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19. DEFENCE MECHANISMS OF THE INNATE SYSTEM: INFLUENCE OF MICROBES

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ESTABLISHMENT OF THE INTESTINAL MICROBIOTA IN INFANCY

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SUMMARY

The large intestinal microbiota represents the most diverse and complex bacterial ecosystem of the human body, estimated to harbour more than 500 different, mostly anaerobic, bacterial species in an adult individual. Its establishment commences at birth, and proceeds in a sequential manner during the first years of life.

The first bacteria to colonise the neonatal intestine are aerobic or facultative anaerobic bacteria, which initially reach high population counts. These bacteria consume the oxygen lowering the redox-potential in the gut, making way for the anaerobes. Successively, a range of different anaerobic species colonises the gut. With the establishment of an increasingly complex anaerobic microbiota, the growth of the facultatives is suppressed, due to the accumulation of toxic metabolites, oxygen depletion and substrate competition.

A number of factors influence the establishment of the intestinal microbiota, including delivery and feeding modes, degree of social exposure and environmental bacterial content. Thus, differences in colonisation pattern are observed between vaginally and *sectio*-delivered infants, between breast- and bottle-fed infants, and between infants in industrialised and developing countries.

This chapter describes the establishment of the intestinal microbiota in human infants, and reviews factors affecting the colonisation process. We have recently characterised the intestinal colonisation pattern in a population of Swedish infants born in the late 1990s. Our findings indicate that the intestinal microbiota of Swedish infants might have changed in the last decades, probably due to an increasingly hygienic life-style.

THE SEQUENTIAL ESTABLISHMENT OF THE INTESTINAL MICROBIOTA

During or after birth the neonate first encounters the world of microbes, and bacteria commence to colonise the skin, and the respiratory, genital and intestinal tracts. This is the starting-point for the successive development of diversified bacterial ecosystems at these sites, of which the intestinal tract harbours the most complex microbiota.

The implantation of different bacteria into the microbiota does not occur at random. It is regulated through limitations in the intestinal milieu, and through bacterial interactions. Thus, the growth of some bacteria makes way for others, whereas yet other bacteria might instead be suppressed. During the neonatal period, however, it is quite easy for a wide range of bacteria to settle in the gut because of limited competition from a yet not fully developed microbiota.

The mother's vaginal, faecal and perineal microbial communities are common sources of bacterial strains colonising the neonate. Bacteria may also be acquired from any other person in contact with the baby, and from environmental sources (*Bettelheim* et al., 1974a,b,c; *Fryklund* et al., 1992; *Tannock* et al., 1990). The development of the intestinal microbiota may be influenced by any factor influencing the spectrum and amount of bacteria encountered by the infant, such as delivery mode, feeding mode, the number of social contacts and the degree of environmental hygiene (*Adlerberth* et al., 1998; *Bennet*, 1987; *Bennet* et al., 1991; *Stark* and *Lee*, 1982).

A number of studies over the last decades have investigated the intestinal colonisation pattern of young infants. Below, we summarise data from studies performed since the 1970s regarding the time of acquisition and origin of the major bacterial groups inhabiting the intestine.

Aerobic and facultative anaerobic bacteria

Facultative bacteria can perform both aerobic and anaerobic metabolism, although more energy is generated in the oxygen-dependent, aerobic metabolism. The first colonisers of the neonatal intestine belong to this group: *E. coli* and other enterobacteria, enterococci, streptococci and staphylococci (*Mata* and *Urrutia*, 1971; *Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982). The intestinal milieu is initially characterised by a positive oxidation-reduction potential (*Grutte* et al., 1965), which favours the metabolism and replication of these bacteria. In the absence of competition from anaerobes, the facultative bacteria commonly reach population levels exceeding 10^{10} colony forming units (CFU)/g faeces, which is roughly 100 times higher than their population levels in adults (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982).

E. coli strains that colonise the neonate may be acquired from the mother during delivery (*Bettelheim* and *Lennox-King*, 1976), but this usually occurs in less than one third of the infants in Western countries (*Fryklund* et al., 1992; *Gothefors* et al., 1976; *Murono* et al., 1993). This has been ascribed to the use of enemas, antiseptics and other measures to reduce bacterial exposure during delivery. However, even during home-deliveries in Pakistan, less than half of the infants acquired maternal *E. coli* strains (*Adlerberth* et al., 1998). Probably, giving birth lying on the back, and mechanical cleaning to avoid faecal contamination is sufficient to reduce exposure of the baby to maternal faecal bacteria.

In maternity wards, neonates may pick up *E. coli* strains spread between infants by the hospital staff (*Bettelheim* and *Lennox-King*, 1976; *Tullus* et al., 1988b). Such transfer is avoided if "rooming in" is practiced, i.e. when only the parents handle the baby (*Bettelheim* et al., 1983).

Other enterobacteria than *E. coli*, e.g. *Klebsiella* and *Enterobacter*, colonise many infants in the first weeks of life and often reach high population levels (*Balmer* and *Wharton*, 1989; *Bennet* and *Nord*, 1987a; *Gothefors* et al., 1976; *Lundequist* et al., 1985). These bacteria are less common than *E. coli* in the in-

testinal microbiota of adults (*Finegold* et al., 1983; *Tannock*, 1995), and the neonatal strains are rarely of maternal origin (*Adlerberth* et al., 1998; *Fryklund*, 1994; *Fryklund* et al., 1992; *Shinebaum* et al., 1979). These enterobacteria are frequently spread between neonates via the hospital staff (*Fryklund* et al., 1992; *Shinebaum* et al., 1979), and may also be acquired from feeds and other non-human sources (*Adlerberth* et al., 1998).

Enterococci are isolated from most neonates and commonly reach high population levels in the gut (*Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982; *Yoshioka* et al., 1983). Their route of transmission to the neonate has not been studied, but most likely both horizontal and vertical transfer occurs. Their natural niche is the intestinal microbiota of humans and animals, but they are also sturdy bacteria that resist various hygienic measures, which make them easily spread in e.g. the hospital milieu (*Kearns* et al., 1995).

Staphylococci, both *S. aureus* and coagulase-negative staphylococci, may colonise the neonatal intestine and reach high population levels in the first days of life (*Balmer* and *Wharton*, 1989; *Lindberg* et al., 2000). Staphylococci are found in the intestine of more than 50% of adults, but in much lower population counts than observed in neonates (*Finegold* et al., 1983). These bacteria are well recognised as members of the skin microbiota, and strains colonising the neonate usually derive from parental skin (*Lindberg* et al., 2004).

Streptococci are found less frequently than enterobacteria, enterococci or staphylococci (*George* et al., 1996; *Rotimi* and *Duerden*, 1981b). Other aerobic bacteria, such as the Gram-negatives *Aeromonas, Pseudomonas* and *Acinetobacter* may transiently colonise the neonate during the first weeks of life (*Adlerberth*, 1996; *Pazzaglia* et al., 1990; *Rotimi* and *Duerden*, 1981b).

Anaerobic bacteria

Obligate anaerobes have an anaerobic metabolism independent of oxygen, and are therefore not dependent on oxygen for growth. In addition, they are often extremely sensitive to the presence of oxygen, because they lack enzymes which detoxify atmospheric oxygen, which is a highly dangerous molecular species (*Gregory* and *Fridovich*, 1973). During the first days of life, the oxygen tension in the infantile gut is quite high, and obligate anaerobes cannot thrive. However, the aerobic and facultative anaerobic bacteria soon consume the oxygen and the redox-potential in the gut changes from positive to negative. This makes way for the anaerobes (*Hoogkamp-Korstanje* et al., 1979; *Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982), and bacteria of the genera *Bacteroides*, *Bifidobacterium* and *Clostridium* soon appear in the microbiota (*Balmer* and *Wharton*, 1989; *George* et al., 1996; *Mata* and *Urrutia*, 1971; *Roberts* et al., 1992; *Rotimi* and *Duerden*, 1981b; *Sepp* et al., 2000; *Stark* and *Lee*, 1982). In classical studies, these bacteria are isolated from a majority of infants in the first week of life and may reach population levels of $10^{9\text{-}11}$/g faeces (*Mata* and *Urrutia*, 1971; *Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982; *Yoshioka* et al., 1983).

Bifidobacteria are aerotolerant anaerobes, and isolates from infants often show some scanty growth under aerobic conditions (*Stark* and *Lee*, 1982). Thus, they may survive quit well outside the intestine, which facilitates their horizontal transfer. Infants' intestinal carriage of bifidobacteria varies between different maternity wards, indicating acquisition from non-maternal sources (*Bezirtzoglou* and *Romond*, 1990a; *Lundequist* et al., 1985; *Mitsuoka* and *Kaneuchi*, 1977). However, transfer of bifidobacteria from mother to infant during delivery also occurs (*Tannock* et al., 1990). As

bifidobacteria may also be part of the oral microbiota of healthy adults, transfer from this source is another possibility.

Bacteroides are strictly anaerobic bacteria that only survive for a short time in the presence of oxygen. Therefore, they require close contact for transfer, and if they are not acquired during delivery, their appearance in the microbiota may be significantly delayed (*Gronlund* et al., 1999; *Neut* et al., 1987). The *Bacteroides fragilis* group (e.g. *B. vulgatus* and *B. thetaiotamicron*) is most common in the intestinal microbiota of newborn infants as well as in adults (*Benno* et al., 1984; *Rotimi* and *Duerden*, 1981a). These bacteria are usually restricted to the intestinal tract and are not part of the normal microbiota at other sites, which may further hamper their spread between individuals.

Clostridia are anaerobes that form spores when the environmental conditions are unfavourable. Clostridia colonising neonates commonly belong to the species *C. perfringens* and *C. difficile*, which are considered quite pathogenic (*Benno* et al., 1984; *Bolton* et al., 1984; *Rotimi* and *Duerden*, 1981b; *Tullus* et al., 1989). *C. perfringens* is common in adults as well, while *C. difficile* is found in less than 4% of healthy adult individuals (*Finegold* et al., 1983; *Rolfe*, 1988). Disturbance of the intestinal microbiota, as in antibiotic treatment, may result in unrestricted expansion of *C. difficile* in the microbiota, leading to antibiotic-associated diarrhoea and, in worst case, pseudomembranous colitis (*Wilson*, 1993). However, for unknown reasons, young infants remain healthy despite high counts of these toxin-producing bacteria in the faeces (*Bolton* et al., 1984; *Tullus* et al., 1989). Colonisation by *C. difficile* usually declines after some months (*Tullus* et al., 1989), reflecting the establishment of a complex microbiota able to suppress their growth.

As clostridia form spores which resist most disinfectants and are ubiquitous in the hospital milieu and other environments (*Wilson*, 1993), clostridiae are easily spread to newborn infants from environmental sources (*El-Mohandes* et al., 1993; *Kato* et al., 1994; *Martirosian* et al., 1995; *Neut* et al., 1987).

The extent to which lactobacilli colonise the intestines of newborn infants is controversial. Most studies from Western countries report quite low *Lactobacillus* colonisation rates in infants (*Balmer* and *Wharton*, 1989; *Ellis-Pegler* et al., 1975; *Gronlund* et al., 1999; *Matsumiya* et al., 2002; *Stark* and *Lee*, 1982) but some find quite high colonisation rates (*Gil* et al., 1986; *Hall* et al., 1990; *Kleessen* et al., 1995). Variations in methodology may account for much of these differences, since lactobacilli are notoriously difficult to identify by traditional biochemical methods. We have recently performed a detailed longitudinal study on the *Lactobacillus* microbiota of more than 100 Swedish neonates, using PCR-based methods for typing of lactobacilli to the species and strain level (*Ahrne* et al., 2005). Lactobacilli were isolated from the stools of at most 45% of the infants at different time-point during the first 18 months of life, and persistent colonisation with a single strain occurred in less than one fifth of the infants. Interestingly, *Lactobacillus* isolation dropped significantly by twelve months, but increased again thereafter. Before one year of age, *L. rhamnosus*, *L. gasseri* and *L. paracasei* dominated, but the first two species disappeared after that age, being replaced by *L. plantarum, L. acidophilus* and *L. delbrueckii* (*Ahrne* et al., 2005).

Lactobacilli are the dominant bacterial group in the vaginal microbiota of healthy women (*Masfar* et al., 1986). Maternal vaginal lactobacilli may tran-

siently colonise the intestine of the baby, but they rarely persist in the microbiota (*Matsumiya* et al., 2002). Other sources of lactobacilli may be the maternal faecal or the parental oral microbiota. In Swedish adults, *L. plantarum, L. rhamnosus* and *L. paracasei* are the most common *Lactobacillus* species in both the oral and intestinal microbiota (*Ahrne* et al., 1998). Most lactobacilli are aerotolerant, and they are probably quite easily transferred between individuals.

Many other anaerobic bacteria form stable populations in the intestinal microbiota of adults, including bacteria of the genera *Veillonella*, *Eubacterium, Fusobacterium, Peptostreptococcus* and *Ruminococcus* (*Finegold* et al., 1983). Only few studies have investigated their establishment in the infantile intestinal microbiota. *Veillonella* are isolated from 10-90% of neonates (*Benno* et al., 1984; *Ellis-Pegler* et al., 1975; *George* et al., 1996; *Kleessen* et al., 1995; *Rotimi* and *Duerden*, 1981b; *Simhon* et al., 1982), and may reach hundredfold higher population levels in young infants than in adults (*Ellis-Pegler* et al., 1975; *Finegold* et al., 1983). Colonisation rates of *Eubacterium* in the microbiota vary between 0 and 40% during the first weeks of life (*Lundequist* et al., 1985; *Rotimi* and *Duerden*, 1981b; *Sepp* et al., 2000; *Stark* and *Lee*, 1982), and <50% are colonised at 9-12 months of age (*Mata* and *Urrutia*, 1971; *Sepp* et al., 1997; *Stark* and *Lee*, 1982). Peptostreptococci generally do not appear until solid foods are introduced (*Benno* et al., 1984; *Stark* and *Lee*, 1982), but are present in a majority of infants at 12 months of age (*Mata* and *Urrutia*, 1971; *Sepp* et al., 1997; *Stark* and *Lee*, 1982). *Ruminococcus* are only occasionally isolated during the first year of life (*Benno* et al., 1984; *Mata* and *Urrutia*, 1971). In one study including the identification of *Fusobacterium* species, less than 20% of infants were colonised by six months of age (*George* et al., 1996). The origin of these strictly anaerobic bacteria successively establishing in the infantile intestinal microbiota has never been studied.

REGULATION OF BACTERIAL POPULATION LEVELS AND COLONISATION RESISTANCE

The successive establishment of various anaerobic species results in a highly diverse microbiota at a few years of age (*Ellis-Pegler* et al., 1975; *Midtvedt*, 1994). During this process, some early anaerobes disappear or decline in numbers, like *C. difficile* and *Veillonella* (*Ellis-Pegler* et al., 1975; *Rolfe*, 1988, 1995). Furthermore, the facultative bacteria are suppressed by the expanding anaerobic populations (*Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982).

However, relatively high numbers of both facultative and anaerobic bacteria may be present during the first months (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982), or even years of life (*Ellis-Pegler* et al., 1975). The quite simple anaerobic microbiota of infants is not capable of suppressing facultative bacteria as effectively as the complex adult anaerobic microbiota, estimated to harbour several hundred different anaerobic species (*Finegold* et al., 1983; *Moore* and *Holdeman*, 1974). In a classical study by *Ellis-Pegler* and coworkers (1975), the mean ratio of anaerobic over facultative anaerobic and aerobic bacteria was 1.5:1 in infants before four months of age, 10:1 in 4-12 months old infants and 50:1 in children 1-4 years old, as compared to a ratio of 200:1 in adults.

The ability of the established microbiota to suppress the growth of the potentially pathogenic facultative bacteria or *C. difficile* is termed "colonisation resistance". Several mechanisms may be involved, including competition for nutrients and binding sites, and production of toxic metabolites (*Freter*, 1992; *Hentges*, 1983). Not only are many potentially pathogenic members in the microbiota kept at bay, but also the implantation of new bacterial strains into the ecosystem is strongly reduced. With increasing age, and with the establishment of an increasingly complex microbiota, it becomes more difficult for newcomers to colonise the gut (*Cooke* et al., 1971; *Jodal* et al., 1977; *Lari* et al., 1990; *Lodinová* et al., 1973).

BREAST-MILK AND THE INTESTINAL MICROBIOTA

Since the pioneering work by *Tissier* (1900) a number of studies have examined the influence of feeding patterns on the early intestinal microbiota. The results vary greatly between studies. Traditional studies reported a pronounced influence by feeding mode on the microbiota (*Bullen* et al., 1977, 1976; *Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982), but in many more recent studies from Western societies, and with the use of modern formulas, there seem to be less difference between the colonisation patterns of breast- and bottle-fed infants.

Mata and *Urrutia* (1971) examined the intestinal microbiota of breastfed indigenous Guatemalan neonates, and found frequent colonisation with and high numbers of *E. coli*, enterococci, clostridia and *Bacteroides* during the first days after birth. However, already by the end of the first week of life the microbiota was completely dominated by bifidobacteria and other bacterial groups were suppressed. *Bullen* and colleagues (1976) while investigating English neonates in the early 1970s, also demonstrated a clear dominance of bifidobacteria in breastfed neonates and low counts of other bacteria. In bottle-fed infants, the bifidobacterial counts were at least one log unit lower than in breastfed infants, and *Bacteroides*, clostridia, *E. coli* and enterococci outnumbered the bifidobacteria. The faecal pH is lower in breastfed than in bottle-fed infants, due to the low buffering capacity of human milk. This was assumed to promote the proliferation of acid tolerant bifidobacteria, whereas *E. coli* and other enterobacteria would be suppressed (*Bullen* and *Tearle*, 1976).

Thus, high bifidobacterial counts were long regarded as the most characteristic feature of the intestinal microbiota of the breastfed infant (*Bullen* et al., 1976, 1977; *Hewitt* and *Rigby*, 1976; *Willis* et al., 1973). However, many of the more recent studies report similar and sometimes very low counts of bifidobacteria in both breast- and bottle-fed infants (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Kleessen* et al., 1995; *Langhendries* et al., 1995; *Lundequist* et al., 1985; *Rubaltelli* et al., 1998; *Simhon* et al., 1982). There is also no correlation between faecal pH and bifidobacterial counts (*Balmer* and *Wharton*, 1989; *Simhon* et al., 1982; *Willis* et al., 1973).

Some, but far from all, studies show more frequent colonisation and higher counts of *Bacteroides* in bottle-fed than in breastfed infants (*Benno* et al., 1984; *Bullen* et al. 1976, 1977; *Long* and *Swenson*, 1977; *Yoshioka* et al., 1983). More persistent differences include lower counts of clostridia and enterococci in breastfed than bottle-fed infants (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Benno* et al., 1984;

Bullen et al., 1976; *Simhon* et al. 1982; *Stark* and *Lee*, 1982). More breastfed than bottle-fed infants may harbour a flora dominated by bifidobacteria, mostly due to lower levels of other bacterial groups (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Benno* et al., 1984; *Rubaltelli* et al., 1998).

Breastfed infants often have higher counts of staphylococci than bottle-fed infants, especially during the first weeks of life (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Lundequist* et al., 1985; *Simhon* et al., 1982). Probably, staphylococci from the nipple are swallowed during breast-feeding. Staphylococci are commonly isolated from the nipples of lactating mothers, and the same strain is often isolated from the infants' stools (*Lindberg* et al., 2004). However, in a recent study, we found no clear association between breast-feeding and *S. aureus* colonisation (*Lindberg* et al., in manuscript).

There is a tendency in many studies that breastfed infants have lower counts of enterobacteria than bottlefed infants, but, apart from the classical studies mentioned earlier (*Bullen* et al., 1976, 1977; *Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982), the difference is usually marginal (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Benno* et al., 1984). However, the enterobacterial flora differs at the species and strain level between breast- and bottlefed infants. Breastfed infants less frequently harbour enterobacteria other than *E. coli*, such as *Klebsiella* or *Enterobacter* spp. (*Adlerberth* et al., 1991; *Balmer* and *Wharton*, 1989; *Bullen* et al., 1976; *Tullus* et al., 1988a; *Yoshioka* et al., 1983; *Örskov* and *Biering-Sörensen*, 1975). They also carry fewer different *E. coli* strains than bottle-fed infants (*Mevissen-Verhage* et al., 1985b; *Örskov* and *Biering-Sörensen*, 1975).

It is commonly stated that breast-feeding would promote colonisation with lactobacilli. However, most studies do not find any differences regarding *Lactobacillus* colonisation between breast- and bottle-fed infants, and, when present, the differences indicate higher numbers of lactobacilli in bottle-fed infants (*Benno* et al., 1984; *Kleessen* et al., 1995). However, we recently reported that colonisation with *Lactobacillus rhamnosus* was significantly more common in Swedish infants at six months age if they still received breast-milk by that time than in completely weaned infants (*Ahrne* et al., 2005). Thus, certain species of lactobacilli may be favoured by breast-feeding.

A number of factors present in breast-milk have been suggested to influence the intestinal microbiota. These include e.g. secretory IgA, lactoferrin, lysozyme, complex oligosaccharides and nucleotides. However, attempts to add lactoferrin, nucleotides or complex oligosaccharides to infant formulas have not changed the intestinal microflora of the infants studied towards a more "breastfed" pattern (*Balmer* et al., 1989, 1994; *Euler* et al., 2005; *Gil* et al., 1986; *Roberts* et al., 1992). Using formulas of low iron supplement supports the establishment of a microbiota more close, but far from identical, to that of breast-fed infants (*Balmer* and *Wharton*, 1991; *Mevissen-Verhage* et al., 1985a). The complexity of factors in human milk is tremendous, and it is unlikely that artificial feedings will ever be able to completely mimic its composition or effects.

CAESAREAN SECTION, ANTIBIOTICS AND NEONATAL INTENSIVE CARE

The mode of delivery, i.e. vaginal birth or caesarean section profoundly influences the establishment of the intestinal microbiota. The *sectio*-delivered neonate is denied the natural exposure to maternal faecal and vaginal bacteria during delivery, and colonisation occurs exclusively from other sources. In Western countries, this results in a delayed acquisition of several of the common early colonisers, e.g. *E. coli, Bacteroides*, bifidobacteria (*Balmer* et al., 1989; *Bennet* and *Nord*, 1987b; *Gronlund* et al., 1999; *Hall* et al., 1990; *Neut* et al., 1987), and in some studies lactobacilli (*Hall* et al., 1990). It seems as if infants catch up with respect to colonisation with bifidobacteria and lactobacilli quite rapidly (*Bennet* and *Nord*, 1987b; *Hall* et al., 1990; *Neut* et al., 1987), whereas colonisation with *Bacteroides* may be delayed for many months (*Adlerberth* et al., 2005; *Gronlund* et al., 1999). The results regarding *E. coli* varies, but these bacteria may be quite rapidly acquired from environmental sources (*Balmer* et al., 1989; *Bezirtzoglou* and *Romond*, 1990b; *Lennox-King* et al., 1976).

Sectio-delivered infants commonly show an increased colonisation with enterobacteria other than *E. coli* (*Balmer* et al., 1989; *Bennet* et al., 1986; *Long* and *Swenson*, 1977). Many *sectio*-delivered infants are initially cared for at a neonatal ward, where spread of these bacteria may be common (*Fryklund* et al., 1992). In addition, delayed acquisition of anaerobic bacteria in these infants probably makes them more easily colonised with non-*E. coli* enterobacteria, which are less well apt than e.g. *E. coli* to establish in the presence of a complex anaerobic microbiota (*Bennet* et al., 1986).

Clostridia, especially *Clostridium perfringens*, are usually the first anaerobes to colonise infants after *sectio* deliveries (*Bezirtzoglou* et al., 1989; *Neut* et al., 1987). Spores of these bacteria are easily acquired from environmental sources. Furthermore, they do not seem to be dependent on a strictly reduced environment for proliferation, which facilitates their early establishment in the gut (*Bezirtzoglou* and *Romond*, 1991; *Bezirtzoglou* et al., 1989).

Not only *sectio*-delivered neonates, but also most infants cared for in neonatal wards and neonatal intensive care units (NICU's) acquire an intestinal flora which differs from that of healthy, vaginally delivered neonates. The enterobacteria isolated usually belong to the genera *Klebsiella* or *Enterobacter* (*Fryklund*, 1994; *Goldmann* et al., 1978; *Tullus* et al., 1988a), and colonisation with anaerobes is delayed (*Gewolb* et al., 1999; *Hall* et al., 1990; *Hallstrom* et al., 2004; *Sakata* et al., 1985).

Infants cared for in NICU's are often treated with antibiotics, and both oral and parental antibiotics markedly affect the intestinal microbiota of neonates (*Bennet* et al., 1986, 2002; *Bennet* and *Nord*, 1987b). Most anaerobic bacteria are profoundly suppressed, although clostridia may remain detectable in some cases (*Bennet* et al., 1986; *Bennet* and *Nord*, 1987b). *E. coli* decreases, whereas other enterobacteria may increase in numbers (*Bennet* et al., 1986; *Goldmann* et al., 1978). Some recent studies of neonates cared for at NICU's indicate a real paucity of bacterial species in their early intestinal microbiota (*El-Mohandes* et al., 1993; *Gewolb* et al., 1999; *Hallstrom* et al., 2004), most likely a result of heavy antibiotic use and strict hygiene. Coagulase-negative staphylococci are the bacteria most frequently isolated from these infants.

GLOBAL DIFFERENCES IN COLONISATION PATTERN

The importance of environmental exposure to bacteria as a determinant of infantile intestinal colonisation is shown by the fact that infants in Western societies are colonised later and have a less varied microbiota than infants in developing countries, and infants in the former socialist countries of Eastern Europe (*Adlerberth* et al., 1991, 1998; *Bennet* et al., 1991; *Rotimi* et al., 1985; *Sepp* et al., 1997, 2000). In Western industrialised societies, strict hygienic hospital standards and general household cleanliness is likely to reduce exposure not only to pathogens, but also to a number of commensal bacteria.

In indigenous Guatemalan infants, maternal faeces commonly contaminate the baby during delivery, and a wide range of intestinal bacteria is present in the baby's faeces during the first days of life (*Mata* and *Urrutia*, 1971). These bacteria initially reach high numbers, but already by the end of the first week they are completely outnumbered by bifidobacteria, and this bifidobacterial predominance persists during the period of breastfeeding (*Mata* and *Urrutia*, 1971). The components of this early microbiota are probably acquired during delivery, transferred directly from the mother. The pronounced effect of breastfeeding on the intestinal microbiota in this population, which is not observed in most modern studies from industrialised countries, could relate to the acquisition of a complex microbiota immediately after delivery, possibly containing bacteria facilitating the growth of bifidobacteria but suppressing other bacteria in the intestinal milieu created by breast milk.

In developing countries, not only vaginally delivered infants but also those delivered by caesarean section may acquire many bacteria very early (*Adlerberth* et al., 1991; *Rotimi* et al., 1985), reflecting pronounced exposure from environmental sources. Within three days after birth, all of both vaginally and *sectio*-delivered Nigerian neonates had acquired *E. coli*, and within 6 days most had *Bacteroides*, bifidobacteria and clostridia (*Rotimi* et al., 1985). Regardless of delivery mode, Pakistani infants harboured enterobacteria within three days after birth, often several species simultaneously, whereas less than 60% of Swedish neonates were colonised with enterobacteria by that age, and rarely with more than one species at a time (*Adlerberth* et al., 1991). During the first six months of life, many different *E. coli* strains occur in succession in the intestinal microbiota of Pakistani infants (*Adlerberth* et al., 1998), whereas among Swedish infants a single *E. coli* strain usually dominates for prolonged periods of time (*Kuhn* et al., 1986; *Nowrouzian* et al., 2003).

Colonisation with enterococci and lactobacilli also occurs earlier in Ethiopian than Swedish neonates (*Bennet* et al., 1991). One-year-old Estonian infants carry lactobacilli and *Eubacterium* spp. significantly more often than Swedish one-year-old infants (*Sepp* et al., 1997). Bifidobacteria may also be less common in infants in Western countries (*Simhon* et al., 1982).

In Western infants, intestinal colonisation with "skin bacteria" like staphylococci and ubiquitous environmental bacteria like *C. difficile* are more prominent, most likely due to reduced competition from more "professional" intestinal bacteria. Thus, *S. epidermidis* is more common in Swedish than Ethiopian neonates (*Bennet* et al., 1991), and one-year-old Swedish children are more often colonised with *C. difficile* than Estonian infants (*Sepp* et al., 1997).

A CHANGING COLONISATION PATTERN IN WESTERN SOCIETIES?

We have recently examined the establishment of the intestinal microbiota of Swedish infants born in the late 1990s. One hundred sixteen infants were followed with regular sampling of the intestinal microbiota and major groups of bacteria were quantitatively cultured. To our knowledge, this is the largest birth cohort ever studied with respect to the establishment of the commensal intestinal microbiota. Although differences in methodology preclude direct comparison with earlier studies, there are some indications that the infantile intestinal colonisation pattern might have changes in Sweden in the last decades.

A striking observation was the ubiquitous isolation of staphylococci from faecal samples during the entire first year of life. Coagulase-negative staphylococci were present in high population counts in the intestinal microbiota of all infants within some days after birth. Colonisation persisted throughout the first year of life in 80% of the infants, although the faecal population counts decreased quite significantly as an indication of the failure of these bacteria to withstand the competition from more "professional intestinal bacteria" (*Adlerberth* et al., 2005). *S. aureus* was also quite common in the infants' microbiota, being isolated from the stools of three out of four infants in the first year of life (*Lindberg* et al., 2000). With the exception of some Japanese studies performed in the 1980s, where staphylococci were isolated from almost all neonates but in quite low counts (*Benno* et al., 1984; *Yoshioka* et al., 1983), studies from industrialised countries in the 1970s and 1980s report much colonisation frequencies for staphylococci (*Bullen* et al., 1976; *McAllister* et al., 1974; *Neut* et al., 1987; *Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982). In studies of Swedish infants in the 1980s, coagulase-negative staphylococci were isolated from 30-70 % in the first week of life (*Bennet* et al., 1986, 1991; *Lundequist* et al., 1985). Thus, staphylococci might have increased in prevalence and numbers in the early intestinal microbiota over the last decades in Sweden. Accordingly, an increase in neonatal staphylococcal colonisation between 1975 and 1995 has been described in France (*Borderon* et al., 1996).

We suggest that an important cause for the expansion of these traditional skin bacteria in the infantile gut microbiota is a lack of competition from "professional" gut bacteria. We found that less than 60% of vaginally delivered infants were colonised with *E. coli* by one month of age (*Adlerberth* et al., 2005; *Nowrouzian* et al., 2003), which is a reduction compared with results from Sweden in the 1970s and 1980s (*Bennet* et al., 1986; *Gothefors* et al., 1976; *Kuhn* et al., 1986; *Lundequist* et al., 1985; *Tullus*, 1988). Short hospital stays, rooming-in and strict hygiene may have reduced exposure of infants to *E. coli* in the hospital. Furthermore, spread of *E. coli* must also be very limited in families and homes, as *sectio*-delivered infants in our study showed delayed acquisition by *E. coli* up to six months of age (*Adlerberth* et al., 2005). Our results suggest that hygienic measures both in hospitals and general life have reduced the circulation of some typical faecal bacteria like *E. coli* in Sweden in the last decades.

In contrast to *E. coli,* the isolation rate of enterococci was as high in the present study as previously reported (*Lundequist* et al., 1985), and *sectio*-de-

livered infants were colonised as early as infants delivered vaginally (*Adlerberth* et al., 2005). As mentioned earlier, enterococci are sturdy bacteria which resist various hygienic measures, and during the last decades they have emerged as important nosocomial pathogens. This indicates that bacteria differ widely in their ability to resist an increasingly hygienic life-style.

Bacteroides colonisation was infrequent compared to Swedish studies performed in the 1980s (*Bennet* and *Nord*, 1987b; *Lundequist* et al., 1985). Thus, only around one third of the vaginally delivered infants harboured *Bacteroides* at one week of age, and this frequency did not increase during the first two months of life (*Adlerberth* et al., 2005). Furthermore, at two months of age, only one of seventeen *sectio*-delivered infants had acquired *Bacteroides*, and not even at one year of age had they caught up with vaginally delivered infants with respect to colonisation by *Bacteroides*. This further indicates a limited circulation of faecal bacteria in the modern Swedish society.

Another finding points towards a poorly developed intestinal microbiota still at one year of age in today's Swedish infants. We observed a persistently increasing *C. difficile* isolation rate throughout the first year of life, and more than 50% of the vaginally delivered infants harboured *C. difficile* by twelve months of age (*Adlerberth* et al., 2005). The incidence was even higher in *sectio*-delivered infants. In a Swedish study performed in the 1980s, colonisation by *C. difficile* peaked at a frequency of 30% around six months of age, and less than 10% of the infants were colonised at twelve months (*Tullus* et al., 1989). As described earlier, *C. difficile* is suppressed by the complex intestinal microbiota that establish with age. The persistent increase of this species in the microflora over the first year of life suggests that there is limited competition from the microbiota.

CONSEQUENCES OF A CHANGED INTESTINAL COLONISATION PATTERN IN EARLY LIFE?

The changes in the early intestinal microbiota indicated by our recent study points towards an increased ratio of Gram-positive to Gram-negative bacteria in the microbiota of young Swedish infants. Colonisation with the Gram-negative enterobacteria (*E. coli, Klebsiella* etc.) and *Bacteroides* seem to have decreased in the last decades, while colonisation frequencies for Gram-positives such as enterococci, bifidobacteria and clostridia remain high and staphylococcal colonisation seem to have increased. As the intestinal microflora is a common source of bacteria causing extra-intestinal infections, it is interesting to note that staphylococci are the most common cause of neonatal septicaemia today (*Kallman* et al., 1997). Furthermore, the intestinal microflora is the major drive for the gut immune system (*Cebra*, 1999). Since Gram-positive and Gram-negative bacteria induce different patterns of immuno-regulatory and inflammatory mediators when they interact with the innate immune system (*Hessle* et al., 2000, 2003, 2005), there is reason to believe that changes in the intestinal colonisation pattern connected to an increasingly hygienic life-style may have global effects on the function of the developing infantile immune system.

NON-CULTURE DEPENDENT METHODS FOR STUDIES OF THE INFANTILE INTESTINAL MICROBIOTA

The data reviewed in this paper on the ecology and diversity of the infantile gut microbiota is based exclusively on studies using traditional culture techniques. Clearly, culture techniques have limitations, since many intestinal bacteria are uncultivable, and they are time-consuming and expensive. However, several alternative approaches for studies of the intestinal microbiota are available today, which may be regarded as useful complements to traditional culturing, and most likely with time will replace culture dependent methods.

