THE NEONATAL INTESTINAL MICROFLORA AND THE IMMUNE SYSTEM

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SUMMARY

Directly after birth, bacteria commence to colonise the skin and the mucous membranes of the respiratory, urogenital and intestinal tract. With time, more or less complex bacterial ecosystems are established at various body surfaces, of which the normal intestinal microflora is the largest and most diverse. Thus, an adult individual harbours more than 400 bacterial species at this site. Most of these bacteria are obligatory anaerobic.

The establishment of the intestinal microflora after birth precedes in a sequential manner, and a complete microflora is not obtained until after several years of age. A number of factors may influence the establishment of the microflora, including delivery and feeding mode, social contacts and the degree of environmental hygiene.

Many bacteria that inhabit the intestine, especially facultatively anaerobic bacteria, are potential pathogens, since they may spread to extra-intestinal sites. High population levels of these bacteria in the intestine in early life make the new-born infant vulnerable to infections, for instance septicaemia. On the other hand, these bacteria may be of great importance for the maturation of the developing immune system. Bacteria colonising the intestine activate both mucosal and systemic immunity, and may contribute to development of oral tolerance towards harmless antigens.

This paper describes the establishment of the intestinal microflora in early life, factors affecting the colonisation process, and influence of the intestinal microflora on the infant's immune system.

ESTABLISHMENT OF THE MAJOR BACTERIAL GROUPS IN THE INTESTINE

The new-born infant is exposed to a wide range of different bacteria, but not all are able to establish and colonise in the neonatal intestine. The implantation of various bacteria into the intestinal microflora is regulated through limitations in the intestinal milieu, which may change with the successive establishment of different bacteria. In summarising what is known of the pattern of colonisation in the neonatal period, it is important to realise that there are considerable variations between studies as to when different bacterial groups settle in the intestine. This likely reflects both differences in methodology as well as study populations.
Aerobic and facultatively anaerobic bacteria

The intestinal milieu is characterised by a positive oxidation-reduction potential during the first days of life (Grutte et al., 1965), which favours the growth of aerobic or facultative bacteria, such as E. coli and other enterobacteria, enterococci and staphylococci (Mitsuoka and Kaneuchi, 1977; Balmer and Wharton, 1989; Bennett et al., 1991). These bacteria often reach population levels of $10^{10}$ bacteria/g faeces in the new-born infant, which is roughly 100 times more than those found in the microflora of adults (Hoogkamp-Korstanje et al., 1979; Stark and Lee, 1982).

E. coli strains that colonise the neonate may derive from the mother's faecal flora, in which case they are often transferred during delivery. Such transfer was frequently observed by Bettelheim and co-workers in the seventies (Bettelheim et al., 1974), whereas most other studies from Western countries (Gotheffors et al., 1976; Fryklund et al., 1992), and even a study of home delivered infants in an urban slum area in Pakistan (Adlerberth et al., 1999a) report that only a minority of neonates acquire their dominant intestinal E. coli strains from their mothers. It is likely that transfer of bacteria from mother to infant during delivery is markedly reduced when mothers give birth laying on their back and faecal soiling of the infant is avoided, whereas a standing or kneeling delivery position, as practised by certain indigenous populations, almost invariably leads to faecal contamination of the infant and most likely to transfer of large amounts of maternal bacteria to the neonate (Mata and Urrutia, 1971).

In maternity and neonatal wards, E. coli strains may be spread between infants via the nurses' hands (Bettelheim and Lenox-King, 1976; Gotheffors et al., 1976). This type of transmission is greatly reduced when "rooming in" is Practised, i.e. when the mothers themselves and not the staff handle the babies (Bettelheim et al., 1983). Later on, E. coli may be acquired from other family members or other persons in contact with the neonate.

Other enterobacteria, such as Klebsiella, Enterobacter and Citrobacter, are isolated from 20-60% of neonates, and in colonised infants reach similar population numbers as E. coli (Lundequist et al., 1985; Balmer and Wharton, 1989). These enterobacteria are less common than E. coli in the flora of adult individuals, and the strains colonising neonates are therefore rarely of maternal origin. Instead they derive from the intestinal microflora of other neonates, transferred via the staff, or from environmental sources (Fryklund et al., 1992; Adlerberth et al., 1999a).

Enterococci (Enterococcus faecalis or E. faecium), are isolated from almost all neonates and commonly reach population levels of $10^{10}$ CFU/g faeces (Rotimi and Duerden, 1981; Stark and Lee, 1982). Some neonates may acquire these bacteria from their mothers, although this has never been particularly studied. In addition, enterococci are very good at spreading and surviving in the hospital milieu.

