

Effect of Oral Application of a Probiotic *E. coli* Strain on the Intestinal Microflora of Children of Allergic Mothers during the First Year of Life

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Received 6 March 2006

Revised version 17 August 2006

ABSTRACT. Influence of intestinal colonization by a probiotic *E. coli* strain on the incidence of bacterial pathogens in stool and allergic symptoms during the 1st year of life was monitored in 3 groups: colonized children of allergic mothers (AC; $n = 52$), noncolonized children of allergic mothers (AN; $n = 50$), children of nonallergic mothers (NC; $n = 42$). Colinfant vaccine was administered within 2 d after birth, 3× a week over a period of 4 weeks. Stool samples were examined after 2 d and at the age of 3, 6 and 12 months. At 3 months *E. coli* was present in 90 %, at 12 months in 73 % of AC. Pathogens were significantly less frequent on day 3 and at 3 months in AC vs. AN (15 vs. 61 %, $p < 0.001$; 15 vs. 38 %, $p < 0.01$) and vs. NC (15 vs. 63 %, $p < 0.001$; 15 vs. 53 %, $p < 0.001$). AC exhibited lower incidence of *Staphylococcus epidermidis* than AN on day 3 (6 vs. 31 %, $p < 0.001$) and of *Klebsiella* strains on day 3 and at 3 months (4 vs. 20 %, $p < 0.05$; 5 vs. 24 %, $p < 0.01$). AC showed a lower incidence of *Pseudomonas aeruginosa* than NC on day 3 (6 vs. 31 %, $p < 0.01$) and *Klebsiella* spp. on day 3 and at 3 months (4 vs. 22 %, $p < 0.05$; 5 vs. 45 %, $p < 0.001$). No significant differences were recorded after 6 and 12 months. The incidence of allergies was 3 % in AC, 26 % in AN ($p < 0.01$), and 10 % in NC.

Abbreviations

AC colonized children of allergic mothers
NC children of nonallergic mothers

AN noncolonized children of allergic mothers
TGF- β transforming growth factor β

Intestinal microflora is a complex ecosystem formed by a number of bacterial populations, which are crucially important for the host (Dai *et al.* 1999). Most of the bacteria (>400 species) colonizing the intestine are strict anaerobes (*Bacteroides* sp., bifidobacteria, eubacteria, propionibacteria, clostridia) while aerobes (enterobacteria, especially *E. coli*, enterococci) and facultative anaerobes form a minor part (2–5 %). The anaerobes-to-aerobes ratio is 1000 : 1. Many bacterial species have not been identified since classical cultivation techniques make it possible to identify only a limited number of bacteria. More than 99 % of cultivable bacteria are constituted by 30–40 species (Fanaro *et al.* 2003).

The postnatal development of neonatal intestinal flora is a gradual process that is affected by a number of external and internal factors (Hanson *et al.* 1997; Dai *et al.* 1999; Kirjavainen *et al.* 1999). Under normal conditions the fetal intestine is sterile, in contact only with amniotic fluid. Following rupture of fetal membranes during birth and after it, the newborn comes into contact with a number of antigens. During a normal birth it meets a vast number of bacteria, especially from the vaginal and perianal regions of the mother. Its gastric contents reflect the maternal cervical flora during the first 5–10 min after delivery. The microbes from the vaginal channel usually do not colonize the intestinal tract of the newborn in contrast to the mother's intestinal microflora (Fanaro *et al.* 2003; Orrhage *et al.* 1999). According to the classical description of colonization of breast-fed children the first to appear in the newborn's intestinal tract are aerobic microorganisms, *i.e.* enterobacteria and streptococci. Gradual consumption of oxygen brings about a lowering of the redox potential which, in turn, causes a prevalence of anaerobic flora. During the first week the intestinal flora is heterogeneous, bifidobacteria becoming then dominant in breast-fed and full-term infants. The intestinal flora of formula-fed children is more variegated (enterobacteria, enterococci, lactobacilli, clostridia, *Bacteroides* spp.), bifidobacteria being less prevalent than in breast-fed children. Environmental factors play the main role in children delivered by Cesarean section. The first to appear are facultative anaerobes, which

are dominant for the 1st 14 d while the incidence of anaerobes is lower than in children delivered spontaneously. Bifidobacteria are low in incidence especially in formula-fed infants (Sakata *et al.* 1985; Ducluzeau 1993; Dai *et al.* 1999; Kirjavainen *et al.* 1999; Fanaro *et al.* 2003).

