

Influence of the Intestinal Flora on the Development of Immune Reactions in Infants

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Breast-fed and artificially fed infants are in contact with the O antigen of *Escherichia coli* from the first days after birth. From the mother, the infant obtains antibodies against nonpathogenic *E. coli* strains in low titer, and the infant begins to form its own antibodies during the 2nd month of life. The transition is known to be continuous even though the transferred antibodies could not be differentiated from the infant's own antibodies. Contact with endotoxin caused sensitization which was detected by the skin test at about 2.5 months, and thereafter the skin test data correlated with the presence of serum antibodies against endotoxin. The newborn infant can be colonized with a different *E. coli* serotype; such an antigenic stimulus evokes the formation of antibodies sooner and at a significantly higher titer than (i) the level of maternal antibodies transferred or (ii) the infant's antibodies normally formed later on against other random *E. coli* serotypes.

During the first days after birth, the infant comes into contact with numerous microorganisms of the environment for the first time, and its immunological reactivity undergoes quantitative and qualitative changes. The complex of antigens of the intestinal microflora which colonizes the hitherto sterile tract of the newborn child represents a powerful stimulus to the maturation of the systems participating in immune reactions.

The problem of whether the bacteria colonizing the intestinal tract can evoke an immune response by production of a biologically reactive component (endotoxin) has been elucidated by the work of Sterzl et al. (8). They proved that germ-free precolostral piglets do not spontaneously form antibodies against gram-negative microbes. When the piglets after birth were colonized by *Escherichia coli* strains in the S form, it was possible to detect antibodies between the 5th and 10th day by means of the bactericidal test. The microbes passed through the intestinal wall and were found in the tissues.

The O antigen of the gram-negative microbes, endotoxin, represents a biologically highly active component among the complex antigens in the intestinal microflora. In recent years, it has been shown that sensitivity to infection is closely correlated with endotoxin sensitivity. The more sensitive the animal is to endotoxin, the higher its resistance to infection is, and vice versa. The reactivity to endotoxin changes with age, young animals being less sensitive than older ones (3, 4).

Stetson assumed that the changes in reactivity to endotoxin occur through natural sensitization of the host by antigens of the intestinal flora (9).

Hypersensitivity to endotoxin can be demonstrated by the skin test or by the presence of natural antibodies in serum (3).

The effect of the intestinal flora on resistance to gram-negative bacteria can be followed in the child, since the first colonization of its intestinal tract occurs in the first days of life (1).

Our work has been concerned with the first contact of the infant with the O antigen of gram-negative bacteria. The investigations were carried out by determining the course of colonization of the intestinal tract by *E. coli* in breast-fed and artificially fed infants, and by studying the transplacental transfer of antibody and the development of antibody against the host's own *E. coli* and pure endotoxin. Further investigations were made to determine, whether contact with the endotoxin in the microbes of the intestinal tract causes the development of sensitization, the age at which sensitization can be first detected, and the relationship between the presence of antibodies against endotoxin and the skin reactivity to endotoxin.

In another phase of this work, we sought to determine whether artificial colonization of the intestinal tract of the infant could be attained by a known nonpathogenic strain of *E. coli* with suitable antigenic properties, which does not occur in the mother, and whether this colonization

would persist in the intestine for several months. We also studied the problem of whether this strain of *E. coli* can evoke the formation of antibodies in the infant and whether these antibodies can be differentiated from maternal antibodies and from those formed by the child against the other *E. coli* types which colonize the intestinal tract.

MATERIALS AND METHODS

All infants under long-term investigation were kept in the department of healthy infants of this Institute during the whole period.

Samples of blood were withdrawn from each child at the age of 1, 2, 4, 8, and 12 weeks, and 4, 5, and 6 months. Blood samples were also taken from the children's mothers during the last months of pregnancy. Eleven of the children were breast-fed.

Feces were collected under sterile conditions from eight breast-fed and five artificially fed infants during the 1st, 2nd, 3rd, 5th, 7th, 9th, 11th, and 13th week, and the 4th and 5th months of life.

The colonization of the intestinal tract was determined quantitatively by the method used by Haenel (2).

The antibodies against *E. coli* were determined by hemagglutination as described by Neter (7) with the use of Takatsy's microtitrator.

The O antigen prepared from *E. coli* isolated from the mother and child was used without further typing. Endotoxin from a mixture of 10 *E. coli* strains prepared by phenol extraction was also used.

The development of hypersensitivity to endotoxin was investigated in another group of 39 children aged 1 to 9 months by means of the skin test. Endotoxin which had been adjusted *ad usum humanum* from the previously mentioned mixture was injected intracutaneously on the volar side of the arm. A dose of 0.1 μ g in 9.1 ml was used; a dose of 9.1 ml of physiological saline was injected into the other arm. After 6, 24, and 48 hr, the extent of erythema and infiltration was read. The test was considered positive when the area of a infiltration erythema was larger than 5 by 5 mm. In 20 children of this group, the antibodies against endotoxin were determined by the hemagglutination test.