Staphylococci, e.g. Staphylococcus epidermidis or S. aureus, also establish in the intestine of many neonates during the first days of life and may reach population levels of $10^{10}$ bacteria/g faeces (Balmer and Wharton, 1989). Although commonly found in low numbers in the intestinal flora of adults, staphylococci are mostly regarded as members of the skin flora, which is probably the origin of many strains colonising the infant.

Aerobic streptococci are also found in the early neonatal microflora (Rotimi and Duerden, 1981), and strains of the Gram-negative genera Aeromonas, Pseudomonas and Acinetobacter may be transiently isolated from neonates dur-
ing the first week of life (Rotimi et al., 1985; Adlerberth et al., 1999a).

Anaerobic bacteria

The establishment and expansion of aerobic and facultatively anaerobic bacteria lower the redox-potential to negative values and "makes way" for obligatory anaerobic bacteria (Hoogkamp-Korstanje et al., 1979; Stark and Lee, 1982). Anaerobes recognised as early colonisers include Bacteroides, bifidobacteria and clostridia, which may reach populations of $10^{9-11}$/g faeces within a week after birth (Mata and Urrutia, 1971; Rotimi and Duerden, 1981; Stark and Lee, 1982).

Infants delivered by caesarean section, who do not come into contact with the maternal faecal, vaginal or perineal flora at delivery, show a delayed colonisation with anaerobic bacteria, especially Bacteroides (Neut et al., 1987; Grönlund et al., 1999). Bacteroides die rapidly in contact with environmental oxygen and, since this limits transfer in the hospital milieu, acquisition of Bacteroides from the mother is probably of great importance.

Bifidobacteria are considerably more aerotolerant than Bacteroides. They are probably spread between neonates in maternity wards, since bifidobacterial colonisation pattern is ward-dependent (Mitsuoka and Kaneuchi, 1977; Lundequist et al., 1985). However, transfer of bifidobacteria from mother to infant also occurs. Tannock and co-workers demonstrated bifidobacterial strains with the same ribotype in mother and infant in two out of five mother/infant pairs (Tannock et al., 1990).

Clostridia are commonly isolated from the intestinal flora of neonates. These bacteria form spores which are spread in the environment and which are resistant to hygienic measures. In accordance, clostridial species are usually the first anaerobes to colonise infants after sectio deliveries (Neut et al., 1987). Strains of e.g. C. difficile colonising neonates are almost never acquired from the mother, but are frequently spread between neonates in maternity and neonatal wards (Martirosian et al., 1995).

In many recent studies, lactobacilli have been detected in as many as 60-80% of one month old infants (Hall et al., 1990; Kleessen et al., 1995), whereas such colonisation was infrequently observed in earlier studies (Mata and Urrutia, 1971; Ellis-Pegler et al., 1975; Stark and Lee, 1982). It is likely that improved bacteriological methods have facilitated the identification of these bacteria, which could have been grouped with e.g. bifidobacteria in earlier studies. Although lactobacilli dominate the vaginal microflora of fertile women, these lactobacilli do not seem to colonise the intestine of the baby (Tannock et al., 1990). The mother's faecal flora is a more likely source of infants' intestinal lactobacilli, as the dominating species in adults' intestine, L. plantarum (Ahrné et al., 1998), dominates also in neonates (Bennet and Nord, 1987). However, lactobacilli are probably easily acquired also from other sources. Within one month, sectio-delivered infants carry lactobacilli at a rate similar to vaginally delivered infants (Hall et al., 1990; Grönlund et al., 1999).

It may take a long time before many other intestinal anaerobic bacteria, including Veillonella, Eubacterium, Peptostreptococcus, Peptococcus and Ruminococcus, establish in the intestine, although there are large variations between different studies (Mata and Urrutia, 1971; Ellis-Pegler et al., 1975; Mitsuoka and Kaneuchi, 1977; Rotimi and Duerden, 1981; Stark and Lee, 1982; Benno et al., 1984; Lundequist et al., 1985; Kleessen et al., 1995; Sepp et al., 1997).

Many of the strict anaerobic bacteria
colonising the intestine can not be cultured in vitro. Little is known about these bacteria and their time of establishment in the microflora. In mice, segmented filamentous bacteria, a group of non-culturable, strictly anaerobic, spore-forming, Gram-positive bacteria (Klaasen et al., 1992), colonise the intestinal tract and become the dominating microbes around the time of weaning (Garland et al., 1982). Related bacteria occur also in humans (Klaasen et al., 1992).

