Chapter 8

IMMUNE FUNCTION

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1. INTRODUCTION

Human milk contains numerous components, which have been linked to immune functions. Initially it was easy to recognise such constituents as classical members of the immune system, for example antibodies and lymphocytes, but today the list is very long. This is due to the fact that we have learnt for one thing that certain nutrients, for instance polyunsaturated fatty acids, and immune components often cooperate and/or direct each other, as exemplified below. Another reason is that the central nervous system and the immune system, which developmentally have different origins, in fact are in several modes intertwined in their functions. This may be illustrated by the fact that hormones such as leptin and growth hormone have a cytokine structure and that certain cytokines such as the pro-inflammatory IL-1β, TNF-α and IL-6 activate the HPA stress axis and induce production of glucocorticosteroids[1], as well as leptin.[2]

Another aspect is based on observations that breast-feeding has several long-term effects on the infant child, such as defence against certain infections and immunological diseases such as allergy. The basis for such effects seems to be both nutrients and immune factors in the mother's milk. Some recent work showing examples of relations between milk components and short, as well as long term effects on the infant child's health are

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described below. These studies also illustrate that the many and and various forms of host defence provided via human milk have one capacity in common: they defend without inducing inflammatory mechanisms, which via cytokine release and phagocyte activation cause clinical symptoms like pain, increased temperature, tissue damage, loss of appetite and increased energy consumption.[3] Some milk components even actively counteract inflammatory processes.

2. ESSENTIAL FATTY ACIDS, BREAST-FEEDING AND IMMUNOLOGICAL EFFECTS

There is much confusion as to the possible role of essential fatty acids (EFAs) in the maternal diet and the risk of the breast-fed offspring in developing allergic diseases. For instance a recent study from Australia did not show that higher levels of *n*-3 fatty acids in maternal milk decreased the risk of developing allergic disease in breast-fed infants.[4] Previously an increased risk of developing allergy has been linked to low levels of *n*-3 fatty acids in the milk.[5-7] It could be, however, that it is not the absolute levels of these EFAs which are crucial, but the ratio between the n-6/n-3 fatty acids. This was suggested by an initial experimental study where we gave various levels of EFAs to rat dams during late pregnancy and lactation and investigated the effect of this diet on the development of neonatal tolerance in the offspring to a foreign protein, ovalbumin (OVA), given perorally to the dams and reaching the rat pups via the milk. A diet deficient in EFAs (7% hydrogenated lard) enhanced development of neonatal tolerance, not seen with a diet sufficient in EFA (7% soybean oil). The tolerance seemed to be mediated by regulatory T cells producing TGF–β. Increased levels of RNA for this suppressive cytokine was found in the local lymph glands.[8] The study was repeated with diets with defined ratios of *n*-6/*n*-3 EFAs. A diet with the ratio of 0.4 resulted in neonatal tolerance against the OVA, but also against an unrelated antigen human serum albumin.[9] This agrees with effects of regulatory T cells functioning via TGF-β. The tolerance included cell-mediated immunity as well as IgM, IgG and IgE antibody responses. With a ratio of 9, in contrast, neonatal tolerance did not develop against the food antigen given.

It seems relevant to test whether or not the ratios of *n*-6:*n*-3 EFAs in the food given in early life to babies via the milk has effects on the risk of developing allergic disease. Formulas today have ratios of *n-6*:*n*-3 closer to 9 rather than 0.4. Likewise the food of breast-feeding mothers may need to be analysed further from this point of view, not just studying *n*-6, or *n*-3 fatty acids as often done.

3. ANTI-SECRETORY FACTOR

This protein was discovered because it prevents the secretory effects of cholera toxin in the gut.[10-11] It also seems to have anti-inflammatory effects in inflammatory bowel disease.[12-13] We have previously shown this hormone-like structure to be present in milk from Guatemalan and Pakistani mothers, but usually not from Swedish mothers.[14]

In a recent small study we investigated the possibility of inducing the anti-secretory factor in the milk of Swedish mothers using a specially treated cereal known to promote the production of anti-secretory factor appearing in serum. Even in the limited group studied we could show how effectively the factor could be induced. Furthermore it turned out to significantly protect against the appearance of mastitis in these lactating mothers.[15] It is of course of major interest to expand these studies to investigate if the anti-secretory factor in the milk may protect the offspring from e.g. diarrhoea. Ongoing studies in Pakistan giving children with diarrhoea egg yolk from hens fed the special cereal and therefore containing the factor, may show us if there is also a treatment effect from giving the ready made factor.