In another group of five children, the intestinal tract was colonized by *E. coli* type O83. This type, which is nonpathogenic for newborn germ-free piglets, has advantageous antigenic properties and does not occur spontaneously in our environment. It consistently cross-reacts with types O22, O32, and O82. A suspension prepared from a 24-hr culture containing 5×10^8 organisms per milliliter was given per os to one child on the 9th day, to another child during the 3rd week, and to three children immediately after birth. The material was given for 3 successive days. A feces sample was obtained before colonization. Further samples were taken on 3 successive days after colonization and finally at intervals of 14 days for a maximum of 26 weeks. Principal *E. coli* types were observed in the samples of feces from mothers and children. Type O83 was detected by agglutination.

The antigens were prepared from the main isolated types. The predominance of type O83 was determined by selecting 10 colonies from each agar plate. Cells from these colonies were agglutinated exclusively by specific antiserum. The antiserum was produced with autoclaved antigen and reacted with native as well as with boiled antigen. Samples of venous blood were obtained from the mother, and cord blood and successive specimens were taken from the infants at weekly intervals early after colonization. Later blood samples were withdrawn at 2-week intervals for 24 to 26 weeks. The titers of the antibodies against the main *E. coli* types and against type O83 were determined by hemagglutination.

RESULTS

In 6 of 11 breast-fed children and in all of those fed artificially, colonization by *E. coli* was demonstrated in the 1st week of life. The number of microbes did not change with age, and no significant difference was observed between the breast-fed and artificially fed infants (Fig. 1).

Antibodies against their own *E. coli* strains were present in all mothers. The average titer was 1:128. Of newborn infants, 85% had antibodies in cord blood, but the average titer was only 1:16. In the first week of life, 71% of newborn infants had antibodies. The titers continued to increase with age. When the mother's and child's antigens prepared from their "own" *E. coli* strains were used (Fig. 2), it was not possible to differentiate the transferred antibodies from the infants own antibodies. This figure also shows that antibodies against the isolated endotoxin were present in most mothers (87%) in an average titer of 1:16. They were detected in the cord blood of 30% of the children. Actively formed antibodies could be detected occasionally after the 4th week; in 46% of the children, they occurred between 3 and 4 months.

In children under 2 months of age, the skin tests were positive in only 8 to 15% of cases. They were positive in 75 to 92% of children be-

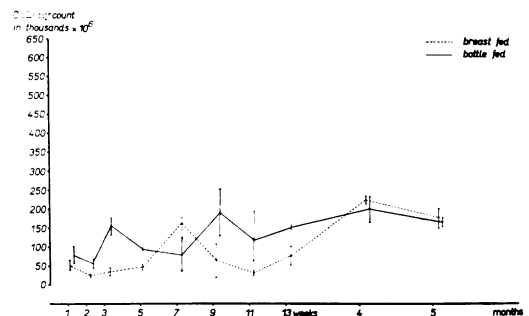


FIG. 1. Number of *Escherichia coli* after colonization of the intestinal tract.

tween 2.5 and 9 months of age. The positive skin test correlated directly with the presence of the serum antibodies against endotoxin (Table 1).

Type O83 *E. coli* could be detected in all children by agglutination from the 2nd day after feeding the bacterial suspension. It persisted in the intestine and predominated over the other types for the entire period of the investigation.

The antibody against *E. coli* type O83 appeared within 14 days after colonization, independently

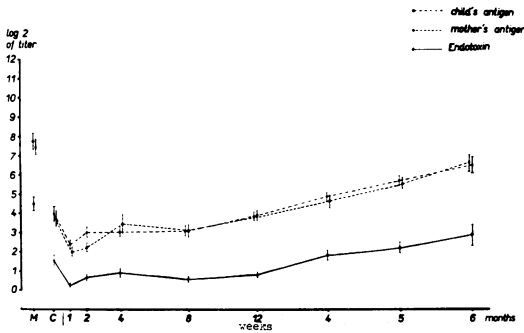


FIG. 2. Level of antibodies in mothers and their children against their own *Escherichia coli* types and endotoxin.

TABLE 1. Correlation between the presence of circulating antibody and dermal reaction to endotoxin

No. of subjects	Age	Percentage with positive skin tests		Presence of antibodies	
		After 6 hr	After 12 hr	No. of subjects tested	Per cent
13	months				
	1-2	8	15	9	0
26	1-9	92	74	11	91

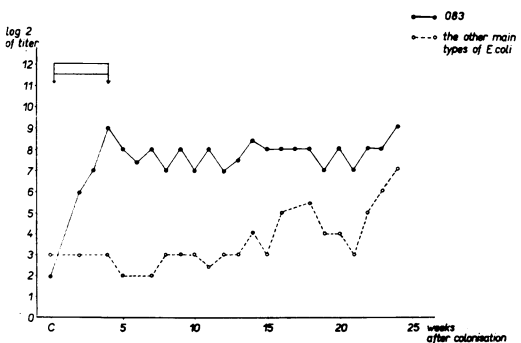


FIG. 3. Level of antibodies against *Escherichia coli* and the other main types (mean titers) in children after artificial colonization with *E. coli* O83