The newborn's intestinal flora is affected also by hygienic conditions and administration of antibiotics during delivery. Geographic differences also exist in its composition between developed and developing countries (Hanson *et al.* 1997; Orrhage *et al.* 1999; Grönlund *et al.* 2000).

The role of optimum intestinal flora is still far from understood. One of its most important functions is maintenance of colonization resistance, prevention of excessive growth of potentially pathogenic microorganisms and anti-inflammatory modulation of the immune system (*see, e.g.*, Kokešová *et al.* 2006). Specific bacteria of the neonatal intestinal microflora affect the development of both local and systemic immunity. The microflora is also assumed to play an important role in the regulation of allergic inflammation. During the early postnatal period, the activity of T_H1 cells becomes prevalent, transforming growth factor β (TGF- β) suppresses T_H2 cells typical for the fetal period and oral tolerance is induced. Production of sIgA, an essential component of mucosal immunity, also increases. The "hygiene hypothesis" of allergy onset explains the atopic disease as being due to a lowered antigenic stimulation of persistence of the T_H2 immune response (Kalliomäki *et al.* 2001, 2003; Kirjavainen *et al.* 1999).

Important for preventing or reducing the symptoms of allergy is the recognition of changes in the composition of intestinal flora in the early postnatal period and/or its regulation by administration of probiotic bacteria. The bacteria most frequently employed for the purpose are strains of lactobacilli and bifidobacteria (Matsuzaki *et al.* 2000; Roberfroid 2000; Björkstén *et al.* 2001; Kirjavainen *et al.* 2002; Blum *et al.* 2003). We used oral administration of a probiotic strain of *E. coli* (Colinfant Newborn vaccine) (*cf.* Trebichavský and Šplíchal 2006).

PATIENTS AND METHODS

One-hundred-forty-four full-term newborns of allergic and nonallergic mothers without complications in the prenatal period were enrolled in this study. Their average birth mass was 3050 g, birth length 500 mm. Mothers with allergic history had to meet the following criteria:

- (a) clinical symptoms of allergy persisting for >2 years,
- (b) documented examination and follow-up by allergologist,
- (c) skin test and/or positive specific IgE,
- (d) positive response to antiallergic therapy.

Groups of infants:

- (1) colonized children of allergic mothers (AC; $n = 52$),
- (2) noncolonized children of allergic mothers (AN; $n = 50$),
- (3) children of nonallergic mothers (NC; $n = 42$).

Colinfant Newborn vaccine was administered within 2 d after birth and then 3 $\times$ a week over a period of 4 weeks. If antibiotics had to be administered, the vaccine was applied repeatedly. Colinfant Newborn, a live lyophilized vaccine, containing a minimum of 8×10^8 *Escherichia coli* serotype O83:K24:H31 cells in 1 mL, was patented in 1992 and registered by the *State Institute of Drug Control* (Prague) in 1997. Characteristics of this probiotic *E. coli* strain were described by Lodinová-Žádníková *et al.* (1991). Its properties, *i.e.* sensitivity to antibiotics and absence of plasmid, have not changed since 1997.

Stool samples were taken and examined on the 3rd–4th d of life and then at 3, 6 and 12 months. Microbiological examination included routine cultivation and detection of the vaccine strain by agglutination with specific rabbit antiserum. All children were immunized according to the scheme common in Czechia (Petráš *et al.* 1998).

Clinical follow-up: The children were regularly examined at 3, 6 and 12 months of life. Clinical symptoms of allergy were diagnosed in collaboration with the pediatric allergologist.

Differences in the incidence of pathogens and allergy between groups were compared using Fischer's exact test. Significance level, *i.e.* type I error rate adopted for all tests was $\alpha = 0.05$.

The study was approved by the *Scientific Council and Ethics Committee of the Ministry of Public Health of the Czech Republic*, and was carried out with a written informed consent of the parents.

