td>2,300</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>181.6</td>
<td></td>
<td>3.00 \times 10^6d &amp;</td>
<td>36,320</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>54.8</td>
<td></td>
<td>1.90 \times 10^5d &amp;</td>
<td>10,960</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>125.8</td>
<td></td>
<td>3.60 \times 10^5d &amp;</td>
<td>25,160</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>45.1</td>
<td></td>
<td>2.10 \times 10^6d &amp;</td>
<td>9,020</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>57.6</td>
<td></td>
<td>5.10 \times 10^6d &amp;</td>
<td>11,520</td>
</tr>
</tbody>
</table>

*a* Expressed as % of the total number of fecal samples that were tested, taken from reference 25.

*b* Present in 50 µl undiluted cell sample.

*c* Taken from reference 29.

*d* Determined by FISH analysis.
TABLE 3. PA ELISA reactivity of liquid RCM cultures of B. bifidum subsp. pennsylvanicum

<table>
<thead>
<tr>
<th>Sample</th>
<th>EI* (n = 2)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total culture</td>
<td>62.1</td>
<td>100</td>
</tr>
<tr>
<td>Cell-free supernatant</td>
<td>23.5</td>
<td>37.8</td>
</tr>
<tr>
<td>Suspended cell pellet</td>
<td>16.0</td>
<td>25.8</td>
</tr>
</tbody>
</table>

* Sample EI was corrected for dilution (20 × to 40 ×), and cell pellet values are also corrected for the five-fold concentration step (from 1,000 µl to 200 µl)

number of cells present in 50 µl undiluted cell sample. So knowing the reactivity per cell, the reactivity per gram of feces can be calculated by multiplying it with the amount of cells present per gram of feces. These fecal numbers were taken from the literature but may vary depending on the method used for quantification (15, 29). Table 2 also shows the EI per gram of feces as measured in the nine collected neonatal fecal samples. All fecal samples exhibited PA ELISA reactivity. Furthermore, all fecal samples contained bifidobacterial species as determined by FISH.

ELISA reactivity of liquid cultures. The result of the ELISA reactivity of the B. bifidum subsp. pennsylvanicum RCM cultures is shown in Table 3. When the undiluted total cell culture was tested in the PA ELISA, no reactivity was observed. However, 40-fold dilution of the samples gave a positive reaction (EI = 1.553). Part of this reactivity was associated with the cells (25.8%) and another part (37.8%) with secreted products. Blank media showed no reactivity. Decaylation of the same samples completely removed ELISA reactivity in contrast to decaylation of GM samples containing 1.5, 3, and 5 ng GM (purified GM was a kind gift from Marc Tabouret, Bio-Rad, Steenvoorde, France) per ml water, which showed a 50% decrease in reactivity upon this treatment (results not shown).

DISCUSSION

Most bifidobacteria showed significant reactivity with the PA ELISA in contrast to other gram-positive bacteria containing LTA or lipomannan or gram-negative bacteria containing LPS (Table 1; Fig. 3). ELISA reactivity was clearly shown to be associated with bacterial lipoglycans containing a β-1,5-galactofuranosyl chain. Only a few bifidobacterial lipoglycans have been structurally analyzed, but also other bifidobacterial species seem to contain reactive residues in their cell wall (7, 18, 30). This specific epitope can be detected as a surface antigen, shown by the reactivity of the suspended cell pellet, but also as an exoantigen, shown by the reactivity of the cell-free supernatant of a liquid culture (Table 3). Secretion of lipoglycans during normal growth was already shown by Op den Camp et al. with B. bifidum subsp. pennsylvanicum (33). The complete removal of PA ELISA reactivity after decaylation of the supernatant, compared to the 50% decrease with GM, suggests that only the acylated micellar form reacts in the PA ELISA.

When our results were extrapolated to the neonatal host, a calculation was made to estimate the PA ELISA reactivity of feces as potential source of cross-reactivity in the neonate. The bifidobacterial numbers found in feces (15, 29) are very high, and the EI per gram of feces is also high, ranging from 4,000 to 100,000. In order to confirm this calculation, FISH analysis was performed on neonatal fecal samples with a genus-specific probe for bifidobacteria. This 16S rRNA hybridization technique is a fast method to quantify bifidobacteria in the human gut (21). The presence of bifidobacteria was associated with PA ELISA reactivity of the fecal samples, but reactivity showed some variation, which probably depends on the bifidobacterial species present, as shown by the calculated examples. The results clearly show that the bifidobacterial community in the gut is a significant source of PA ELISA reactivity and that, considering the dilution in the blood volume, the serum concentration could become high enough after transmucosal passage to be detected with the PA ELISA. Furthermore, false-positive PA reactivity in fully breast-fed neonates could not come from galactomannan since breast milk reacts negatively in the PA ELISA (results not shown).