The development of the intestinal ecosystem can be followed by assessing biochemical reactions performed by the intestinal bacterial population. Anaerobic bacteria in the human colon produce short chain fatty acids (SCFAs), such as acetate, propionate and butyrate, and the variety of SCFAs increases as a more complex microflora is established (*Midtvedt*, 1994; *Midtvedt* et al., 1988). Other functional activities of intestinal bacteria include, for example, the conversion of cholesterol to coprostanol, transformation of bilirubin to urobilins, degradation of mucins, and inactivation of pancreatic trypsin. By assessing various biochemical parameters in faeces information is achieved on whether bacterial groups responsible for certain key metabolic reactions have established or not. In Swedish children, some biochemical functions characterising a highly complex microbiota are not established yet at five years of age (*Midtvedt*, 1994; *Norin* et al., 1985).

Another approach to study the intestinal microbiota is to perform gas-liquid chromatography of bacterial cellular fatty acids in faecal samples (*Eerola* and *Lehtonen*, 1988; *Vaahtovuo* et al., 2001). Each bacterial species has a typical cellular fatty acid composition, and the cellular fatty-acid profile of a faecal sample thus consists of cellular fatty acids of all bacteria present in that sample. This method may be used to assess changes in the microbiota over time, and differences between individuals or groups of individuals, but cannot directly show which bacteria account for the differences observed. As a technical procedure, GLC is inexpensive and rapid. The method was recently used to indicate differences in intestinal microbiota between vaginally and *sectio*-delivered neonates, and changes in the microbiota occurring with the development of symptoms of necrotising enterocolitis in premature neonates (*Hallstrom* et al., 2004).

With the development of molecular genetic techniques, a variety of methods have recently been established for studies of the intestinal microbiota, most of which are based on the detection of bacterial 16S rRNA genes. So far, however, only a limited number of studies on the infantile intestinal microbiota have been performed with molecular techniques.

Fluorescent in situ hybridisation (FISH) probes targeting 16S rDNA of specific bacterial groups has been used to study the intestinal microbiota of infants. Whole bacterial cells are permeabilised to allow the probes to reach their target. If the 16S rRNA contains a sequence complementary to the fluorescently labelled probe, a hybrid is formed, causing the whole cell to fluoresce, and the fluorescing bacteria can be visualised in the microscope. Although only bacterial groups recognised by the probes currently available can be detected, the number of probes is rapidly increasing (*Blaut* et al., 2002). Using this method, *Harmsen* and co-authors (2000) demonstrated that bifidobacteria dominated the microbiota in breastfed but also in most bottle-fed infants, and

that *E. coli* and *Bacteroides* seemed to constitute a relatively larger proportion of the microbiota in bottle-fed infants. Thus, this study largely confirmed previous results obtained with conventional culture techniques. Another study by the same group demonstrated that formula-fed neonates had higher numbers of bacteria belonging to the *Coriobacterium* group, which includes e.g. *Eggerthella lenta* (formerly *Eubacterium lentum*) and *Collinsella aerofaciens* (formerly *Eubacterium aerofaciens*). This agrees with a previous study by *Benno* et al. (1984) showing higher counts of *Eubacterium* in bottle-fed than in breastfed neonates.

PCR and denaturing gradient gel electrophoresis (DGGE) combined with sequencing of the major ribotypes was used in one study to analyse the development of the intestinal microbiota of two healthy babies. This method involves the extraction of bacterial DNA followed by PCR amplification of fragments corresponding to region V6 to V8 of the 16S rRNA gene, using universal bacterial primers. Subsequent separation of the PCR products in DGGE results in a fingerprint of the microbiota where each band represents a specific bacterium. The identities of the bands are determined by cloning and sequencing of the amplified PCR products. This method permits the identification of hitherto unrecognised bacteria, but only dominant groups of intestinal bacteria are detected. The authors confirmed the early appearance of bifidobacteria in the infantile intestinal microbiota, but *Ruminococcus* was also identified as an early coloniser in these infants (*Favier* et al., 2002). Many of the cloned rDNA sequences exhibited less than 97% identity with sequences of known bacteria, indicating the presence of bacteria not previously identified in the infantile intestinal microbiota.

In a study by *Wang* and co-workers (2004), the faecal microflora of two Swedish infants was monitored over time by terminal restriction fragment length polymorphism analysis (T-RFLP) of amplified 16S rRNA genes from faecal samples. Bacterial DNA from faecal samples was isolated and 16S rRNA genes were amplified in PCR using fluorescently labelled primers. The PCR products were digested with restriction enzymes, and the fluorescently labelled terminal restriction fragments were separated and detected. 16S rDNA clone libraries were constructed from the same faecal samples, and the T-RFLP patterns of the clones were compared with those of the corresponding faecal samples. In this way, dominant bacterial groups present in the samples were identified. The bacterial groups detected most frequently in the early samples were *Enterobacteriaceae, Veillonella, Enterococcus, Staphylococcus* and *Bacteroides*. Bacteria of the genera *Bifidobacterium, Clostridium, Ruminococcus* and *Eubacterium* were identified in both infants, the three latter genera mainly after the first months of life. *Enterobacteriaceae* and *Bacteroides* predominated in both infants during breast-feeding (*Wang* et al., 2004).

As the two infants analyzed above participated in our longitudinal study of the establishment of the intestinal microflora in Swedish infants, we could compare the results using T-RFLP with the results of traditional culture (*Lindberg*, 2004). We found that culture, by use of selective media, was superior to T-RFLP for detection of sub-dominant groups, including facultative bacteria such as *E. coli*, *S. aureus*, as well as anaerobes of low population numbers, mainly lactobacilli. Bacteria that instead were commonly missed by culture were mainly strictly anaerobic bacteria, e.g. those belonging to the genera *Eubacterium*, *Veillonella*, *Ruminococcus* and *Fusobacterium*, for which no selective media

were used. In addition, a wider spectrum of *Bacteroides* species was detected with T-RFLP than with culture.

So far, the results obtained when using molecular methods for analyses of the infantile intestinal microbiota have mostly confirmed what has been found using conventional culture techniques. Although the relative proportion of some bacterial groups in the early microbiota seem to be underestimated by culture (*Harmsen* et al., 2000), a high proportion of the early intestinal colonisers seem to be detectable with traditional culture methods. However, with age the complexity of the microbiota increases, and in samples from older infants it is likely that culturing only detects a fraction of all different bacterial species present. Further development and application of molecular methods in studies of the establishment of the intestinal microbiota will greatly increase our knowledge on intestinal ecology in early life.

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MATERNAL AND NUTRITIONAL FACTORS SUPPORTING HOST DEFENCE IN THE INFANT

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SUMMARY

The neonate receives support for its host defence as transplacental IgG antibodies. These protect especially in blood and tissues, by activation of complement and neutrophils, both of which are of reduced capacity in the neonate. The inflammation still induced via these mechanisms causes pain, fever, reduced appetite increased energy consumption etc. This may impair growth and development in the young infant.

Breastfeeding instead provides protection via numerous mechanisms which act without inducing inflammation, or like lactoferrin, which also actively hinders development of inflammation. Lactoferrin and lactoferrin fragments are resorbed and excreted in urine, suppressing experimental urinary tract infections The secretory IgA antibodies, like many milk oligosaccharides, prevent infections by blocking microorganisms from attaching to mucosal tissues. The anti-secretory factor, which may be induced in milk by certain foods and by bacterial enterotoxin exposure, can prevent mastitis in mothers and acute diarrhoea in children.

Lactoferrin, as well as α-lactalbumin reorganised after exposure to low pH, seem to be able to kill tumour cells. The biological relevance of this is not yet known.

Human milk carries numerous signals from mother to infant, like cytokines, chemokines, hormones etc. which may help explain why the enhanced protection in the breastfed against several infections like otitis media, respiratory tract infections, septicaemia, diarrhoea etc. in some cases seem to last for some years. Such signals may help explaining the stimulation of certain vaccine responses seen in breastfed babies.

Studies in pregnant and lactating rats suggest that polyunsaturated acids with high n-6/n-3 ratio may impair development of neonatal oral tolerance and mediate untoward effects of immunoactive leptin. Intrauterine growth retardation is a severe condition of unknown origin. Deficient content of immunosuppressive IL-10 in decidua suggest inade-

quate suppression of the potentially harmful maternal anti-foetal immune response. Fatty acids are very important for the normal formation of the placenta.

PRO-INFLAMMATORY IgG ANTIBODIES VIA PLACENTA

The effective transfer of IgG antibodies from the maternal circulation to that of the infant via the Fcn receptor, or Brambell receptor after its discoverer, provides the newborn with serum IgG antibodies at somewhat similar concentrations as in the mother's serum. They have a slightly longer half-life in the infant and remain in trace amounts during the second half of the first year, explaining why measles vaccination has to be delayed till the second year of life not to be inhibited by the remaining maternal IgG anti-measles antibodies. On the other hand anti-idiotypic antibodies are also included among the transferred IgG, and they can function as immunogens inducing the corresponding idiotype (*Hanson* et al., 2003).

IgG antibodies can bind and neutralise toxins and eventually viruses to some extent on mucosal membranes, but in tissues they need to activate phagocytes like neutrophils via the complement system to support efficient host defence. The problem is that the complement system is not fully functional until about 3 months of age (*Berger*, 1996). In addition the neutrophils are fewer in the bone marrow in early life and they respond poorly to chemotaxis; they aggregate less well and are less efficiently activated than such cells in the adult (*Schelonka*, 1998; *Uguz* et al., 2002). Macrophages/monocytes and lymphocytes in the neonate often show somewhat unbalanced cytokine responsiveness, bringing a risk for more inflammation-induced tissue damage and less efficient protection (*Schultz* et al., 2004). Furthermore, the inflammation resulting from this form of defence in the young infant causes listlessness, fever, pain, tiredness due to the pro-inflammatory cytokines produced, which also increase leptin levels inducing a loss of appetite (*Hanson*, 2004). These symptoms are untoward in the young infant who needs all energy available for normal growth and development. The consequences are most evidently seen among infants in poor countries, where frequent infections forcefully add to the undernutrition, increasing the risk of mortality (*Pelletier*, 1994). These facts brought together suggest that the young infant may not be optimally protected by the maternal IgG antibodies via the placenta.

ANTI-INFLAMMATORY PROTECTION VIA MOTHER'S MILK

The secretory IgA (SIgA) antibodies

SIgA antibodies are produced in the mammary glands by plasma cells, which via the "enteromammaric link" have migrated there from the gut mucosa (*Roux* et al., 1977). As a consequence they will primarily be directed against the microbes of the mother's intestinal flora, which the baby normally is colonised with at delivery since it takes place close to the mother's anus. The main function of the milk SIgA antibodies on the breastfed infant's mucosal membranes in the upper respiratory tract and gastrointestinal tract is to prevent microbes from attaching to and penetrating the mucosal epithelium where they normally would meet the inflammatory response of the innate and specific immunity, which as mentioned is still subop-

timal during the first few months of life. This seems to be the background to the fact that milk SIgA antibodies have a significant role in protecting the young infant against infections and by preventing the induction of symptom-inducing and energy-consuming IgG- and T cell-mediated tissue defence (*Hanson*, 2004). The SIgA antibodies have also been shown via their carbohydrate side chains to promote the growth of type 1-piliated *Escherichia coli* which are of low virulence, and also to promote the formation of a biofilm on an epithelial surface, possibly enhancing normal microbial colonisation in the gut (*Nowrouzian*, 2004; *Bollinger*, 2003).

Lactoferrin (LF)

LF is one of the major milk proteins and is bacteriostatic and for certain bacteria also bactericidal. This bactericidal effect is primarily due to a surface-exposed cationic region located in the N-terminal end of the molecule. Certain peptides from this region are strongly bactericidal (*Haversen*, 2004). LF, as well as LF peptides, is taken up by the gut mucosa bringing LF and LF peptides into the circulation and also into the urine. In an experimental model we could show that via this mechanism orally given LF and certain LF peptides protected against experimental urinary tract infections in mice (*Haversen*, et al. 2000).

LF is also anti-inflammatory, presumably because it interferes with the signalling pathway of NF-κB, the transcription factor inducing production of the pro-inflammatory cytokines IL-1ß, IL-6 and TNF-α (*Haversen* et al., 2002). The likely clinical significance of the anti-inflammatory capacity of LF and its peptides was illustrated in a model of experimental dextran sulphate-induced colitis in mice. It was found that LF and certain LF peptides decreased the appearance of blood in the stools, protected the morphology of the crypts and decreased the number of $CD4^+$ cells and infiltrating F4/80 positive macrophages, as well as TNF-α and IL-10 producing cells (*Haversen* et al., 2003).

LF is a protein with multiple functions, which may support the breastfed infant in many ways. Our ongoing studies showing that LF has some capacities in similarity to heat shock proteins (HSPs) (*Moisei*, 2004), such as binding ATP and having ATPase activity, reveal also that LF binds the protein-kinase 2 (CK2) enzyme which is strongly involved in phosphorylation processes of proteins (*Olsten* and *Litchfield*, 2004) and in signal transduction (*Pawson* and *Nash*, 2000). The findings that LF binds and is phosphorylated by CK2 (*Hatomi* et al., 2000) may provide clues to elucidate the effects of LF in cells and their nuclei [when it enters there (*Haversen* et al., 2002)] and also add a new perspective to the well known properties of the secreted LF.

A possible role in primary defence against tumourigenesis has also been suggested for LF (*Campbell* et al., 1992; *Shau* et al., 1992; *Bezault* et al., 1994; *Yoo* et al., 1997). Thus it has been shown that lactoferrin suppresses the growth of tumour cells *in vitro* and strongly inhibits experimental metastases in mice (*Shau* et al., 1992; *Yoo* et al., 1997). The reported anti-proliferative effects of LF on breast carcinoma cells and colon epithelial cells occur via modulations of the key protein that regulates the G1 to S transition of cell cycle (*Damiens* et al., 1999). Though it seems that LF could act both by enhancing NK activity and directly on tumour cells, the mechanism responsible for its anti-tumour activity is still not well understood.

Quite recently *Roseanu* et al. (2003) found that LF is cytotoxic to murine melanoma B16-F1 cells by affecting the cell viability and morphology (Figures 1A, B, and C). A cytotoxic and apoptotic effect of human milk lactoferrin in L929

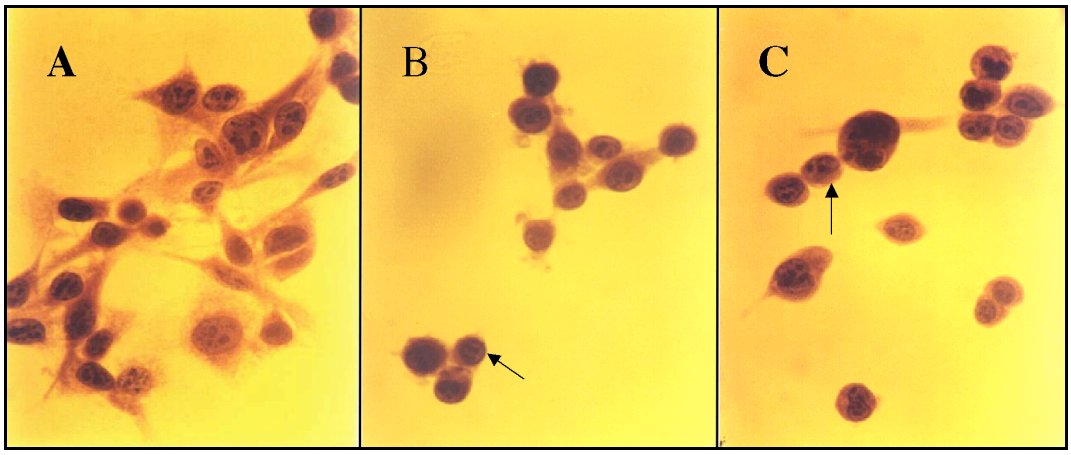

Figure 1: **A)** B16-F1 cells – control 24 h. **B)** Small groups of atypical round cells (arrow) with a rim of cytoplasm. (500 μg/ml of LF, 24 h.). **C)** Groups of hyperchromatic round cells (arow) with pyknotic nuclei (500 μg/ml of LF, 24 h.). Magnification 800x.

(mouse fibroblasts) and HL-60 (human promyelocytes) has also been reported by *Kanyskova* et al. (2003).

Our present studies show that LF binds to B16-F1 cells and is taken up by these cells (Figure 2). Moreover, preliminary experiments suggest that LF has thecapacity to modulate the expression of phosphorylated forms of p38, JNK and ERK 1/2 kinases. These proteins belonging to the MAPKinases family are known to be involved in apoptosis.

Further studies are under way to elucidate the mechanism underlying the action of LF on B16-F1 cells.

α-Lactalbumin killing tumours

α-Lactalbumin is a major milk protein which appears in large complexes which have the capacity to cause apoptosis in transformed lymphoid and embryonic cells, but have no effect on mature epithelial cells (*Hakansson* et al., 1995). At the low pH in the stomach α-lactalbumin aggregates, unfolds and forms a receptor site for oleic acid. This

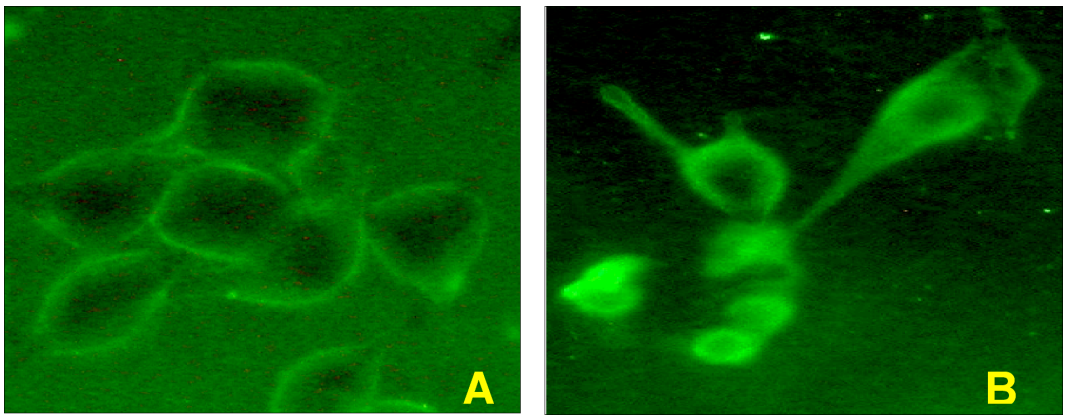

Figure 2: A) Binding of LF to B 16-F1 cells. **B)** Internalisation of LF into B 16-F1 cells.

compound, arising from the degradation of milk triglycerides, binds to the complex of the reorganised α-lactalbumin, which in this appearance has been found to kill many various forms of cancer cells, but not normal differentiated cells. This form of the molecule, called HAMLET, from **h**uman **α**-lactalbumin **m**ade **le**thal to **t**umour cells, has significant activity against certain human tumours (*Gustafsson* et al., 2004). It is presently not clear whether or not the α-lactalbumin via mother's milk can provide any anti-tumour defence in the offspring, although some reports, but not others, suggest that breastfeeding may reduce the risk of childhood leukaemia (*Hanson*, 2004). The significantly reduced risk of breast cancer mediated by breastfeeding might possibly be related to effects of the milk α-lactalbumin, possibly aided by LF as mentioned above.

Protective effects of the anti-secretory factor (AF)

AF can be induced and made to appear in human milk if the mother encounters in her milieu microbes like *Escherichia coli* or *Vibrio cholerae*, which produce enterotoxins. As a consequence milk samples from females in poor communities in Pakistan usually have detectable AF (*Hanson* et al., 2000). In a Swedish population no or very little AF is found in the milk, but giving the mothers a specially processed cereal to eat, production of the AF is induced and the milk will contain AF. Its site of production is unknown, but it may be produced in the intestinal epithelium by gut lymphocytes and/or in the mammary glands.

In a preliminary study in Sweden we found that the levels of AF induced in the milk significantly reduced the prevalence of mastitis, which appears in about 1/3 of lactating women in the West (*Svensson*, 2004). Among village women in Pakistan mastitis is hardly known although usually it is a very painful condition difficult to miss (*Jalil* et al., unpublished observations). It seems that the levels of AF induced in the Swedish study and which protected against mastitis often appears after natural exposure in the Pakistani village mothers and therefore mastitis is no problem there.

Since AF protects against diarrhoea in piglets (*Lonnroth* et al., 1988) it was of interest to determine if such protection may also occur in man. Inducing AF so that it occurs in the mothers' milk, or giving it directly to the offspring might be used to study protection against diarrhoea in children. We choose the latter mode for a double blind randomised study of 120 Pakistani children 6-24 months old with defined acute diarrhoea (7 days of passing 3 or more loose watery stools per 24 hours before admittance) (*Zaman*, 2005). One half of the group received egg yolk containing AF obtained by feeding hens the AF-inducing food. The other half obtained egg yolk without AF. The consistency of the stools improved, normalising significantly faster in the group obtaining AF from day 2 on of hospitalisation compared the control group ($p<0.01$). The mean frequency of the stools was significantly reduced day 2 ($p<0.006$) and 3 ($p<0.02$) compared to the control group. The effect of the AF on the diarrhoea was also illustrated by the fact that the mean number of days of hospitalisation was diminished from 3.1 to 2.2 ($p<0.002$).

From these studies it seems that AF in the young children can be clinically useful for protection against acute diarrhoea (*Zaman*, 2005). Our ongoing study on the possible effect of AF on prolonged diarrhoea will be completed within the next few months and is awaited with interest, since in this group the mortality can presently be considerable.

Cytokines, hormones and other signals in the milk, which may have short and long term effects on the breastfed offspring

Breastfeeding has a number of immediate and long-lasting effects on the offspring which may be linked to some of the many signal substances present in the milk. Thus the thymus, the central organ in the immune system, gets twice as large in an exclusively breastfed than in a non-breastfed infant (*Hasselbalch* et al., 1996; 1999). It is considered that the IL-7 from the milk can be one factor behind that rather striking effect (*Ngom*, 2004). This cytokine also promotes the formation of the cryptopatches in the gut from which the Tγδ lymphocytes originate (*Laky* et al., 2003).

The presence of TGF-ß in the milk may enhance production of SIgA and possibly counteract production of IgE antibodies and symptoms of allergy in the infant (*Saarinen* et al., 1999; *Oddy* et al., 2003). The IL-6 in milk may, like TGF-ß, promote differentiation of B lymphocytes (*Kono* et al., 1991). It also seems to promote phagocyte production of α1-antitrypsin which is found in the stools of breastfed infants (*Schanler*, 2001).

The fact that longer duration of breastfeeding seems to be linked to higher levels of protective serum IgG2 antibodies against *Haemophilus influenzae* type b (Hib) bacteria may at least partly be due to the TGF-ß transferred via the milk (*Silfverdal* et al., 2002). Phenomena of this kind may help us understand how it may be possible that there is evidence that breastfeeding can have long term effects on the offspring seen as promotion of certain vaccine responses. There is also evidence for remaining enhanced protection against various infections like otitis media, respiratory tract infections, *Haemophilus influenzae* type b infections, diarrhoea, and urinary tract infections for some years after the termination of breastfeeding. This data was recently summarised (*Hanson*, 2004).

NUTRITIONAL FACTORS INFLUENCING THE IMMUNE SYSTEM OF THE OFFSPRING

Undernutrition and the immune system of the offspring

Undernutrition can impair many aspects of host defence. This is most clearly demonstrated by the fact that among the many cases of deaths caused by infections especially in underpriviledged populations, undernutrition contributes to the deaths in 56% (*Pelletier*, 1994). Most of the cases dying are children with mild to moderate undernutrition. The most common form of undernutrition is protein-calorie malnutrition (PEM) which reduces lymphoid tissues, circulating T cells, complement levels and the appetite-regulating hormone leptin. This hormone has a structure similar to IL-6 and binds to class 1 cytokine receptors. It upregulates or modifies haematopoietic cells, monocytes/macrophages and T cell responses, increasing Th1 and suppressing Th2 cytokine production (*Hanson*, 2005). Some vaccine responses are impaired by PEM, others not.

Micronutrient deficiencies may also contribute to inadequate host defence with vitamin A deficiency (VAD) being the most common. Its subclinical form occurs in about 125 million children. VAD impairs phagocytosis, antibody production and T cell functions. Vitamin A supplementation to children in poor areas reduces morbidity and mortality significantly, especially in diarrhoea. Zinc deficiency may lead to lymphoid atrophy and reduced responses to T cell dependent antigens. Providing

zinc to undernourished children has enhanced their immune functions, reduced the appearance of diarrhoea and supported growth. Deficiencies of other micronutrients like iron, cupper, vitamins E, D and K, as well as selenium and also nucleotides affect the immune system. This vast area of research was recently reviewed (*Hanson*, 2005)

The effects of n-6 and n-3 polyunsaturated fatty acids (Pufas) on leptin levels and on oral tolerance in early life

The essential fatty acids are needed as building stones of all cell membranes and for their functional capacity. They are required for production of cell receptors and their functions and also for essential signals like leukotrienes and prostaglandins. On this basis they are necessary for the function of cells both in the innate and the specific immune system.

In recent studies using a rat model we found that a diet deficient in Pufas to rat dams reduced the levels of leptin in serum of the pups (*Korotkova*, 2001). The inguinal white adipose tissue was reduced and so was its leptin mRNA in the suckling pups (*Korotkova*, 2002). Feeding a diet to the rat dams during late pregnancy and early lactation that varied in the ratio of n-6/n-3 fatty acids showed that using a low ratio of 0.4 (linseed oil) resulted in lower serum leptin in the pups than a ratio of 9 (soybean oil) (*Korotkova* et al., 2002). The latter ratio, which is common in our food today, resulted in higher body weight, larger body length, higher inguinal fat pad weight and larger adipocyte size compared to the pups given the low ratio fat provided by linseed oil.

The two sides of the immune system, the defence against infections and the immunological tolerance are equally important for our health. Just as much as we need to mount efficient host defence it is vital to become tolerant in order to avoid allergic and autoimmune diseases. Tolerance develops already early in life and may then be somewhat easer to attain than in adult life. Our understanding of how tolerance appears is still inadequate, but presently the role of regulatory T cells is considered central. Such T cells, which function via cytokines like TGF-ß or IL-10, will obviously induce antigen non-specific tolerance (*Dahlman-Höglund* et al., 1995; *Lundin*, 1999). In contrast will tolerance caused by T cell anergy or clonal deletion be antigen specific.

It has been demonstrated that dietary intake of Pufa can influence tolerance induction in adult mice and that the content of n-6 and n-3 fatty acids may play a role (L 2003). In adult rats we obtained oral tolerance both with an essential fatty acid deficient and an essential fatty acid replete diet and it was antigen non-specific; thus presumably due to cytokine producing regulatory T cells (*Korotkova*, 2004). In the offspring of rat dams on the diets with either deficient or adequate content of Pufa we noted that the deficient diet resulted in neonatal tolerance in the pups against ovalbumin given to the lactating rat dams. We compared the effect of diets to the dams with either high (9), or low (0.4) ratio of n-6/n-3 fatty acids as to the immune response of their pups to the ovalbumin given to the dams during lactation (*Korotkova* et al., 2004). The diet with the high n-6/n-3 ratio did not result in a capacity of the rat pups to develop tolerance against the ovalbumin reaching them via the milk from their mothers. In contrast those with mothers on the low n-6/n-3 ratio diet became tolerant to the ovalbumin both in the cell- and the antibody-mediated immune responses. They were also tolerant to an unrelated antigen suggesting that the tolerance resulted from cytokine-producing regulatory T cells. This was supported by increased production of TGF-ß by enlarged regional lymph glands.

The possible effects of Pufas on intrauterine growth retardation, potentially a failure of maternal tolerance to the foetus?

Pufas are important for the normal development of the placenta. In cases of intrauterine growth retardation (IUGR) aberrations in Pufas have been described (*Cetin*, 2002). The mechanisms behind IUGR are still unknown. It is well appreciated that the maternal immune response against the foetus is an important driving and regulatory force during pregnancy. We have investigated the possibility that the maternal immune response against the foetus may not have been adequately controlled and regulated in cases of IUGR so that the formation of the placenta via the outgrowth of the trophoblast from the ovum has become insufficient, resulting in a placenta which cannot fully support the growth of the foetus.

IUGR occurs in about 1-4% in Sweden, but in developing countries it is much more common, like in Pakistan, where we have registered IUGR among around 15% of pregnancies (*Amu*, 2005). We have studied the levels of mRNA for certain cytokines in the decidua and trophoblasts from placentas from cases of IUGR from Sweden as well as Pakistan. We found in 20 placentas from IUGR pregnancies of Swedish mothers that the mRNA for IL-10 was significantly reduced in the decidua compared to the non-IUGR controls ($p<0.05$), whereas the mRNA for the pro-inflammatory IL-8 was significantly increased ($p<0.05$) (*Hahn-Zoric* et al., 2002). Also if the mothers had pre-eclampsia the mRNA for IL-10 was significantly low in the decidua, but then the pro-inflammatory IL-6 was increased as well mRNA for the multifunctional TGF-ß was very high in all placentas with or without IUGR.

Comparing 45 cases of IUGR in Pakistani mothers with 55 non-IUGR controls we found again decreases of mRNA for IL-10 ($p<0.0001$) and also IL-12 ($p<0.008$) in the decidua (*Amu*, 2005). These observations may be taken to suggest that the foetus is inadequately protected against the maternal immune reactivity, which is normally a driving force for the course of pregnancy. However, TGF-ß was increased ($p<0.009$). The significance of this is not clear at present, but this cytokine which is usually regarded as down-regulatory, has recently been shown to induce expression of CD103, which makes $CD8^+$ T cells destructive in graft-versus-host reactions after bone marrow transplantation in man (*El-Asady*, 2005).

If the reduced presence of immunosuppressive IL-10 in placentas adds to the risk of developing IUGR, it might be considered to investigate whether or not IL-10 can be used for treatment as soon as IUGR is discovered during pregnancies.

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MUTUALISM BETWEEN THE MUCOSAL IMMUNE SYSTEM AND COMMENSAL INTESTINAL BACTERIA (PART I)

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SUMMARY

Members of the ordinarily benign, 'normal' intestinal microbiota stimulate the development and maintenance of the adaptive and innate gut immune system. Comparisons of antigen-free (AF), germ-free (GF), and conventionally-reared (CNV) mice indicate that absolute cell numbers, cell subset distributions, and activation states of both mucosal and systemic elements of immunity can be differentially affected by particular gut microbes. Colonisation by Gram-positive segmented filamentous bacteria (SFB) vs. Gram-negative *Morganella morganii* will be used to illustrate effects. A further type of experiment using such gnotobiotic mice is aimed at determining whether continuous gut colonisation by either SFB or the pair of Gram-negative bacteria (Schaedler's *M. morganii* plus *E. coli*) is required to maintain the steady state level of 'natural' and specific IgA production in the gut. A dietary 'shift down' to an AF diet eliminates SFB but not the two Gram-negative microbes from the gut without affecting IgA production over 4-7 weeks in either case. Further, a role for gut microbes in driving the development of IgA blasts in μMT gene segment (-/-) mice will be supported.

Use of comparisons of GF vs. gnotobiotic mice will be presented illustrating roles for gut microbes in: (a) contributing to 'homeostatic' and 'greater than homeostatic' proliferation of T cells; and (b) the role of particular gut microbes in upregulating expression of various products of gut epithelial cells (ECs). In the latter, gene-profiling analyses were used to analyze expression of RELM-β (goblet cells) and Reg III (autocrine growth factor, ECs) stimulated by particular gut microbes. Finally, the striking finding of pauci-dispersity of gut IgA plasma cells in even CNV mice will be considered. The roles and mechanisms by which microbial TI-1 and TI-2 Ags may stimulate a pronounced oligoclonality of gut IgA cells expressing germ-line or near germ-line Ig V-genes amongst the overall population of IgA blasts will be discussed and model systems using chronic colonisation of mucosal surfaces by microbes expressing such TI Ags will be presented.