RESULTS

AC children were colonized after birth with the probiotic strain of *E. coli* ($n = 52$) (Table I). On the 3rd d after birth the *E. coli* vaccine strain was present in 71 %, out of which in 19 % as a monoculture. In 8 % cases it appeared together with known pathogens (*P. aeruginosa*, *Klebsiella* spp., *S. epidermis*), in 44 %

Table I. Incidence of *E. coli* O83:K24:H31 in the stool of the group of colonized children during the follow-up period

Bacterial strain	3 days $n = 52$		3 months $n = 52$		6 months $n = 51$		1 year $n = 33$	
	%	n	%	n	%	n	%	n
Total O83 ^a	71	37	90	47	67	34	73	24
O83 ^b	19	10	21	11	14	7	15	5
O83 + pathogens ^c	8	4	13	7	10	5	18	6

^aPresence of *E. coli* O83:K24:H31 in samples of stool.

^bPure incidence of the *E. coli* O83:K24:H31 strain only (regarding our tested panel of bacterial strains).

^cSimultaneous incidence of *E. coli* O83:K24:H31 and tested pathogenic bacterial strains.

cases together with commensal intestinal flora. At 3 months, *E. coli* was found in the stool of 90 % children. The strain was detected in 21 % children as monoculture (regarding the tested panel of bacterial strains), in 13 % cases in a mixed culture with pathogens, and in 56 % cases together with commensal intestinal flora. At 6 months the strain was found in 67 %, at 12 months in 73 % cases. The *E. coli* strain was detected as a monoculture even in 15 % of 1-year-old children. Colonization was repeated in 3 cases after antibiotic therapy (1× at 3 months, 2× at 1 year).

Incidence of colonization by pathogenic intestinal microflora was significantly lower on day 3 and at 3 months in AC group as compared with both the noncolonized group (AC vs. AN, 15 % vs. 61 %, $p < 0.001$, and 15 % vs. 38 %, $p < 0.01$) and with the control NC group (AC vs. NC, 15 % vs. 63 %, $p < 0.001$, and 15 % vs. 53 %, $p < 0.001$). At 3 months, the incidence of pathogens in groups NC vs. AN was the highest in the stool of NC group (53 vs. 38 %) (Table II). Comparing AC group with AN group on day 3, the incidence of *Staphylococcus epidermis* was significantly lower in the former group (AC vs. AN, 6 vs. 31 %, $p < 0.001$). The same comparison performed on day 3 and at 3 months showed a significantly lower colonization with *Klebsiella* spp. (AC vs. AN, 4 vs. 20 %, $p < 0.05$ and 5 vs. 24 %, $p < 0.01$). AC group compared

Table II. Incidence of occurrence of pathogens in the stool during the follow-up expressed as counts and incidence rates (%)^a

Pathogen ^b	Group ^c	3 days			3 months			6 months			1 year		
		n	n_p	%	n	n_p	%	n	n_p	%	n	n_p	%
<i>P.a.</i>	NC	32	10	31**	38	4	11	41	6	15	33	5	15
	AN	50	7	13	50	6	12	49	7	14	34	7	21
	AC	52	3	6	52	6	11	51	3	6	33	4	13
<i>S.e.</i>	NC	32	6	19	38	2	5	41	1	2	33	0	0
	AN	50	16	31***	50	2	3	49	0	0	34	2	6
	AC	52	3	6	52	0	0	51	3	6	33	1	3
<i>K.s.</i>	NC	32	7	22*	38	17	45***	41	9	22	33	2	6
	AN	50	10	20*	50	12	24**	49	7	14	34	9	26
	AC	52	2	4	52	3	5	51	5	10	33	3	10
All	NC	32	20	63***	38	20	53***	41	15	37	33	7	21
	AN	50	31	61***	50	19	38**	49	14	29	34	16	47
	AC	52	8	15	52	8	15	51	11	22	33	9	28

^a n – total number of children in followed group, n_p – number of children with detected pathogenic bacterial strain; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

^b*P.a.* – *Pseudomonas aeruginosa*, *S.e.* – *Staphylococcus epidermidis*, *K.s.* – *Klebsiella* spp., All – all pathogens.