When the anaerobic bacterial populations expand in the intestine, facultative bacteria are suppressed and decline in numbers (Mata and Urrutia, 1971; Stark and Lee, 1982). Within a few weeks or months, reduced counts of e.g. Klebsiella, Enterobacter and staphylococci are observed (Ellis-Pegler et al., 1975; Rotimi and Duerden, 1981; Balmer and Wharton, 1989; Kleessen et al., 1995; Adlerberth et al., 1999a). Other facultative bacteria, such as E. coli and enterococci retain quite high population numbers for longer periods of time (Bennet, 1987). Thus, high levels of both facultatives and anaerobes may co-exist during the first months (Hoogkamp-Korstanje et al., 1979; Stark and Lee, 1982), or even years of life (Ellis-Pegler et al., 1975). This relates to the fact that a diversified anaerobic microflora is required to suppress e.g. the numbers of E. coli in the intestine. In mice mono-associated with E. coli, population levels of $10^{10-11}$ bacteria/g faeces are obtained. To bring down the E. coli population to the levels found in conventional animals, 95 different anaerobic strains isolated from conventional mice are required (Freter, 1992).

In humans, the successive establishment of different anaerobic species into the intestinal microflora proceeds over a period of several years (Ellis-Pegler et al., 1975; Midtvedt, 1994), finally resulting in a pronounced anaerobic predominance. The ratio of anaerobes to aerobes has been calculated to be 1.5 before 4 month's age, 10 between 4 and 12 month's age, 50 between 1 and 4 year's age and 200 in an adult (Ellis-Pegler et al., 1975).

THE INFLUENCE OF BREASTFEEDING ON THE INTESTINAL MICROFLORA

A great number of studies have investigated the intestinal microflora in breastfed and bottlefed infants. The results vary considerably between studies, but some differences between breastfed and bottlefed infants are quite consistently observed. Thus, most studies find lower counts of clostridia and enterococci in breastfed than in bottlefed infants (Stark and Lee, 1982; Benno et al., 1984; Lundequist et al., 1985; Balmer and Wharton, 1989; Kleessen et al., 1995), whereas breastfed infants tend to have higher counts of staphylococci than bottlefed infants, especially during the neonatal period (Lundequist et al., 1985; Balmer and Wharton, 1989). The reason may be that staphylococci colonising the nipple are swallowed during breastfeeding (Gothefors, 1975).

High bifidobacterial counts were a long time regarded as the most characteristic feature of the intestinal flora of the breastfed infant (Bullen et al., 1976). However, most studies from the eighties and onwards report similar counts of bifidobacteria in breastfed and bottlefed infants (Lundequist et al., 1985; Balmer and Wharton, 1989; Balmer et al., 1994; Kleessen et al., 1995). Nevertheless, more breastfed than bottlefed infants may harbour a flora dominated by bifidobacteria, most-
ly due to lower levels of other bacterial groups (Balmer and Wharton, 1989). Lactobacilli, which is another group of acid tolerant bacteria, are not favoured in the intestine of breastfed babies (Benno et al., 1984; Kleessen et al., 1995).

Only a minority of studies report higher counts of Bacteroides or enterobacteria in bottlefed than in breastfed infants (Bullen et al., 1976; Benno et al., 1984; Balmer and Wharton, 1989). However, the composition of the enterobacterial flora differs at the species and strain level between breastfed and bottlefed infants. Breastfed infants less often carry enterobacteria other than E. coli, for example Klebsiella or Enterobacter (Örskov and Biering-Sørensen, 1975; Bullen et al., 1976; Balmer and Wharton, 1989; Adlerberth et al., 1991). They also have a more stable enterobacterial flora than bottlefed infants, with fewer different E. coli serotypes being present concomitantly (Örskov and Biering-Sørensen, 1975; Mevissen-Verhage et al., 1985) and over time (Mevissen-Verhage et al., 1985). It seems reasonable to assume that differences at the species and strain level between breastfed and bottlefed infants may exist also for other major bacterial groups in the intestinal microflora.

A number of different factors in breastmilk has been suggested to contribute to differences in the bacterial flora between breastfed and bottlefed infants. These include the low buffering capacity of human milk (Bullen et al., 1976), and factors like secretory IgA, lactoferrin, lysozyme, complex oligosaccharides and nucleotides (Wold and Hanson, 1994). However, feeding with formulas supplemented with e.g. lactoferrin or nucleotides or formulas with reduced buffering capacity have not resulted in a "breastfed" type of microflora in the infants studied (Balmer et al., 1989a; 1989b; 1994).

GLOBAL DIFFERENCES IN INTESTINAL COLONISATION PATTERN - IMPACT OF HYGIENIC CONDITIONS

The intestinal colonisation pattern is strongly influenced by the degree of bacterial exposure and, thus, varies considerably between infants in developing and industrialised societies.