GENERAL HISTORIC INTRODUCTION

'Natural', benign microbial colonisers of the gut have long been thought to have a role in stimulating the normal development of both innate and adaptive elements of the mucosal immune system and in maintaining its 'natural state' of activation. The main support for this role has come from comparing the numbers and phenotypic/functional properties of various subsets of cells of the mucosal immune system – B cells, T cells, NK cells, etc. – found in the intestine of germ-free (GF) or antigen-free (AF) hosts vs. those reared conventionally (CNV) (*Crabbe* et al., 1970; *Crandall* et al., 1967; *Cebra* et al., 2005; *Bos* et al., 2003). The general finding is that many fewer of such cells – for instance IgA-blasts, α/β TCR^+ T cells, cytotoxic NK cells, CD45RBlow/$CD4^+$ T cells, etc. – are found in the germinal centres (GCs), lamina propria, or intraepithelial leukocyte (IEL) spaces of the guts of GF vs. CNV mice. Our group and others have compared the gut mucosal system of GF mice vs. mice monoassociated with a variety of ordinarily benign, non-invasive, single species of bacteria (*Shroff* et al., 1995; *Talham* et al., 1999; *Umesaki* et al., 1995). Generally, all such species of commensal microbes stimulate the development of GC reactions (GCRs), the production of IgA blasts which accumulate in the lamina propria and secrete both 'natural (total)' and specific IgA. Individual species of normal gut colonisers seem to differ in the maximal amount of total IgA they induce and the relative amount of this that can be shown to be specific vs. the microbial Ags of the inducer (*Bos* et al., 2001). All seem to induce a waxing and long term waning of the secreted IgA and a much more rapid development and decline of GCRs. An example of such a perturbation of the humoral response is given by segmented filamentous bacteria (SFB, related to the Clostridia), which becomes a major gut coloniser of the distal ileum of CNV as well as GF mice between weaning and puberty. Colonisation of formerly GF weanlings results in transient GCRs, the populating of lamina propria by IgA plasma blasts, and levels of IgA production about 50-70% of that seen normally in CNV mice. Only about 1% of this IgA can be shown to be specific for the SFB (*Talham* et al., 1999; *Jiang* et al., 2001). Colonisation of GF mice with SFB also shifts the prevalent ratio of $CD4^+$ subsets from about 65:35 CD45RB-high:CD45RB-low cells to the reverse ratio of 35:65. The specificities of these 'activated' $CD4^+$ T cells for microbial Ags has not been shown, as is the case for most activated gut T cells presumably induced *in vivo* by microbial products. We have used neonatal/young adult GF mice, monoassociated with SFB, to address a number of follow-up questions concerning host mucosal immune system/microbial coloniser interactions:

1) Can mice monoassociated with SFB for long periods develop a new round of GCRs in the gut upon super-colonisation with a different commensal microbe? After about 100 days following monoassociation with SFB, mice were super-colonised with *Morganella morganii* (Gram-negative, facultative anaerobic rod). GCRs again developed, as did a novel, specific IgA Ab response vs. *M. morganii*. Little change in overall production of total IgA in the gut was detected although the maximal, specific responses to *M. morganii* were about 20x greater than found vs. SFB. Thus, chronic GCRs, observed in CNV mice, are likely due to overlapping, following continuous exposure of the gut to novel microbial Ags (*Talham* et al., 1999);
2) Although the GCRs waxed and waned following monoassociation of

formerly GF mice with a particular microbe, the increased level of lamina propria plasma cells and the output of 'natural' and specific IgA in the gut changed only gradually, if at all, even over periods greater than 100 days post-colonisation. The more rapid decline of GCRs could be attributed to the 'blocking' or 'shielding' of B cell follicles from microbial Ags by the effective production of specific IgA Abs. However, the productive IgA plasma blasts, both specific and 'natural', persist for much longer periods. This finding raises the possibility that long-term persistence of the bacteria in the gut can provide the necessary, continuing stimulation of the previously initiated specific and 'natural' IgA production. In order to test this possibility we performed a dietary 'shift down'. This approach was based on our observations that SFB could not colonise the gut of mice maintained on a chemically defined (CD) diet (*Pleasants* et al., 1986), while a pair of facultative anaerobes, *E. coli* (Schaedler) and *M. morganii*, could. After colonisation of GF mice raised on regular mouse chow (conventional, CNV), groups of colonised mice were periodically shifted to the CD diet (so-called AF-diet) and analyzed for gut bacterial counts and gut IgA production in comparison with littermates that continued on CNV diet. After colonisation with either type of microbe, total IgA production by gut fragments rises over 3-4 weeks and remains rather constant up to 7 weeks. The total IgA output stimulated by the two Gram-negative microbes is about half that given with SFB, and specific IgA Ab follows the same time course except that the specific Abs given by the pair of Gram-negative enteric rods was about 40x higher than that in SFB colonised mice (absolute levels) or 20x higher on a basis relative to total IgA produced. Upon 'shifting down' to the CD, the faecal level of *E. coli* dropped by about one order of magnitude (E+10 to E+09) while the level of *M. morganii* increased by about 1.0 to 1.5 orders of magnitude to E+09. By the end of the experiment both mice on CD and CNV diets had rather balanced bacterial populations of 10^9/g of each species. The SFB quickly dropped to non-detectable over the 2-3 days following the dietary shift, although mice maintained on the CNV diet retained a near constant level of faecal SFB. In both groups of mice colonised with a particular organism(s), the overall level of total IgA and specific Ab remained unperturbed over the period of dietary shift and for four weeks beyond, even though the SFB were effectively eliminated while the coliform were not. Thus, the maintenance of levels of total IgA and specific IgA anti-microbial Ags was not affected by the dietary shift, whether the gut bacteria was effectively eliminated or not. These observations suggest that continuous, overt colonisation is not necessary to maintain rather long-lived production of IgA in the gut, although Ag-retention on dendritic cells, such as FDC or IDC, cannot be excluded as a source of continuous stimulation. It now seems relevant to revisit the half-lives and turnover of IgA plasmablasts in the gut;

3) Finally, we have addressed the issue of whether mucosal Abs, obtained by neonates during suckling, could affect the numbers and whereabouts of particular gut commensal microbes. Since SFB appears in the small intestine only after weaning and rises rapidly to become one of the most abundant enteric bacteria there before disappearing at 8-12 weeks, we suspect a role for mucosal IgA and/or IgM in these changes. Pups born of

immunodeficient (SCID) mothers show a precocious rise in SFB in the terminal ileum and its persistence there to adulthood compared with offspring of immunocompetent mothers (*Jiang* et al., 2001). By conducting swaps of pups at birth which were either immunocompetent or SCID to nurse mothers that were either immunocompetent or SCID, we demonstrated that suckling on immunocompetent nurse mothers forestalled the small gut colonisation compared with pups having SCID nurse mothers. The genotype of the pups: scid/scid vs. scid/+ only had an effect after weaning to determine the persistence or not of SFB in the ileum. The persistence of the SFB was only evident in pups who themselves were (scid/scid). Recently, mice with the AID k.o. have been used to argue that these effects of nursing on SFB colonisation depended on Ig isotype switching and/or the point mutational process leading to affinity maturation (*Suzuki* et al., 2004). A likely target for a specific Ab effect could be those 'hold-fast' structures that anchor the SFBs to the brush border of ileal epithelial cells, allowing their persistence at this turbulent site.

PARTICULAR EXAMPLES OF THE *IN VIVO* EFFECTS OF COMMENSAL BACTERIA/HOST MUCOSAL IMMUNE SYSTEM INTERACTIONS

Some common approaches to implicating members of the 'normal' murine microbiota in initiating or maintaining 'normal' cellular responses evident *in vivo*:

1. Compare the particular response in CNV vs. GF mice or in gnotobiotic (defined flora, DF) vs. specific pathogen free (SPF) mice;
2. If the response is qualitatively or quantitatively different between the pairs of mice being compared, attempt to mono- or oligoassociate GF mice with particular, defined members of the 'normal' microbiota (make DF or gnotobiotic mice) to try to mimic the condition of full 'natural' colonisation and its effect on the particular phenomenon of interest;
3. If one or several defined microbial species can initiate the effect, attempt to identify the microbial Ags/ligands and their target mammalian cells/receptors that are involved in the initiation and/or maintenance of the phenomenon;
4. Try to establish any 'cross-talk; that may lead to indirect effects on cell types not directly interactive with the microbial product;
5. Try to understand the operative mechanisms and the consequences of the host cell responses for normal homeostasis or resistance/protection vs. pathogens. For instance, if a mammalian cellular product is induced by a microbial stimulus, does it act in an autocrine manner, does it target other host cells and/or does it have anti-microbial effects?

Recently, attempts to genetically 'knock out' particular, potential microbial agonists or host cell receptors/products have culminated such studies as outlined above. However, often these have been frustrated by redundancies of genes for relevant products and common signalling pathways.

The status of four projects carried out along the lines summarised above, which have progressed to varying levels of understanding, will be presented. It is postulated that in each case, microbial products either induce, amplify, or contribute to the maintenance of particular mammalian, cellular responses. It is ex-

pected that the description of these phenomena will stimulate much discussion and further work.

A. In 2001 a paper by *Macpherson* et al. (2001) appeared which described a 'leakiness' of IgA production in CNV μMT (-/-) mice. These mice have a homozygous deletion of the gene segment encoding the transmembrane portion of the μ-heavy chain components of their BCRs. Thus their B cell development was blocked at the pre B cell stage. Nevertheless, at about 5 weeks of age most but not all of these pups expressed about 1-50% of normal serum IgA levels. These mice carried the so far undefined SPF flora of the Zurich mouse colony. Our own studies utilised several lots of μMT breeding stock from the Jackson Labs SPF colony, directly obtained from the founder colony in Köln (*Kitamura* et al., 1991). Our CNV μMT mice and W/T littermates were maintained in Trexler isolators using those sterile procedures appropriate for GF mice. Our findings agreed qualitatively but not temporally or quantitatively with those of the Macpherson group. We found only sporadic expression of serum IgA in a small fraction of mice up to 7 months of life and the concentrations were usually <1% of normal, W/T mouse levels. After one year of age, more mice (about 50%) were serum positive for IgA but again at around >1% of normal levels. Such low and sporadic expression of IgA suggested that successful transit of B cells through the pre-B cell stage was a rare event in these mutant mice and may be reflected in the mono- or oligo-clonality of their IgA blasts. Thus we tested cell samples from different regions of the gut for cells expressing the same CDR3 sequences (clonal relatives) and the occurrence of point mutations in the Vα region of IgA using PCR cloning and DNA sequencing. The surprising result from analyses performed on samples from one 13 month old mouse was that most relevant, cloned cDNA sequences from its productive, recombined Vα genes were clonally related and differed by only 1-2 VH mutations from the GL sequence. This GL sequence, from our previous studies, is expressed in IgA antibodies reactive with α1_6 dextran, a microbial product. Of course, we must develop these observations further, but our surmise is that active VDJ gene, associated with the truncated Cμ gene, can be translocated to the usual switch recombination region 5' to the intact Cα gene, even in the absence of external stimulation via BCRs. Such rare B cells, now expressing membrane-associated IgA as BCR, may be 'rescued' specifically by gut microbial products and be expanded to form rather large clones. Thus, appreciable rescue events would depend on a rather large repertoire of microbial ligand and the temporal and quantitative difference between our findings and those of the Zurich group could well be due to a very different, more diverse microbiota in the mice used by the latter. Further, one might expect that those specificities of B cells we detect as clonal products may signify those very common microbial ligand - matched to GF gene VJH/VL pairs - that specifically stimulate very early TI-responses. Finally, an attempt to enhance the detection of 'leakiness' in our mice by superimposing *Trichuris muris* infection on their normal SPF flora was unsuccessful.

B. Transfer of congenic, unfractionated or naïve (CD44low T cells), labelled with a vital fluorescent dye (CFSE),

into 6 Gy irradiated hosts results in a rather slow outgrowth of T cells. Reactivity with self-peptide ligands was implicated as a stimulus for such expansion, called 'homeostatic proliferation' (*Jameson*, 2002; *Moses* et al., 2003; *Kieper* et al., 2004). More recently, we found that such cell transfers into non-irradiated, immunoincompetent hosts [H-2^b RAG (-/-) or H-2^d C.B17 SCID mice] led to a much more extensive proliferation of both $CD4^+$ and $CD8^+$ donor T cells (*Kieper* et al., 2005). Many of these, when analyzed after proliferation and loss of detectable CFSE, were CD44high, CD25positive, and expressed TNF-α, γ-interferon, or both. Further, we found that transfer of CFSE-labelled T cells into GF SCID mice compared with CNV SCID hosts resulted in a marked diminution of expansion to 'homeostatic levels'. Thus, it seems likely that gut microbial products may markedly augment the stimulus by 'self' peptides for cells to proliferate *in vivo*. So far, GF SCID mice monoassociated with one of several common commensal bacteria provide a rather small increment of 'extra' expansion of T cells. Possibly, the marked expansion we observed in CNV SCID mice reflects the superpositioning of many specific T cell responses to gut microbial Ags? Obviously, we should use a variety of oligoassociated SCID mice (*i.e.*, DF) to try to detected an additive effect in stimulating T cells outgrowth *in vivo*.

It has become popular to search for tissue-specific products whose expression may be upregulated by gut microbes using first Northern blotting developed with cDNA probes and then gene expression profiling via cDNA arrays on microchips. We have studied two such products expressed in mouse large intestine and apparently up-regulated by colonisation of formerly GF mice with whole SPF microbiota or particular gut commensal microbes.

C. Using cDNA probes, Northern blotting, and a polyclonal anti-peptide antiserum, we have shown the increased presence of RELM-β (resisten-like molecule) (*Steppan* et al., 2001) in the secretory granules of goblet cells from the large intestine upon colonising formerly GF BALB (immunocompetent) or C.B17 SCID mice with SPF microbiota (carried by Jackson Lab. mice) (*He* et al., 2003).

The prodigious amount of product, RELM-β, can be easily extracted from faecal pellets or isolated from colonic washes. The rise in expression of RELM-β can easily be detected within 24 hours of exposure of GF immunocompetent or SCID mice to SFB microbes. Thus, an adaptive immune system is not required to participate in this up-regulation of RELM-β expression induced by colonisation with the Jackson SPF microbiota. At the level of RELM-β mRNA expression, about a 7-8 fold increase is also noted. Of interest and a cause for some chagrin, is that monoassociation of GF BALB mice with SFB, *Helicobacter muridarum*, *E. coli*, or oligoassociation with the 'altered Schaedler's flora' fail to result in any increase in expression of RELB-β. Thus, it would appear that identification of microbial agonists might be stymied. However, it has recently been shown that supercolonisation of CNV mice with embryonated eggs of helminths, such as *Trichuris muris*, result in the induction of very high levels of RELM-β, such as 40x the levels of expression of mRNA found in CNV mice bearing the complete SPF microbiota. The fate of these recently hatched worms is expulsion in 10-18 days, coinciding with a marked Th2 T cell response. Along with the adaptive

immune system, it seems possible that the copious RELM-β produced may also play a role in expulsion by accumulating in the bacillary pores of the worm (*Artis* et al., 2004). However, it is relevant to note that SCID mice, which also somewhat more modestly up-regulate RELM-β expression when colonised with *T. muris*, develop a chronic helminth infection.

D. Using gene expression profiling via cDNA arrays on microchips we compared fold induction of mRNA from GF, monoassociated, oligoassociated, and CNV mice that were either immunocompetent BALB or SCID (C.B17). We found a set of genes, RegIIIβ and RegIIIγ, that were upregulated in the colon about 9-11 fold in SFB monoassociated and in DF (altered Schaedler's flora) colonised SCID mice compared with expression in colons of GF SCID mice (*Keilbaugh* et al., 2005). The RegIII gene family is related to human pancreatitis associated protein (PAP), which induces cellular proliferation and inhibits apoptosis (*Clark* et al., 2000). The intestinal versions of this gene family may be autocrine growth factors made by gut epithelial cells. Because γ-interferon, and several other genes upregulated by γ-interferon such as IRG47, Rab6-kinesin like, UPase, guanylate nucleotide binding protein, were also up-regulated in the colons (2-6 fold) of the same group of colonised mice, we initially supposed that colonisation of SCID mice led to activation of components of the innate immune response – for instance NK cells which produced γ-interferon – and the subsequent up regulation of the RegIII genes occurred secondarily. Thus, we assessed the effect of monoassociating GF mice with *E. coli* (Schaedler) on expression of mRNA transcribed from RegIII genes and γ–interferon gene in the colon, since we had found that colonisation of GF mice with *E. coli* was particularly effective at activating NK cells in the IEL compartment. Our findings were:

1) that increases in relative expression of all these genes was much greater in formerly GF SCID mice vs. GF immunocompetent mice – suggesting an antagonistic effect of the specific, adaptive immune systems in immunocompetent mice;
2) that the up-regulation of γ-interferon expression was temporally distinct from that of the RegIII genes; and
3) that the natural mouse *E. coli* was more effective than other microbes used to colonise GF SCID mice in up-regulating RegIII expression (*Keilbaugh* et al., 2005).

Thus, it seemed that the host response to colonisation by *E. coli* was based on its innate immune system, but we are unclear about the mechanisms of action of its microbial products. We further pursued the possibility that activated NK cells would play an intermediate role in the increased expression of RegIII genes. Although we found an increase in activated NK cells expressing γ-interferon in the IEL spaces of *E. coli* colonised SCID mice, pre-treatment of these mice with MAb anti-γ-interferon did not depress expression of RegIII genes but did markedly decrease expression of the colonic γ-interferon gene and of the γ-interferon dependent UPase gene (*Keilbaugh* et al., 2005). Recently, some further relevant findings have been reported by Lora Hooper and colleagues at the ASM Symposium: Beneficial Bacteria (*Cash* et al., 2005). These included:

1) that RegIIIβ and γ genes are also up-regulated in the small intestine of CNV compared with GF mice and Paneth cells seemed to be

major producers of protein product;
2) these RegIII proteins appear to be animal lectins, reactive with peptidoglycan (especially its N-acetyl glucosamine component); and
3) these proteins appear to selectively inhibit clonal outgrowth of Gram-positive but not Gram-negative bacteria, using *in vitro* culture procedures.

It remains to be determined whether such anti-bacterial action is manifest *in vivo* and whether any particular gut commensal bacteria of the SPF-mixture can induce expression of the RegIII proteins in small intestinal Paneth cells.

POSSIBLE ROLES FOR COMMENSAL, MICROBIAL ANTIGENS/LIGANDS IN EXPANDING, SHAPING, AND MAINTAINING THE 'NATURAL' MUCOSAL IgA ANTIBODY REPERTOIRE

Relative to the systemic 'natural' and 'specific' antibody repertoire, the IgA antibodies made by the abundant IgA plasmablasts in the gut show striking differences. For instance, using CDR3 length spectrotype analyses of recirculating, follicular, splenic IgM^+ B cells, their productive Vμ genes show a symmetric distribution of CDR3 lengths. However, a similar set of analyses of productive, intestinal IgA-blasts show an oligodisperse distribution of CDR3 lengths (*Stoel* et al., 2005). This observation is the same whether monoassociated SCID recipients of B cells are analyzed or whether CNV immunocompetent mice of several common strains are examined: BALB/c, C57BL/6, and CBA. Likewise the findings are the same whether CBA (*xid*/*xid*) mice, deficient in B1 cells, or wooley mice, deficient in B2 cells are similarly analyzed. These rather qualitative observations were supported by DNA-sequencing of CDR3 regions and then VH-regions from productive IgA-plasmablasts. Surprisingly, out of 15-20 sequences examined from various types of mice, two or more likely clonal relatives were found, based on identical V/D/J junctional sequences. The relationship was verified by finding that these usually differed in Vα-sequences and/or were GL or near-GL, with unrelated point mutations that were not clustered in CDR-regions and did not show significant increase in R/S type mutations (*Stoel* et al., 2005).

It seemed to us that if microbial, polyclonal stimuli accounted for the development of these B cells likely making 'natural' IgA antibodies, then polydisperse B blasts would be expected. Perhaps either B1- and/or B2-cells, stimulated specifically by TI-1 and/or TI-2 Ags via their BCRs, allow for an oligodisperse population of IgA-blasts making 'cross-reactive' but <u>not</u> polyreactive natural antibodies. Perhaps such B blasts, with randomly recombined V/D/J segments but without appreciable N-additions or point mutations in Vα regions, are selected in the gut by relatively few TI antigens?

We have tried to first evaluate this possibility by assessing whether either or both B1- and B2- cells making IgA in the mucosa show any evidence of having been selectively and specifically stimulated by microbial TI Ags, based at least in part on their BCR? Particularly:

1. Can B1-cells be selectively and specifically stimulated by a TI-1 Ag – such as LPS bearing a particular antigenic determinant – via their BCRs and go on to secrete IgA Abs?
   In order to approach this issue in a way that permitted identification of B1- or B2- cells *ex vivo*, we created

Igh-allotype distinctive, congenic chimaeric mice by transferring B1- or B2-cells from different sources (C.B20, Ighb vs. BALB/c, Igha) into formerly GF SCID (C.B17) mice. In order to provide any necessary, bystander T-cell help we made reciprocal mixtures of purified B1- cells from peritoneal cavity cells (PeC) of one Igh allotype and added these to the congenic, B1-cell depleted Pec (including B2-cells, T cells, APCs, etc.) of the other Igh allotype and then transferred these mixtures. In order to distinguish the effects of possible BCR-specific determinants borne on an LPS from its polyclonal effects, we collaborated with the Weiser group, who prepared two genetically-manipulated strains of *Haemophilus influenzae*: One that constitutively added the phosphocholine prosthetic group (PC) to its LPS and the other that lacked the necessary, functional enzymes to attach PC to the same LPS moiety (*Lysenko* et al., 2000). Thus we could monoassociate B-cell reconstituted SCID mice with one or another of the *H. influenza* strains. We found (*Lysenko* et al., 2000):

a) That neither the PC^+ not the PC^- strain chronically colonised the intestine of SCID mice but that each colonised the upper respiratory tracts about equally for up to six months;
b) At two weeks after stable colonisation of 8-10 weeks old GF SCID mice with either the PC^+ or the PC^- strains of *H. influenzae*, lymphoid cells were transferred. At 10 weeks after cell transfer, mice monoassociated with either the PC^+ or PC^- bacteria showed 'natural' or 'total' IgA production by their NALT and respiratory lymphoid tissue (RALT) *ex vivo*. Those mice colonised with the PC^+ strain showed roughly 10x higher levels of total IgA production;
c) Most of this 'natural' IgA production was made by the B1-cells in the Igh-allotype chimeras – about 10x as much as could be attributed to B2-cells – if the recipients had been colonised with the PC^+ strain of *Haemophilus*. If the PC^- strain was used for colonisation, B1- cells showed almost no contribution to the total IgA output, while the B2-cells produced about the same amounts of 'natural' IgA as did their counterparts from PC^+ colonised mice;
d) Examination of the same tissue fragment culture supernates as used above to detect 'total' IgA for detection of specific anti-PC antibodies showed that only mice colonised with PC^+ *Haemophilus* made detectable specific Abs, and B1-cells contributed about 30x more of these than did B2-cells. Much of the 'total' IgA made by B1-cells could be accounted for by the anti-PC component;
e) Perhaps surprisingly, almost all the B1-cell derived anti-PC was positive for the T15-idiotype, using MAb A.B1 for detection (*Benedict* and *Kearney*, 1999). This MAb specifically reacts with Abs expressing the VH/VL pair of germ-line (GL) or near GL-gene associated with the dominant type of anti-PC made by these mice;
f) Finally, the extent of colonisation of upper respiratory tract remained about the same for both PC^+ and PC^- strains of bacteria, whether or not the hosts were making anti-PC in RALT or NALT.

Thus, the LPS of the PC^+ vs. PC^- strains did not contribute detectably to the anti-PC response by polyclonal stimulation. Instead, the PC-prosthetic group on PC-LPS seemed to

selectively and specifically activate B-cells, at least in part, via their BCRs. Of course, LPS interactions with the CD14/TLR4 complex may have played a co-stimulatory role *in vivo*.

2. Can either B2-and/or B1-cells be selectively and specifically stimulated *in vivo* by microbial TI-2 Ags based on their BCR and then go on to generate IgA Ab secreting cells?

   In order to approach this issue with any likelihood for quantitatively detecting IgA Abs specific for microbial TI-2 determinants we believe it necessary to:

   a) Identify enteric microbes that carry TI-2 determinants, known or shown to react with IgA MAbs that expressed GL- or near GL-V genes;
   b) Show that such microbes could chronically colonise the guts of GF mice;
   c) Show that such microbial colonisers could stimulate an appropriate and detectable, specific IgA Ab response *in vivo*. Of course, such a finding would be followed up by a molecular genetic analysis to discern oligoclonality and assess the pattern of point mutations among any clonal relatives detected (see above). Such a response might implicate chronic bacterial TI-2 Ag stimulation of the host's B1- and/or B2-cell populations in establishing the partly oligoclonal occurrence of 'natural' IgA producing cells in the guts of mice not deliberately challenged by enteric Ags.

   The earlier attempts to establish Ag-specificities for the 'spontaneous' IgA plasmacytomas that arose in BALB/c mice as well as our own evaluation of a set of eight MAb hybridomas (*Bos* et al., 1996) have indicated that many of these express GL- or near GL-V genes and may react with determinants found on microbial poly- or oligosaccharides (TI-2 Ags). Examples of such specificities include α1-6 dextrans, β2-1 fructosans and PC-borne by teichoic acids. Indeed MAbs from plasmacytomas and hybridomas are available for testing such specificities borne by particular microbes and Ags suitable for ELISA or RIA assays are available for evaluating gut IgA responses made by mice chronically colonised by candidate microbes that might express IgA Abs of such specificities.

Thus far, our results have been meager in attempts to identify suitable colonising organisms. The experimental protocol discussed above needs to begin with GF or gnotobiotic mice in an attempt to exaggerate any response to TI-2 Ags. We have found an encouraging approach to search for commensal gut bacteria that may bear determinants of the above listed TI-2 Ags. We have used a set of defined flora (DF: *Lactobacillus salivarus*, *M. morganii*, SFB, and *Bacteriodes distasonis*) to colonise GF IgA (-/-) mice (H-2b haplotype). After two weeks bacteria are harvested from gut, presumably expressing *in vivo* characteristics, and stained with PI plus MAb reactive with β2-1 fructosyl or PC-determinant. Because the host mice are IgA (-/-), they do not show the usual 'endogenous' coating of nearly all gut bacteria with their own IgA. We find that MAb ABPC47, reactive with β-fructosyl, stains 16% and MOPC603 or TEPC15 MAbs, reactive with PC, stain 2.7% and 12% of the live bacteria respectively. Thus, it might be possible to relate the expression of these TI-2 determinants to particular bacterial species.

However, monoassociation of GF mice with particular single bacterial species – *M. morganii*, Schaedler's *E. coli*, *L. salivarus*, SFB – has failed to result in IgA Abs detectable above the general rise in 'natural' IgA and reactive with either PC^-, β2-1 fructosyl or α1-6 dextran. Possibly we have not so far identi-

fied an example of a gut bacterial species that does specifically stimulate BCRs related to those commonly found to be expressed by 'spontaneously' or 'randomly' arising plasmacytomas, hybridomas, or 'leaky' B cells in μMT mice (see above). Possibly, one should try gut colonisation with *Aerobacter levanicum*, a free living bacterium that ordinarily does not colonise animals, since it is the source of the prototypic fructosan Ag that has been delivered parenterally to mice to study the stimulation of B cells expressing anti-fructosyl antibodies (*Boswell* and *Stein*, 1996).

We hope that our many imperfect and incomplete *in vivo* experiments seeking to better understand reactivity of microbial TI Ags with mammalian lymphoid cells will stimulate further, more sophisticated attempts to understand the relationships between 'natural' and 'adaptive' immune responses. We especially hope that we have been persuasive of the benefits of using GF and gnotobiotic mice compared with different conventionally reared mice such as 'SPF' with imperfectly defined but partially perturbed gut microbiota.

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MUTUALISM BETWEEN THE MUCOSAL IMMUNE SYSTEM AND COMMENSAL INTESTINAL BACTERIA (PART II)

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SUMMARY

Mammals normally coexist with a large load of commensal bacteria in the lower intestine that are not normally pathogenic. Commensal bacteria and their products shape mucosal and systemic immunity, epithelial differentiation and gene expression, and probably influence many other body systems. Although mammals are highly adapted to the presence of commensal intestinal bacteria, relatively little is known about the mechanisms of these adaptations or their functional significance.

We used *Enterobacter cloacae*, originally isolated from the intestinal flora of pathogen-free mice, as a model organism to investigate the mechanisms of immune handling of commensal bacteria. Intestinal challenge doses of *E. cloacae* resulted in penetration of live bacteria into dendritic cells within the Peyer's patches and these bacterial-loaded DC traffic to the mesenteric lymph nodes, but not into systemic secondary lymphoid tissues. Although live intestinal bacteria persist in DC, they are very rapidly killed by macrophages. IgA^+ B cells are induced in *ex vivo* cultures in the presence of intestinal DC loaded *in vivo* with live, but not heat-killed, bacteria. Repeated intestinal challenge *in vivo* with live intestinal commensal bacteria induces intestinal and serum IgA, but the response is absent if heat-killed organisms are used. Experiments with mice following mesenteric adenectomy show that mesenteric lymph nodes are important to avoid live bacteria reaching the spleen and priming of serum IgG responses to the organism. Thus under normal conditions, in the presence of mesenteric lymph nodes, priming by challenge doses of live bacteria is largely confined to the mucosal immune system, so mucosal immune adaptation can be achieved in the face of systemic immune ignorance of intestinal bacteria.

The function of intestinal IgA secretion has been investigated by challenging SPF mice with different doses of *E. cloacae*, and by parallel recolonisation of germ-free wild-type and ($J_H^{-/-}$) antibody-deficient mice using the same SPF sentinel. In each case antibody secretion did not affect the luminal densities of intestinal bacteria but reduced the levels of live bacterial translocation through the epithelial layer.

ADAPTATION OF THE IMMUNE SYSTEM TO COMMENSAL INTESTINAL BACTERIA

Interest in the mutualism between commensal intestinal bacteria and the mammalian body has a long history. Over a century and a quarter ago, micro-

scopic studies were published showing that the meconium of new-born babies was without visible microorganisms, but these appeared when the first yellow stools were passed (*Billroth*, 1874). Escherich confirmed these observations with microbiological cultures, showing that initially the postnatal intestinal discharges were sterile, but started to contain culturable bacteria within a few days (*Escherich*, 1885). The high densities of intestinal bacteria were also recognised to be non-pathogenic as long as they were contained within the lower intestinal lumen. Harvey Cushing and his collaborators showed with both clinical observations and a series of animal experiments, that accidental or belligerent perforation of the lower small intestine or the colon were likely to lead to peritonitis and septicaemia (*Cushing* and *Livingood*, 1900).

Study of the biology of mutual relationships between mammals and their intestinal bacteria also has a long history. Classical observations showed that the nutrition of humans and experimental animals influences the composition of the intestinal flora, especially when breast-feeding is substituted by formula feeding in human infants. Pasteur considered that microbes would be essential to the long-term viability of plants and animals. This provoked the challenge of raising animals in an aseptic environment, which was initially accomplished over several weeks, and later over a full life span (*Cohendy*, 1912, 1914; *Glimstedt*, 1936). A program that was able to successfully maintain experimental animals germ-free long term was started at Notre Dame University in 1928 (*Reyniers*, 1959).

The benefits of the lower intestine as a habitat for microorganisms are fairly obvious. The temperature is a steady 34-40 °C and there is a reliable supply of carbon sources, vitamins, minerals and water. The availability of germ-free animals in the early axenic and gnotobiotic programs allowed the impact of the microflora on their host to be investigated. For example, early studies showed that animals kept in sterile conditions suffered nutritional (especially vitamin K) deficiencies and had immature lymphoid structures and low levels of serum gammaglobulins (*Gustafsson*, 1959). Subsequently it has been shown that there are many changes, both immune and non-immune, within the mucosa (short range interactions) and elsewhere in the body (long range interactions) that differentiate germ-free animals from those colonised with intestinal bacteria (reviewed in *Macpherson* et al., 2005)[1]. These changes can be recapitulated in a germ-free animal within days or weeks of bacterial colonisation, and this can be done very simply, merely by placing a sentinel with an intestinal flora in the same cage as the germ-free animals. For example, there is only a sparse content of lamina propria IgA secreting cells in the mucosa of germ-free animals, and the mucosal lymphoid structures (Peyer's patches), are hypoplastic with few B cell follicles or germinal centres. The content of some intestinal lymphocyte subsets, particularly intestinal epithelial cells bearing the CD8αβ heterodimer and double positive $CD4^+$ $CD8^+$ subsets are reduced in germ-free circumstances. $CD4^+$ lamina propria lymphocytes are also deficient in germ-free animals. Immunity in the rest of the body, away from the mucosa is also shaped by the presence of commensal bacteria: systemic secondary lymphoid structure, high endothelial morphology, polyclonal responses to immunisation and serum isotype-switched immunoglobulins are all deficient or abnormal in animals living in germ-free conditions.

[1]Detailed references to the primary literature are given in this review.

We can refer to these short and long range changes of the immune system when germ-free animals are colonised as being adaptive to the commensal flora. However, there is very little evidence that the adaptation of the immune system has functional benefits. The low pathogenicity of commensal bacteria and the fact that innate immunity is sufficient to protect the animal from these organisms complicates assessment of functionality. The role of innate immunity is demonstrated by the viability of profoundly immunodeficient mice in specific pathogen-free (SPF) animal houses, where they are colonised by a simple (modified Schaedler) intestinal flora, but are screened to be free of pathogens[2]. For example SPF *scid/scid* and $RAG^{-/-}$ mice which both lack the majority of both B and T cells, are perfectly viable. Only if phagocytes are profoundly deficient in microbiocidal activity by introduction of two genetic targeted lesions of $gp^{91}phox^{-/-}$ and inducible nitric oxide $(NOS_2)^{-/-}$, which jointly abolish generation of oxygen and nitric oxide radicals, do the mice start to experience systemic sepsis with commensal organisms after they have been weaned (*Shiloh* et al., 1999). Conversely, consider the mechanisms used by endocytosed bacterial pathogens to survive in the host. The relatively small pathogenicity islands which distinguish their genome from commensals, predominantly encode proteins that subvert microbiocidal killing by phagocytes (*Sansonetti*, 2001).

MUCOSAL IMMUNITY COMPARED WITH SYSTEMIC IMMUNITY TO COMMENSALS

Our experiments have mainly used the model commensal aerobe *Enterobacter cloacae* present in the flora of our specific pathogen-free mice in Zürich. Western blots of *E. cloacae* cell wall proteins probed with purified secretory IgA from unmanipulated mice showed a poly-specific binding pattern of intestinal IgA, whether or not the strain of mice had T cells (*Macpherson* et al., 2000). In contrast, there was no binding of serum IgG even in wild type C57BL/6 mice. This was shown to be a result of immunological ignorance rather than tolerance, because an experimental injection of 10^4 to 10^6 organisms into the tail vein induced a strong, reproducible and specific immune response to a narrow range of cell wall proteins 14 days later (*Macpherson* et al., 2000). This suggests that specific pathogen-free mice are systemically ignorant rather than tolerant of their flora. We also found that there was evidence of spontaneous serum IgG priming against *E. cloacae* proteins, without prior deliberate infection, where the strains were deficient in intestinal IgA production, for example in the alymphoblastic mouse that has no secondary lymphoid structure outside the spleen and lacks IgA, or the $IgA^{-/-}$ mouse, which is deficient in IgA as a result of a targeted genetic lesion (*Macpherson* et al., 2000). This spontaneous (low level) systemic IgG priming was assumed to be the result of translocation of bacteria across the epithelial cell layer to reach deeper systemic tissues as a result of deficient intestinal secretory antibody, although the evidence was entirely indirect.

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[2]It is important to note that specific pathogen-free (SPF) mice have a simple limited bacterial flora unlike germ-free mice which contain no intestinal bacteria. SPF mice are screened to be free of murine pathogens.