E. lenta is, like Bifidobacterium species, a gram-positive, obligatory anaerobic, non-spor-forming rod and has been found in adult feces at concentrations of 10^6 to 10^7 cells/g in 4 out of 12 subjects that were tested (41). Comparative studies of species-specific oligonucleotide probes might be helpful with future studies of E. lenta in the human intestine (19, 41).

Positivity of fecal samples has already been shown (1) but was always correlated with consumption of food containing fungal GM from contaminated sources (1, 23). In addition to the host's own microflora as a source of bifidobacteria, ELISA reactivity of certain food products is more likely to result from bifidobacterial lipoglycan than from fungal GM. Because of their health-promoting effects, bifidobacteria are widely used as probiotics and food additives and are present in fermented foods like milk, olives, sauerkraut, yogurt, butter, and cheese (3). Especially B. lactis and Bifidobacterium animalis are often used, and amounts of 10^7 to 10^9 of bifidobacteria per gram of food can be found (20, 40, 50). As can be seen in Fig. 3, B. lactis (recently reclassified as a subspecies of B. animalis [50]) and B. animalis show high reactivities and could act as a source of ELISA serum reactivity after consumption of food products. Furthermore, bifidobacteria are used as fecal indicator organisms and have been found on meat and meat products and in raw milk (2, 11). Especially meat products are often GM positive (23) but are more likely contaminated with bifidobacteria instead of fungal GM.

The frequent isolation of bifidobacteria from clinical infections in recent years has raised debate whether the bacteria are actually infective (3, 17, 36). However, even if there's a lack of pathogenicity in immunocompromised patients (3), bifidobacteria can invade the host by bacterial translocation (17). This phenomenon is caused by a diminished intestinal barrier, resulting in the passage of bacteria or bacterial components or products across the mucous membrane and epithelium. Not only intestinal mucosal injury by for instance cytotoxic chemotherapy or an immature intestinal mucosa but also immuno-
deficiency in the host, overgrowth of intestinal bacteria, and treatment with antibiotics and/or immunosuppressive agents have been shown to promote translocation of intestinal bacteria (i.e., bifidobacteria) (6, 17). The actual translocation of reactive components across the intestinal wall remains to be proven but seems even more likely for an exocellular lipoglycan molecule of 10 kDa. Penicillin treatment of B. bifidum subsp. pennsylvaniae resulted in an increase of lipoglycan excretion (33). This was not the result of bacteriolysis, as was also observed for acylated or deacylated LTAs of other bacteria and for other inhibitors of cell wall synthesis (33, 35, 43, 49). Consequently, antibiotic treatment of immunocompromised patients will increase the free lipoglycan pool in the intestine which may easily translocate to the blood.

In the blood, lipoglycan might bind to different components, resulting in a mixture of Galf-containing molecules that react in the PA ELISA, as is the case with GM (27). LTAs and lipoglycans spontaneously bind to mammalian cell membranes. In a study on B. bifidum subsp. pennsylvaniae it was shown that its lipoglycan bound reversibly to human colonocytes (8.3 × 10^6 binding sites/cell) and erythrocytes (2.1 × 10^6 binding sites/cell) (34). The lipid part of the molecule was shown to be responsible for binding. Furthermore, the lipoglycan of B. bifidum subsp. pennsylvaniae exhibited strong binding to the macrophage scavenger receptor, comparable to the LTA of Staphylococcus aureus (13). In order to analyze the nature and structure of the cross-reacting molecule(s) present in (false-) positive serum samples, a method is needed that specifically isolates reactive components because of their small amounts present. Several techniques (28) can then be used for further analysis to discriminate between false- and true-positive PA ELISA results and thereby prevent unnecessary preemptive treatment of patients.

The Bifidobacterium lipoglycan offers an explanation for the occurrence of a high rate of false PA ELISA reactivity among neonates and infants. Furthermore, together with E. lenta, these bacteria might also be the cause of false-positive reactivity in adult patients.

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FALSE REACTIVITY OF Platelia ASPERGILLUS ELISA