In general, infants born in poor areas in developing countries are earlier colonised with a number of different bacteria than infants in rich and highly developed societies. In indigenous Guatemalan infants, meconium passed at 4-7 hours after birth often contained bacteria, most commonly enterobacteria and streptococci (Mata and Urrutia, 1971). The mothers gave birth in a kneeling position, and maternal faeces commonly contaminated the infant during delivery (Mata and Urrutia, 1971), facilitating direct transfer of bacteria from mother to infant. However, under poor hygienic conditions, sectio-delivered neonates as well acquire bacteria almost immediately after birth (Rotimi et al., 1985; Adlerberth et al., 1991), reflecting heavy environmental exposure to bacteria. In Pakistan, infants from underprivileged groups, whether delivered in hospital or at home and regardless of delivery mode, acquire enterobacteria earlier and harbour a more diverse enterobacterial flora than Swedish infants (Adlerberth et al., 1991; 1999a). In an ongoing study, we have observed than at least 40% of Swedish neonates have not yet acquired any enterobacteria at one week of age, and that it takes a month before all infants harbour enterobacteria in their faecal flora (Adlerberth et al., 1999b). This shows the severely restricted circulation of enterobacteria in
a highly hygienic modern society. Pronounced environmental exposure to enterobacteria also leads to a high turnover of enterobacterial strains in the microflora. Thus, different *E. coli* strains replace each other in rapid succession in the intestinal flora of Pakistani infants (Adlerberth et al., 1999a) whereas in Swedish infants a single *E. coli* strain usually dominates the enterobacterial microflora for prolonged periods of time (Kuhn et al., 1986).

Colonisation with many other bacterial groups, e.g. enterococci, lactobacilli and eubacteria is similarly delayed in Western infants (Bennet et al., 1991; Sepp et al., 1997). The fact that more Swedish than Estonian one year old infants carry *C. difficile* (Sepp et al., 1997) indicate that Swedish infants have a poorly developed intestinal microflora by that age, as *C. difficile* is common in the intestinal microflora of young infants, but usually disappear when a complex anaerobic microflora is established.

In the absence of competition from e.g. enterobacteria, intestinal colonisation with "skin bacteria" like *S. epidermidis* have become more prominent among neonates in Western societies. Thus, early colonisation with *S. epidermidis* is more common in Sweden than in Ethiopia (Bennet et al., 1991). Similarly, in French neonates (Borderon et al., 1996), *S. epidermidis* rather than *E. coli* or enterococci are the first bacteria to colonise the intestine. Today, all Swedish infants seem to be colonised with staphylococci within three days after birth (Adlerberth et al., 1999b).

**BACTERIAL TRANSLOCATION**

Certain bacteria that colonise the intestinal tract have the capacity to translocate, i.e. pass viable over the intestinal barrier to reach mesenteric lymph nodes, blood or other organs (Berg, 1983b). Translocating bacteria include *E. coli* and other enterobacteria, staphylococci, enterococci and lactobacilli. Most obligate anaerobes seem unable to translocate - most likely they do not survive in the oxygen-rich milieu of viable tissues. Translocation may occur when bacteria with the capacity to translocate reach high population levels in the intestine, i.e. more than 10^8 bacteria/g faeces (Berg, 1995), as occur e.g. during treatment with antibiotics (Berg, 1983b). It is further promoted by deficiencies in the host immune system and damage to the intestinal mucosal barrier (Berg, 1995). Although mostly studied in experimental animals, bacterial translocation has been shown to occur also in humans (Brooks et al., 1993), and has been suggested to be the mechanism behind septicaemia due to enteric bacteria in immunocompromised patients (Tancrede, 1992). Furthermore, bacterial translocation may explain the propensity of enterobacteria to cause septicaemia in new-born, especially premature infants (Van Camp et al., 1994). As discussed above, facultative bacteria reach high population levels in the intestinal flora during the neonatal period which, in combination with immature immune functions, may predispose for bacterial translocation and subsequent septicaemia (Van Camp et al., 1994). A considerable frequency of asymptomatic bacteraemia, occurring at the time for intestinal colonisation, has been observed in neonates (Albers et al., 1966).

Neonatal septicaemia is especially common in developing countries (Dawodu and Alausa, 1980; Khan et al., 1993), where intestinal bacteria such as *E. coli*, *Klebsiella* and other enterobacteria, enterococci and *Pseudo-
monas are responsible for up to 80% of the cases (Dawodu and Alausa, 1980; Bhutta et al., 1991).