SAMPLING OF BACTERIA AT THE INDUCTIVE SITES OF MUCOSAL IMMUNITY

In order to try and resolve the question of how there could be induction of the mucosal immune system by commensal bacteria independently of the mucosal immune system, allowing a significant IgA response in the former but leaving the latter ignorant of these organisms, we attempted to carry out functional experiments to measure the levels of culturable bacteria in macrophages. The intention was to determine whether translocated bacterial loads differed as a function of whether or not the mouse strain concerned was capable of secreting IgA. Following an oral dose of 10^9 *E. cloacae* given into the intestine by gavage, we found culturable organisms in washed mesenteric leukocytes between 6 and 60 hours after the dose was given. To our initial surprise, we were unable to recover any live bacteria in the sorted $CD11b^+$ $CD11c^-$ macrophage fractions. However, the dendritic cell fraction ($CD11b^+$ $CD11c^+$) contained a remarkable enrichment of live bacteria (*Macpherson* and *Uhr*, 2004). By carrying out macrophage killing assays, we could show that the explanation for being unable to culture live organisms in the macrophage fraction, was that our experimental protocols for flow cytometric sorting lasted approximately 5-6 hours, and in that time macrophages are able to kill almost all phagocytosed commensal organisms (*Macpherson* and *Uhr*, 2004). In contrast, dendritic cells are rather deficient in microbiocidal activity (*Delamarre* et al., 2005), so live organisms persist for several days (*Macpherson* and *Uhr*, 2004).

Although we had studied mesenteric leukocytes, as these are sterile by bacterial culture unless the intestine receives a challenge dose of commensal bacteria, the question arose whether dendritic cells in the mesenteric lymph nodes had taken up free *Enterobacter cloacae*, or whether *E. cloacae* were carried within dendritic cells as they travelled to the mesenteric lymph nodes. To resolve this question *in vivo*, we made two strains of *E. cloacae* that were antibiotic resistant either to nalidixic acid (Nal^R) or to rifampicin (Rif^R), both as a result of a chromosomal mutation. We also constructed isolated small intestinal loops by disconnecting segments of the small intestine and anastomosing both ends to the skin as mucous stomas. The vascular and lymphatic connections of these isolated loops of small intestine were not disturbed. Our experiment was to challenge one loop with *E. cloacae* carrying the Nal^R and the other loop with *E. cloacae* carrying the Rif^R and to isolate mesenteric leukocytes 18 hours later. The leukocytes were plated out at low density on bacterial culture media, so that any bacteria growing had arisen from a single cell. We established that approximately 30% of cells contained more than one bacteria, by lysing the cell suspension with deoxycholate prior to plating. We could now see whether each bacterial colony from a single unlysed cell growing on unselective media contained one or both antibiotic resistances by a replica plating technique. We found that if the Nal^R and Rif^R *E. cloacae* organisms were given into different intestinal loops, each cell from the mesenteric lymph nodes would always contain just one antibiotic resistance. As a control, if both antibiotic resistances were given into a single loop, a proportion of master colonies for mesenteric leukocytes had both antibiotic resistances. This result demonstrates that, *in vivo*, intestinal dendritic cells are capable of sampling bacteria at the intestinal surface and carrying them to the mesenteric lymph nodes, otherwise if *E. cloacae* were freely penetrating *either* antibiotic resistance would be just as likely to be taken up by

those dendritic cells within the mesenteric lymph nodes containing more than one organism.

Further direct evidence that intestinal dendritic cells can sample bacteria in the Peyer's patches themselves, was obtained by deriving a fluorescent form of *Enterobacter cloacae*, with green fluorescent protein (GFP) expression driven by the constitutive ribosomal rpsM promoter. Following intestinal challenge by gavage with GFP^+ *E. cloacae*, we were able to demonstrate a proportion of $CD86^+$ activated dendritic cells from the Peyer's patches that were positive for green fluorescence, this positive dendritic cell fraction was absent in mice treated with an identically transformed *E. cloacae*, but lacking the coding sequence for GFP. Thus low numbers of intestinal bacteria can be sampled after penetrating the intestinal epithelium, and persist as live organisms within dendritic cells, whereas they are very rapidly killed if phagocytosed by macrophages.

In these experiments where challenge doses of commensals are given into the intestine, the live organisms are seen only in the local mesenteric lymph nodes, and not in other systemic secondary lymphoid structures including central lymph nodes and the spleen. The presence of the mesenteric lymph nodes is critical to avoid this, because in wild type C57BL/6 mice where the lymph nodes have been removed by mesenteric adenectomy along the superior mesenteric artery, and allowed to recover with re-anastomosis of the lymphatic vessels, an intestinal challenge with *Enterobacter cloacae* subsequently results in culturable organisms detectable in the spleen. The presence of the mesenteric lymph nodes to protect the systemic secondary lymphoid system from exposure to commensal bacteria is also vital to preserve systemic ignorance, as specific priming of serum IgG, splenic enlargement and the hypertrophy of the splenic marginal zones all occur following repeated intestinal bacterial challenge of mice following mesenteric adenectomy.

Flow cytometry experiments with Peyer's patch and mesenteric lymph nodes leukocytes from animals treated with GFP^+ *E. cloacae* suggest that only a small proportion of the bacteria that are taken up across the intestinal epithelium are actually delivered to the mesenteric lymph nodes by dendritic cell trafficking.

The mechanism by which bacteria penetrate the M cell epithelial layer overlying the Peyer's patches to reach the underlying dendritic cells in the dome layer is still uncertain. In cell culture dendritic cells have been shown to be able to protrude processes between epithelial cells and sample the apical environment, and this is a pathway for the penetration of pathogenic bacteria including *Salmonella*. It has also been shown that the particular subepithelial dendritic cell subset that expresses the chemokine receptor CX_3CR1, is also able to sample intestinal organisms in the lower small intestine (ileum). In our experiments translocation across the Peyer's patches was a quantitatively far greater pathway than translocation through the villous intestinal epithelium. However, we were carrying out experiments with luminally delivered challenge doses of *E. cloacae* so these bacteria may have failed to penetrate the mucus layer overlying the villous epithelial cells as readily as they were able to penetrate the M cells overlying Peyer's patches. It is possible that subepithelial DCs away from the Peyer's patches are principally responsible for sampling bacteria that form in biofilms of the mucous layer overlying the epithelium, whereas the Peyer's patches with a reduced surface mucous layer and thin epithelial glycocalyx are principally responsible for sampling planktonic bacteria in the luminal contents.

INDUCTION OF IgA

Since the landmark papers of Cebra, Gowans, and their colleagues it has been known that IgA is induced in the Peyer's patches, and that IgA plasmablasts recirculate through the lymphatic and blood vascular systems to home back to the mucosa as they differentiate into IgA secreting plasma cells (*Craig* and *Cebra*, 1971; *Husband* and *Gowans*, 1978; *Pierce* and *Gowans*, 1975). Brandtzaeg and his colleagues showed that large amounts of secretory IgA are continuously secreted across the epithelial cell layer into the intestinal lumen coupled to the specific polymeric immunoglobulin receptor transport protein (*Brandtzaeg*, 1978; *Brandtzaeg* and *Prydz*, 1984). *Ex vivo* experiments have previously shown that dendritic cells are critical for class switch recombination to IgA. Although the factors responsible for signalling alpha specific class switch recombination are incompletely understood, TGF-β, IL-4, IL-5, IL-2 and IL-10 are know to be able to promote the IgA class switch *in vitro* or *in vivo* (reviewed by *Macpherson* et al., 2001) and *Johansen* and *Brandtzaeg*, 2004), and specific interaction between dendritic cells and B cells or T cells (*Cebra* et al., 1991; *Fayette* et al., 1997) leading to IgA induction might be promoted by the TNF family members B cell activating factor (BAFF) and APRIL (*Litinskiy* et al., 2002). *In vivo*, APRIL deficient mice have decreased spontaneous levels of IgA and reduced specific switching with T dependent and T independent immunisation protocols (*Castigli* et al., 2005).

To evaluate the importance of DC loaded with commensal bacteria in IgA induction, we set up cell cultures of B ± T cells isolated from mesenteric lymph nodes of unmanipulated mice with dendritic cells purified from Peyer's patches of mice that have been pulsed *in vivo* with 10^9 *E. cloacae*. Control experiments were carried out with dendritic cells purified from the Peyer's patches of mice that had received *E. cloacae* that were heat-killed prior to administration. Measurement of IgA^+ B cells after 3 days of culture, or levels of supernatant IgA after 6 days, both showed induction of IgA only in the presence of DC loaded *in vivo* with commensal bacteria. IgA induction was always greatest in the presence of T cells, although B cells and DC loaded with live commensals were sufficient for the response: In contrast no IgA induction occurred in cultures where DC were isolated from mice challenged *in vivo* with heat-killed *E. cloacae* (*Macpherson* and *Uhr*, 2004).

To see whether IgA was also induced *in vivo* by challenge with commensal bacteria, we carried out a protocol of repeatedly dosing mice by gavage with 10^8 *E. cloacae* every third day for a month. Control mice were gavaged with the same preparation of *E. cloacae* that had been split, and heat-killed, prior to administration. Immunohistochemistry and quantitative ELISPOT assessments of the small intestinal mucosa after this conditioning protocol showed that intestinal IgA levels were increased over 4 fold as a result of treatment with live, but not heat-killed, *E. cloacae* (*Macpherson* and *Uhr*, 2004). We have not yet determined whether the reason that heat killed *E. cloacae* does not work in this induction protocol, is that the heat killed organism is less effectively translocated epithelium into the dendritic cells, or whether it is important that live organisms per say are taken up by dendritic cells for induction of IgA by the adjacent B cells.

We did evaluate the IgA response to repeated treatments with *E. cloacae* in C57BL/6 mice that had been treated over a month previously by mesenteric adenectomy. The levels of IgA following *E. cloacae* conditioning were essentially identical in C57BL/6 mice whether or

not they had mesenteric lymph nodes, suggesting that the critical phase of the IgA induction occurred in the Peyer's patches, or that in the absence of mesenteric lymph nodes other secondary lymphoid organs are able to take over any maturation or amplification process that normally occurs within the mesenteric lymph nodes (*Macpherson* and *Uhr*, 2004). However, as described in the previous section, in the absence of the mesenteric lymph nodes it is possible for bacterially loaded dendritic cells to traffic to systemic secondary lymphoid organs, with the result that there is dramatic lymphoid hyperplasia in both the spleen and lymph nodes, and specific serum IgG responses against commensals are induced.

Our interpretation of these experiments was that normally the inductive response for IgA is focused on the Peyer's patches because of the local exposure to live commensal bacteria at that level. Mesenteric lymph nodes are not vital for *in vivo* induction of IgA per se, but are required to prevent trafficking of bacterial-loaded dendritic cells to central lymphoid sites where they provoke superfluous, and possibly harmful, immunologic reactions.

FUNCTIONAL CONSEQUENCES OF INTESTINAL IgA SECRETION

To examine the functional consequences of IgA induction we have carried out two different sorts of experiments. In the first, we used bacterial recolonisation of previously germ-free mice to examine the way in which production of secretory antibodies effects the penetration of intestinal bacteria. Experiments like this have been carried out previously, for example by Cebra and his colleagues who inoculated *Morganella morganii* into germ-free mice and followed the kinetics of bacterial translocation and IgA induction (*Shroff* et al., 1995). In order to extend these observations to the functional consequences of intestinal antibody secretion *in vivo*, we first rederived the $J_H^{-/-}$ antibody deficient strain [on the C57BL/6 background (*Chen* et al., 1993)] germ-free by aseptic embryo transfer. We then housed germ-free wild type C57BL/6 and $J_N^{-/-}$ mice together in a single cage and introduced a single C57BL/6 mouse carrying an SPF flora. We used an SPF sentinel to introduce a balanced bacteria flora, to avoid the possibility that a single species would behave unphysiologically without any microbial competition during recolonisation. We found that there was a short period of overgrowth of both aerobes and anaerobes at day 2, after which luminal bacterial densities settled down to steady state levels, that were not significantly difference whether or not the animals secreted antibodies. However, in the antibody-deficient germ-free animals undergoing recolonisation, we did measure increased aerobic bacterial translocation to the mesenteric lymph nodes peaking at day 6 and lasting until day 60 (*Macpherson* and *Uhr*, 2004). This shows that the natural IgA (produced prior to the inductive effect of commensal bacteria colonising the intestine) as well as induced IgA is effective in limiting the penetration of commensal bacteria beneath the epithelial cell layer. It also shows that although antibodies are reduce the levels of bacterial penetration during the recolonisation process, the tissues (at the level of the mesenteric lymph nodes) become sterile by day 70, indicating that antibody secretion is only one of a number of redundant mechanisms capable of limiting the ingress of bacteria below the epithelial layer.

A further experimental setup to look at the effect of antibodies in limiting

penetration of commensal organisms was to challenge either wild type C57BL/6 mice or antibody-deficient SPF $J_H^{-/-}$ mice with different doses of *E. cloacae* given into the intestine. Because in this second experiment all mice were SPF they already contained a limited flora of intestinal bacteria prior to the challenge dose of *E. cloacae* being given. The results were that with each of the graded challenge doses of *E. cloacae*, a smaller proportion of organisms penetrated as far as the mesenteric lymph nodes in mice that were secreting intestinal antibodies (*Macpherson* and *Uhr*, 2004). Moreover, if the experiment was done with C57BL/6 mice where the IgA levels had been increased by the repeated conditioning protocol referred to above, the numbers of organisms reaching the mesenteric lymph nodes was consistently lower at all doses than unconditioned C57BL/6 mice. This also indicates that antibody secretion is protective against overall penetration of commensal bacteria. We did not find evidence in our experiments for alterations in luminal densities of commensal organisms, either spontaneously or after challenge. However, these experiments are measuring overall levels of penetration, and do not exclude an effect of IgA in promoting uptake of bacteria through small areas of specialised epithelial such as the M cells.

CONCLUSIONS

Our data suggest that dendritic cells in the inductive lymphoid structures of the intestine sample live commensal bacteria, and that these loaded dendritic cells are then responsible for induction of mucosal immune responses. The experiments have been carried out with challenge doses of intestinal bacteria, and it is currently an assumption that the lower levels of spontaneous translocation of intestinal bacteria induce the mucosal immune system by a similar mechanism. Intestinal delivery of live bacteria is required to induce IgA although this may be because of better translocation rather than a requirement for endocytosis of live organisms per se. Even with high recurrent challenge doses of commensals, live organisms within dendritic cells do not penetrate further than the mesenteric lymph nodes, so the inductive effect is focused primarily on the mucosal immune system, rather than in systemic lymphoid structures. IgA has an overall protective effect against commensal bacterial exposure, but this is just a part of a multilayered system that adapts the mucosa to the presence of the microflora.

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THE INNATE AND ADAPTIVE IMMUNE SYSTEM OF THE INTESTINAL EPITHELIUM

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SUMMARY

It is increasingly being appreciated that innate immune mechanisms play a key role for the tuning of adaptive immunity in the gut and maintenance of mucosal homeostasis. Innate responses use preformed and rapidly synthesised effectors and sensors that lead to spontaneous activation or modulation of cellular functions, aiming at elimination of microbial challenges and return of the local tissue to a basal state with minimal pathology. Efficient adaptive immunity depends on postnatal 'education' of specific immune cells and induction of immunological memory in processes that are significantly modulated by innate immune responses. The intestinal immune system has through evolution developed two layers of adaptive non-inflammatory defence: Immune exclusion provided by secretory antibodies to limit epithelial penetration and host invasion of microorganisms as well as other potentially dangerous antigens; and immunosuppressive mechanisms to inhibit overreaction against innocuous luminal antigens – often referred to collectively as 'oral tolerance'. Both these strategies depend on co-operation of adaptive immune mechanisms with the innate defence system, including crosstalk between the intestinal epithelium and various lamina propria cells. This review focuses on immunological mechanisms taking place at the epithelial level.

INTRODUCTION

The body is under constant threat of attack by viruses, bacteria and parasites. Evolution has therefore provided mammalians with several complex and potent layers of defence. Microorganisms have inhabited Earth for at least 2.5 billion years, and the power of immunity is a result of co-evolution in which particularly the commensal bacteria have shaped the body's defence functions (*Hooper* and *Gordon*, 2001; *Bäckhed* et al., 2005). In humans, the critical role of the immune system becomes clinically apparent when it is defective. Thus, inherited and acquired immunodeficiency states, or more subtle immunoregulatory defects, are characterised by increased susceptibility to infectious diseases that are sometimes caused by the commensal microbiota which is normally considered to be non-pathogenic (*Haller* and *Jobin*, 2004; *Sansonetti*, 2004; *Yan* and *Polk*, 2004).

The immune system can be divided into two general arms: Innate (natural or non-specific) and adaptive (acquired or

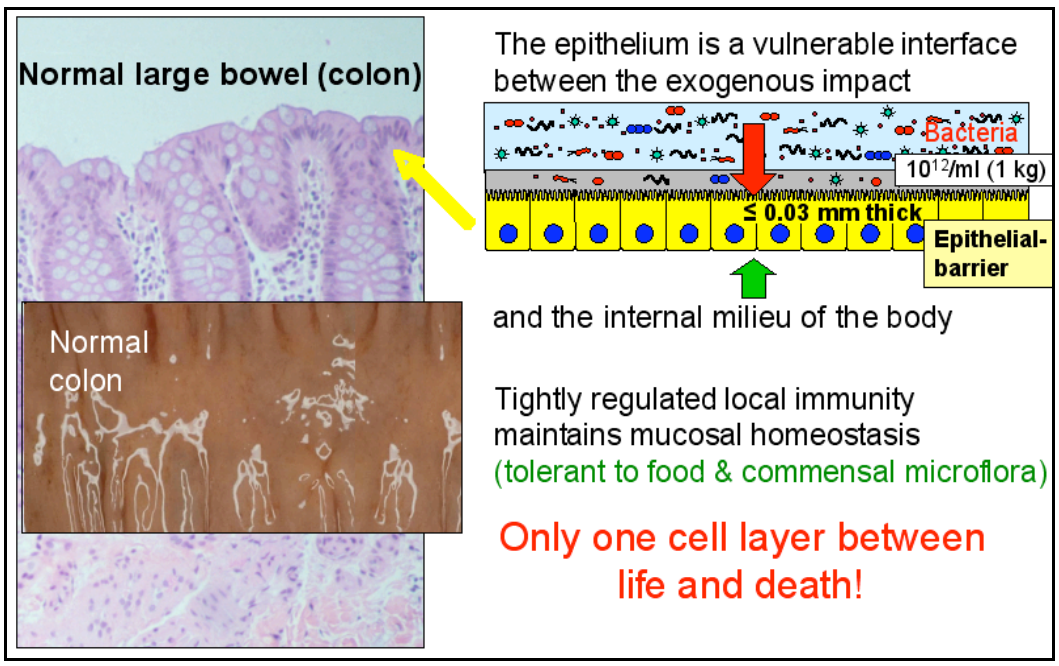

Figure 1: Mucosal homeostasis is maintained in the gut by tightly regulated immune mechanisms. Left panel: Histology and macroscopic picture of normal colon. Right panel: Schematic depiction of the monolayered epithelium covered by mucus and its enormous bacterial challenge, which can cause deadly disease if the epithelial barrier deteriorates.

specific) immunity, which work together synergistically (*Chaplin*, 2003; *Sompayrac*, 2003). Notably, the adaptive immune system developed rather late in the phylogeny, and most species survive without it. However, this is not true for mammalians, which have an extremely sophisticated adaptive immune system of both systemic and mucosal (local) type. There appears to be a great redundancy of mechanisms in both systems providing robustness to ensure that essential defence functions are preserved.

The success of such a complex overall strategy is evidenced by the fact that most humans have a normal gut. This is indeed remarkable because the enormous surface area (some 300 m^2 in an adult) is covered only by a monolayered, quite vulnerable epithelium exposed to an enormous load of commensal bacteria (Figure 1). Tightly regulated local immunity is clearly needed to maintain mucosal homeostasis (*MacDonald* and *Monteleone*, 2005).

INNATE IMMUNITY

The attempt of an infectious agent to enter the body will immediately be recognised and counteracted by the innate immune system (*Chaplin*, 2003), which comprises surface barriers, soluble factors, professional phagocytes, and dendritic cells (DCs). Together, these functions constitute a primary layer of natural defence against invading microorganisms, with the common goal of restricting their entry into the body by providing: (a) physical/structural hindrance and clearance mechanisms (epithelial linings of skin and mucosae,

mucus, ciliary function, peristalsis); (b) chemical factors (pH of body fluids, numerous antimicrobial proteins and peptides such as lysozyme, lactoferrin, peroxidase, and defensins); and (c) phagocytic cells (e.g. neutrophils, eosinophils, monocytes/macrophages, and immature DCs). Challenges of the innate system often lead to activation of adaptive immunity, which aids substantially the recovery from infection, as discussed below.

Triggering of innate immunity

The recognition molecules involved in innate immunity are encoded in the germline (*Chaplin*, 2003). Basically, this system is therefore quite similar among healthy individuals and shows no apparent memory effect; re-exposure to the same pathogen will normally elicit more or less the same type of response. The actual cellular receptors sense microbial molecular structures that are conserved and often essential for survival of the microorganisms. Such structures include for instance endotoxin or lipopolysaccharide (LPS), teichoic acid, peptidoglycan, and unmethylated CpG motifs of DNA (*Beutler* and *Rietschel*, 2003); together they are traditionally called pathogen-associated molecular patterns (PAMPs) but they also occur in commensal bacteria (*Medzhitov*, 2001) and are therefore preferably called microbe-associated molecular patterns (MAMPs).

It remains unclear whether the intestinal microbiota through its MAMPs induces a distinct molecular program in the innate immune system, which could explain that the indigenous microbiota is normally tolerated by the host (*Nagler-Anderson*, 2001; *Philpott* et al., 2001). The relative absence of microbial recognition structures from the apical surface of normal gut epithelium (see later), as well as potential attenuation of their signalling by certain commensal bacteria (e.g., NF-κB-mediated responses), also appear of crucial importance to this end (*Kobayashi* et al., 2002; *Haller* and *Jobin*, 2004; *Rakoff-Nahoum* et al., 2004; *Kelly* et al., 2004, 2005).

The cellular receptors of the innate immune system that recognise PAMPs or MAMPs as activation signals are called pattern recognition receptors (PRRs), many of which belong to the so-called Toll-like receptors (TLRs). PRRs are expressed mainly by macrophages and DCs, but also by a variety of other cell types including T and B cells as well as epithelial cells (see later).

Engagement of PRRs with their intracellular signalling pathways causes cellular activation; in the case of DCs, this leads to maturation accompanied by production of various cytokines and up-regulation or down-regulation of surface molecules according to strictly defined kinetics (*Ricciardi-Castagnoli* and *Granucci*, 2002). Such cellular modulation will critically influence further development of both innate and adaptive immunity.

In summary, there are both stereotypical and selective responses of innate host cells to different types of microorganisms. In this manner, they can imprint their 'signatures' on the immune system. Thus, the plasticity of the innate system prepares the ground for a targeted and powerful function of subsequent adaptive immune responses (*Liew*, 2002).

ADAPTIVE IMMUNITY

T and B cells

In peripheral blood, the lymphocytes comprise 20-25% of the leukocytes. All T lymphocytes (or T cells) express selectively cluster of differentiation (CD)3, and all B lymphocytes (or B cells) ex-

press selectively CD19 and CD20 molecules. A particular subset of T lymphocytes usually called T-helper (Th) cells expresses CD4, whereas most cytotoxic T cells express CD8. Adaptive immunity depends on the functional properties of T and B cells and is directed by their antigen-specific surface receptors, which show a random and highly diverse repertoire (*Chaplin*, 2003; *Sompayrac*, 2003).

Lymphocytes originate in the bone marrow from a common lymphoid stem cell. Further development and maturation of T and B cells occur in the thymus and bone marrow, respectively (so-called primary lymphoid organs). Mature but yet unprimed ('naïve') T and B cells enter the bloodstream and become disseminated to secondary lymphoid organs such as the spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT).

Certain adhesion molecules and receptors for chemokines (chemo-attractant cytokines) enable adherence of immune cells to specialised vascular endothelium and their migration into the lymphoid organs, which are anatomically and functionally organised to facilitate interactions between professional antigen-presenting cells (APCs) such as mature DCs and lymphocytes (*Kunkel* and *Butcher*, 2002; *Brandtzaeg* and *Pabst*, 2004; *Brandtzaeg* and *Johansen*, 2005). Antigens are carried into these immune-inductive structures from epithelial surfaces via draining lymph – passively as soluble molecules or particles (e.g. infectious agents), and actively by migrating DCs – as well as directly from mucosal surfaces by 'membrane' or 'microfold' (M) cells in MALT (*Neutra* et al., 2001).

Each T and B cell bears antigen receptors with a certain specificity, which differs between individual clones of lymphocytes (*Chaplin*, 2003). A clone consists of daughter cells derived by proliferation from a single ancestor cell ; so-called clonal expansion. The total population of T and B cells in a human may be able to recognise some 1011 different antigens. This remarkably diverse antigen receptor repertoire is generated during lymphocyte development by random rearrangement of a limited number of receptor genes. Even without priming, the adaptive immune system would thus be able to respond against an enormous number of antigens, but the detection of any single antigen could be limited to relatively few lymphocytes, perhaps only 1 in 1,000,000.

It follows from the above that a primary immune response usually generates an insufficient number of specific lymphocytes to eliminate the invading pathogen. However, when an antigen receptor is engaged by its corresponding antigen, the lymphocyte generally becomes activated (primed), ceases temporarily to migrate, enlarges (blast transformation) and proliferates rapidly so that, within 3-5 days, there are numerous daughter cells – each specific for the antigen that initiated the primary immune response.

Such antigen-driven clonal expansion accounts for the characteristic delay of several days before adaptive immunity becomes effective in defending the body against an infection. In addition to the effector cells generated by clonal expansion and differentiation, also so-called memory cells are generated; these may be very long-lived and are the basis of immunological memory characteristic of adaptive immunity (*Chaplin*, 2003). Functionally, immunological memory enables a more rapid and effective secondary (or 'anamnestic') immune response upon re-exposure to the same antigen. In contrast to innate immunity, the antigen recognition profile of the adaptive immune system reflects the individual's lifetime exposure to stimuli from infectious agents and other antigens, and will consequently differ among individuals.

Immune response and immune reaction

Induction of immune responses

The purpose of adaptive immunity is primarily to combat infections by preventing colonisation of pathogens and keeping them out of the body (immune exclusion), and also to seek out specifically and destroy invading microorganisms (immune elimination). In addition, specific immune responses are, through regulatory mechanisms, involved in avoidance of overreaction (hypersensitivity or allergy) against harmless antigens as well as discrimination between components of 'self' and 'non-self' (*Chaplin*, 2003). Autoimmunity occurs when the latter control mechanism breaks down.

Both the primary and secondary adaptive immune responses depend on professional APCs, which express major histocompatibility complex (MHC) class II determinants (in humans: HLA-DR, -DQ and -DP) as genetically determined restriction elements for $CD4^+$ Th cells (*Chaplin*, 2003). In this manner, the T-cell receptors can specifically recognise short immunogenic peptide sequences of the APC-processed antigen that each is presented in the polymorphic grove of an MHC molecule. The immune response may also involve polymorphic MHC class I molecules and $CD8^+$ T cells with cytotoxic and/or suppressive potential (*Chaplin*, 2003). All of these cell categories are present in secondary lymphoid organs and at immunological effector sites where the primed immune cells extravasate by means of homing molecules, which differ markedly between the systemic and the mucosal immune system (*Kunkel* and *Butcher*, 2002; *Brandtzaeg* and *Johansen*, 2005).

A long-lasting secondary immune response gives rise to abundant differentiation of effector cells and release of biologically active substances, aiming at neutralisation and elimination of antigens through a variety of targeted strategies. Such immunological effector mechanisms, and the non-specific biological amplification often triggered by them via hyperactivation of innate immunity, are collectively referred to as immune reactions. Adaptive immunity is thus based on specific immune responses but expressed by an array of cellular and humoral immune reactions.

The effector cells of the B-cell system are the terminally differentiated antibody-producing plasma cells that provide so-called humoral immunity. Antibodies are constituted by five immunoglobulin (Ig) classes (IgG, IgA, IgM, IgD, and IgE). The antigen-specific receptor on the surface of the B cells is a membrane-bound form of Ig produced by the same cell (*Chaplin*, 2003; *Sompayrac*, 2003). Engagement of surface Ig by corresponding antigen will – in cooperation with 'help' provided by cognate Th cells – initiate B-cell differentiation and clonal expansion. The resulting effector B cells can then differentiate into plasma cells that secrete large amounts of antibody with the same specificity as that of the antigen receptor expressed by the progenitor B lymphocyte. While IgM (primary response) and IgG (secondary response) dominate systemic humoral immunity, IgA is normally the predominant antibody class of mucosal immunity (see later).

Immune reactions

Immune reactions aim at immune elimination – a term that refers to mechanisms involved in removal of foreign material that has penetrated the epithelial barrier. Thus, when required, immune elimination can constitute a 'second line' of mucosal defence that depends partly on systemic immune effectors such as serum-derived antibodies in addition to locally produced antibodies, often operating in combination with cytotoxicity intraepithelial $CD8^+$ T cells. Immune elimination is generally enhanced by innate non-specific amplifi-

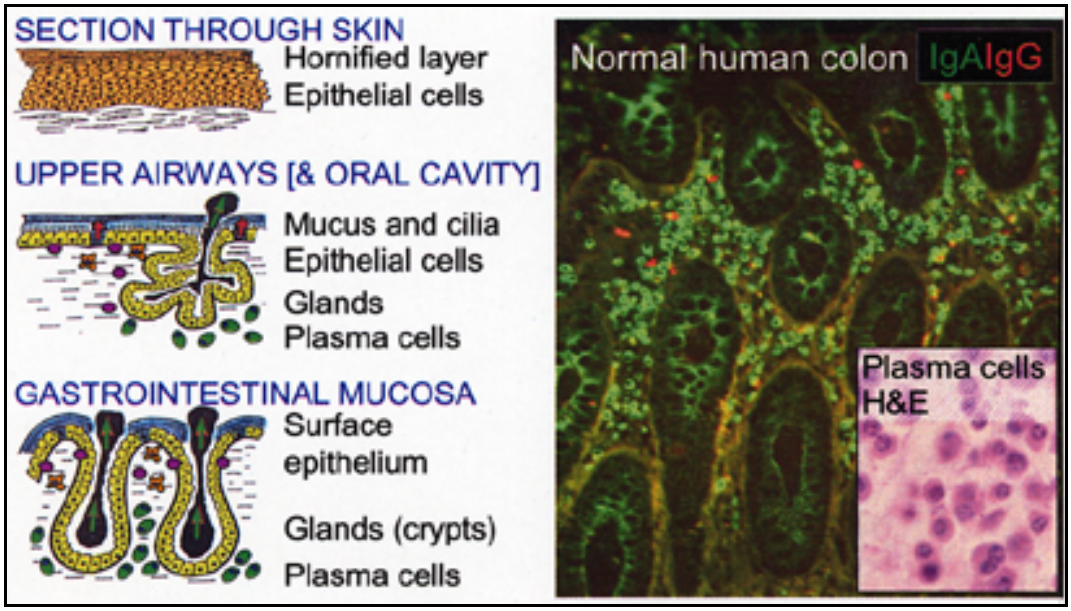

Figure 2: The cellular basis of secretory immunity. Left panel: Schematic depiction of cross-section through gastrointestinal and airway mucosae compared with skin. Because of their specialised function, mucosal epithelia are generally monolayered and protected by various innate defence mechanisms and a sophisticated B-cell system with IgA-producing plasma cells that co-operate with an export system for secretory antibodies (green arrows). Right panel: Paired immunofluoresence staining of IgA- and IgG-producing plasma cells in normal human colon, showing the remarkable dominance of the former class and its export through crypt epithelium outside of the goblet cells which are black. Immunofluoresence photograph from the author's laboratory.

cation mechanisms generating various themes of inflammation.

This scenario may evolve into overt immunopathology and inflammatory disease if satisfactory antigen removal and immunoregular control are not rapidly achieved (*Brandtzaeg* et al., 2005; *MacDonald* and *Montelone*, 2005). Such an adverse development is apparently part of the pathogenesis in various gut disorders including food allergy, coeliac disease, and inflammatory bowel disease (IBD).

MUCOSAL IMMUNITY

Homeostasis-promoting mechanisms

Mucosal immunity can be viewed as a first line of defence that aims at reducing the need for systemic immunity, which can engage forceful pro-inflammatory mechanisms to enable immune elimination when required to save life. This scenario can be reviewed as a 'two-edged sword' which may cause immunopathology and tissue damage as discussed above.

During evolutionary modulation, the mucosal immune system has generated two non-inflammatory layers of defence: (a) immune exclusion performed by secretory antibodies to restrict epithelial

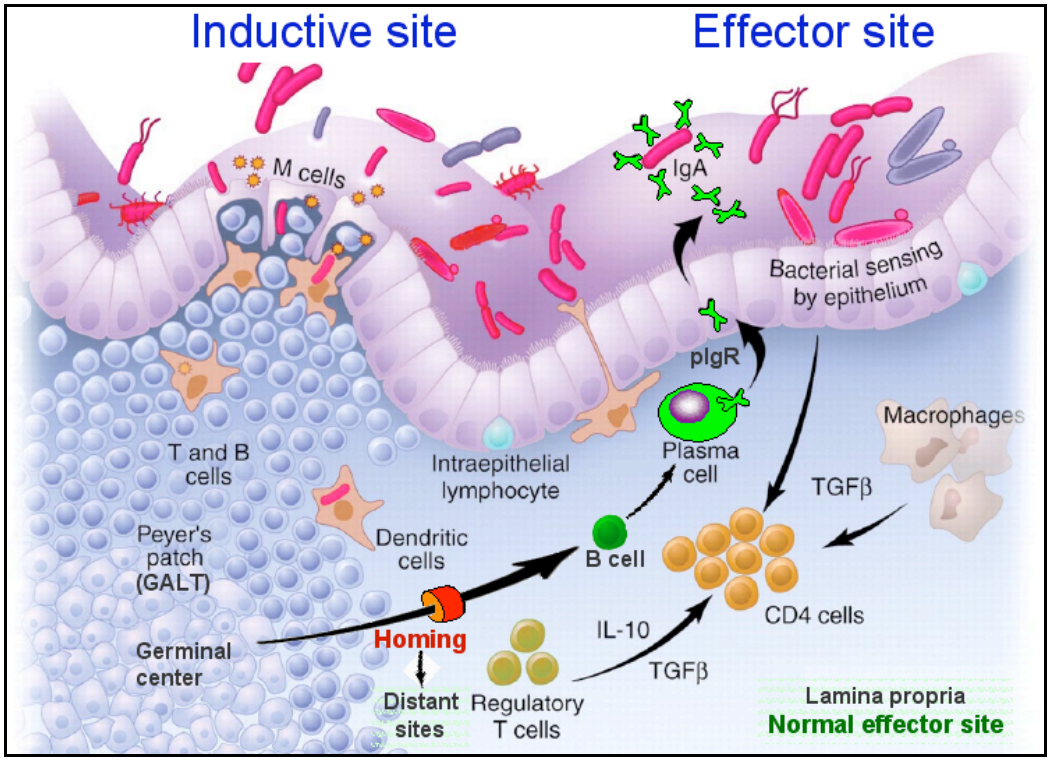

Figure 3: Illustration of inductive site for intestinal immunity (left) represented by Peyer's patch which in numbers up to 250 makes up a major part of the human gut-associated lymphoid tissue (GALT). The follicle-associated epithelium contains antigen-sampling M cells. The germinal centre of activated follicle generates memory/effector B cells which, together with primed mucosal T cells, home to mucosal effector sites, constituted largely by the intestinal lamina propria. Here the T cells perform immune regulation and provide various cytokines which, together with bacteria sampled from the lumen by dendritic cells as indicated, provide second signals for B cells. The latter differentiate into plasma cells mainly producing dimeric IgA which is exported to the lumen by the polymeric Ig receptor (pIgR) to become secretory IgA. Antibodies of this class then coat commensal bacteria. The antibody export may be enhanced by cytokines and epithelial sensing of bacteria (see Figure 7). Modified from *MacDonald* and *Monteleone* (2005).

penetration and host invasion of microorganisms as well as other potentially dangerous antigens; and (b) immunosuppressive mechanisms to avoid local and peripheral hypersensitivity to innocuous luminal antigens. The latter mechanisms are collectively referred to as 'oral tolerance' when induced via the gut (*Brandtzaeg*, 1996; *Mowat*, 2003) and probably explains why overt and persistent allergy to food proteins is relatively rare (*Bischoff* et al., 2000). A similar down-regulatory tone of the immune system normally develops against antigenic components of the commensal intestinal microbiota (*Duchmann* et al., 1997; *Helgeland* and *Brandtzaeg*, 2000; *Moreau* and *Gaboriau-Routhiau*, 2000).