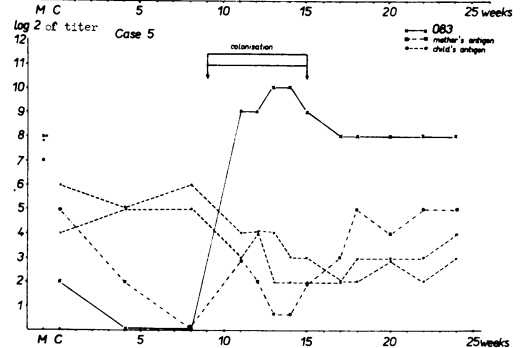
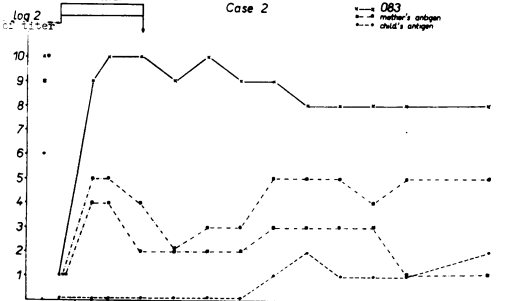
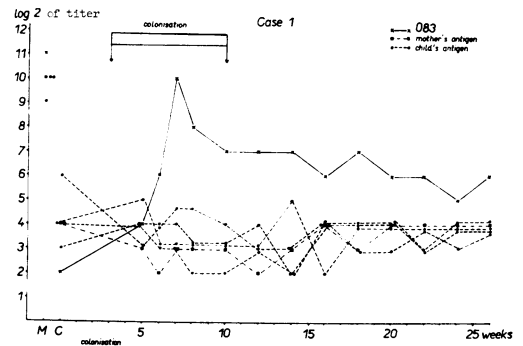


FIG. 4. Antibody levels against the main *Escherichia coli* types isolated from mothers and their infants and against the type *E. coli* O83.

of the age of the child. The mean titer for a group of five infants is shown in Fig. 3. This titer is significantly higher than that of the antibodies against the other main types isolated from mothers and infants. The higher titer persisted over the entire period of investigation. Typical individual cases are depicted in Fig. 4.

DISCUSSION

The findings reported here on the fecal flora, even in breast-fed infants, from the first days of life confirm that the infant comes into contact with endotoxin-containing gram-negative bacteria at a very early age.

By what mechanism the endotoxin is released from the microbes in the intestine and how it may

permeate parenterally is unknown. Miller (4) observed that endotoxin was present in individual organs after its introduction into the intestine of young animals. However, although increased resistance resulted, formation of antibodies did not ensue. Tlaskalova (*personal communication*) has recently detected endotoxin in the organs after monocontamination by whole microbes. Our experiments, using the skin test, are relevant to the general issue of whether hypersensitivity against endotoxin develops in children and whether it can in fact be demonstrated. Although the difference between the reactive capacity of the skin of small children and of adults certainly plays a role, the child at the age of about 2.5 months appears to be capable of manifesting a hypersensitivity reaction (as judged by the skin test) as early as 6 hr after administration of the test material. This positive skin test correlates directly with the presence of serum antibodies against the endotoxin. Nejedla (6), who applied intracutaneously a vaccine prepared from an *E. coli* culture, did not obtain any local reaction or increased serum antibody level. This may have occurred because, on administration of the vaccine, composed of whole microbes, phagocytosis took place before the antigen-antibody reaction could occur. The ability of a newborn infant, and of premature infants as well, to manifest the development of delayed hypersensitivity by a positive skin test has been demonstrated by Uhr (10).

In this study, antibodies against *E. coli* were observed in a larger number of children (85% in the cord blood, 71% in the 1st week) than reported by Neter and Stuhlberg (7) for pathogenic *E. coli* strains and by Nejedla (5) for nonpathogenic strains. However, the present method, in which the antigens were prepared from the host's own *E. coli* strains without further typing, did not permit differentiation of the antibodies transferred from the mother from those produced by the infant, as the antigen was actually a mixture of various cross-reacting *E. coli* types.

When pure endotoxin was used for the passive hemagglutination tests, antibodies were found to be present in the cord blood of only 35% of the children. During the first weeks of life, they could not be detected.

The group of children in which colonization with type O83 *E. coli* was carried out is rather small in number, and this work is being continued. Nevertheless, some of the present results appear to be significant. No clinical symptoms

have appeared in 10 children given the *E. coli* by mouth. Type O83 *E. coli* rapidly adapted to the intestine, even when the suspension was administered during the 9th week of life, a time when the child had been colonized by other *E. coli* types. Type O83 *E. coli* remained in the intestine during the entire period of investigation and continued to be the predominant type even when the suspension was not reintroduced again. Formation of antibodies could be detected 14 days after the first colonization when bacteria were introduced directly after birth. In this case, antibody formation started considerably earlier than is observed in natural colonization. These antibodies could be distinguished from maternal antibodies, since the titer was significantly higher and remained so even after the formation of antibodies against the other types had begun.

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