The mechanisms behind bacterial translocation are only partly understood. It is likely that bacteria are taken up via the Peyer’s patches and other lymphoid follicles lining the intestinal tract, in which case translocation may be regarded as a physiological process related to the sampling of luminal contents by the gut immune system, causing disease only if the host defence systems are overridden. In addition, enterocytes under certain conditions may permit passage of live bacteria.

THE NEONATAL IMMUNE SYSTEM

The new-born infant is more susceptible to infections than older children and adults because certain functions of the immune system are to a greater or lesser extent immature in the neonate.

Some aspects of innate immunity are not fully developed at birth. Concentrations of the complement factors C8 and C9 only reach 20 and 10%, respectively, of adult levels (Ballow et al., 1974), and the plasma levels of C1, C2, C3, C4, C6 and C7 are also low in the new-born infant (Fireman et al., 1965; Adinolfi and Beck, 1976). In accordance, the sera of neonates show decreased bactericidal activity (Lassiter et al., 1992).

The number of phagocytes in the blood are as high in infants as in adults, but the total pool of granulocytes that can be mobilised during infection is small. Neutrophils show reduced adherence and chemotaxis and lower enzymatic activity than in adults (Klein et al., 1977; Kovarik and Siegrist, 1998). Chemotactic activity of monocytes may be impaired, and there is a clearly diminished influx of monocytes into sites of inflammation (Wilson et al., 1996). Natural killer cells are present in normal numbers at birth, but show decreased cytotoxic activity compared with cells from adults (Wilson et al., 1996).

The specific immune system is fairly well developed at birth. However, although the neonate has the capacity to mount a specific immune response to most antigens, a primary immune response has to take place for each new antigen that is encountered. Thus, neonatal T cells are almost entirely of the naive phenotype (Lewis et al., 1991), which means that they are more difficult to activate than memory cells (Wilson et al., 1996). Like naive T cells from adults, neonatal T lymphocytes produce high levels of IL-2 when stimulated with mitogens, but very little IFN-γ and IL-4 (Bodeker et al., 1982; Lewis et al., 1991). They also show an inability to express the CD40 ligand following activation (Nonoyama et al., 1995), which limits their capacity to deliver help to B cells (Wilson et al., 1996).

The cytotoxic capacity of sensitised T cells, defined as the capacity to lyse allogeneic lymphocytes, is not fully developed in term neonates (Granberg and Hirvonen, 1980). At least partly, this could reflect the absence of memory CD8+ T cells in neonates, as such cells kill more efficiently than naive CD8+ T cells (McFarland et al., 1992; Wilson et al., 1996).

Neonatal B cells function is fully mature at birth with regard to IgM formation but not with regard to IgG or IgA formation (Andersson et al., 1981). Thus, B cell activation in vivo in response to antigens mainly results in IgM production (Gathings et al., 1977). This is most likely at least partly related to the inability of neonatal T cells to provide efficient help for B cell differentiation and isotype switch, as discussed above. Neonatal B cells respond poorly to bac-
terial polysaccharides, which are antigens not requiring contact-dependent T cell help (Wilson et al., 1996). However, the limited capacity of neonatal T cells to produce IFN-γ could also be of importance in this matter (Wilson et al., 1996), as IFN-γ enhances the B cell response to bacterial capsular polysaccharides (Peeters et al., 1992).

Another specific feature of the foetal and neonatal B-cell repertoire is the relative preponderance of B cells expressing CD5 (Bhat et al., 1992). B cells of the CD5+ or B1 phenotype represent a separate lineage, precursors for which are found only early during development (Herzenberg et al., 1986). They typically produce polyspecific antibodies, most often of the IgM isotype, which are often reactive with self-antigens as well as bacterial antigens (Casali and Notkins, 1989; Barbouche et al., 1992). These antibodies may be produced independently of exogenous antigen stimulation, and are thought to play a role in regulation and development of the immune system in early ontogeny and also to act as a first line of defence against invading micro-organisms, before specific immune responses have evolved (Casali and Notkins, 1989; Avrameas, 1991).

The serum levels of immunoglobulin, except IgG, are low in neonates (Allansmith et al., 1968). Due to the active transport of IgG antibodies over the placenta, IgG levels in cord blood exceed those found in maternal blood (Allansmith et al., 1968). IgG synthesised by the baby is present only in low amounts (Martensson and Fudenberg, 1965). Immunoglobulins of the IgA class may also be found in cord blood, in concentrations of less than 50 μg/ml (Allansmith et al., 1968; Wilson et al., 1996). IgM is present in sera of all healthy neonates, in concentrations of 110-150 μg/ml, which resemble approximately 10% of adult levels (Allansmith et al., 1968; Wilson et al., 1996). Much of the IgM antibodies present at birth are likely to be polyspecific antibodies, produced by CD5+ B cells in the absence of exogenous antigenic stimulation.