Oral tolerance appears be a rather robust adaptive immune function in view of the fact that more than a ton of food may pass through the gut of an adult every year. This results in a substantial uptake of intact antigens, usually without causing harm. However, the neonatal period is particularly critical, both with

regard to infections and priming for allergic disease, because the mucosal barrier function and the immunoregulatory network are poorly developed for a variable period after birth (*Holt*, 1995; *Brandtzaeg*, 2002). Notably, the postnatal development of mucosal immune homeostasis depends on the establishment of a normal microbiota as well as on adequate timing and dose of dietary antigens when first introduced (*Brandtzaeg*, 1996, 1998; *Moreau* and *Gaboriau-Routhiau*, 2000).

The intestinal IgA system that provides secretory immunity is the best-studied part of mucosal immunity (Figure 2). In fact, the gut mucosa contains at least 80% of the body's activated B cells – terminally differentiated to Ig-producing blasts and plasma cells (*Brandtzaeg* and *Johansen*, 2005). Their generation depends on complex mechanisms of B-cell induction and homing as discussed below.

Immune stimulation in mucosa-associated lymphoid tissue

Inductive mucosal tissue structures

Gut-associated lymphoid tissue (GALT) constitutes a major part of MALT and comprises Peyer's patches, the appendix and numerous solitary or isolated lymphoid follicles (*Brandtzaeg* et al. 1987, 1999; *Brandtzaeg* and *Pabst*, 2004; *Brandtzaeg* and *Johansen*, 2005). All these structures are believed to represent inductive sites contributing to intestinal immune responses, while the lamina propria and epithelial compartment principally constitute effector sites (Figure 3).

The domes of GALT are covered by a characteristic follicle-associated epithelium, which contains antigen-sampling M cells. These very thin and bell-shaped specialised epithelial cells transport effectively live and non-proliferating antigens (especially particles) from the gut lumen into the organised lymphoid tissue (*Neutra* et al. 2001). Many enteropathogenic infectious agents use the M cells as portals of entry, so they represent extremely vulnerable parts of the surface epithelium. However, such 'gaps' in the epithelial barrier are needed to facilitate efficient induction of mucosal immunity.

GALT structures resemble lymph nodes with B-cell follicles, intervening T-cell zones and a variety of APCs such as macrophages and DCs, but there are no afferent lymphatics (*Brandtzaeg* and *Pabst*, 2004). Exogenous stimuli therefore come directly from the epithelial surfaces mainly via the M cells, probably aided by DCs which may penetrate the epithelial layer with their processes (*Rescigno* et al. 2001). Induction and regulation of mucosal immunity hence takes place primarily in GALT but also to some lesser extent at the effector sites (Figure 3).

Priming and dispersion of intestinal B cells

Antigens are presented to naïve T cells in GALT by APCs after intracellular processing (degradation) to immunogenic peptides. In addition, luminal peptides may be taken up and presented by B-lymphocytes and epithelial cells to subsets of intra- and sub-epithelial T lymphocytes (*Brandtzaeg* et al. 1999). Both professional mucosal APCs, B cells, and the small-intestinal villous epithelium, as well as the follicle-associated epithelium of GALT surrounding the M cells, express MHC class II molecules – in humans particularly HLA-DR (see later) – in addition to classical and non-classical MHC class I molecules (*Brandtzaeg* et al. 1987; *Christ* and *Blumberg*, 1997). As discussed previously, such molecules are essential for an antigen-presenting function (*Chaplin*, 2003). Interestingly, MHC class II-expressing naïve and memory B-lymphocytes abound juxtaposed to the M cells. Such B cells may present antigens efficiently to T cells in

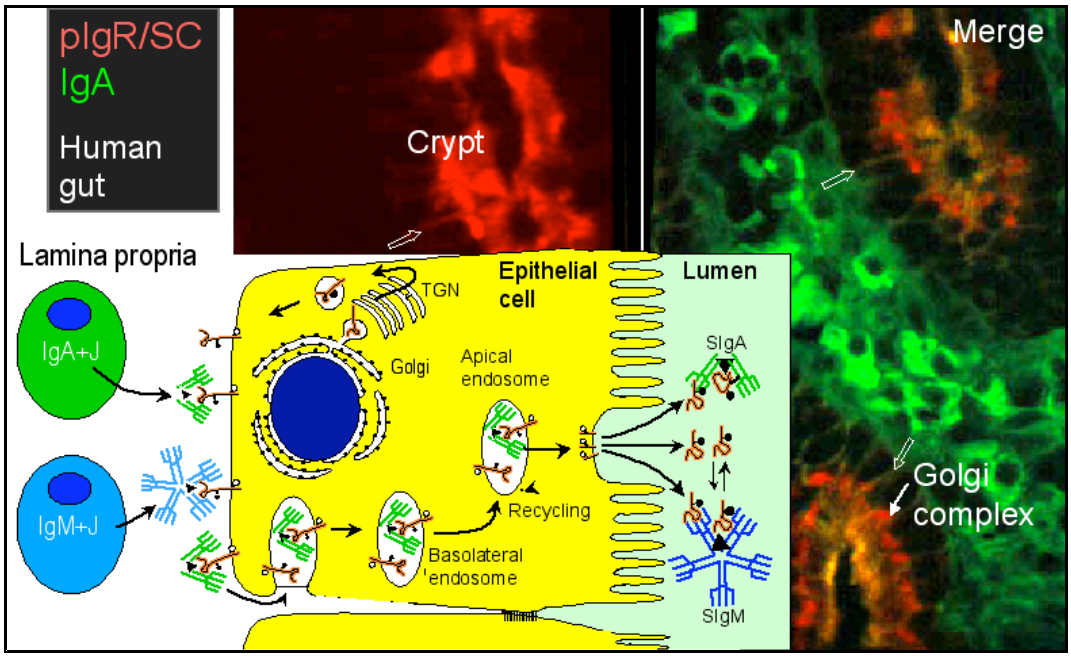

Figure 4A: Epithelial export of secretory antibodies. In the background is shown paired immunofluoresence staining for pIgR/SC (left panel) together with IgA (right panel, merge) from crypt region of human gut mucosa. Superimposed is a schematic representation of the various steps involved in the generation of SIgA and SIgM as explained in the text. Transmembrane SC is synthesized in the rough endoplasmic reticulum and sorted through the Golgi complex/trans-Golgi network (TGN) where it is revealed as pure red immunofluoresence (right panel, solid arrow). The mature form is terminally glycosylated (solid dots), them phosphorylated (open dots) and expressed basolaterally as functional pIgR (open arrows in both immunofluoresence panels). J chain-containing dimeric IgA (IgA+J) and pentameric IgM (IgM+J) are produced by lamina propria plasma cells, and both ligands are endocytosed and transcytosed by pIgR to reach recycling apical endosomes (yellow colour in right panel). Here pIgR is cleaved to release SIgA and SIgM to the lumen complexed with bound SC as well as free SC derived from unoccupied pIgR. Covalent stabilisation of SIgA occurs by disulfide exchange with bound SC (one S-S bridge indicated), whereas free SC in excess is needed to retain non-covalently bound SC in SIgM (dynamic equilibrium indicated).

cognate down-regulatory or immunostimulatory interactions (*Brandtzaeg* et al. 1999).

T cells primed in GALT release cytokines such as transforming growth factor (TGF)-ß and interleukin (IL)-10, which may drive the differentiation of mucosal B cells to predominantly IgA-committed plasma blasts, although their regulation still remains unclear (*Brandtzaeg* et al. 2001; *Fagarasan* and *Honjo*, 2003; *Brandtzaeg* and *Johansen*, 2005). Most B cells primed by 'first signals' in GALT structures migrate rapidly via draining lymphatics to mesenteric lymph nodes where they are further stimulated (*Brandtzaeg* and *Pabst*, 2004; *Brandtzaeg* and *Johansen*, 2005); they may then reach peripheral blood and become seeded by preferential homing mechanisms into distant mucosal effector sites, particularly the intestinal lamina propria where they finally develop to Ig-producing plasma cells (Figure 3).

This terminal differentiation is modulated by 'second signals' from lamina propria $CD4^+$ T cells, antigen-

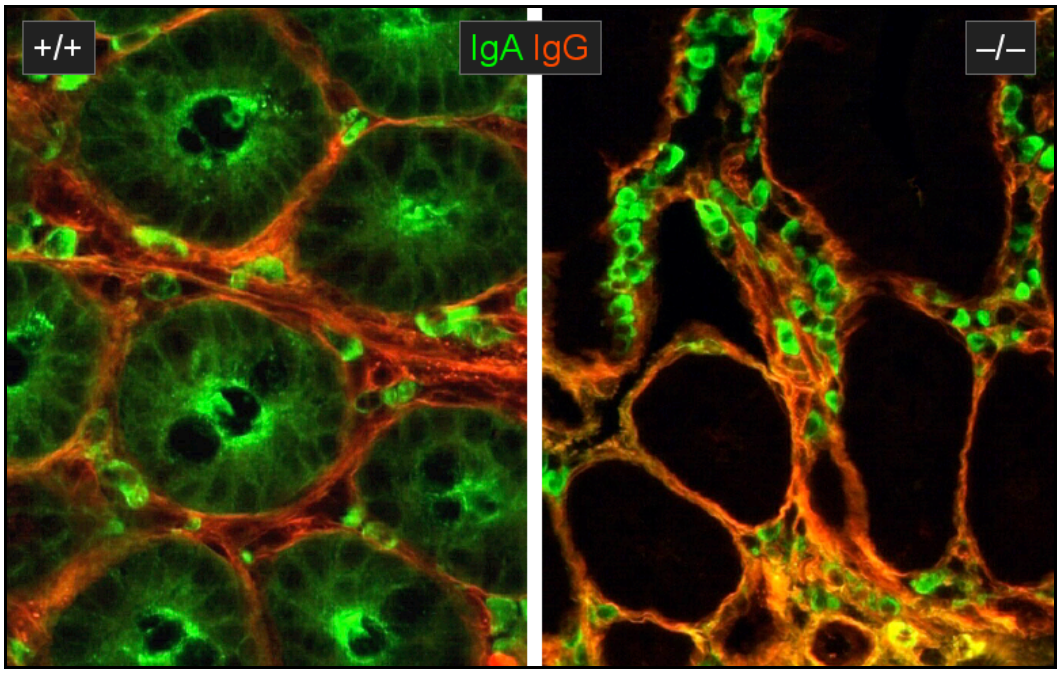

Figure 4B: Epithelial export of secretory antibodies. Paired immunofluoresence staining for IgA and IgG in gut of wild-type mouse (+/+) and pIgR knockout mouse (-/-), the latter showing no epithelial uptake of IgA in cross-section of colonic crypts despite a large number of IgA-producing plasma cells in the lamina propria. There is a high level of serum-derived IgG surrounding the crypts, but no visible epithelial uptake of this isotype. Immunofluoresence photographs from the author's laboratory.

sampling DCs, and available cytokines (Figure 3). Most B cells included in the homing to mucosal effector sites apparently belong to clones of an early maturation stage, as indicated by their high level of J chain (see later) regardless of concomitant isotype – although the IgA class normally predominates (*Brandtzaeg* et al., 1999). J chain-containing dimeric IgA and pentameric IgM are finally translocated to the lumen as secretory IgA (SIgA) and secretory IgM (SIgM) by the polymeric Ig receptor (pIgR) as discussed below. In the lumen, SIgA will coat commensal bacteria (Figure 3) as first demonstrated almost 40 years ago (*Brandtzaeg* et al., 1968). This coating reduces their access to the epithelial surface and protects against bacterial overgrowth and invasion (*Macpherson* et al., 2005).

Antibody-mediated mucosal defence

Secretory immunity and immune exclusion

As alluded to above, the unique and efficient export of SIgA and SIgM antibodies to the epithelial surfaces is mediated by pIgR, also known as membrane secretory component (SC), which is cleaved and sacrificed as bound SC to stabilise the secretory antibodies in the lumen (Figure 4A). The ligand site specific for this receptor depends on the small peptide called 'joining' or J chain that is incorporated selectively into dimeric IgA and pentameric IgM (*Brandtzaeg*, 1974a,b; *Brandtzaeg* and *Prydz*, 1984; *Brandtzaeg* and *Johansen*, 2005). It has been estimated that such pIgR-mediated transport of dimeric IgA on average results in the daily delivery of approximately 3 g of SIgA into the

Table 1: Antimicrobial effects of SIgA antibodies

- Perform non-inflammatory extracellular and intracellular immune exclusion by inhibiting epithelial adherence and invasion
- Polyreactive ('innate-like') activity provides cross-protection
- SIgA (particularly SIgA2) is quite stable (bound SC stabilizes both isotypes)
- Mucophilic and lectin-binding properties (via bound SC in both isotypes and mannose in IgA2)
- Dimer provides efficient agglutination and virus neutralisation

intestinal lumen of an adult human (*Conley* and *Delacroix*, 1987). SIgA acts as the first line of adaptive immune defence against microorganisms and other antigens, and regulates the host response to pathogens as well as commensal bacteria.

Notably, however, in face of the indigenous microbiota, the intestinal IgA system of mice behaves in a somewhat 'primitive' or 'innate-like' way by exhibiting high capacity, broad specificity, and low affinity (*Macpherson* et al., 2005; *Brandtzaeg* and *Johansen*, 2005). Even in humans, SIgA antibodies show a substantial level of cross-reactivity (*Bouvet* and *Fischetti*, 1999). The mucosal IgA system is therefore well designed to provide antibody-mediated immune exclusion of the extremely diverse intestinal microbiota with a multitude of redundant epitopes, while at the same time allowing a persistent host-commensal bacterial mutualism.

Defence functions of SC and secretory antibodies

Free SC is generated by apical cleavage and epithelial release of unoccupied pIgR (Figure 4A). Interestingly, free SC has been shown to possess several innate immune properties (*Phalipon* and *Corthésy*, 2003). This fact suggests that it phylogenetically has originated from the natural defence system like many other proteins involved in adaptive immunity. Importantly, SC has been exploited as the only identifiable epithelial receptor involved in secretory immunity as documented by the phenotype of pIgR knockout mice (*Johansen* et al., 1999), which have no active external transport of dimeric IgA and pentameric IgM (Figure 4B).

A crucial protective role of secretory antibodies is supported by the fact that such mice have 'leaky' mucosal epithelia (*Johansen* et al., 1999). Also, similarly to J-chain knockout mice that likewise lack both SIgA and SIgM, they show decreased resistance against bacterial toxins and early colonisation of the gut epithelium by pathogens (*Lycke* et al., 1999; *Uren* et al., 2005).

Altogether, animal and cell culture experiments have suggested that SIgA antibodies promote intestinal homeostasis by neutralising viruses and bacterial products through non-inflammatory mechanisms at various levels (Table 1). In addition to traditional luminal neutralisation, it has been demonstrated that dimeric IgA antibodies – when exported by pIgR – can remove antigens from the lamina propria and neutralise viruses within the epithelium or block their transcytosis through polarised epithelial cells (*Mazanec* et al., 1993, 1995; *Burns* et al., 1996; *Bomsel* et al., 1998; *Alfsen* et al., 2001; *Robinson* et al., 2001; *Feng* et al., 2002; *Huang* et al., 2005). It has also been reported that dimeric IgA can neutralise bacterial LPS within intestinal epithelial cells (*Fernandez* et al., 2003), suggesting a novel intracellular, non-cytotoxic and anti-inflammatory role for this antibody class during its export.

Although SIgA is the chief effector of immune exclusion, SIgM also contributes – particularly in the new-born

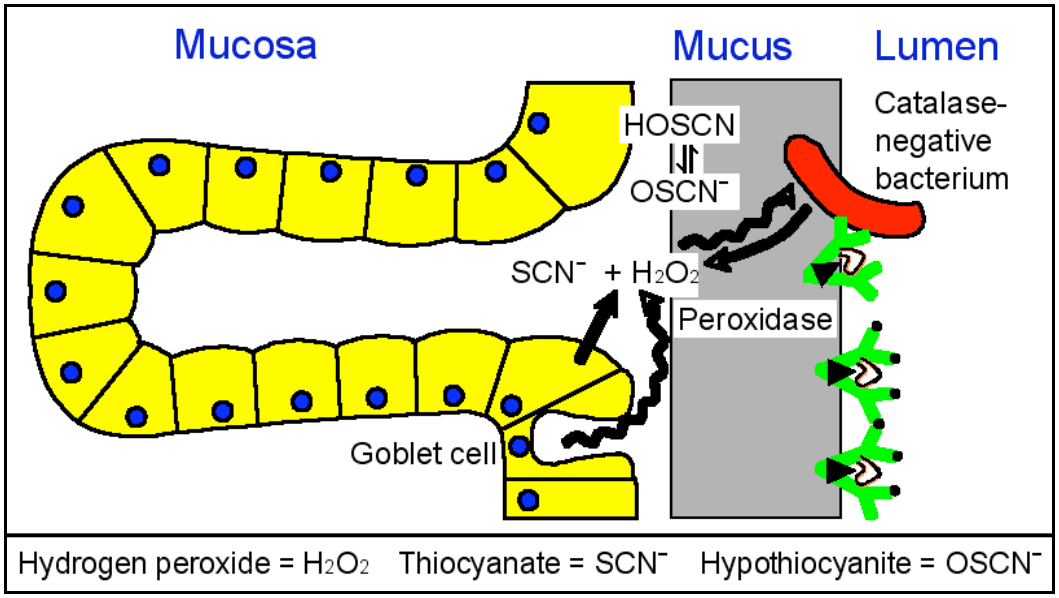

Figure 5: Schematic illustration of the co-operation between SIgA and the peroxidase defence system in mucus layer of colonic mucosa. Details are discussed in the text.

period and in IgA deficiency (*Brandtzaeg* and *Nilssen*, 1995). In addition, there may be some contribution to immune exclusion by serum-derived or locally produced IgG antibodies transferred passively to the lumen by paracellular leakage (*Persson* et al., 1998) or perhaps to some extent exported by the neonatal Fc receptor (FcRn) expressed by the gut epithelium (*Yoshida* et al., 2004). Notably, however, because IgG is complement-activating, its contribution to surface defence is potentially pro-inflammatory, which could jeopardise the epithelial barrier function (*Brandtzaeg* and *Tolo*, 1977).

Interactions between secretory immunity and innate defence factors

Several studies have shown that mucosal immunity may be enhanced by co-operation between SIgA and innate defence factors. Thus, SIgA antibodies can clearly promote the bacteriostatic effect of lactoferrin, apparently by inhibiting bacterial production of iron-chelating agents which may interfere with its function (*Rogers* and *Synge*, 1978).

SIgA can also promote the broad antimicrobial spectrum of the peroxidase defence system, independently of antibody specificity (*Tenovuo* et al., 1982) – probably because of the mucophilic properties of bound SC in SIgA (*Phalipon* and *Corthésy*, 2003) which, by its cross-reactivity, retains bacteria for prolonged and focused action of the enzyme with its biocidal product hypothiocyanate (Figure 5). Human milk is rich in both lactoperoxidase and leukocyte-derived myeloperoxidase (*Moldoveanu* et al., 1982) which survives enzymatically active in gastric juice (*Gothefors* and *Marklund*, 1975). Moreover, peroxidase is produced by colonic goblet cells and therefore appears to be part of the innate intestinal defence (*Venkatachalam* et al., 1970).

There is a possibility that catalase-positive bacteria may resist the oxidising effects derived from H_2O_2 (Figure 5). However, bacterial catalase is restricted to the cytoplasm or periplasm and would therefore not be expected to alter sub-

Table 2: Effect on bacterial overgrowth of co-operation between SIgA and other defence mechanisms as revealed in various clinical conditions with (+) or without (-) defective adaptive or innate immunity

Variables					
• SigA	+	+	-	-	-
• Peristalsis	+	-*	+	+	-*
• Gastric Acid	+	-**	+	-**	-**
Result					
• Jejunal colonisation	-	-	-	-	+

*Complete vagotomy
**Gastric drainage (gastroenterostomy) or pernicious anemia

Modified from *McLoughlin* et al. (1978)

stantially the availability of H_2O_2 as substrate for peroxidase, and some bacteria may even produce H_2O_2. Importantly, catalase-positive pathogens have been shown to be cleared efficiently from the airways *in vivo* by the peroxidase defence system (*Gerson* et al., 2000).

Immune reactions that take place at the epithelial surface may furthermore stimulate the release of mucus from goblet cells and thereby reinforce the mucosal barrier against penetration of soluble molecules and microorganims (*Walker* et al., 1982; *Walker* and *Bloch*, 1983). Through its affinity for bound SC, the mucus may further enhance its 'flypaper' effect – topically retaining SIgA antibodies with complexed antigens (*Lim* and *Rowley*, 1982; *Phalipon* and *Corthésy*, 2003).

Antigens present in immune complexes trapped in the mucus layer are more rapidly degraded by proteolytic enzymes than free antigens (*Walker* et al., 1975). It has been proposed that IgA1 is especially miscible with mucus because of certain structural homologies, whereas the function of IgA2 may take place mainly in the external fluid (*Clamp*, 1980). This may be particularly relevant in the distal gut where there is a predominance of local IgA2 production (*Kett* et al., 1986); and this subclass is also more resistant to proteolytic degradation than IgA1 (*Kilian* et al., 1996).

The co-operation between SIgA and the important innate defence function provided by the 'germicidal barrier' of gastric acid, as well as by intestinal peristalsis, was strikingly demonstrated in a study of bacterial overgrowth in the jejunum of vagotomised patients; mucosal protection deteriorated when IgA deficiency was combined with a suboptimal function of these two non-specific protective mechanisms (*McLoughlin* et al., 1978). As always when IgA is selectively lacking (*Brandtzaeg* and *Johansen*, 2005), the study subjects necessarily had compensatory SIgM in their gut; however, although several antimicrobial activities have been identified for this antibody class (*Brandtzaeg* et al., 1987), it can clearly not adequately replace SIgA (Table 2). This is also shown by the fact that IgA-deficient subjects have raised levels of serum IgG antibodies to dietary antigens and an increased frequency of allergic and autoimmune disorders, including coeliac disease (*Brandtzaeg* and *Nilssen*, 1995). The same is claimed to be true for Crohn's disease, with some 20-fold increased incidence (L. Hammarström, personal communication).

Neonatal mucosal immunity

In contrast to several animal species, the human foetus acquires circulating

Table 3: Colonisation of germ-free mice with *Bacteriodes thetaiotaomicron* increases intestinal expression of genes involved in epithelial barrier function and nutritient absorption

Gene product	Fold Δ over germ-free
Barrier function	
• Polymeric Ig receptor (pIgR/SC)	2.6 ± 0.7
• Small proline-rich protein 2a (sprr2a)	205 ± 64
• Decay-accelerating factor (DAF)	5.7 ± 1.5
Nutrient absorption	
• Na+/glucose co-transporter (SGLT-1)	2.6 ± 0.9
• Co-lipase	6.6 ± 1.9
• Liver fatty acid-binding protein (L-FABP)	4.4 ± 1.4

Modified from Hooper et al. (2001)

maternal IgG via the placenta and not as a result of breast-feeding. Also notably, intestinal uptake of SIgA antibodies from breast milk is of no importance for systemic immunity in humans, except perhaps in the preterm neonate (*Brandtzaeg*, 2002). Although so-called 'gut closure' normally occurs in humans mainly before birth, the mucosal barrier function may not be fully established until after 2 years of age; the different variables involved in this process remain poorly defined.

Only occasional traces of SIgA and SIgM occur in human intestinal fluid during the first postnatal period, whereas some IgG is often present – reflecting bulk transfer from the lamina propria, which after 34 weeks of gestation contains readily detectable maternal IgG (*Brandtzaeg* et al., 1991). IgA-producing plasma cells are normally undetectable in the intestinal mucosa before the infant is 10 days of age, but thereafter a rapid increase takes place – IgM often remaining predominant up to 1 month. Little increase of intestinal IgA production usually takes place after 1 year. A much faster establishment of secretory immunity may be seen in developing countries because of a more massive mucosal exposure to microorganisms (*Brandtzaeg* et al., 1991).

Critical role of breast-feeding in infancy

At least 90% of all pathogens use the mucosae as portals of entry. Thus, mucosal infections are a major killer below the age of 5 years – being responsible for more than 14 million deaths of children annually in developing countries. In those parts of the world, infants are highly dependent on SIgA antibodies from breast milk to protect their mucosae; epidemiological data suggest that the risk of dying from diarrhoea is reduced 14-24 times in sucklings (*Anonymous*, 1994).

Although the value of breast-feeding in westernised countries is clinically most apparent in preterm infants, population studies show that exclusively breast-fed infants are in general better protected against a variety of infections and probably also against allergy, asthma and coeliac disease (*Brandtzaeg*, 2002). This strongly suggests that the mucosal barrier function in newborns can be reinforced by breast-feeding. Experiments in neonatal rabbits have convincingly demonstrated that SIgA is a crucial anti-microbial component of breast milk (*Dickinson* et al., 1998), in addition to a variety of other factors that may enhance mucosal homeostasis (*Brandtzaeg*, 2002).

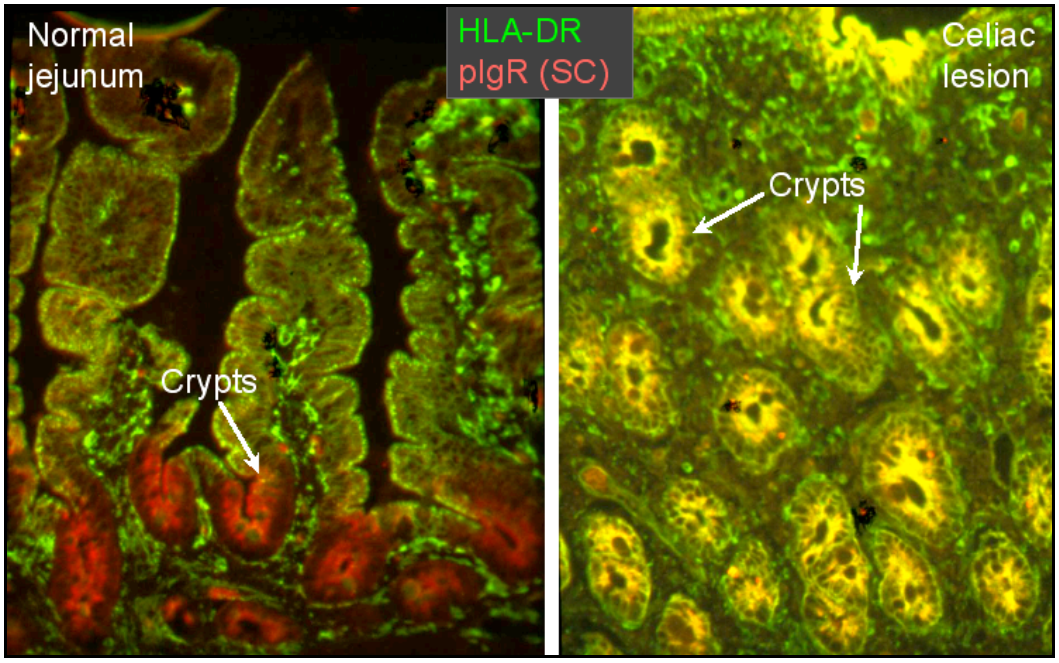

Figure 6: Paired immunofluoresence staining for HLA-DR and pIgR/SC in section of normal human jejunum (left) and jejunal lesion of untreated coeliac disease (right). In the normal state, DR is expressed apically by the villous epithelium as well as by numerous macrophages and dendritic cells in the lamina propria, while pIgR/SC expression is typically restricted to the crypt epithelium. In the coeliac lesion the hyperplastic crypts as well as the surface epithelium show co-expression of DR and pIgR/SC (yellow colour). Both components are presumably up-regulated by cytokines, particularly IFN-γ, derived from activated T cells and macrophages in the lesion (see Figure 7). Immunofluoresence photographs from the author's laboratory.

Regulation of pIgR expression

The postnatal colonisation of commensal bacteria is important both to establish and regulate an appropriate intestinal epithelial barrier function in the gut, including enhanced expression of pIgR (*Neish* et al., 2000; *Hooper* et al., 2001). This has been clearly demonstrated in mouse experiments (Table 3). Epithelial synthesis of pIgR/SC starts in human foetal life as early as around 20 weeks of gestation, but there is a peak of expression during the first couple of postnatal months – best revealed in salivary glands (*Brandtzaeg* et al., 1991) – most likely reflecting microbial colonisation of the mucosae which starts already during the vaginal delivery process.

Collectively, these observations show that the expression of pIgR is both constitutive and subjected to inductive transcriptional up-regulation, as first documented at the molecular level by our laboratory (*Johansen* and *Brandtzaeg*, 2004). Because one molecule of pIgR is consumed for every ligand of dimeric IgA or pentameric IgM transported, regulation of pIgR expression must be crucial for maintenance of intestinal homeostasis.

Coeliac disease is a good example of how the pIgR expression and IgA export can be indirectly enhanced via cytokines produced by activated mucosal T cells and macrophages (Figures 6 and 7, left panel). There is a remarkable level of interferon (IFN)-γ in the untreated

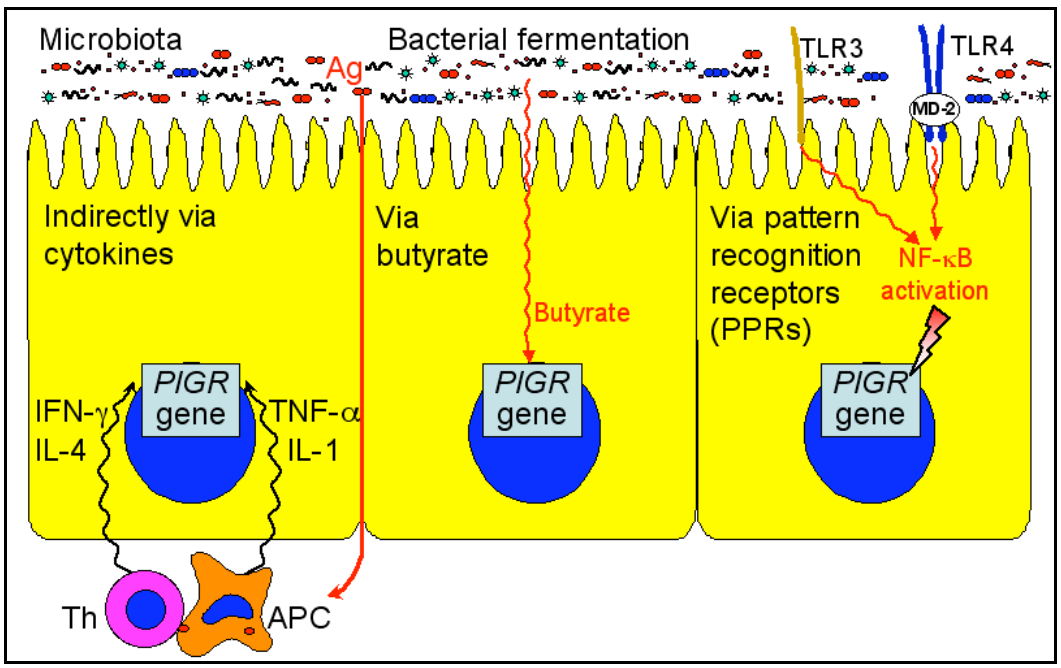

Figure 7: Schematic illustrations of three possible manners in which pIgR expression can be up-regulated by activation of its gene locus *PIGR*. Details are discussed in the text.

coeliac lesion, derived from gluten-specific $CD4^+$ lamina propria T cells (*Nilsen* et al., 1995, 1998) and activated intraepithelial $CD8^+$ T cells (*Olaussen* et al., 2002). Similar up-regulation of pIgR and epithelial IgA export is seen in Sjögren's syndrome and chronic gastritis – supporting the notion that cytokines provide an immunoregulatory link between increased local IgA production and enhanced output of SIgA during low-grade inflammation and infection (*Brandtzaeg* et al., 1992).