4. LACTOFERRIN

This major human milk protein has been much studied and is well known for its anti-microbial effects, although there are no clinical studies to prove its protective capacity in the breast-fed infant. Such studies would be difficult to do. In recent work we have defined the bactericidal capacity of the whole molecule and of various peptides based on the 12-15 aminoacid long surface-exposed αβ region at the N-terminal end of the molecule, which have such effects.[16] Using these peptides, as well as the whole molecule, we have studied their anti-infectious and anti-inflammatory effects in mouse models. Giving dextran-sulphate to induce colitis in mice we could show that lactoferrin and certain of its peptides given perorally significantly decreased the number of CD4 positive cells, F4/80 positive macrophages and TNF-α producing cells in the colon mucosa. The level of IL-1β in the blood was also reduced, as was the typical shortening of the colon.[17] In parallel there was a reduced number of cells producing the immunosuppressive cytokine IL-10 in the colonic submucosa. The anti-inflammatory effect of lactoferrin is presumably due both to its anti-infectious effect and its capacity to inhibit the transcription factor NFκB, which is instrumental in bringing about the production of the main pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in leucocytes.[18]

In a model of urinary tract infections caused by *Escherichia coli* in mice orally given human lactoferrin, it appeared in the urine 2 hours later and killed bacteria. The urinary tract infection was significantly prevented compared to animals just given the vehicle ($p< 0.009$ for kidneys, $p< 0.0001$ for bladder). The number of urinary leucocytes and IL-6 levels in blood and urine were also reduced. One of the tested lactoferrin peptides also provided perorally gave significant protection.[19] These experimental data agree well with clinical observations showing that breast-feeding protects against urinary tract infections.[20]

It is likely that the protection against urinary tract infections via lactoferrin is also supported by the milk secretory IgA antibodies since they coat the gut bacteria, which are the major cause of such infections.[21] Such coating would prevent the bacteria from passing through the gut mucosa infecting via the blood as may occur in early infancy. It would also prevent the gut bacteria from climbing the urinary tracts from below, which is the most common route of infections in the urinary tract.

Lactoferrin has been reported to have several enzyme activities, including that of ribonuclease.[22] We asked the question whether or not bacteria killed in the gut, for instance by lactoferrin, might leak nucleotides and that these may be degraded and taken up in the intestine. In preliminary experiments in germfree rats we could actually see that addition of nucleotides to the diet enhanced their weight gain significantly and increased mitosis in enterocytes.[23] Human milk in addition of course contains nucleotides.

In further ongoing studies we find evidence that lactoferrin shows some capacities in similarity to heat shock proteins (HSPs), which are very conserved structures appearing in cells under stress. In similarity to HSPs lactoferrin seems to bind to ATP and it also has ATP-ase activity, although weak.[24] It might be possible that lactoferrin can interact with ATP coming from bacteria killed by anti-microbial agents, possibly such as lactoferrin.

Many components of human milk influence the microbial gut flora of the breast-fed infant, contributing to the microflora, which is the main stimulus for the expansion of the immune system, which is tiny, but more or less complete at birth.[25] This flora also has a central role in making the expanding immune system able to develop immunological tolerance against foods, pollen, autoantigens etc. This is the background to the Hygiene hypothesis, claiming that today neonates are often not permitted to be colonised with the proper microflora, originating mainly from being delivered next to the mother´s anus, like all mammals.[25] In addition many milk components contribute to the development of this flora. Lactoferrin for instance seems to promote growth of Bifidobacteria and hinders adherence of enteropathogenic *E. coli* to the gut mucosa.[26] Through its serine protease activity lactoferrin cleaves two colonisation factors of *Haemophilus influenzae*,[27] presumably

decreasing their appearance on, and pathogenic attachment to respiratory epithelium.