**THE MUCOSAL IMMUNE SYSTEM IN THE NEONATE**

The gut associated immune system contains the vast majority of all lymphoid cells in the human body. This includes lymphoid cells dispersed in the lamina propria, intraepithelial lymphocytes, and organised lymphoid tissue, such as Peyer's patches and colonic lymphoid follicles as well as the mesenteric lymph nodes that drain the intestinal tract. At birth, lymph nodes and Peyer's patches contain only primary follicles with mainly IgM+ and IgD+ cells, but very few IgA+ cells (Russell et al., 1990). The lamina propria contains very few immunoglobulin-containing cells (Perkkiö and Savilathi, 1980; Russell et al., 1990), which are mainly IgM+ and almost never IgA+ (Iwase et al., 1987; Russell et al., 1990). In accordance, no or only small amounts of secretory IgA are detected in foetal gut content or in meconium (Rule et al., 1971; Petit et al., 1973).

CD4+, CD3+ and CD8+ cells are readily identified in the lamina propria of both foetal and neonatal intestine. Many of the CD4+ cells have the morphological appearance of macrophages or dendritic cells (Russell et al., 1990). Very few intraepithelial lymphocytes are present at the time of birth, and no epithelial MHC class II expression is observed (Russell et al., 1990).
DEVELOPMENT OF THE IMMUNE SYSTEM AFTER BIRTH - RELATION TO INTESTINAL COLONISATION

Systemic immunity

In healthy neonates, there is a rapid increase in serum IgM during the very first weeks after birth (Allansmith et al., 1968). Thereafter the levels increase more slowly and 60-100% of adult levels are achieved at one year of age (Stiehm and Fudenberg, 1966; Allansmith et al., 1968). IgG production in the neonate is most likely initiated during the first weeks of life (Allansmith et al., 1968). By two months of age, the amount of circulating IgG synthesised by the infant equals the amount derived from transplacental transfer, and by one year of age, almost all circulating IgG is synthesised by the infant (Wilson et al., 1996). Sixty to 80% of adult IgG levels are reached by one year's age (Allansmith et al., 1968; Stiehm and Fudenberg, 1966) and adult levels by 7 years' age (Allansmith et al., 1968). Of the IgG subclasses, the production of IgG2 and IgG4 increase more slowly than that of IgG1 and IgG3 (Morell et al., 1972). The levels of IgA in serum also increase gradually after birth. At one year of age, serum levels of IgA are approximately 20-25% of adult levels, which are not achieved before 12 years of age (Stiehm and Fudenberg, 1966; Allansmith et al., 1968). The most pronounced increase in serum IgA levels is observed during the first 3 months after birth (Stiehm and Fudenberg, 1966).

The initiation of IgG and IgA production after birth is likely to be a response to bacterial colonisation of the gastrointestinal tract and other mucosae. The importance of the intestinal microflora as a stimulus for antibody production is illustrated by the fact that serum immunoglobulin levels in germfree animals are only 10 to 20% of those in conventional animals (Sell and Fahey, 1964; Kim et al., 1966; Wostmann et al., 1971). When germ-free animals are colonised by an intestinal flora, immunoglobulin concentrations in serum and secretions rise and antibodies appear towards the colonising micro-organisms (Carter and Pollard, 1971). In comparison with the strong stimulus afforded by the microflora, the diet contributes very little antigenic stimulation, despite the fact that bacterial components are present in standard animal feed (Midtvedt and Gustafsson, 1981). Thus, serum IgA and IgG are slightly increased in germfree mice fed a commercial diet compared with those fed an antigen-free diet, but much lower than in conventional mice (Hashimoto et al., 1978).

It is less clear how serum IgM levels depend on exogenous antigenic stimulation. A high proportion of the IgM in serum may represent "natural" antibodies, which may be formed as a consequence of endogenous antigenic stimulation by self antigens or antibodies of maternal origin (Berg, 1983a). Thus, germfree mice fed an antigen free diet possess normal levels of serum IgM (Hooijkaas et al., 1984). However, the very rapid increase in serum IgM observed in human infants during the first weeks of life is most likely a response to antigenic, probably microbial stimulation after birth (Allansmith et al., 1968).

Mucosal immunity

Secretory IgA responses develop earlier and independently of serum IgA antibody responses (South, 1971). Neonatal secretions contain no or only low levels of secretory IgA (Rule et al., 1971; Petit et al., 1973; Burgio et al., 1980; Gleeson et al., 1982; Mellander et al., 1984) but relatively more IgM than in older children (Gleeson et al., 1982; Mellander et al., 1984). This is due to the fact that IgM, in the absence of
dimeric IgA, can bind to secretory component and be transported out into mucosal secretions (Hanson et al., 1999).

