Intestinal Microbiota in Exclusively Breast-Fed Infants with Blood-Streaked Stools

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ABSTRACT. Intestinal microbiota in exclusively breast-fed infants with blood-streaked stools and in healthy exclusively breast-fed babies was compared. Total anaerobes, bifidobacteria, lactobacilli, coliform bacteria, enterococci and clostridia were quantified by cultivation methods in feces of 17 full-term exclusively breast-fed patients (aged 16.3 ± 7.4 weeks) with blood-streaked stools and in the control group of 22 healthy full-term exclusively breast-fed infants (13.7 ± 6.4 weeks). Specific fluorescence in situ hybridization kits for \textit{Bifidobacterium spp.} were used for the quantitative detection of bifidobacteria in samples. Control samples had significantly \((p < 0.05)\) higher counts of total anaerobes. Bifidobacteria were not detected in patients’ samples in 65 % and in controls in 36 % \((p < 0.01)\). Bifidobacteria counts were also significantly higher in the control group \((p < 0.01)\). Furthermore, clostridia strains were detected only in feces from bifidobacteria-negative infants reaching counts >8 log CFU/g. Lactobacilli were not detected in 65 % patients and in 45 % control samples. However, this difference was not significant as well as the difference in lactobacilli counts. Eosinophilia was observed in 35 % of patients, low IgA concentration in 71 % and also low IgG concentration in 71 %. pANCA positivity was found in 53 % of patients. In conclusion a significant low proportion of bifidobacterial microbiota in patients with blood-streaked stools was shown in comparison with controls.

Abbreviations

\begin{tabular}{ll}
ANA & antinuclear antibody \\
ANCA & antineutrophil cytoplasmic antibodies \\
ASCA & antibodies to \textit{Saccharomyces cerevisiae} \\
C3, C4 & complement components \\
CFU & colony forming units \\
CRP & C-reactive protein \\
ENA & extractable nuclear antigens \\
FISH & fluorescence in situ hybridization \\
IgG, IgA, IgM, IgE & immunoglobulins G, A, M, E
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The most common cause of non-infective colitis in infants is eosinophilic proctocolitis (syn. allergic colitis, benign dietary protein proctitis, breast-milk induced proctocolitis). Other, not so common, noninfective colitides are nonspecific colitis, autoimmune enterocolitis, ulcerative colitis, combined immunodeficiency and Crohn’s disease.

Eosinophilic proctocolitis has been repeatedly observed in young babies having gross blood in stool without involvement of general health status and with a good prognosis. Formerly, the disorder was demonstrated in exclusively breast-fed babies (Lake 1982), later in children fed by cow’s milk formula or soy formula, but also in babies receiving hydrolysate formula (Machida \textit{et al.} 1994). Approximately 50 % of all babies are exclusively breast-fed (Lake 2003).

Eosinophilic proctocolitis begins in the first months of life with blood-streaked stools, which is always a stressful event for parents. Systemic clinical symptoms are absent and infants appear well in contrast with other types of colitis in which clinical features are present. Endoscopic examination reveals petechial hemorrhages, focal erosions and nodularity with striking eosinophilic infiltration especially in the lamina propria. Allergy to cow’s milk protein transferred to the infant \textit{via} the breast milk was believed to be the cause of inflammation. Protection against potentially harmful agents is ensured by a number of factors including intestinal microbiota, which can play an important role in mucosal physiology, barrier function, and mucosal and systemic immunological and inflammatory responses in young infants.

The aim of our work was to compare intestinal microbiota in exclusively breast-fed infants with gross blood in stools in comparison with healthy, also exclusively breast-fed babies.
MATERIALS AND METHODS

Patients. The criteria for including of patients were the following: infants had to be younger than 6 months, without any previous exposure to cow’s milk formula, soy formula, partially or extensively hydrolyzed formula or amino-acid-based formula. All patients had to be exclusively breast-fed at the moment of examination. Patients had no positive history of hemorrhagic disease. Neither mothers nor patients had been treated with antibiotics.

None of the patients had systemic features and no anal fissures were found. All infants with positive pathological or potentially pathological microbial or viral findings in their stools were excluded. In the group of patients were 17 exclusively breast-fed infants (11 girls and 6 boys) aged 16.3 ± 7.4 weeks. All patients were full-term with a birth mass of 3113 ± 389 g.