Table 4 summarises characteristics of the regulation of human pIgR/SC, and the various putative regulatory DNA elements identified in the PIGR locus are schematically depicted in Figure 8A (*Johansen* and *Brandtzaeg*, 2004). Our laboratory has recently characterised an enhancer region ('enhanceosome') in intron 1 of the human pIgR gene that is sufficient for induction by IL-4 and also contributes to tumour necrosis factor (TNF)-α responsiveness (Figure 8B). The minimal enhancer for IL-4-induced

Table 4: Characteristics of transcriptional regulation of the human *pIgR* gene (locus *PIGR*)

- Constitutively expressed in mucosal and glandular epithelia
- Expression upregulated by immunoregulatory (IFN-γ, IL-4, IL-13) and pro-inflammatory (TNF-α, IL-1) cytokines
- Upregulation of pIgR by cytokines depends on *de novo* protein synthesis
- Both promoter elements and intronic enhancer ('enhanceosome') contribute
- Increased expression correlates with enhanced ability to transport polymeric Igs which contain J chain (dimeric/trimeric IgA and pentameric IgM)

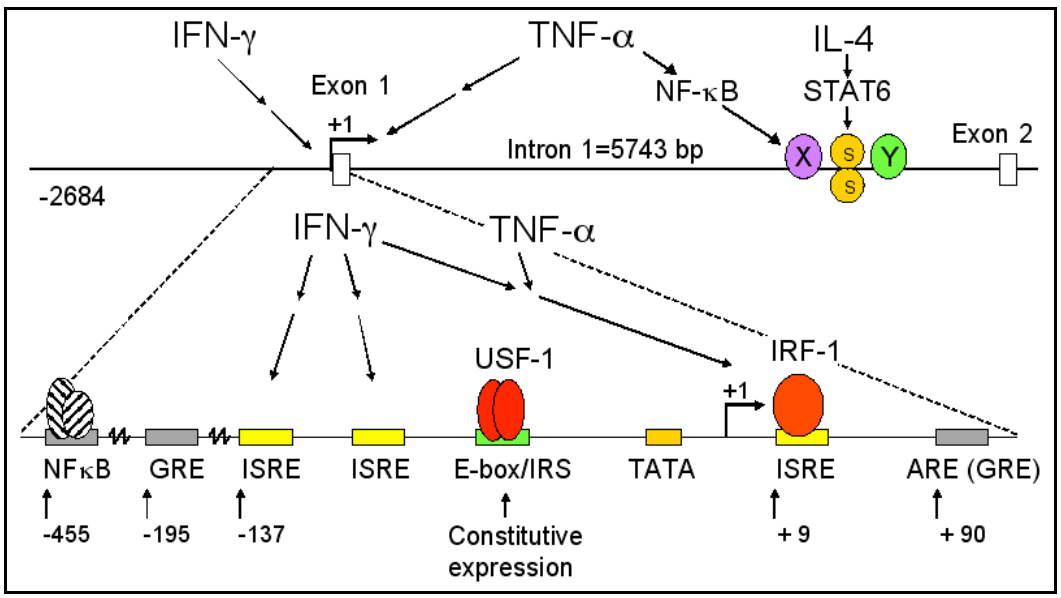

Figure 8A: Regulatory DNA elements in the human *pIgR* gene (locus *PIGR*) and their constitutive and cytokine-induced transcriptional activation. At the top is shown the overall orientation of the promoter, exon 1, and the extensive intron 1 with its pIgR enhancer element that binds signal transducer and activator of transcription (STAT)6 (S/S) and other potential transcriptional regulators (X and Y). DNA elements with some of the identified transcriptional factors in the upstream proximal promoter region and exon 1 are detailed below (GRE = glucocorticoid response element; ISRE = interferon-stimulated response element; USF = upstream stimulatory factor; IRF = IFN regulatory factor; ARE = androgen response element). Based on data from *Schjerven* et al. (2001).

transcription is a 250 bp region located more than 4 kb downstream of the transcription start site and containing at least seven target elements for different DNA-binding factors (*Schjerven* et al., 2000, 2001, 2003; *Johansen* and *Brandtzaeg*, 2004). A binding site for STAT6 (signal transducer and activator of transcription 6) is a crucial element in this enhancer (Figure 8A).

MICROBIAL IMPACT ON MUCOSAL IMMUNE REGULATION

The extended hygiene hypothesis

It is possible that suboptimal development of the SIgA-dependent mucosal barrier function and inadequate tolerance mechanisms together may explain the increasing frequency of certain diseases in industrialised countries – particularly allergies and autoimmune inflammatory disorders (*Brandtzaeg*, 2002; *Yazdanbakhsh* et al. 2002). Interestingly, we have recently demonstrated that milk-allergic children who do not 'grow out' of their allergy after a milk-free diet, have a relatively low level of $CD4^+CD25^+$ regulatory T (Treg) cells (*Karlsson* et al., 2004). This could explain their persistently deficient oral tolerance to cow's milk proteins.

Homeostatic effects of probiotics

On the basis of the so-called extended hygiene hypothesis (*Rautava* et al., 2004), several studies have evaluated the beneficial clinical effect of probiotic

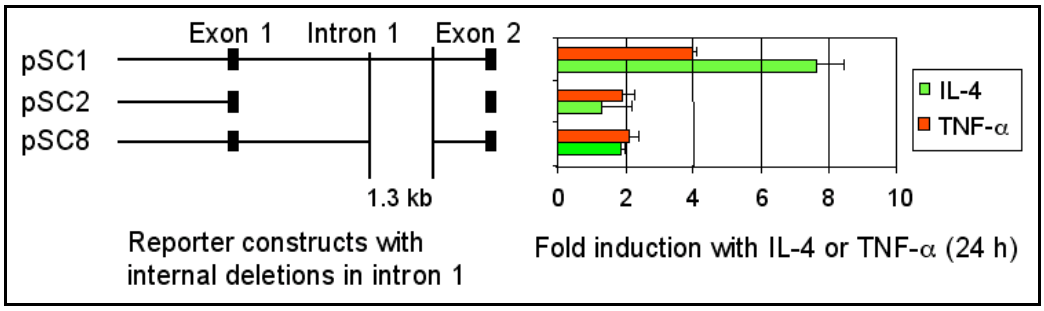

Figure 8B: Regulatory DNA elements in the human *pIgR* gene (locus *PIGR*) and their constitutive and cytokine-induced transcriptional activation. Luciferase reporter assay performed with HT-29 cells transiently transfected with the indicated constructs from the *PIGR* locus (left) and treated with IL-4 or TNF-α to observe induction of pIgR mRNA (right). Deletion of the 1.3-bp intronic enhancer abolishes responsiveness to both cytokines. Based on data from *Schjerven* et al. (2001).

bacterial preparations (*Collins* and *Gibson*, 1999; *Kirjavainen* and *Gibson*, 1999; *Isolauri* et al., 2001). Especially certain strains of commensal intestinal lactic acid bacteria (LAB), particularly lactobacilli and bifidobacteria, have been reported to enhance IgA responses (both in humans and experimental animals – apparently in a T cell-dependent manner (*Yasui* et al. 1995; *Malin* et al. 1996; *Prokesová* et al. 1998, 1999; *Moreau* and *Gaboriau-Routhiau*, 2000). A recent double-blind study of children with a family history of atopy, reported the prevalence of atopic eczema to be reduced by 50% at the age of 2 years after receiving the probiotic Lactobacillus GG strain daily for 6 months (*Kalliomäki* et al. 2001). It remains unknown whether this beneficial effect was mediated via SIgA enhancement or promotion of oral tolerance.

Similarly, there is some hope that immunisation with mycobacterial antigens or bacterial CpG oligonucleotides might skew the cytokine profile towards Th1 and thereby – through cross-regulation – dampen Th2-dependent allergic (atopic) symptoms (*von Reyn* et al., 1997; *Hopkin* et al., 1998; *Wohlleben* and *Erb*, 2001). New-borns are in fact able to mount a Th1-type immune response when appropriately stimulated (*Marchant* et al., 1999). It is also possible that DNA from probiotic bacteria may induce Treg cells that enhance the immunoregulatory homeostasis because certain strains of LAB appear to act as well by subcutaneous or peritoneal injection as by the oral route (*Sheil* et al., 2004; *Foligné* et al., 2005a,b).

Microbial enhancement of pIgR expression

Up-regulation of pIgR expression by microbes and their metabolic products is another intriguing possibility to enhance homeostatic immune functions in the gut as alluded to above. A role for commensal bacteria to this end was suggested by the observation that intestinal pIgR mRNA levels were increased almost three-fold when germ-free mice were colonised with *Bacteroides thetaiotaomicron*, a prominent bacterium of the intestinal flora (Table 3). Also, it was recently observed that infection with reovirus up-regulates expression of pIgR in the human colonic epithelial cell-line HT-29 (*Pal* et al., 2005); this might reflect interaction with epithelial PRRs as discussed later. Changes in composition of the intestinal microflora may thus explain the reported roles for passive and adaptive immunity in the ontogeny of pIgR expression in mice (*Jenkins* et al., 2003).

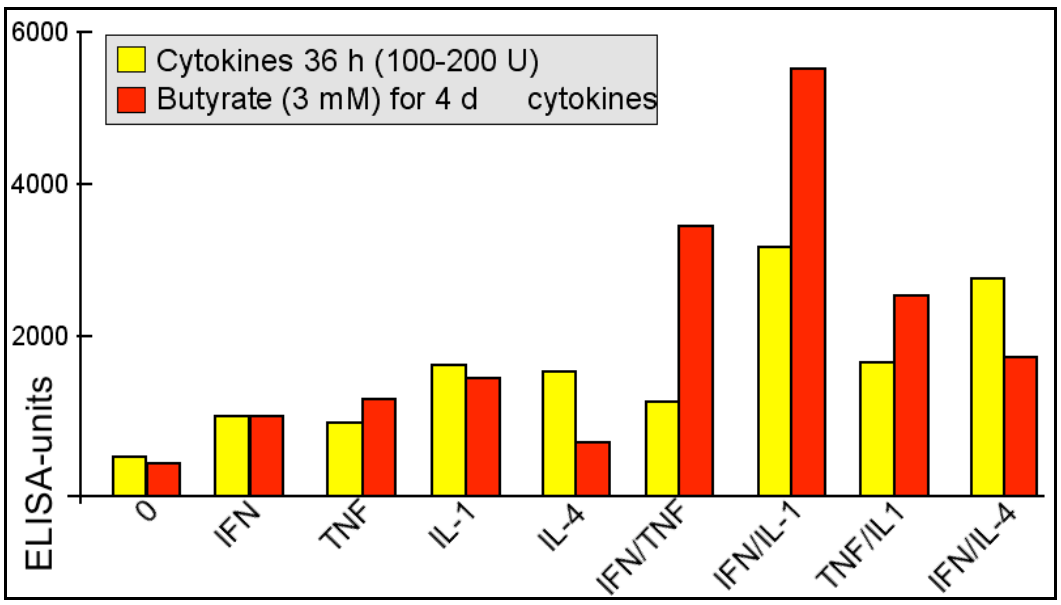

Figure 9A: Effects of butyrate and prebiotics on pIgR expression in gut epithelial cells. Pre-incubation of HT-29 cells for 4 days with butyrate as indicated enhances the pIgR-inducing effects (shown as ELISA-units) of TNF-α and IL-1 or these cytokines in combination with IFN-γ, whereas the effect of IL-4 is reduced (based on data from *Kvale* and *Brandtzaeg,* 1995).

Altogether, commensal bacteria may be important for maintaining the 'tone' of intestinal pIgR expression above its constitutive level. This notion is in keeping with a role for certain strains of commensal gut bacteria to enhance homeostatic immunoregulatory mechanisms – observed directly by administration of probiotics such as LAB (see earlier) or indirectly by adding prebiotics to the diet. Oligosaccharides, for instance, can both promote the growth of LAB and act as a substrate for intestinal formation of butyrate (C-C-COONa) – a fermentation product of many anaerobic bacteria in the normal colonic microbiota. Butyrate is an important energy source for colonic epithelial cells and, notably, it can increase gene transcription levels through specific DNA sequences (*Glauber* et al., 1991).

In this context (Figure 7, middle panel), it is of considerable interest that when we pre-treated HT-29 cells with butyrate, the effect on pIgR/SC expression induced by some cytokines, and particularly various combinations of cytokines, was remarkably enhanced (Figure 9A) – with the exception of the effect of IL-4 which was reduced (*Kvale* and *Brandtzaeg*, 1995). In line with our observations, suggesting an overall positive effect in the distal gut, *Nakamura* et al. (2004) reported that mouse pups receiving dietary fructo-oligosaccharides showed significantly enhanced pIgR expression in ileal and colonic epithelium (Figure 9B), as well as increased export of SIgA into ileal loops and faeces.

Intestinal pattern recognition receptors

The Drosophila gene called Toll gave name to TLRs, which are trans-membrane proteins functioning as PRRs to recognise a variety of microbial constituents or products and trigger innate

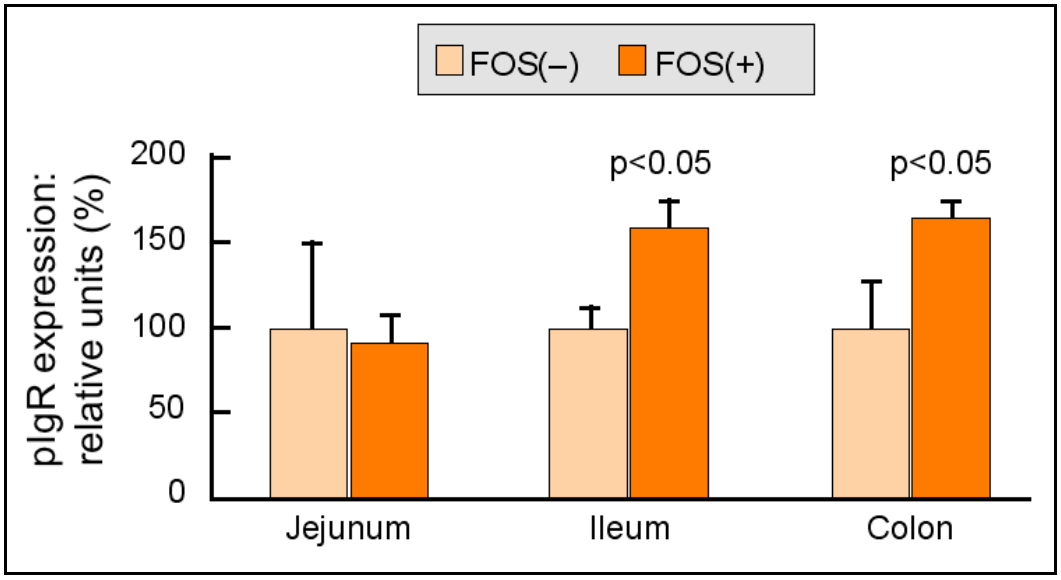

Figure 9: Effects of butyrate and prebiotics on pIgR expression in gut epithelial cells. Effect of dietary fructo-oligosaccharides (FOS) on the expression of pIgR (shown as relative units) in the distal gut of mouse pups (based on data from *Nakamura* et al., 2004).

cellular responses (*Medzhitov*, 2001; *Akira*, 2003). To date, at least 11 TLRs acting singly or in combination have been identified (*Beutler*, 2004; *Cario*, 2005). TLR4 and TLR2 act as the sole conduits for signalling from LPS – an integral component of the outer membranes of Gram-negative bacteria. The classical LPS receptor CD14 is anchored in the cell membrane by glycosylphosphatidyl-inositol; in complex with its ligand and TLR4 or TLR2, it represents an important link between innate and adaptive immunity. The same is true for other TLRs which recognise additional PAMPs or MAMPs (Figure 10).

This immunoregulatory link operates partly via 'shared' and partly via 'specific' signalling pathways generally leading to NF-κB activation, DC maturation, release of pro-inflammatory cytokines (*Modlin*, 2000; *Kaisho* and *Akira*, 2001; *Medzhitov*, 2001), including the Th1-inducing IL-12 and IL-18, and expression of chemokines and co-stimulatory molecules (*Manigold* et al., 2000; *McInnes* et al., 2000; *Cario*, 2005). Moreover, certain unmethylated CpG motifs of bacterial DNA have been shown to promote Th1-cell activity through interaction with TLR9 (*Klinman* et al., 1996; *Kadowaki* et al., 2001; *Peng* et al., 2001).

Although not yet extensively studied in the human gut, sub-epithelial APCs reportedly express certain TLRs. Thus, mRNA for TLR2 and TLR4 has been detected in isolated lamina propria macrophages (*Smith* et al., 2001), but the proteins were undetectable by immunohistochemistry in the normal state although a substantial fraction of the sub-epithelial macrophage-like putative APCs showed positive staining in IBD lesions (*Hausmann* et al., 2002). Also, only extremely low levels of CD14 are normally present on these cells, and their cytokine response is usually poor after LPS stimulation (*Rugtveit* et al., 1997a; *Smith* et al., 2001).

In IBD, on the other hand, expression of CD14 on recently recruited monocyte-like macrophages is strongly elevated, while the co-stimulatory mole-

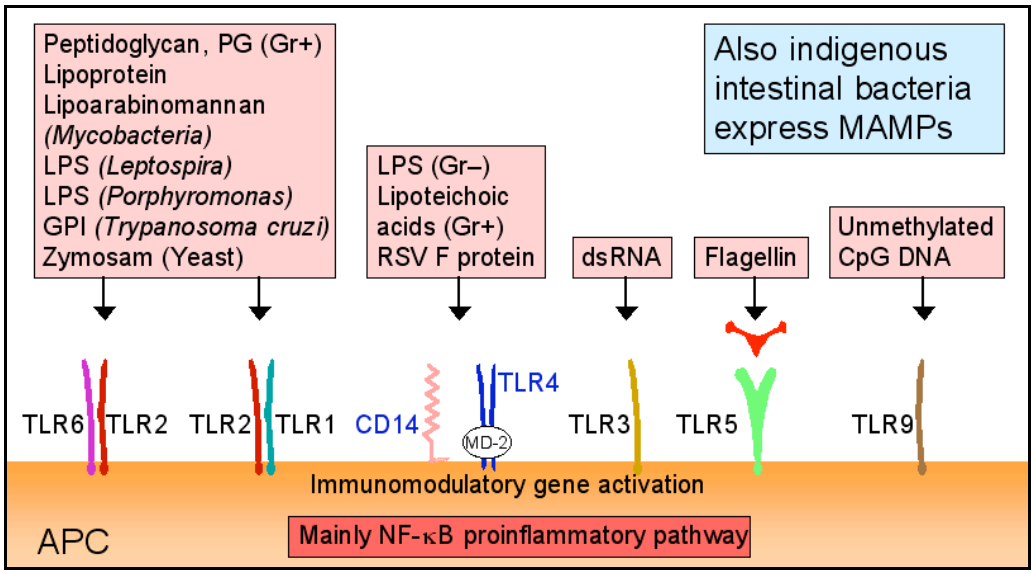

Figure 10: Ligand specificities of Toll-like receptors (TLRs). A variety of pathogen-associated molecular patterns (PAMPs), preferably called microbe-associated molecular patterns (MAMPs), are recognised by highly conserved TLRs expressed on the surface of antigen-presenting cells (APCs) and a variety of other cell types. Recognition of endotoxin or lipopolysaccharide (LPS) from Gram-negative bacteria by TLR4 is aided by two accessory proteins, CD14 and MD-2. TLR2 recognises a broad range of structurally unrelated ligands and functions in combination with several (but not all) other TLRs, including TLR1 and TLR6. TLR3 is involved in recognition of double-stranded RNA (dsRNA). TLR5 is specific for bacterial flagellin, whereas TLR9 is a receptor for unmethylated CpG motifs which are abundant in bacterial DNA. Gr+: Gram-positive; Gr-: Gram-negative; GPI: glycophosphoinositol; RSV: respiratory syncytial virus. Adapted from *Medzhitov* (2001).

cules B7.1 (CD86) and B7.2 (CD80) are up-regulated on putative APCs below the surface epithelium (*Rugtveit* et al., 1997b). The LPS-induced pro-inflammatory cytokine response of macrophage-like cells isolated from IBD lesions is concomittantly increased (*Rugtveit* et al., 1997a). Presumably, also the antigen-presenting potential in the lesions is increased because monocyte-derived macrophages can be skewed towards DC differentiation under the influence of TNF-α (*Chomarat* et al., 2003).

Epithelial sensing of microorganisms

The intestinal epithelium appears to have inherent mechanisms to protect itself against activation from the luminal side unless production of pro-inflammatory cytokines and chemokines is needed in defence against invading microorganisms (*Philpott* et al., 2001; *Sansonetti*, 2004; *Yan* and *Polk*, 2004). Thus, epithelial cells apparently possess sensing systems that allow discrimination between pathogenic and non-pathogenic bacteria in order to initiate an inflammatory reaction only when elimination of invading pathogens is needed (Figure 11). Interestingly, non-pathogenic *Salmonella* strains are able to block the NF-κB transcription pathway in human gut epithelial cells *in vitro* and thereby reduce basolateral IL-8 secretion in response to pro-inflammatory stimuli, including apical infection with wild-type *S. typhimurium* (*Neish* et al., 2000).

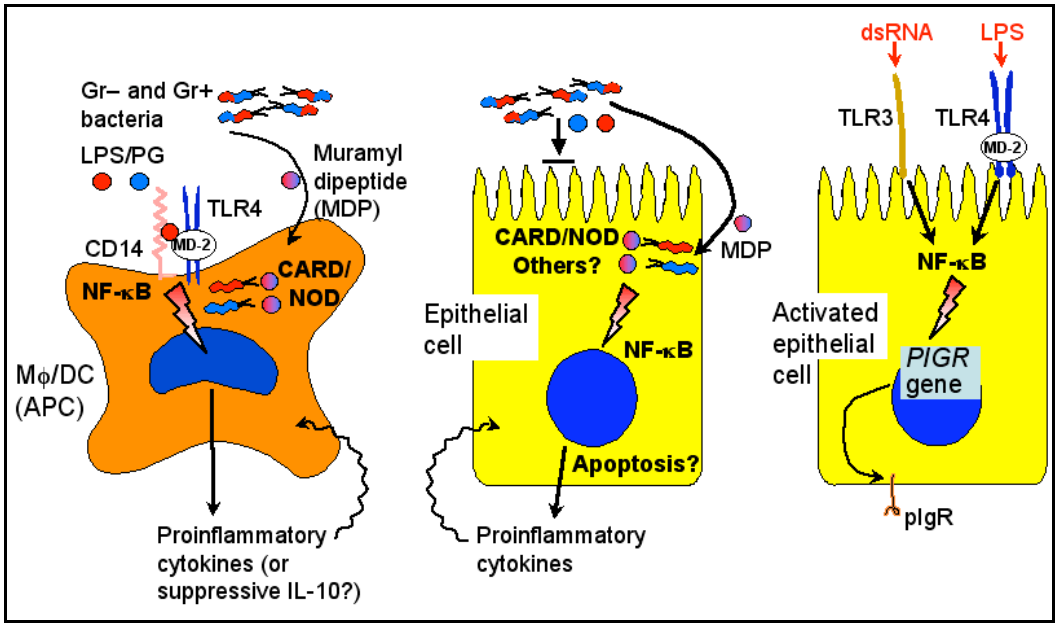

Figure 11: Comparison of the sensing of bacteria and bacterial products by professional antigen-presenting cell (APC) and epithelial cells. Left: TLRs and CD14 expressed by APCs sense Gram-negative (Gr-) and Gram-positive (G+) bacteria or their bacterial products such as lipopolysaccharide (LPS, red dots), peptidoglycan (PG, blue dots), and muramyl dipeptide (MDP, red/blue dots) by APCs; subsequent NF-κB signals may lead to inflammation, apoptosis or immunosuppression. Middle: The mechanisms by which epithelial cells recognise and respond to bacteria remain rather unclear, but they are normally quite refractory to signals from the luminal site; this is probably important for tolerance to commensal bacteria. However, inflammation in response to invasive pathogens promotes bacterial clearance and host survival. One relevant intracellular recognition system is represented by CARD/NOD molecules which sense Gr- or Gr+ bacteria or their MDP motifs. This event leads to secretion of proinflammatory cytokines and cellular activation. Right: Activated epithelial cells show enhanced expression of TLRs. Recognition of double-stranded RNA (dsRNA) and LPS by TLR3 and TLR4, respectively, induces enhanced pIgR expression via activation of the *PIGR* gene (see Figure 12). MØ = macrophage, DC = dendritic cell.

Studies on the expression of CD14 and TLRs on human intestinal epithelial cells have provided inconsistent results, but the HT-29 adenocarcinoma cell line expresses low levels of TLR3 and TLR 4 (*Schneeman* et al., 2005). Double-stranded RNA (dsRNA), a by-product of viral replication, has been identified as a ligand for TLR3 (Figure 10), and epithelial cells can apparently be activated by LPS via TLR4 in a CD14-independent manner (*Böcker* et al., 2003).

The expression of both TLR3 and TLR4 has been shown to be up-regulated on epithelial cells in IBD lesions, particularly so for TLR4 (*Cario* and *Podolsky*, 2000), although others have failed to confirm this by immunohistochemistry (*Hausmann* et al., 2002). While the signalling cascades for TLR3 and TLR4 are similar, recent studies have shown that differential usage of adaptor molecules may result in different biological responses (*Yamamoto* et al., 2002, 2003; *Hoebe* et al., 2003; *Oshiumi* et al., 2003).

In collaboration with our laboratory, Dr. C.S. Kaetzel's group recently showed that pIgR mRNA and protein expression was strongly up-regulated in

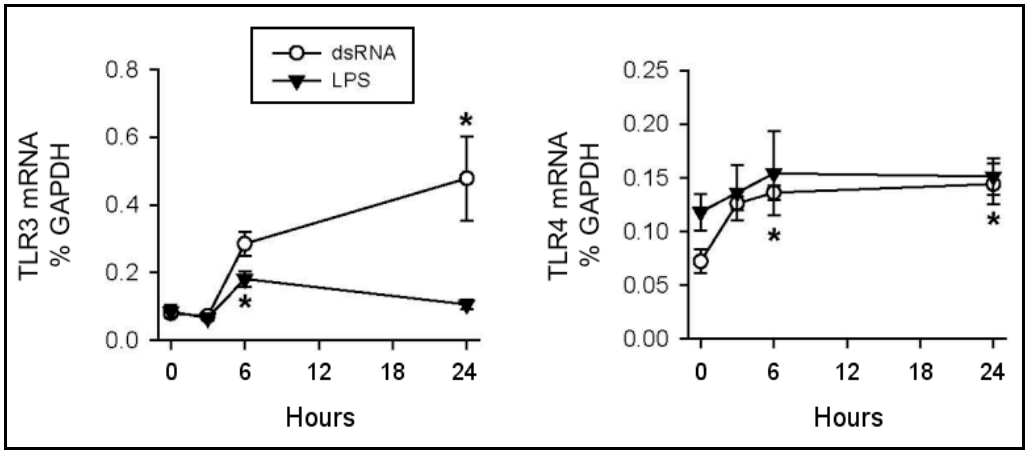

Figure 12A: Differential expression of mRNA for TLR3 and TLR4 and their ligand-induced effect on pIgR expression in HT-29 cells via the *PIGR* intronic-1 enhancer. Levels of TLR3 (left) and TLR4 (right) mRNA in HT-29 cells stimulated with double-standard RNA (dsRNA, 100 μg/ml) or lipopolysaccharide (LPS, 1 μg/ml) for 24 hours, normalised to the mRNA level of 'house-keeping' gene (GAPDH).

response to both dsRNA/TLR3 and LPS/TLR4 signalling in HT-29 cells (*Schneeman* et al., 2005). By contrast, dsRNA but not LPS increased the mRNA level for TLR3 and TLR4 (Figure 12A), although the protein expression of both receptors was enhanced by the respective ligands; this suggested that TLR4 could have been transported to the cell surface from intracellular stores.

A binding site for NF-κB in the previously discussed intron 1 enhancer of the pIgR gene (Figure 8) was shown by reporter assays with differently mutated cDNA constructs from the PIGR locus to be critical for transcriptional activation in response to TLR3 and TLR4 signalling (Figure 12B). Analysis of several cytokine/chemokine gene products, including IL-8, demonstrated that TLR3 signalling resulted in a more pronounced pro-inflammatory response than did TLR4. These data suggested that signalling through TLR4 up-regulates pIgR expression while minimising initiation of inflammation.

Altogether, epithelial TLR engagement by microbes and their products might serve to augment pIgR expression (Figure 11, right panel) and thereby enhance export of SIgA, thus linking the innate and adaptive immune responses to viruses and bacteria. The differential epithelial activation induced via TLR3 and TLR4 implies that particularly the latter receptor may promote IgA-mediated homeostasis in the presence of commensal Gram-negative bacteria.

Experiments in mice have suggested that also other epithelial TLRs are involved in maintenance of intestinal homeostasis (*Rakoff-Nahoum* et al., 2004; *Bambou* et al., 2004). In addition, evidence is accumulating to suggest that such homeostasis is significantly influenced by cross-talk between epithelial cells and underlying lamina propria cells, particularly macrophages and DCs (*Haller* et al., 2000; *Rimoldi* et al., 2005).

Defects in innate immune mechanisms may jeoparise mucosal homeostasis

It follows from the available information that defects in innate immune mechanisms should predispose to abro-

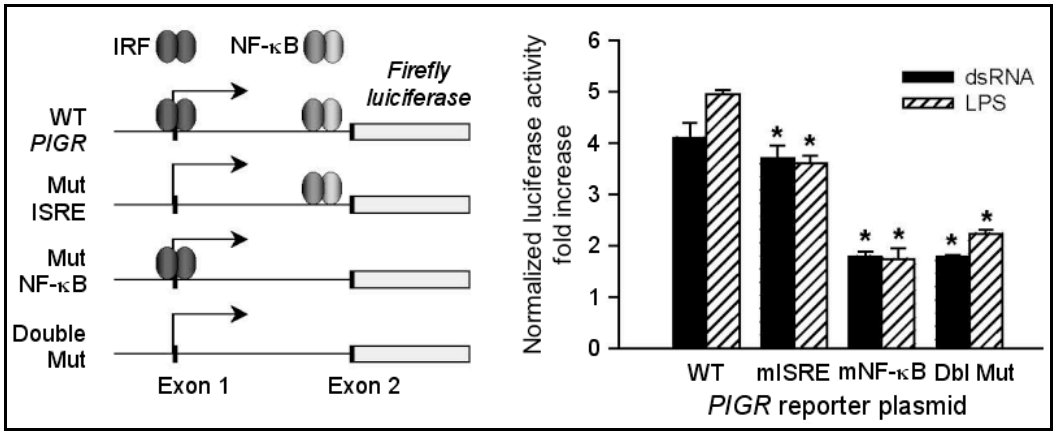

Figure 12B: Differential expression of mRNA for TLR3 and TLR4 and their ligand-induced effect on pIgR expression in HT-29 cells via the *PIGR* intronic-1 enhancer. Lusiferase reporter assay performed in HT-29 cells transiently transfected with the indicated constructs from the *PIGR* locus (left), wild-type (WT) or mutated (Mut/m), and treated for 24 hours with dsRNA (100 μg) or LPS (1 μg/ml) to observe induction of pIgR mRNA (right). Although reduced pIgR expression was observed after mutating the interferon-stimulated response element (ISRE) that binds IFN regulatory factor (IRF) in exon 1, a more striking effect was obtained by mutating the NF-KB element in the intronic enhancer, with no further effect seen after double (Dbl) mutation of the two elements. Asterisks indicate significant differences from untreated cells ($p<0.05$). Based on data from *Schneeman* et al. (2005).

gated mucosal homeostasis. The best proof of principle in this respect has been provided by the NOD2 family of intracellular sensor molecules carrying a C-terminal leucine-rich repeat domain; its PRR activity recognises unique muramyl dipeptide (MDP) motifs of peptidoglycans from both Gram-negative and Gram-positive bacteria (*Cario*, 2005).

The NOD2 (CARD15) gene is encoded by the IBD susceptibility locus (IBD1) on chromosome 16, and certain mutations of this gene is associated with clinical subsets of Crohn's disease patients, showing a striking dose effect of mutations that affect the PPR function of the molecules (*Hugot* et al., 2001; *Ogura* et al., 2001; *Abreu* et al., 2002; *Ahmad* et al., 2002; *Cuthbert* et al., 2002; *Hampe* et al., 2002; *Mathew* and *Lewis*, 2004). These seminal observations provide strong support for the possibility that aberrant 'sensing' of the intestinal microbiota is an early pathogenic event – perhaps involving deficient induction of NF-κB-mediated activation of epithelial Paneth cells leading to insufficient defensin and cryptidin production (*Lala* et al., 2003; *Kobayashi* et al., 2005) and impaired intestinal barrier function (Figure 13A).

Imbalanced triggering of innate immunity may in fact be a common theme for disease initiation on a polygenic susceptibility background in the pathogenesis of a spectrum of clinical IBD entities. In this scenario, it has been suggested that NOD2/CARD15-gene mutations may cause deficient signalling for down-regulatory mechanisms in the gut (*Judge* and *Lichtenstein*, 2002; *Bouma* and *Strober*, 2003), which in the normal state maintain lamina propria APCs/DCs in a quiescent state (Figure 13B). One possibility is that the function of NOD2 is to limit the pro-inflammatory effects mediated by TLR2 stimulation by peptidoglycan at the APC

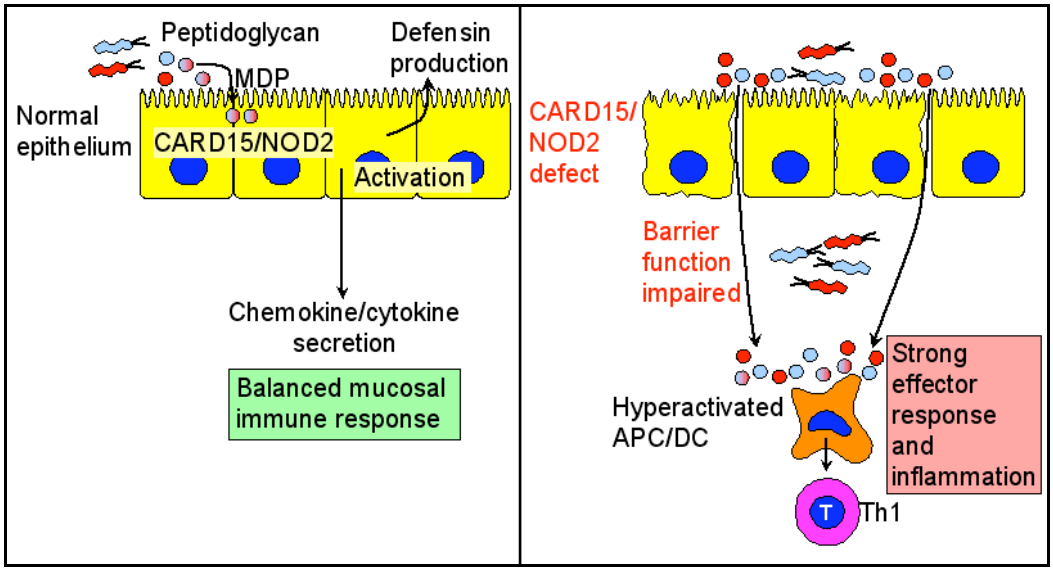

Figure 13A: Intestinal homeostasis depends on the epithelial barrier function and the balance between regulatory T (Treg) cells and Th1/Th2 effector cells as modulated by antigen-presenting cells (APCs), particularly conditioned dendritic cells (DCs). Normal epithelium senses muramyl dipeptide (MDP) motifs from Gram-positive and Gram-negative bacteria via intracellular CARD15/NOD2 molecules, which leads to activation with secretion of protective defensins and cytokines/chemokines (left). Mutations of CARD15/NOD2 may result in defective barrier function and APC hyperactivation which causes strong effector responses with mucosal inflammation (right). Modified from *Bouma* and *Strober* (2003).

surface (*Watanabe* et al., 2004); mutant NOD2 is unable to sense MDP, which leads to 'gain-of-function' for the TLR2 pathway with enhanced IL-12 and inflammation (Figure 13B).