During the first weeks or months of life, there is a marked increase in secretory IgA in saliva, where-after the levels may decrease slightly and then remain fairly constant for years (Gleeson et al., 1982). Adult levels of secretory IgA are reached at the age of 6-8 years (Burgio et al., 1980).

Immunohistochemical examinations of human postnatal intestine show the appearance of secondary lymphoid follicles, increasing numbers of IgA positive cells in the lamina propria, increasing numbers of intra-epithelial lymphocytes and the expression of MHC class II antigen on enterocytes (Perkkiö and Savilathi, 1980; Russell et al., 1990; Rognum et al., 1992; Machado et al., 1994). Expansion of mucosal dendritic cells also occurs postnatally (MacDonald, 1996). Levels of soluble IL-2 receptors, secreted by activated T cells and macrophages (Rubin and Nelson, 1990), rise within days after birth presumably reflecting mucosal immune responses (Spear et al., 1995).

The appearance of IgA positive cells in the lamina propria and the rapid increase in secretory IgA levels after birth is most likely primarily the result of colonisation of mucosal surfaces by commensal bacteria. Germfree animals have approximately one tenth as many IgA-producing cells in the intestinal lamina propria as conventional animals (Crabbé et al., 1968; Hashimoto et al., 1978). Koopman et al. (1982) demonstrated an increase in IgA-producing cells in the ileal mucosa and Peyer's patches after associating germfree mice with an intestinal microflora. In contrast, food antigens are very poor inducers of secretory IgA production (Wold et al., 1989).

Animal studies have shown that also the presence and activation state of intra-epithelial lymphocytes as well as the expression of MHC class II molecules on enterocytes are strongly dependent on the presence of a normal microflora (Umesaki et al., 1993; 1995). In response to luminal bacteria, intra-epithelial lymphocytes start to produce IFN-γ, which in turn upregulates the expression of MHC class II molecules on intestinal epithelial cells (Matsumoto et al., 1999).

In humans, 24-74% of intestinal bacteria are coated with IgA in vivo (van der Waaij et al., 1996). The capacity of a bacterial strain to induce strong immunity is linked to its ability to colonise and to invade the Peyer's patches (Hohmann et al., 1979). When a bacterial strain successfully colonises the intestine and reaches numbers high enough to permit translocation, a stimulation of the Peyer's patches resulting in the formation of germinal centres occurs. Activated B cells leave the patches and home to the lamina propria where they differentiate into IgA-producing plasma cells. The secretory IgA so produced coats the bacteria in the intestinal lumen, which most likely prevents further translocation. Thus, despite the continued presence of the microbe in the gut flora, there will be no, or only minimal, further stimulation of the immune system (Shroff et al., 1995). Therefore, it is likely that a persistent activation of the mucosal immune system depends on the continuous acquisition of new bacterial strains in the microflora.

Only a limited portion of the secretory IgA produced in response to a microbe colonising the intestine seem to be specific for this microbe (Cebra, 1999). A significant proportion of the secretory IgA produced may, in fact, represent polyreactive antibodies (Vassilev and Veleva, 1996; Quan et al., 1997). It is possible that a portion of intestinal lamina propria IgA plasma cells are derived from B1 cells residing in the peritoneal cavity (Kroese et al., 1995). In addition, it has been shown that IgA secret-
ing hybridoma clones obtained from mouse Peyer’s patches may also produce polyreactive antibodies (Shimoda et al., 1999). Polyreactive antibodies could possibly act as a first line of defense at mucosal surfaces, before the induction of specific immune responses (Quan et al., 1997).

The pattern of intestinal colonization may influence the "maturation" of the immune system

As mentioned above, it is likely that a persistent activation of the mucosal immune system requires a high turnover of bacterial strains in the intestinal microbiota. Pakistani infants, who are early colonized and constantly acquire new enterobacterial strains in their intestinal flora, have higher secretory IgA levels in saliva, and higher anti-"E. coli" antibody levels than Swedish infants of the same age (Mellander et al., 1985). Also, Nagao and co-workers (1993) found that children living in slum areas in Sao Paulo had higher levels of salivary IgA than Brazilian middle-class children, most likely reflecting differences in microbial exposure between the groups.