The control group consisted of 24 healthy exclusively breast-fed infants (9 girls and 15 boys) 13.7 ± 6.4 weeks of age. The difference in age of patients and control group was not significant. All babies were full-term with a birth mass of 3542 ± 488 g. The criteria for inclusion in the study were the same as in the group of patients. The microbial evaluation in the stools was the same in the patients and in the control infants. Rectosigmoidoscopy was performed with flexible endoscope.

Sample collection. Fecal and serum samples were obtained in all patients at the time of the first clinical examination at the gastroenterological unit. Fecal samples of control infants were collected by primary care pediatrician. Freshly collected fecal samples were transferred to the Wilkins–Chalgren broth (Oxoid, UK) and immediately transported to the microbiology laboratory.

Media and agar used. Wilkins–Chalgren broth was used for the dilution of fecal samples. Modified TPY (MTPY) agar, consisting of trypticase phytone yeast extract (TPY) agar (Sharlau, Spain) supplemented with mupirocin (100 mg/L) and glacial acetic acid (1 mL/L) was used for the isolation of bifidobacteria (Rada et al. 2000). Lactobacilli were enumerated using Rogosa agar (Oxoid). Wilkins–Chalgren agar (Oxoid) was used for the enumeration of total anaerobic bacteria. Reinforced clostridial agar (Oxoid) was used for the enumeration of clostridia. MacConkey agar and Slanetz–Bartley medium (both Oxoid) were used for the enumeration of coliform bacteria and enterococci, respectively.

Microbiological evaluation. Freshly collected fecal samples were transferred to the Wilkins–Chalgren broth and serially diluted in the same medium under anaerobic conditions. Appropriate dilutions (1 mL) were transferred to sterile 90-mm Petri dishes. The dishes were immediately filled with the media for bifidobacteria, clostridia, lactobacilli, and anaerobes. Bifidobacteria, clostridia, and anaerobic bacteria were incubated for 3 d in anaerobic jars (Anaerobic Plus System; Oxoid) at 37 °C. Lactobacilli were incubated for 2 d aerobically at 37 °C. Petri dishes with MacConkey agar and Slanetz–Bartley medium were inoculated with 0.1 mL of an appropriate dilution. Inoculated plates were incubated aerobically at 37 °C for 1 d (coliform bacteria) or 2 d (enterococci).

FISH kits for Bifidobacterium spp. by the fluid method (RiboTechnologies, The Netherlands) were used for the quantitative detection of bifidobacteria samples. FISH was quantified using epifluorescence microscopy (Eclipse E-800; Nikon, Japan).

Immunological analyses. We examined blood count and CRP. Commercially available kits were used for examination of IgG, IgA and IgM (Beckman Coulter, USA), IgE, specific IgE antibodies to cow’s milk, peanut and egg white (DPC, USA), ENA (Orgentec, Germany), C3 and C4 (Beckman Coulter, USA), ANA (Euroimmun, Germany), pANCA (Binding Site, UK), antibodies ASCA-IgG and ASCA-IgA (Binding Site, UK). For ethical reasons the blood laboratory examinations were not carried out in the control group.

Statistical analysis. Means and SD of bacterial counts and enzyme activity were calculated. One-sample Kolmogorov–Smirnov test of composite normality was used to confirm a normal distribution of data. The significance of differences between patients and control group was evaluated by the t-test. Nonparametrical test ($\chi^2$ test) was used for statistical evaluation in samples which had undetectable levels of clostridia, lactobacilli and bifidobacteria; value of $p < 0.05$ was considered as statistically significant.

Ethical considerations. The testing protocol was approved by the Committee on Ethics of 2nd Medical School of the Charles University of Prague. All parents signed an informed consent form.

RESULTS AND DISCUSSION

Clinical and immunological examinations. The clinical characteristics of patients with gross blood in their stools appeared well. Rectosigmoidoscopy was performed in 47 % (8/17) of infants with typical endoscopic and microscopic findings of eosinophilic infiltrations (Table I). ASCA as well as ANA, ENA, EMA,