^cNC – nonallergic controls (children of nonallergic mothers)

AN – allergic noncolonized (children of allergic mothers)

AC – allergic colonized (colonized children of allergic mothers).

with NC group exhibited a lower incidence of *P. aeruginosa* on day 3 (6 vs. 31 %, $p < 0.01$) and also a significantly lower incidence of *Klebsiella* spp. on day 3 and at 3 months (4 vs. 22 %, $p < 0.05$ and 5 vs. 45 %, $p < 0.001$). No statistically significant differences in the incidence of pathogenic and potentially pathogenic microorganisms were recorded at 6 and 12 months. Nevertheless, colonization with pathogens during the 1st year of life was the highest in AN group (47 % cases).

No statistically significant differences between groups were in the length of breast feeding, number of infections or antibiotic therapy. The median breast feeding period in the NC, AN and AC groups was 34, 28 and 26 weeks. Numbers of infants breast-fed at different periods of follow-up stratified by group are shown in Table III. Percentages are calculated from the total number of subjects whose follow-up time extends beyond the lower limit of a given time period in each group. Therefore, they do not necessarily add up to 100.

Table III. Number (*n*) of infants breast-fed in the follow-up period (in weeks)

Group ^a	<6		6–12		12–26		26–52	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
NC	3	6.7	5	11.1	3	6.7	9	23.1
AN	4	6.8	6	10.3	15	25.9	18	40.9
AC	0	0	6	10.5	15	26.3	16	32.0

^aSee footnote of Table II.

Within the AC group only 1 child suffered from 3 months of age from atopic eczema confirmed by pediatric allergologist. AN group showed a significantly increased incidence of allergies during the 1st year. After 3 months, allergy appeared in 6 children (AC vs. AN, 2 vs. 12 %, $p < 0.05$). In 2 cases the diagnosis determined allergy to cow's milk proteins, 4 children had atopic eczema. At 6 months another 3 cases of atopic eczema and 1 case of food allergy appeared (AC vs. AN, $p < 0.01$). Examination at 1 year did not reveal any increase in the number of allergies although the difference remained statistically significant (AC vs. AN, 3 vs. 26 %, $p < 0.01$). Even the control group (NC) exhibited symptoms of allergy more often than did colonized infants (AC), the difference being not statistically significant (Table IV).

Table IV. Cumulative incidence of allergies during the follow-up expressed as incidence rates (%) and total number (*n*) of subjects at risk in each group^a

Group ^b	3 months		6 months		1 year	
	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>
NC	2	42	7	42	10	42
AN	12*	50	20**	50	26**	38
AC	2	52	2	51	3	33

^a* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

^bSee footnote of Table II.

DISCUSSION

The intestinal microflora plays a very important role in immunomodulation of the naïve immune system of the newborn since >70 % cells are located in the intestine (Grönlund *et al.* 2000; Rautava *et al.* 2002).

Probiotic *E. coli* strain administered orally after birth remained dominant in the intestine for several months. Prolonged presence of the strain in the intestine reduced the presence of bacterial pathogens in the intestine and other mucous body surfaces (Lodinová-Žádníková *et al.* 1997, 2004). According to our results, the probiotic strain of *E. coli* positively affects the composition of intestinal microflora and maturation of the newborn's immune system, and provides an efficient and long-term protection against the development of allergy (Matsuzaki *et al.* 2000; Lodinová-Žádníková *et al.* 2003). Up to 6 months of age, the lowest presence of pathogens and potential pathogens in the stool of AC was detected. After 1 year the number of pathogens was comparable with that found in NC group.

From 3 months allergy appeared in only 1 child from the AC group. Although the colonization of this child with the probiotic *E. coli* strain was repeated, the strain was not detected in regular microbiological examinations in the stool after 3 months.

The AN group, which exhibited an increased incidence of pathogenic and potentially pathogenic microorganisms, showed also increased incidences of allergy during the 1st year (10 cases). Although a similar microbiological finding was recorded in the stool of NC group, no significant rise in allergic symptoms was observed here (4 cases). Effect of genetic factors in the development of allergies in children of allergic mothers could be suggested.

Our findings cannot be explained by differences in nutrition of the infants or antibiotic therapy but, rather, by early colonization of children of allergic mothers with probiotic *E. coli* strain. For this reason, colonization of the intestine after birth in children of allergic mothers by oral Colinfant Newborn vaccine appears to be important for preventing allergy. (Matsuzaki *et al.* 2000; Edwards *et al.* 2002).

This work was supported by the *Ministry of Public Health of the Czech Republic* (Internal Grant Agency project 7525-3).

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