Breastfeeding, the intestinal microbiota and the immune system

Breastfed infants may experience less translocation, partly because they are colonised with fewer different strains, but mainly because secretory IgA, which is present in high concentrations in the milk, directly prevents translocation of gut bacteria (Maxson et al., 1995). A reduction of translocation may be a major reason for the strong protection against sepsis afforded by breastfeeding (Winberg and Wessner, 1971; Ashraf et al., 1991). Thus, it is likely that breastfeeding reduces the load of different microbial antigens reaching the intestinal immune system. Accordingly, immunocompetent mouse pups nursed by SCID/SCID mothers, and therefore are not supplemented with IgA via the milk, undergo an accelerated development of IgA responses (Kramer and Cebra, 1995; Cebra, 1999).

Many prospective studies show a more rapid and prominent increase of salivary IgA after birth in bottlefed than in breastfed infants (Stephens, 1986; Gleeson et al., 1986). Serum IgA responses may also be elevated in bottlefed neonates (Sarrinen et al., 1979), and Stephens et al. (1984) demonstrated significantly higher levels of IgM anti-"E. coli" antibodies in bottlefed compared to breastfed babies from the sixth day of life. The baseline activation of lymphocytes also seems to be higher in bottlefed than in breastfed infants, as reflected by a higher integrin expression and pronounced proliferative responses in the absence of antigen (Pabst et al., 1997). The antibody response to mucosal vaccines (e.g. live poliovirus and rotavirus) is often lower in breastfed than in bottlefed infants, which is generally attributed to an inhibition of virus replication in the gut or an enhanced clearance of virus from mucosal surfaces due to antiviral secretory IgA antibodies and other factors in breastmilk (Pichichero, 1990; Rennels, 1996).

However, breastmilk contains a host of factors that might modulate the developing immune system (Wold and Hanson, 1994). Large amounts of both inflammatory (IL-1, TNF-α) and anti-inflammatory (TGF-β, IL-10) cytokines are present in breastmilk (Wold and Hanson, 1994). Animal experiments show that cytokines survive and retain biologic activity during the passage through the gastrointestinal tract and that they may even be taken up into the circulation and thus affect immune functions (Rollwagen and Baqar, 1996). Breastmilk also contains large numbers of macrophages and activated T lymphocytes (Wold and Hanson, 1994) which could also influence the infant’s immune system. Indeed, radiolabelled
human breastmilk leukocytes fed to new-born baboons are taken up into the circulation (Jain et al., 1989). The transient tuberculin positivity observed in breastfed infants born to tuberculin-positive mothers provides indirect evidence that functionally active T cells are taken up from the maternal milk by the infant (Schlesinger and Covelli, 1977). Furthermore, a range of hormones, such as thyroxin, insulin and corticosteroids, and growth factors, e.g. epidermal growth factor, nerve growth factor and insulin-like growth factor, are present in human milk (Koldovsky and Thornburg, 1987). Human milk also contains considerable amounts of mononucleotides, which may augment proliferative responses by lymphocytes (Carver et al., 1990). Thus, although reducing antigenic stimulation, breastfeeding may have other stimulating effects on the neonatal immune system.

The intestinal microflora and oral tolerance

Food proteins and other harmless soluble antigens usually induce a state of specific unresponsiveness, termed oral tolerance, when presented to the immune system via the oral route (Telemo et al., 1997). To uphold a state of tolerance to these antigens is an important task of the immune system; in the absence of such mechanisms inflammatory and hypersensitivity reactions might occur.

The presence of a normal bacterial flora in the gut facilitates the induction of oral tolerance to food antigens (Moreau and Corthier, 1988; Sudo et al., 1997). Further, the administration of LPS together with food antigens increases the tolerising effect of feeding (Kim and Ohsawa, 1995). The mechanisms behind these interactions have not been defined but could possibly involve effects of the intestinal microflora on antigen presenting cells (Wold et al., 1998). Several factors secreted by macrophages in response to bacterial products have been shown to decrease the antigen presenting capacity of dendritic cells (Holt et al., 1993; Chouaib et al., 1985), which are the cells likely to present soluble protein antigens to T cells (Steinman, 1991; Wold et al., 1998). A downregulation of the antigen presenting capacity of dendritic cells could be crucial for the induction of oral tolerance to soluble protein antigens (Wold et al., 1998).

It is possible that the early establishment of high population levels of certain bacteria in the neonatal intestine provides stimulus for the developing immune system of importance for the induction of tolerance to e.g. food proteins and inhaled environmental antigens. Thus, in Western societies, where colonisation with certain bacterial groups is delayed and the intestinal microflora of infants seem to be less diverse than in developing countries, too little stimulation of the immune system could hamper tolerance induction (Sepp et al., 1997; Wold et al., 1998). The incidence of atopic allergy is steadily increasing in Western countries (Björkstén, 1994). An interesting speculation is that the high incidence of allergy relates to an inadequately developed intestinal microflora of infants in these societies (Sepp et al., 1997; Wold et al., 1998).

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