However, this theory needs substantiation (*Cario*, 2005). It has alternatively been suggested that mutated NOD2 itself achieves gain-of-function whereby its N-terminal CARD domains become capable of activating Caspase-1 (*Maeda* et al., 2005). This enzyme may then cleave off the pro-domain of IL-1, leading to secretion of mature IL-1 which promotes the inflammatory process (Figure 13B). Admittedly, further studies are needed to discern the precise role of NOD2 in MDP recognition and subsequent intracellular signalling and cytokine/chemokine production. However, the near future will undoubtedly see a body of evidence also for other PRR mutations predisposing to intestinal inflammation.

Secondary alterations in the pIgR gene with defect transcription can furthermore result in deterioration of the intestinal barrier function, which may appear as patchy lack of pIgR/SC expression and absence of SIgA export (Figure 14). In ulcerative colitis we have observed a relationship between down-regulated pIgR/SC expression and the degree of hyperplastic epithelial lesions – overt dysplasia showing the most reduced immunohistochemical staining (*Rognum* et al., 1982). Expression of pIgR/SC protein and mRNA correlated and both variables were negatively related to the histological grade of dysplasia (*Krajci* et al., 1996), suggesting that this defect is a rather late event in the IBD process.

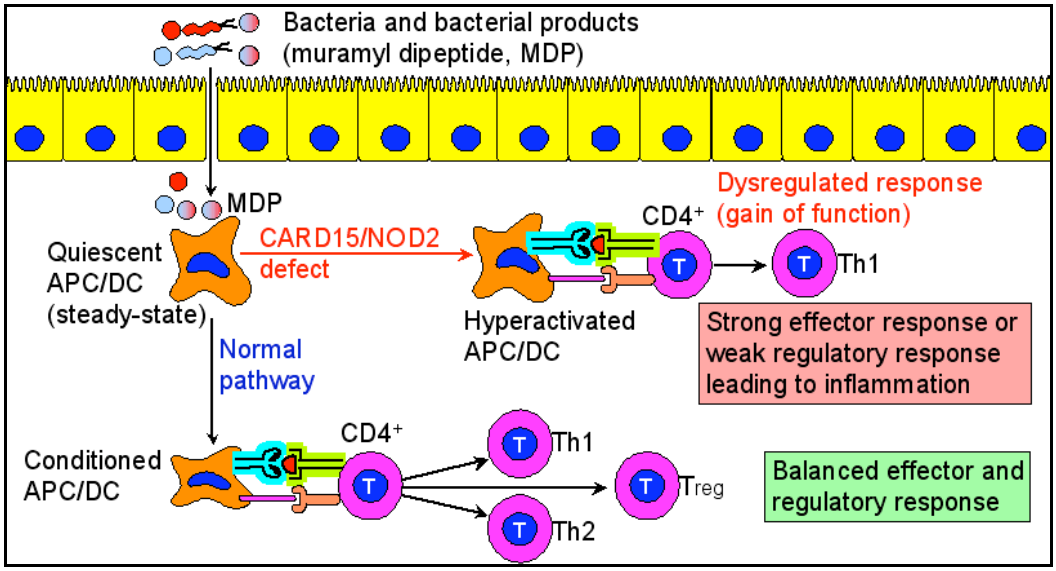

Figure 13B: Intestinal homeostasis depends on the epithelial barrier function and the balance between regulatory T (Treg) cells and Th1/Th2 effector cells as modulated by antigen-presenting cells (APCs), particularly conditioned dendritic cells (DCs). Mucosal APCs are normally quiescent and migrate in a steady state to mesenteric lymph nodes to become conditioned for induction of a balanced immune response. Mutations of CARD15/NOD2 may by different molecular mechanisms as discussed in the text, provide APCs with 'gain-of-function' that leads to disruption of the homeostatic balance between effector and regulatory cells. Modified from *Bouma* and *Strober* (2003).

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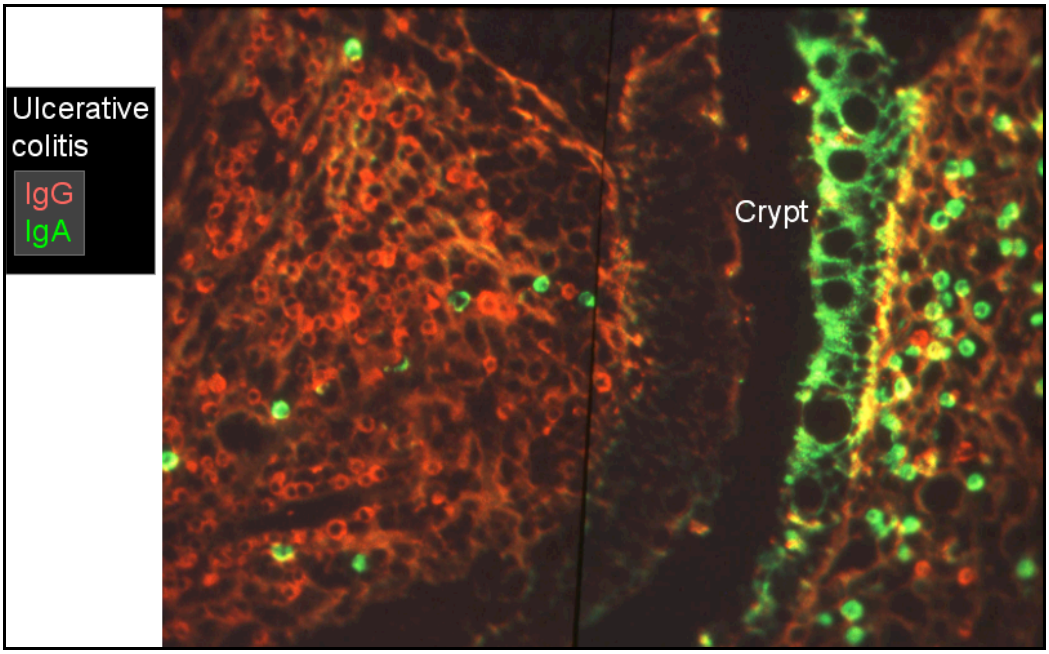

Figure 14: Patchy inflammation-induced defect in pIgR expression causes lack of epithelial IgA transport. Paired immunofluoresence staining for IgG and IgA in ulcerative colitis shows localised absence of IgA in the epithelium on one side of colonic crypt facing inflammatory infiltrate dominated by IgG-producing blasts and plasma cells. On the other side of the crypt, there is less inflammatory activity with more remaining IgA-producing cells, and the epithelium exports large amounts of IgA outside of the goblet cells which are black. Immunofluoresence photograph from the author's laboratory.

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DIVERSE EFFECTS OF TLR SIGNALLING ON INTESTINAL INFLAMMATION

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SUMMARY

Probiotics are live commensal microorganisms of the intestinal tract that confer multiple gastro-intestinal health benefits to the host. Yet, the exact mechanisms by which probiotics ameliorate experimental colitis in animals and human inflammatory bowel disease are largely unknown. We tested whether the attenuation of experimental colitis by live probiotic bacteria is mediated by Toll-like receptor signalling and whether non-viable probiotics are similarly effective. Administration of probiotic DNA ameliorated the severity of experimental colitis whereas methylated probiotic DNA, calf thymus DNA and DNAse treated probiotics had no effect. The colitis severity was attenuated to the same extent by delivery of non-viable, γ-irradiated or viable probiotics, but not by heat-killed probiotics, in wild type mice, in mice deficient in Toll-like receptor 2 or in mice deficient in Toll like receptor 4. In contrast, we did not observe any inhibition of experimental colitis by probiotics, in mice deficient in MyD88 or Toll-like receptor 9.

In subsequent studies, we identified that Toll-like receptor 9-induced type-1 IFN mediates the anti-inflammatory effects in experimental colitis. The addition of neutralisation antibodies to type-1 IFN abolished the anti-inflammatory effects whereas the administration of recombinant IFN-β mimicked the anti-inflammatory effects induced by Toll-like receptor 9 agonists.

Taken together, these results indicate that the protective effects of probiotics are mainly mediated by their own DNA rather than by their metabolites or their ability to colonise the colon. These finding underscore the diverse effects of indigenous microbial TLR ligands in intestinal homeostasis and intestinal inflammation and suggest that strategies, which modulate type-1 IFN may be of therapeutic value for intestinal inflammatory conditions.

INTRODUCTION

Inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis, both of which are characterised by flare up periods with possible life-long relapses. Clinical and experimental evidence suggest that the aetiology of IBD is multifactorial involving susceptibility genes and environmental factors, such as intestinal microflora or their products, and it is the interaction of these factors with the immune system that leads to dysregulated mucosal immunity

and chronic intestinal inflammation (*Shanahan*, 2001).

Probiotics are live commensal microorganisms of the intestinal tract that confer health benefits to the host by one or more of the following means: Production of various anti-microbial metabolites, competitive exclusion of enteric pathogens, neutralisation of dietary carcinogens, and modulation of mucosal immune responses. Current probiotic therapy is mainly advocated for its immunomodulatory properties and anti-inflammatory activities at mucosal sites (*Hooper* et al., 2001, *Shanahan*, 2001).

The rationale for using probiotics in IBD is based on evidence implicating enteric bacteria in the pathogenesis of various models of murine colitis and IBD in humans (*Podolsky*, 2002). Indeed, probiotic therapy has been effective for the attenuation of experimental colitis (*Madson* et al., 2001), prevention of pouchitis, and maintenance of remission of pouchitis, Crohn's disease and ulcerative colitis (*Hart* et al., 2003). Despite these beneficial effects, the exact mechanisms and the molecular pathways by which probiotics ameliorate experimental colitis and IBD are largely unknown.

Toll-like receptors function as sentinels of innate immunity. By recognising signature microbial compounds they trigger the transcriptional activation of pro-inflammatory cytokines (e.g., IL-12, TNF-α) and chemokines (e.g., RANTES), as well as co-stimulatory molecules (e.g., CD40). The activation of this pro-inflammatory program initiates defence mechanisms that are vital for host survival (*Takeda* et al., 2003). Indeed, various $TLR^{-/-}$ mice as well as mice deficient in TLR-related adaptor proteins (e.g., MyD88) fail to mount protective responses and succumb to various microbial infections (*Kopp* et al., 2003). Bacterial DNA and its synthetically derived immunostimulatory DNA sequence oligonucleotides (ISS-ODN, also known as CpG-ODN) contain unmethylated CpG dinucleotide motifs within consensus sequences and are ligands of TLR9. Like other TLR ligands such as LPS, ISS-ODN has a broad range of activities on the mammalian innate immune system. In particular, ISS-ODN induces the secretion of Th1-type cytokines and up-regulates the expression of co-stimulatory molecules on antigen presenting cells (*Krieg* 2002). These immunostimulatory characteristics of ISS-ODN have been utilised to elicit Th1-dependent immune responses (*Roman* et al., 1997) and mucosal immunity (*Horner* et al., 1988), leading to an enhanced host defence against invading pathogens.

Since ISS-ODN mimics the immunomodulatory activities of bacterial DNA, we hypothesised a role for probiotic DNA in the inhibition of colonic inflammation and therefore conducted studies which explored the mechanisms by which probiotics ameliorates experimental colitis

DISCUSSION

Persuasive evidence indicates that intestinal microflora play an important role in the initiation and the perpetuation of murine experimental colitis and human IBD (*Podolsky*, 2002). However, the molecular mechanisms by which probiotics exert their therapeutic effects have not been identified. The impact of probiotics on intestinal barrier function, their diverse metabolic activities, their competitive exclusion of intestinal indigenous microflora, and their interaction with the mucosal immune system have all been implicated in mediating their therapeutic effects (*Hooper* et al., 2001, *Shanahan*, 2001).

In a recent study, we provided biochemical, immunologic and genetic evidence that implicated TLR signalling, especially TLR9, in mediating the protective effect of probiotics (VSL-3) on experimental colitis (*Rachmilewitz* et al., 2004). The administration of γ-irradiated probiotics effectively ameliorated experimental colitis, as did the administration of viable probiotics. Since the irradiated probiotics were unable to grow in culture, it is unlikely that either their metabolites or their competitive inhibition with indigenous microflora were responsible for the protective effects on the colonic mucosa. Therefore, we reasoned that the anti-inflammatory activities could be the product of the activation of innate immunity (e.g., via TLR) by structural microbial probiotic components (*Rachmilewitz* et al., 2004).

To further verify the role of TLR signalling in the probiotic-induced amelioration of experimental colitis, mice deficient in TLR2, TLR4, TLR9 and MyD88 were treated with dextran sodium sulphate (DSS) and irradiated probiotics. The administration of γ-irradiated probiotics ameliorated the clinical, biochemical, and histological parameters of colitis in TLR2 and in TLR4 deficient mice but not in TLR9 and MyD88 deficient mice indicating the involvement of the TLR9 signalling pathway in the observed amelioration of colonic inflammation (*Rachmilewitz* et al., 2004). The inhibition of colonic inflammation by probiotic DNA or by ISS-ODN was reproduced in DSS- and TNBS-induced colitis as well as in spontaneous colitis in IL-10 deficient mice (*Rachmilewitz* et al., 2002, 2004). Thus, in contrast to the current paradigm related to the pro-inflammatory role of TLR-activated innate immunity, our data indicated that TLR9 signalling results in the activation of an anti-inflammatory program that attenuates inflammation in different models of experimental colitis.

Subsequent studies addressed the molecular basis for the anti-inflammatory effects induced by TLR9 signalling in models of experimental colitis. We found that two genetically distinct, but phenotypically similar animals responded differently to ISS-ODN administration (*Katakura* et al., 2005). While DSS-induced colitis in $RAG^{-/-}$ mice was inhibited by ISS-ODN, colitis in SCID mice was not. We utilized these ISS-responsive and ISS-resistant phenotypes to dissect the anti-inflammatory role of TLR9 signalling in colonic inflammation (*Katakura* et al., 2005). Analysis of the response to ISS-ODN of these two mouse strains revealed defective TLR9-induced type-1 IFN production in SCID mice. Furthermore, we observed that IFN-a/$bR^{-/-}$ mice are extremely sensitive to colitis inflicted by DSS and that the administration of ISS-ODN to these mice increased their mortality. In addition, the lack of inhibition of DSS-induced colitis in ISS-treated wild type mice adoptively transferred with bone marrow derived macrophages (BMDM) from IFN-α/$\beta R^{-/-}$ but not with BMDM from wild type mice also suggests that TLR9-induced type 1 IFN inhibits the inflammatory response of activated macrophages. Finally, the administration of recombinant IFN-β to DSS-treated mice mimicked the anti-inflammatory effects on colonic inflammation induced by ISS-ODN (*Katakura* et al., 2005). Collectively, these set of data indicate that type-1 IFN has a physiological and protective role on colonic injury and that it also cross-regulates the other pro-inflammatory activities induced by TLR9 triggering (*Katakura* et al., 2005). Indeed, in subsequent preliminary studies we observed that basolateral administration of IFN-α to polarised monolayers of intestinal epithelial cells protected the cells against apoptosis and disruption of the epithelial tight junctions. Thus, type-I IFN may protect against colonic inflammation by preventing epithelial barrier dysfunction.

Recent study documented the protective effect of other TLR ligands on colonic injury (*Rakoff-Nahoum* et al., 2004). Our previous studies identified the anti-inflammatory effects of TLR9 agonists on experimental colitis and identified that TLR-induced type-1 IFN mediates these protective effects on colonic inflammation. These findings and the hypersensitivity to DSS observed in IFN-$\alpha/\beta R^{-/-}$ mice expand the already known activities of type-1 IFN and indicate an important role for type-1 IFN in intestinal homeostasis. Taken together, these results suggest that strategies designed to trigger a type-1 IFN response in the intestinal tract, by the administration of certain TLR ligands (e.g., ISS-ODN, probiotic DNA) or probiotics are of therapeutic value for intestinal inflammatory conditions.

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MEDIATORS AND OUTCOMES OF BACTERIA-EPITHELIAL CELL INTERACTIONS

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SUMMARY

Intestinal mucosal surfaces are in continuous contact with heterogeneous populations of commensal microorganisms, which collectively make up the intestinal microbiota. Historically, the barrier function of intestinal epithelial cells (IEC) has been considered to be important in preventing or limiting the interaction of non-invasive bacteria from making contact with, and activating the mucosal immune system, and therefore maintaining immune (oral) tolerance to commensal bacteria. More recent investigations suggests that IEC may also play a role in sensing the external environment and communicating this information to the local immune system to affect appropriate responses. In this review the basis of IEC recognition of microorganisms and how disruption or breakdown in the interaction between IEC and the commensal microbiota are thought to underlie the development of chronic intestinal inflammation are discussed. In particular, the function of the cell surface pattern recognition receptors (PRR), Toll-like receptors, and the cytosolic nucleotide binding site plus leucine-rich repeat protein, NOD2, in IEC and the outcome of the triggering of these PRRs for IEC function are discussed.

INTRODUCTION

A single layer of epithelial cells is all that separates the host from the external environment. These cells are constantly interacting with a vast number of bacteria that are resident in the colon and distal small intestine and whose products such as endotoxin would in other sites of the body cause inflammation and tissue injury. Yet in the intestinal tract there is usually no evidence of any inflammation and instead commensal bacteria exist in a mutually beneficial or symbiotic relationship with the host. Although the nature of this relationship and the mechanisms by which it is regulated are poorly understood, its importance is evident from numerous studies demonstrating that dysregulation of this interaction is associated with chronic inflammation of the type seen in patients with inflammatory bowel disease (IBD). An important question therefore is how are pro-inflammatory responses to members of the commensal microbiota avoided while maintaining the capacity to orchestrate vigorous defence to any microorganisms that pose a threat to the host, and what role do IEC play in sensing and discriminating between innocuous versus dangerous bacteria in the gut?

INTESTINAL EPITHELIAL CELLS ARE MORE THAN A PHYSICAL BARRIER

The intestinal epithelium forms a highly impermeable barrier to most enteric antigens including microorganisms (*Didierlauerent*, 2002). Barrier function can broadly be described as being either physical, biochemical or immunological in nature all of which are to some extent dependent upon commensal bacteria for their establishment and/or efficient operation. These barriers must be breached by pathogenic microorganisms in order to invade and cross the epithelium.

The integrity of the physical intestinal barrier depends on specialised structures involved in cell-cell contacts known as tight junctions and adherens junction. The tight junction located at the apical region of epithelial lateral membranes provide a barrier that is selectively permeable to certain hydrophilic molecules, ions and nutrients, whereas the adherens junctions mediates strong cell-to-cell adhesions between adjacent IEC (*Cereijido* et al., 1998; *Liu* et al., 2000; *Mitic* and *Anderson*, 1998; *Moncrief* et al., 1995; *Obsio Jr.* et al., 1997; *Wu* et al., 1998). Tight junctional complexes are targets of pathogens such as *Bacteroides fragilis*, *Clostridia* sp. and enteropathogenic *Escherichia coli* (*Berkes* et al., 2002). Conversely, certain strains of probiotic lactobacilli have been shown to enhance epithelial barrier function (*Isolauri* et al., 1993; *Mao*, 1996) and to attenuate epithelial invasion by pathogenic bacteria (*Madsen* et al., 2003; *Resta-Lenert* and *Barret*, 2003). Compromised or leaky junctional complexes that facilitate translocation of commensal bacteria have been associated with the pathogenesis of IBD (*Gassler* et al., 2001). The major biochemical barrier of the intestinal epithelial layer is the mucus layer that overlies it that comprises mucin glycoproteins and members of the trefoil factor family. In addition, antimicrobial peptides (AMP) produced by Paneth cells in the crypts of the small intestine also contribute to the biochemical and anti-microbial defences of the epithelium. Induction of expression of the potent AMP, Angiogenin-4, by Paneth cells has been shown to be under the influence of commensal bacteria which together with the species specificity of Angiogenin-4 anti-microbial activity (*Hooper* et al., 2003) suggests that bacterial interactions with Paneth cells help shape the composition of the commensal microbiota. The major immunological defence mechanism of the intestinal epithelium is IgA, which is transported from the lamina propria and secreted into the lumen by IEC where it acts to prevent commensal bacteria breaching the mucosal barrier. IgA is not, however, exclusively produced in response to pathogenic microorganisms. In a seminal study *Schroff* and colleagues (1995) showed that upon colonisation of the gut commensal bacteria elicit adaptive an immune response which by virtue of coating bacteria in the intestinal lumen with IgA and preventing further translocation and stimulation of gut lymphoid tissue are naturally self-limiting. The generality of this phenomena is however uncertain since not all commensal bacteria are equally effective at inducing "protective" IgA (*Cebra*, 1999; *Moreau* et al., 1978) which may be related to differences in expression of pathogenicity factors such as molecules that mediate adhesion and invasion or that are endotoxic. How and where IgA production is initiated in the gut associated lymphoid tissues (GALT) has recently been established. Intestinal dendritic cells (DCs) have been shown to transport live commensal bacteria from the gut lumen to the intestinal mesenteric lymph nodes where IgA^+ lymphoblasts are activated enabling them to migrate to the lamina propria where they become

plasma cells (*Macpherson* and *Uhr*, 2004). IEC are thought to contribute to this process by producing cytokines in response to commensal bacteria that recruit DCs to the gut mucosa, acting therefore as a link between the innate and adaptive immune systems. How then is IEC recognition of enteric microorganisms mediated?

IEC RECOGNITION OF MICROORGANISMS

Host cells use distinct receptors (designated pattern-recognition receptors; PRR) for recognition of highly conserved structures of microorganisms, which have been called pathogen-associated molecular patterns (PAMPS) (*Medzhitov* and *Janeway*, 2002). However, considering that both pathogenic and non-pathogenic microbes can express the same molecular patterns these molecules are better described as microbe-associated molecular patterns (MAMPS) (*Didierlauerent*, 2002). Based upon their sub-cellular distribution, mammalian PRR can be divided into two types, the cell surface Toll-like receptor family (TLR) which recognise various PRRs of bacteria (lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), flagellin, methylated DNA), viruses (single and double stranded RNA) and yeast (zymosan), and the cytoplasmic nucleotide-binding oligomerisation domain (NOD) family of proteins which recognise components of PGN (*Inohara* et al., 2002; *Medzhitov*, 2001). IEC expression of functional TLRs and NOD proteins is contentious and by no means clear.

TLR FUNCTION IN IEC

Most studies investigating PRR expression by IEC have relied on established intestinal epithelial cell lines which have provided evidence for both the presence and absence of TLRs (*Abreu* et al., 2002; *Bocker* et al., 2003; *Cario* and *Podolsky*, 2000; *Cario* et al., 2000; *Melmed* et al., 2003). Demonstrating that any TLRs expressed by IEC are functional is critical. Although some IEC lines have been shown to express TLR mRNA production of TLR protein and the co-factors required for recognition of certain MAMPs have been shown to be negligible rendering them functionally unresponsive (*Abreu* et al., 2001; *Funda* et al., 2001). The inability to detect TLR expression by IEC *in vivo* has led to the notion that IEC are normally unresponsive or ignorant of commensal bacteria (*Cario* and *Podolsky*, 2000). The cellular localisation of any TLR expression by IEC may also be significant in terms of discriminating between commensal and pathogenic microbes. Expression of TLR5, which recognises bacterial flagellin, in the intestinal epithelium has been shown to be restricted to the basolateral surface of IEC (*Gewirtz*, 2001) and may therefore be effectively hidden from all enteric microbes except those (pathogenic strains) able to subvert the barrier function of IEC. Not all studies of TLR5 expression by IEC however, agree with this finding (*Sierro*, 2001). The finding that expression of TLR4 and the co-receptors (MD-2 and CD14) required for recognition of LPS in crypt epithelial cells are primarily intracellular within the Golgi apparatus (*Hornef* et al., 2003), is thought to enable IEC to selectively respond to internalised MAMPs from invasive bacteria while remaining tolerant

of the excessive amounts of MAMPs and LPS present in the lumen. Conflicting evidence suggest that TLR4 trafficking occurs across the epithelium implying that IECs have the capability of sensing and sampling luminal bacterial antigens using receptors localised to one surface polarised IECs (*Cario* et al., 2002). The variability in TLR expression and responsiveness seen among IEC lines may at least in part be explained by observations in mouse models of spontaneous or induced intestinal inflammation that IEC responses to MAMPs vary according to their anatomic location along the intestine and on whether they are in the lower crypt or villous tip (*Ortega-Cava* et al., 2003; *Suzuki* et al., 2003).

There is also uncertainty regarding the consequences of TLR-MAMP interactions and the downstream signalling pathways utilised in IEC. The intracellular signalling pathways downstream of these PRR have principally been mapped using haematopoietic cell lines and ultimately result in nuclear factor-kappa B (NF-κB, p65) activation leading to the production of antimicrobial factors, cytokines and chemokines (*Aderem*, 2001; *Akira* et al., 2001; *Irie* et al., 2000). Two divergent pathways have been identified (*Akira*, 2003). In the first, stimulation of PRR by MAMP leads to the recruitment of the adaptor molecule, myeloid differentiation factor 88 (MyD88) that binds to the conserved Toll/Interleukin-1 receptor (TIR) domain (*Means* et al., 2000) enabling phosphorylation of serine kinase IL1-receptor-associated kinase (IRAK), which in turn leads to recruitment and activation of TNF-associated factor 6 (TRAF-6). The IRAK-TRAF6 complex then interacts with another complex containing TGFß-activated kinases leading to the activation of IKK and finally NF-κB activation. Alternative or MyD88-independent pathways activate caspase-1 (IL-1ß converting enzyme; ICE) that converts the inactive form of pro-inflammatory cytokines, IL-1β and IL-18, into the secreted and active form (*Akira* et al., 2001; *Seki* et al., 2001). Other MyD88-independent pathways involve the induction of IFN-inducible genes such as the CXC chemokine IP-10 (*Akira* et al., 2001; *Irie* et al., 2000; *Kawai* et al., 2001). Several inhibitory molecules have recently been identified that interfere with TLR signalling at various points in the signalling cascade. One of these, Toll-interacting protein (TOLLIP) which binds to MyD88 and suppresses IRAK phosphorylation (*Miyake*, 2004), has been shown to be expressed at high levels in IEC lines after exposure to LPS (*Otte* et al., 2004) resulting in hypo-responsiveness to not only TLR4 ligands but also TLR2 ligands. TLR homologues lacking signalling domains have also been identified in antigen presenting cells and dendritic cells (*Divanovic* et al., 2005). A Probiotic strain of *Bacteroides thetaiotaomicron* has been shown to induce expression of the peroxisome-proliferator-activated receptor-γ (PPAR-γ) in IEC lines, which is a negative regulator of NF-κB activation (*Kelly* et al., 2004) accounting perhaps for the bacterium's anti-inflammatory role.

To attempt to redress the conflicting data concerning TLR expression by IEC our own studies have focused on examining the functionality of TLRs expressed by primary IEC *in vivo* and *in vitro* using a novel IEC culture system capable of supporting populations of murine colonic epithelial cells (CEC) that maintain the properties and characteristics of their *in vivo* counterparts during prolonged culture (*Baumgart* et al., 1998; *Telega* et al., 2000). CEC from wild type adult mice analysed directly *ex vivo* express detectable levels of both TLR2 and TLR4 (*Singh* et al., 2005). In response to LPS, LTA, PGN or the

synthetic TLR2 ligand, Pam3Cys, CEC proliferated *in vitro* increasing in number by more than twofold compared to cells cultured in media alone (*Singh* et al., 2005). In addition, MAMPs and in particular LPS, up-regulated production of the cytokines IL6 and MCP-1 by primary CEC (*Singh* et al., 2005) both of which in addition to being involved in activation of the inflammatory response (*Conti* and *DiGioacchino*, 2001; *Hibi* et al., 1996; *Mukaida* et al., 1998; *Shephard*, 2002) also contribute to epithelial growth and homeostasis (*Grossmann* et al., 1989; *Naka* et al., 2002; *Yoshizaki* et al., 1990) and epithelial restitution (*Low* et al., 2001). These findings are consistent with the recently proposed role for TLR-mediated response in IEC homeostasis (*Rakoff-Nahoum* et al., 2004) with the balance between promoting inflammation and epithelial homeostasis perhaps being determined by the strength and duration of the MAMP stimulus.

IEC CAN DISTINGUISH DIFFERENT COMMENSAL BACTERIA

A CEC:bacteria co-culture system was used to compare the ability of different representative colonic commensal bacteria to modulate the production of different cytokines (n=15) by primary CEC. Exposure of primary CEC to *Bacteroides ovatus*, *E. coli* (SLF) or *Lactobacillus rhamnosus* all of which were human or rodent colonic isolates induced or up-regulated different patterns of cytokine production and secretion (*Lan* et al., 2005). *E. coli* selectively induced production of MIP-1α and MIP-1β and defensin 3 whereas *B. ovatus* and *L. rhamnosus* exclusively induced MCP-1 and MIP-2α expression, respectively. Other cytokines (TNFα, RANTES and MEC) were induced or up-regulated in response to some but not all three of the bacteria whereas others (IP-10, ENA78) were up-regulated in response to all bacteria. Evidence of bacterial interference and suppression of CEC cytokine production was also obtained from mixed bacterial:CEC co-cultures. Probiotic *L. rhamnosus* suppressed *E. coli*- and *B. ovatus*-induced production of pro-inflammatory cytokines, identifying CEC as a potential cellular target for probiotoic bacteria *in vivo*. Although the ability of probiotic bacteria to down modulate pro-inflammatory cytokine (TNFα and IL-6) production has been demonstrated previously (*Borruel* et al., 2002; *Schultz* et al., 2003), the identity of the cells effected by the bacteria and the cellular source(s) of the cytokines was not established in these studies. Although the mechanism(s) of action of probiotics remains unclear (*Ghosh* et al., 2004) our findings suggest a mechanism by which non-pathogenic or probitoic bacteria might suppress or limit the ability of other "pathogenic" bacteria to promote or sustain inflammatory responses. How *L. rhamnosus* interferes with the ability of *E. coli* or *B. ovatus* to induce cytokine production by CEC is not yet known but may include the production of AMPs (e.g. bacteriocins), expression of MAMPs of higher density and affinity by *L. rhamnosus*, and the induction of TLR antagonistic signalling pathways or molecules. The CEC:bacteria co-culture system we have developed should be of value in investigating these potential mechanisms in more detail.

IEC TLR RESPONSIVENESS CHANGES DURING THE DEVELOPMENT OF CHRONIC INFLAMMATION

Since the regulation of TLR expression in the intestinal epithelium may change during the development of chronic inflammation in patients with IBD (*Cario* and *Podolsky*, 2000), we examined the responsiveness of primary CEC in a murine model of ulcerative colitis during the development of chronic inflammation. Before or coincident with disease onset, CEC from Interleukin-2-deficient ($IL2^{-/-}$) mice demonstrated a switch in TLR responsiveness from being TLR4 dominant in healthy wild type mice to decreased TLR4 responsiveness and increased TLR2 responses resulting in exacerbated IL6 and MCP-1 secretion in $IL2^{-/-}$ mice (*Singh* et al., 2005). Changes in TLR responsiveness or underlying abnormalities in regulation of TLR-mediated responses appear therefore be associated with the development of chronic inflammation and presumably reflect differences or changes in the interaction of CEC with commensal bacteria in the healthy versus inflamed intestine.

NOVEL TLR SIGNALLING PATHWAYS IN IEC

Based upon the analysis of haematopoietic cells and immortalised epithelial cell lines the downstream signalling pathways from TLRs have been shown to ultimately result in MyD88-dependent NF-κB (p65) activation (*Akira*, 2003). In primary IEC, however, MyD88-independent pathways are primarily utilised for TLR signalling (*Singh* et al., 2005). Following stimulation of CEC with MAMPs several MAPK family members are activated in association with transient caspase-1 activation and predominance of the inhibitory, p50, subunit of NF-κB (*Singh* et al., 2005). This is consistent with the divergence of TLR-mediated intracellular signalling pathways (*Kawai* et al., 2001; *Muzio* et al., 1998) that may be selectively used by different cell types (haematopoietic versus epithelial). The differences in TLR-signalling pathways used by primary versus established IEC may be explained by the developmental control of TLR expression and function. Analysis of NF-κB activation in the colonic mucosa has shown that expression of the p65 subunit is restricted to rapidly dividing cells within the crypts and that the inhibitory p50 subunit predominates in mature enterocytes (*Inan* et al., 2000). MAMP responsiveness by IEC may therefore, be restricted to specific developmental or maturational stages, with less differentiated cells being less or hyporesponsive while fully differentiated cells can respond to MAMPs (*Cario* et al., 2002). This could be mediated by the action of specific negative regulators of TLR signalling such as PPAR-γ (*Kelly* et al., 2004), single immunoglobulin-IL-1 receptor related molecule (*Wald* et al., 2003) or of TLR cofactors (*Akashi* et al., 2000; *Shimazu* et al., 1999) or functionless TLR homologues (*Divanovic* et al., 2005) the expression of which would be developmentally regulated.

The importance of regulating TLR-mediated signalling is demonstrated by the sustained expression and lack of down-regulation of caspase-1 activity in primary CEC during the development of intestinal inflammation (*Singh* et al., 2005). The failure or breakdown in the regulation of caspase-1 activity in CEC may therefore play a role in the development or maintenance of intestinal inflammation. The observation that $caspase\text{-}1^{-/-}$ animals are resistant to

chemically-induced colitis (*Siegmund* et al., 2001b) and that anti-IL-18 antibodies ameliorate colitis in a number of murine models of IBD (*Kanai* et al., 2001; *Siegmund* et al., 2001a; *ten Hove* et al., 2001; *Wirtz* et al., 2002) are consistent with this interpretation. Of note, up-regulation of IL-18 expression has been demonstrated in the inflamed lesions of the intestine of IBD patients localised primarily to macrophages and epithelial cells, and caspase-1 has been identified as a potential therapeutic target for IBD (reviewed in *Siegmund*, 2002).

NOD2 FUNCTION IN IEC

Both NOD1/CARD4 and NOD2/CARD15 are members of the caterpillar gene family that encodes proteins with a variable but limited number of N-terminal domains, followed by a nucleotide-binding domain (NBD) and leucine-rich repeats (LRR) that are highly conserved in plants and vertebrates (*Ting* and *Davis*, 2005). NOD1 and NOD2 are capable of detecting bacterial peptides of PGN with NOD1 detecting muropeptide found mainly in Gram-negative bacteria, whereas NOD2 detects muramyl dipeptide, which is present on all bacteria containing PGN (*Inohara* and *Nunez*, 2003; *Inohara*, 2002). The interaction of these peptides with the LRR domain of NOD proteins leads to oligomerisation and recruitment of the adapter protein RICK (RIP-like CARD containing domain) which in haematopoietic cells results in NF-κB activation (*Kobayashi* et al., 2002; *Ogura* et al., 2001). NOD2 expression is restricted to the cytoplasm of monocytes, epithelial cells and Paneth cells in the small intestine (*Lala*, 2003). Although there is strong data linking NOD2 gene polymorphisms and mutations in the LRR domain of NOD2 with Crohn's disease that results in defective signalling in response to MAMPs (*Bonen*, 2003), the precise mechanisms of action of NOD2 is uncertain. Three different models of NOD2 function have been proposed based upon studies carried on transgenic mice with targeted mutations or deletion of the NOD2 gene (Figure 1). In the first model, based upon the analysis of spleen cells from mice deficient in exon 1 of the NOD2 gene preventing the NOD2 protein from being expressed, *Wantanabe* and colleagues (2004) propose that the interaction of NOD2 with MDP normally limits TLR2 signalling activated by PGN at the cell surface. This affects activation of the NF-κB subunit c-Rel, leading to an increase in the production of IL-12. Mutant NOD2 in Crohn's disease is unable to sense MDP and thus removes a constraint on the TLR2 pathway thereby invoking inflammation. In the second model based upon the outcome of infection of mice in which exon 3 had been deleted with the enteric pathogen *Listeria monocytogenes, Kobayashi* and co-workers (2005) propose that NOD2 plays an important role in the regulation of α-defensins made by Paneth cells enabling protection against pathogenic bacteria. Mutations in NOD2 cause a decrease and therefore loss of the α-defensins and loss of protection from invading pathogenic bacteria. Interestingly, in contrast to the Wantanabe study, analyses of bone marrow-derived macrophage (BMDM) responses in mice generated by Kobayashi failed to detect any increase in the production of pro-inflammatory cytokines, including IL-12, in response to various TLR ligands, such as PGN. The third model based upon the analysis of BMDM from mice expressing a mutated form of NOD2 protein (NOD^{2939iC}) homologous to the major mutation in human Crohn's disease resulting in a truncated NOD2 protein, led *Maeda* and col-

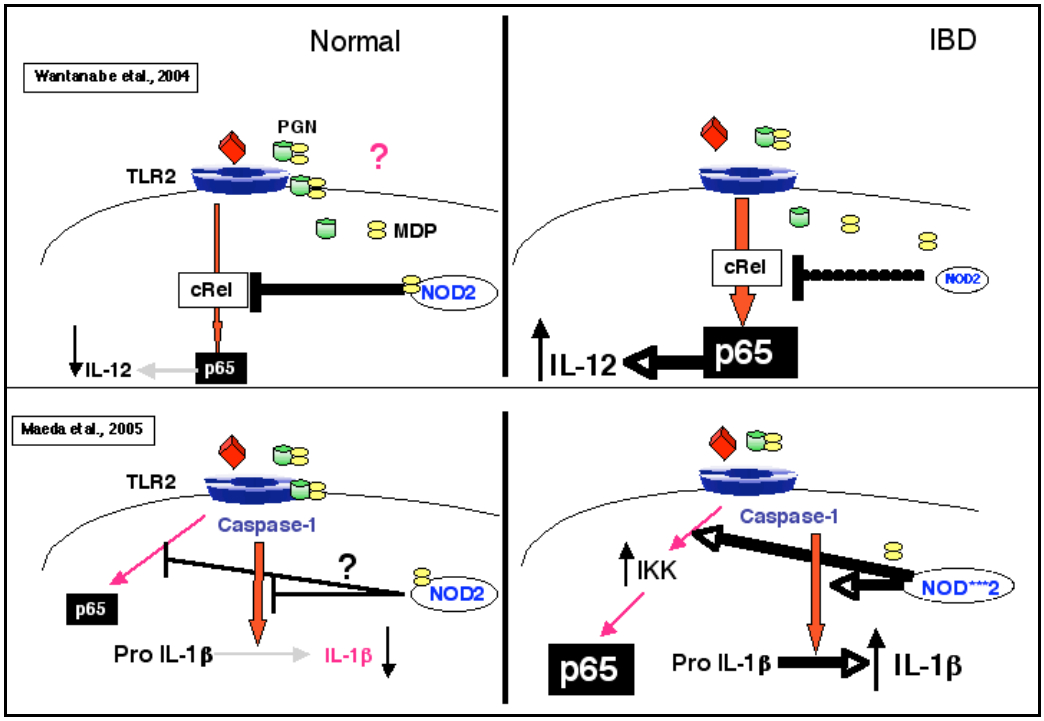

Figure 1: Models of NOD2 function based upon studies of haematopoietic cells. In the model proposed by *Wantanabe* and co-workers (2004) NOD2 activation by MDP reduces or interferes with TLR2-mediated IL-12 production by interfering or blocking activation of the c-Rel subunit of NF-κB. The mutated forms of NOD2 in Crohn's diseases patients are unable to provide this restraining effect and IL-12 production proceeds unabated leading to inflammation and disease. The model proposed by *Maeda* and colleagues (2005) is based on a gain-of-function of mutated forms of NOD2 that acquire the ability to activate IKK and caspase-1 which acts on the precursor form of IL-1β (and IL-18) enabling the secretion of biologically active cytokine leading to inflammation.

leagues (2005) to propose that the mutation in NOD2 results in a gain-of-function. Specifically, the N-terminal CARD domains of the mutated protein are now capable of activating caspase-1, which acts on the precursor form of IL-1β which is then secreted and can drive the inflammatory response in Crohn's disease patients.

Whilst all three models clearly identify a link between NOD2 activation and pro-inflammatory cytokine production there is no consensus as to how NOD2 influences cytokine production and whether NOD2 activation up- or down-regulates cytokine production. The reasons for these conflicting results are not clear but must be related to differences in the mutations introduced into the NOD2 gene (deletion of different exons or knock-in of a frame shift mutated gene) and the affect they now have on proteins and signalling complexes that NOD2 interacts with or is a part of. Importantly, none of these studies have examined the function of NOD2 in IEC, which is surprising since they are the first point of contact with enteric microbes, the outcome of which is of importance in determining the nature of the host response. Our own studies therefore, have focused on examining the function of NOD2 in primary CEC and have provided evidence for an anti-in-

flammatory role for this cytoplasmic PRR that may help explain the pathogenesis of chronic intestinal inflammation.

ROLE OF NOD2 IN PRIMARY CEC

The conditions under which NOD2 is expressed by primary CEC and the affects its activation has on their immune function were examined first. In a series of yet to be published studies NOD2 is expressed at low levels by CEC *in vivo* with expression localised to crypt regions and villous tips in normal healthy adult mice. This expression was shown to be dependent on the presence of commensal bacteria since it was not possible to detect any expression in the colonic mucosa of germ-free animals. Expression was dramatically and transiently up-regulated both *in vivo* after peroral infection with the bacterium, *L. monocytogenes* and the protozoan parasite, *Toxoplasma gondii* and *in vitro* upon exposure to pro-inflammatory cytokines (TNFα), MAMPS (PGN and MDP) and to varying degrees by different commensal bacteria.

Clues as to the function of NOD2 in CEC were obtained from analyses of cytokine production by CEC in response to MDP and other MAMPs. Activation of NOD2 in cultured primary CEC abrogated constitutive production of pro-inflammatory cytokines by CECs and counteracted the ability of TLR2 ligands to up-regulate cytokine production. The suppression of cytokine production by NOD2 activation was associated with expression of the p50 inhibitory subunit of NF-κB. Although these findings need to be corroborated by additional functional studies incorporating NOD2 deficient CEC, they suggest that NOD2 acts as an anti-inflammatory protein in CEC by regulating cytokine production. The transient nature of NOD2 expression by CEC in response to infection and exposure to MAMPs suggests that NOD2 gene transcription is normally tightly regulated and restricted to specific stages of the host response. Disruption of this regulation and sustained expression of NOD2 may therefore effect how it interacts with its associated downstream signalling molecules leading to qualitative or quantitative changes in NF-κB activation resulting in sustained or increased pro-inflammatory cytokine production.

It is interesting to note that elevated and sustained expression of NOD2 is associated with intestinal chronic inflammation (*Lala*, 2003; *Rosenstiel*, 2003) and increased levels of pro-inflammatory cytokine production, particularly IL6, by IECs in IBD patients (*Jones*, 1993; *Kusugami*, 1995; *Shirota*, 1990). Since the highest levels of NOD2 protein in the inflamed colon are localised to the crypts it may influence or be a consequence of the increased proliferation and IEC turnover that is required to repair and regenerate an intact epithelial barrier. It may also reflect disruption of NOD2 regulation, or a futile attempt to control and contain the inflammatory response. In an anti-inflammatory role, the high levels of pro-inflammatory cytokines and sustained NF-κB (p65) activation seen in Crohn's disease could be explained by the loss of inhibitory signals normally provided by NOD2 activation that restrict or prevent NF-κB activation in IEC. In view of the diversity of cellular functions associated with caterpillar proteins that range from influencing differentiation, proliferation and cell death (*Ting* and *Davis*, 2005) it is possible that there may be regional differences in NOD2 function in IEC in the colon; influencing or promoting epithelial anti-microbial defence (α-defensin production) and IEC differ-

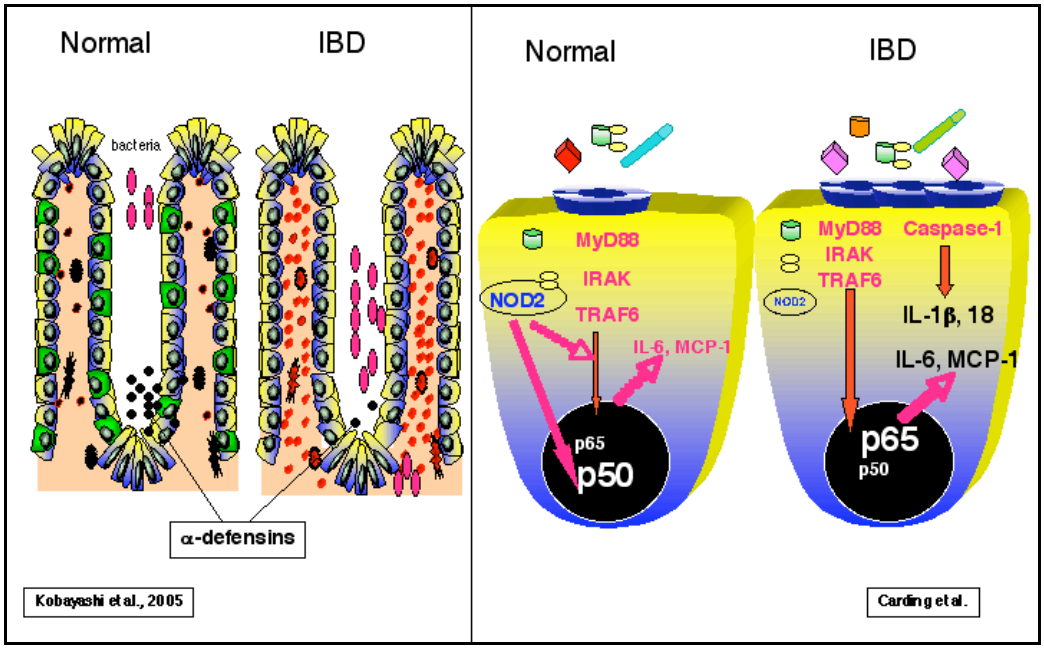

Figure 2: Models of NOD2 function in IEC. In the model proposed by *Kobayashi* et al. (2005) NOD2 regulates α-defensin production by Paneth cells in the crypts of the villi of the small intestine. Mutations in NOD2 lead to a decrease in defensin levels which compromises protection against invading bacteria. An alternative model based upon our own work (*Carding* et al., unpublished observations) is that NOD2 is an anti-inflammatory protein in CEC acting to suppress or restrict the production of NF-κB (p65)-dependent pro-inflammatory cytokines (e.g. IL6, MCP-1) induced by TLR-mediated recognition of commensal bacteria or enteric MAMPs. The loss-of-function of NOD2 in Crohn's disease effectively removes this control mechanism increasing the responsiveness of TLR-mediated signalling resulting in NF-κB (p65)- and caspase-1-dependent responses to commensal bacteria and inflammation and disease.

entiation and proliferation in the crypts, and apoptosis in IEC at the villus tips.

Although there are some similarities between our own findings and model of NOD2 function in primary IEC with those already described (Figures 1 and 2) they are not identical. The differences most likely reflect cell type (haematopoietic versus epithelial cells) or cell stage (immature versus mature) specific differences in NOD2 function that could for example, be mediated by differences in NF-κB subunit activation (p65 vs. p50) and types of cytokines produced (IL-12 or others). Considering that IEC are the first points of contact with enteric microbes the regulation of NOD2 function in IEC may be of central importance in determining the outcome of host-bacteria interactions and in mounting appropriate responses to commensal versus pathogenic bacteria. This does not exclude a (different?) role for NOD2 in other cell types once the epithelial barrier has been breached and they are exposed to MAMPs in the lamina propria or other tissues. Another important point that emerges from these studies is that it is unwise to extrapolate findings based upon one cell type to another unrelated cell type and in comparing responses of immortalised cells with that of primary cells.

CONCLUDING REMARKS

It is now clear that IEC express functional PRRs and that they are used to recognise and respond to microorganisms and microbial antigens in the gut lumen. This interaction or dialog between the two cell types is important in epithelial homeostasis, anti-microbial defences and influencing host innate and adaptive immune responses. This interaction is complex in that the response of IEC to microbial challenge is not stereotypical and they appear to possess the capacity to distinguish between different bacteria and to mount different (pro- or anti-inflammatory) responses. Disruption of this interaction and associated downstream signalling pathways may underlie the pathogenesis of chronic intestinal inflammation and IBD. Among the many questions that remain unanswered that relate to IEC-bacteria interactions, or conversations, are:

- What is the basis of the dialog between commensal bacteria and IEC? Face-to-face (cell-cell contact), long distance (soluble factors), or do they require a third party operator or interpreter such as intestinal DC that are often found in close association with IEC and can gain access to lumen contents via dendritic process that protrude between adjacent IEC?
- Which genes (lexicon) in each partner are required for this dialog?
- Are all bacteria and IECs equally equipped to converse? Are language skills developmentally acquired and are there different regional IEC dialects?
- How might this dialog be different in individuals prone to develop IBD, especially in the majority of IBD patients that have an intact and functional NOD2 gene? Deaf and/or dumb IEC perhaps? Are other known or unknown PRRs involved?
- Do probiotic bacteria converse with IEC in a different language to that of other enteric bacteria?
- Can this dialog be interrupted or corrected for prophylactic and therapeutic benefit?

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INFLAMMATORY IMMUNITY AT BIRTH

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BIRTH, A TRAUMA-LIKE EVENT

Birth constitutes one of the most dynamic but also potentially hazardous events in the human life cycle. It involves huge changes in most organ-systems of the mammalian body. Cardio-respiratory, endocrine-metabolic, nutritive, nervous and immune functions are affected, including an activation of immune-inflammatory pathways (*Marchini* et al., 2000, 2005a). In many ways this process have striking similarities to "trauma-stress" (*Souba*, 1994). Birth also marks a transition from foetal, sterile life to a terrestrial life in co-existence with the microbial world. Meeting a rapid colonising commensal microflora for the first time, results in a potential danger of a microbial invasion through the epithelial linings. This is one of the reasons why an activation of the immune system, in particular an induction of innate immunity, makes good sense from an evolutionary point of view. Skin and mucosal surfaces are rapidly colonised at birth and a preparedness of the immune system increases the capability to resist the predation of potentially invading microbes.

BIRTH, AN ACUTE PHASE REACTION

IL-6, the principal cytokine mediating the acute-phase reaction, is up-regulated already during normal parturition (*Marchini* et al., 2000). The release of IL-6, a pleiotrophic cytokine with many effects (*Heinrich* et al., 1990; *Molloy* et al., 1993), including that on the hypothalamic-pituitary-adrenal stress axis (*Masturakos* et al., 1993) is followed by a peak induction of acute phase proteins such as C-reactive protein, serum amyloid A and pro-calcitonin during the early postnatal days (*Marchini* et al., 2000). An increase in body temperature, reflecting the interactions between immune and nervous system is also notes in new-born infants (*Marchini* et al., 2000) as part of the of the systemic inflammatory response maybe to the colonisation and/or tissue "damage" connected to birth. Increases in temperature is believed to be important in host defence (*Kluger* et al., 1996). The infant's capacity to secrete gastric acid soon after birth is well described (*Avewry* et al., 1966) as is the importance of the acidification of the gastric content for the microbial ecology of the gastro-intestinal tract (*Wolfe* and *Soll*, 1988). A huge recruitment of circulating leukocytes are found at birth (*Moshfegh* et al., 2005). The enhanced reactivity of neonatal leukocytes, as reflected by an increased transmigrations capacity *in vitro* of eosinophils (*Moshfegh* et al., 2005) as well as increased cytotoxic responsiveness of neutrophils (*Koening* et al., 2005) clearly points out the potential role of inflamers immunity for health and disease in the human new-born (*Diodovich* et al., 2004; *Jiang* et al., 2004; *Thorton* et al., 2004).

SKIN IMMUNE SYSTEM

Protecting the body from infection is critical for survival and the mammals have developed a number of defence systems to reinforce epithelial linings, including the skin immune system. Immediately after birth the human new-born is partly covered by a cream-like white substance, *vernix caseosa*. This substance is to large extent constituted by water but contains also foetal keratinocytes and antibacterial peptides/proteins such as LL-37, psoriasin and lysozyme. (*Marchini* et al., 2002; *Yoshio* et al., 2003). It disappears spontaneously within a few hours after birth and it may have a physiological role in immunoprotection in the immediate postnatal period, before humoral and cellular components are recruited into the skin. Furthermore, there is a powerful upregulation of innate immunity in the skin of the healthy new-born infant during the early days of life (*Marchini* et al., 2001, 2002, 2003). This immune activity may be seen as a rash, known as *Erythema Toxicum Neonatorum* and is most probably a response to the commensal colonisation of the skin at birth. One-day old, healthy new-borns are colonised mainly with coagulase-negative *Staphylococci* (84%), remaining with *Staphylococcus aureus*, Alfa-streptococcus, group B streptococcus, *Enterococcus* species and *Micrococcus* species. Transmission electron microscopy analysis of a typical lesion of *Erythema Toxicum* has revealed microbial-like material into phagosomes of epithelial cells and into immune cell located in proximity to the hair follicle, indicating a penetration of microbes into the skin at birth (*Marchini* et al., 2005b). The findings also suggest that the hair follicle immune system may constitutes the physiologic "room" where microbes are presented to immune competent cells. This exposure may provide signals, possibly mediated by the Toll-Like receptors (TLRs) that alert the immune system and promotes a protective immune response. Preliminary results indicate that TLR 2 and TLR4 are indeed expressed on keratinocytes of newborns. Other factors that may affect immune functions are the high levels of steroid hormones normally present at birth (*Marchini* et al., 2005b; *De Peretti* and *Forest*, 1976). Steroid hormones may influence immune functions both locally in the skin and other epithelial linings, but also in a systemic way (*De Peretti* and *Mappus*, 1983; *Pelletier* and *Ren*, 2004; *Thornton*, 2002).

Previous and recent results clearly indicated that the healthy human newborn mounts an acute and powerful inflammatory immune response in order to face the commensal colonisation of epithelial linings. This event may have implications for colonisation resistance and the induction of tolerance.

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DEFENCE MECHANISMS OF THE INNATE SYSTEM: NO AS A GASTROINTESTINAL ECO-REGULATOR

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SUMMARY

Nitrogen (N) constitutes around 80% of the air. The air gas di-nitrogen is transformed by soil microorganisms into organic material which is utilised by all other organisms. Denitrification is an important microbiological process for the return of fixed nitrogen to the atmosphere. Non-microbial life exists in between these two major microbial processes.

In most organisms, nitrogen is a limited source and consequently, they have developed systems for excretion and recycling. In ureotelic organisms, as mammals, urea is the major excretion product; uric acid is dominating in birds, and ammonium in organisms as amphibian. In most mammals, parts of urea will be excreted into the gastrointestinal tract where it is used for protein synthesis by the microbial flora. The proteins thus formed might be utilised by the host. Nitrate constitutes an important dietary form of nitrogen.

Despite having no host enzymes capable of nitrate utilisation, nitrate is rapidly – often within some minutes - absorbed and concentrated by a factor of 10 in the saliva. In the oral cavity, anaerobes will utilise nitrate as a terminal respiratory electron acceptor, and nitrite will be formed.

A further transformation of nitrite to nitric oxide may be partly microbial partly non-microbial. The latter will take place in the stomach at low pH.

Recently, comparative studies in germ-free and conventional rats have highlighted the important role of the oro-intestinal flora. In some ongoing studies, we are screening probiotic strains for their capabilities of forming nitric oxide.

LIFE IS NOT POSSIBLE WITHOUT BACTERIA (*L. PASTEUR*)

Background

Nitrogen (N) is an essential part of all life. As the gas dinitrogen (N_2) it constitutes the major part of the air. However, only some soil microorganisms are the only organisms capable - by dinitrogen fixation - of transforming atmospheric nitrogen into organic material. In the other end of the chain, denitrification is an important biological mechanism for the return of fixed nitrogen to the atmosphere. Again, this process is carried out by microorganisms, and involved four consecutive reactions in which nitrate is reduced to dinitrogen gas by the metallo-enzymes, nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. Although many molecular aspects of microbial denitrification recently have

been elucidated, the eco-physiology of this important process is hardly understood (*Van de Pas-Schoonen*, 2004).

Whatsoever, Pasteur was right. In a broader scale: Life on Earth is not possible without bacteria.

Excretion and recycling of nitrogen

All multi-cellular organisms have mechanisms for excretion – and recycling – of nitrogen. The three major excretion products are ammonium (amphibian), uric acid (birds) and urea (mammals). However, it should be taken into consideration that all three compounds are found in all macroorganisms.

Under physiological conditions, urea is the major excretion product in mammals. In all mammals, a considerable part is excreted into the gastrointestinal tract, especially the stomach. This gastrointestinal excretion is strongly species-related. In man, it may account for around 20-25%, whereas the gastric excretion in camels may reach 90%.

It seems reasonable to assume that this extra-renal excretion has several functions. In camels, the high excretion gives ruminant microorganisms enough nitrogen for a proper digestion of cellulose-rich desert plants. Additionally, it will be less urea for renal excretion, thereby saving valuable water for the camel.

Many microorganisms in the GI tract, as most of the *Helicobacter* species, many enterobacteria and lactobacilli, including many probiotic strains, produce ureases. On the other hand, endogenous mammalian ureases do not exist. When urea is excreted into the GI tract it will immediately be utilised by the microorganisms for their own metabolism. The breakdown of urea will go through production of ammonium. However, it should be taken into consideration that the toxicity of ammonium is pH dependent. The ammonium thus formed may influence upon the local pH, thereby also influencing upon the ecological balance. Under physiological conditions the microbes themselves will utilise the formed ammonium. If this microbial utilisation of urea takes place in the upper part, the microbes may undergo lyses further down in the GI tract, thereby supplying the macroorganisms with N-containing material, as amino acids, peptides, nucleotides, etc.

The nitrate-nitrite-nitric oxide pathway

Nitrate is an important anion in the GI tract. Vegetables are the main dietary source of nitrate, accounting for up to 2/3 of the dietary intake. Once ingested, nitrate is rapidly absorbed from the gastrointestinal tract and mixed with endogenously produced nitrate. The latter is mainly coming from the L-arginine NO pathway (*Leaf* et al., 1989). The absorption of orally given nitrate is very rapid, i.e. within some few minutes after ingestion. The salivary glands are actively "extracting" nitrate from plasma, the resulting salivary nitrate concentration can easily be at least 10 times higher than the concentration in plasma.

Salivary nitrate is rapidly reduced to nitrite by commensal bacteria in the oral cavity, and at least three types of nitrate reductases may catalyse this reduction (*Potter* et al., 2001). All three types are molybdoproteins and some bacteria, as *Paracoccus pantotrophus,* possess all three (*Richardson* et al., 2001).

During more than half a century, nitrate has gained a bad reputation, preliminary due to its assumed association with development of some types of cancer (*Fraser* et al., 1980) and methaemoglobinaemia. The last condition mostly affects infants up to one year of age and is caused by oxidation of nitrite or nitric oxide, of haemoglobin in red blood cells to an abnormal form, methaemoglobulin, that cannot bind or transport oxygen. In most countries, regulatory authorities have expended great efforts to minimise

dietary intake of nitrate. They may have forgotten that possible deleterious effects of human health are not primarily related to the nitrate ion itself. That ion has a remarkable low toxicity. They may also have forgotten that on a typical Western diet, endogenous formed nitrate may account for as much as 50% of the total daily nitrate load. In fact, there is still no clear evidence for a link between nitrate intake and gastric cancer in humans. In fact, some studies show either no relationship or even an inverse relationship between a high intake of nitrate and the occurrence of gastric cancer (*McKnight*, 1999).

When salivary nitrite is reaching the stomach, it is rapidly converted no nitric oxide by non-enzymatic mechanisms. Once formed, nitric oxide exhibits a variety of biological activities and is assumed to be involved in regulation of blood flow, gut motility, secretory and immunological functions (*Bjorne* et al., 2004, *Herulf* et al., 1999), and it may be toxic to several bacteria and viruses (*Duncan*, 1997)

Comparative studies in germfree (GF) and conventional (Conv) rats

In a recent, comparative study (*Sobko* et al., 2004) in GF and Conv rats we found that the concentration of NO in Conv rats differed profoundly along the GI tract; 4000 ppm, 20 ppm, 200 ppm, 20 ppm in the stomach, small intestine and colon, respectively. Gastric NO increased greatly after a nitrate load. Concentration of NO was significantly lower in all 4 compartments in GF rats (31 ppm, 10 ppm, 9 ppm, 13 ppm in the stomach, small intestine, coecum and colon, respectively), and gastric NO remained low after a nitrate load. Giving a NOS inhibitor to Conv rats, we could reduce the NO concentration in the colon, without affecting NO in the stomach and in the coecum.

In a previous comparative study in GF and Conv rats we found no significant difference in NO in exhaled air (*Persson* et al., 1994). Additionally, in another study in newborn babies we found that nitric oxide was present in faeces first after some days (*Sobko* et al., 2005). In an ongoing study we are investigating nitrate and nitrite reducing capacity in strains, including probiotics, belonging to species known to be present in the gastrointestinal flora under physiological conditions.

Taken together, our results clearly show that when microbes and nitrate are present in the same compartment at the same time, the possibility exists for an increased luminal concentration of NO. The physiological and patho-physiological consequences of this microbial production should be further investigated.

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STRESS, BACTERIA AND THE HPA AXIS

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The effect of gut microbiota on a wide variety of systemic and behavioural activities is well characterised. Gut microbes are even responsible for locust swarming (*Dillon* et al., 2000): Three organisms commensal to the host all produce guiacol, an essential component of the locust aggregation pheromone, from vanillic acid derived from plants on which they feed. Many other examples exist and include the fact that conventionalisation of germ-free mice increases fat storage by 60% through suppression of synthesis of a natural epithelial inhibitor of lipoprotein lipase (fasting intestinal adipocyte factor) (*Backhed* et al., 2004). Monoassociation of germ-free animals completes full maturation of T and B lymphocyte immune populations as well as organised lymphoid tissue (*Jiang* et al., 2004). Monoassociation of germ-free mice stabilises an exaggerated HPA axis response to stress (*Sudo* et al., 2004).

Bacteria communicate with each other through signalling molecules. Quorum sensing is a mechanism whereby bacteria can sense their environment including the bacterial biomass. These signalling molecules (auto-inducers) were first identified in marine bacteria such as *Vibrio fischeri* in the Hawaiian squid (*Euprymna scolopes*) (*Visick* et al., 2000). A particular auto-inducer (A1-2) is essential for the generation of the bioluminescent light organ in the squid. Moments after hatching the *V. fischeri* monospecifically colonises the light organ of its symbiotic partner at high density. The bacteria are essential for normal development of the light organ and the bacteria must cause swelling of the epithelium lining the crypts of this structure since mutants that colonise but do not cause swelling induce poor light organ development (*Koropatnick* et al., 2004). A fragment of the bacterial surface peptidoglycan, tracheal cytotoxin and LPS are responsible. This cytotoxin is pathogenic in humans and causes extensive damage to local tissues in whooping cough and gonorrhoea. The A1-2 acts as a cell density sensing signal and regulates expression of up to several hundred genes. Pathogenic *E-coli* 0157 also make A1-3 which regulates flagellar motility and secretion of proteins and restores a mutant *V. fischeri* (LUX S mutant), deficient in A1-2 and A1-3 to full protein secretion and motility.

It is therefore particularly interesting that catecholamines, part of the HPA axis response to stress, have been known since the 1930's to promote bacterial growth (*Lyte*, 2004). Nor-epinephrine restores the LUX S mutant to full capacity and this action is blocked by both ∀ and ∃ adrenergic receptor antagonists but this blockade did not affect growth (*Lyte*, 2004; *Sperandio* et al., 2003; *Winzer* and *Williams*, 2003). Since these adreno-receptors are not found in the organisms, A1-3 and nor-epinephrine are presumed to be recognised by the same bacterial receptors but these must be distinct from the ∀ and ∃ adrenergic receptors. *Lyte* (2004) has for a number of years suggested that activation of the HPA axis and production of catecholamines produces up-regulation of growth of potentially pathogenic organisms and activation of

their virulence genes, which in turn, through a complex cascade may lead to sepsis in susceptible individuals such as in patients undergoing surgery or in intensive care.

The stress response in germ-free mice is exaggerated relative to conventional animals (*Sudo* et al., 2004). Monocontamination of these animals leads to a relatively blunted, but normal, HPA axis response with respect to ACTH and corticosterone levels when animals are subjected to restraint stress. This exaggerated response can be restored to normal by monocontamination with certain organisms or with faeces from SPF animals. This restoration only occurs in animals which have been so conventionalised within 3 weeks of birth. Similarly handling of neonates dampens the adult HPA axis in response to stress if this occurs in the same neonatal period (*Meaney* et al., 1988). Maternal deprivation in conventional neonates however results in an increased HPA axis response to stress in adults. The effect of maternal deprivation in monkeys has been studied by *Bailey* and *Coe* (1999) who have shown a decrease in the number of aerobic lactobacilli in faeces of such animals within 3 days of separation. Prenatal maternal stress alters intestinal microbiota in Rhesus monkeys and this has been shown to occur particularly if the stress occurs late in pregnancy, i.e., between 15 and 24 weeks (*Bailey* et al., 2004). Anaerobic bifidobacteria were only reduced if stress occurred late in life whereas anaerobic lactobacilli were reduced in number both in early as well as in late stress.

The role of the HPA axis in susceptibility to experimental arthritis induced by streptococcal cell wall has been shown to be crucially important by *Sternberg* et al (1989). Lewis rats are susceptible to induction of arthritis whereas Fischer rats are not. The Lewis strain has impaired plasma corticotropin releasing hormone (CRH) as well as corticosterone responses to streptococcal cell wall, IL-1 and CRH. The hypothalami of Lewis rats respond poorly relative to Fischer hypothalami in CRH development to IL-1, acetylcholine, norepinephrine and a 5HT agonist (*Calogero* et al., 1992). Thus the HPA axis may be blunted because of a genetic background and render the animal susceptible to inflammatory events. These observations may have far reaching implications for inflammatory diseases (*Shanks* and *Lightman*, 2001).

Shanks et al. (2000) has additionally shown that exposure of Sprague Dawley rats to endotoxin in the neonatal period alters the adult HPA and immune responses to stress and inflammation. Specifically, rats injected on day 3 and 5 with endotoxin had greater mean corticosterone levels in adulthood but greater suppression of splenocyte proliferation to LPS. Most importantly Lewis rats were protected from adjuvant arthritis development if pre-treated in the neonatal period with endotoxin, suggesting that early encounters with LPS in the neonatal period could set the "tone" of the HPA axis in adult life.

SUMMARY AND CONCLUSIONS

Thus enteric microbiota can influence the HPA axis at a critical period in neonatal life and cause a permanent change as exemplified by these experiments and even in animals genetically predisposed to lowered HPA axis responses to stress. Similarly psychological stress (maternal deprivation) of neonates results in increased HPA axis responses in adulthood and this can be prevented by handling the animals during deprivation in the neonatal period. Amazingly the

molecular basis for these observations has been recently identified (*Weaver* et al., 2002). *Weaver* et al. (2004) have shown that increased grooming/licking in rats altered the offspring epigenome, at a glucocorticoid receptor gene promoter in the hippocampus. This particular epigenetic effect was reversible by cross-fostering and again persisted into adulthood. The effects of stress both prenatally and postnatally on the numbers of enteric commensal bacteria such as bifidobacteria and lactobacilli have also been observed (*Bailey* and *Coe*, 1999; *Bailey* et al., 2004). Some of the hormones induced by stress such as catecholamines may have both direct and indirect effects on enteric bacterial type and number as well as their functional activation. The known effects of catecholamines on the quorum sensing system of certain bacteria may in part explain this latter phenomenon.

Thus the interaction between bacteria and the HPA axis is bidirectional and should be taken into account when explorations of the HPA axis are undertaken and examined.

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