5. HUMAN MILK ANTIBODIES

The only milk components proven in clinical studies to protect against infections are secretory IgA (SIgA) antibodies directed against various intestinal pathogens such as *Campylobacte* and *Vibrio cholerae*. [25] It is likely that there is similar protection against respiratory infections and, as already mentioned, urinary tract infections, due to the capacity of the milk SIgA antibodies in preventing microbes attaching to the various cellular structures on mucosal membranes, which they use to initiate the contact with the host. Many milk oligosaccharides and glycoconjugates act in a similar way by appearing as analogues to the structures on mucosal epithelium that microbes need to bind to for starting an infection.[28] In the next step the microbes would invade deeper tissues where they meet circulating antibodies like IgM and IgG, as well as T cell mediated immunity. These forms of defence always activate phagocytes and result in production of pro-inflammatory cytokines giving clinical symptoms such as local pain, fever, loss of appetite, inflammatory tissue damage and increased energy consumption.

Human milk contains numerous anti-inflammatory factors.[29] Defence against both infection and inflammation is provided via human milk. Host defence via serum antibodies, the complement system and phagocytes regularly induce inflammation when activated. In early infancy the complement system, as well as the phagocytes are not fully functional. [25] Thus the transplacental IgG antibodies may not provide efficient defence. This may be why neonatal septicaemia/meningitis is such a threat to non-breast-fed infants. [30]

Due to the entero-mammaric link of the lymphoid cells producing the milk SIgA antibodies, they are directed against the microbes passing through the mother's intestine.[31] These include microbes not only in the gut microflora, but also swallowed respiratory tract pathogens. As a result the maternal milk provides protection against a very broad spectrum of microbes present in the mother's milieu. It is remarkable how SIgA antibodies of a very broad spectrum of specificities remain present throughout lactation, for instance against numerous *E. coli* O and K antigens, regardless of bacteria with those antigens were present in the gut at the time of sampling the milk.[32-33] It is not quite clear whether this represents antibodies also produced by memory cells to maintain significant levels against very many antigens throughout lactation. Usually SIgA responses are expected to be of

a relatively short duration and be dependent on re-exposure to be kept up. Whereas Swedish infants usually showed a rather stable microflora in the gut with respect to *E. coli* strains, Pakistani children had a rapid turnover of various serotypes of *E. coli*.[34-36] It is probably of great help for the infant that the milk can contain SIgA antibodies of such a wide range of specificities and remain throughout lactation.

The milk SIgA antibodies promote growth of *E. coli* with type 1 adhesins, because they bind to carbohydrate side chains of such antibodies.[37-38] These *E. coli* are generally of low virulence. Recently it has also been suggested that SIgA antibodies favour the formation of a biofilm of microbes on an epithelial surface.[39] This may favour normal microbial colonisation of the breast-fed infant.

We found SIgA and IgM antibodies to *E. coli* and poliovirus in saliva and meconium samples obtained during the first day of life in Swedish newborns.[40] It should be added that poliovirus does not exist in Sweden because of general vaccination with killed poliovirus vaccines. These antibodies did presumably not originate from their mothers, since they were also present in the offspring of a mother with hypogammaglobulinemia, (lacking IgA and IgM) and of mothers with IgA deficiency.[41] Normally IgA and IgM do not pass across the placenta. The SIgA and IgM antibodies in the neonates may have been induced by anti-idiotypic antibodies specific for *E.coli* and poliovirus, because we found such antibodies in serum and milk from Swedish mothers.[42] In an experimental model we even found evidence that anti-idiotypic antibodies given in the neonatal period could enhance specific immune responses over two generations.[43] Such long-term enhancement of immune responses may take place via transfer of maternal anti-idiotypic antibodies via the placenta, as well as the milk.

6. α-LACTALBUMIN

Recent work by Svanborg *et al.* has shown that the major milk protein α–lactalbumin can take the shape of HAMLET, human α-lactalbumin made lethal to tumour cells. In small aggregates of α-lactalbumin exposed to the low pH in the stomach, the protein is unfolded and forms a binding site for oleic acid, which may originate from triglycerides degraded in the stomach. In this form HAMLET kills cancer cells. This activity has been demonstrated against some 40 malignant human cell lines.[44] *In vivo* it was found that HAMLET killed human glioblastoma cells in an animal model. Most recently a clinical effect was found against human papilloma.[45] Human glioblastoma cells in brain xenografts were also killed by HAMLET.[46]

Presently it is not clear whether the fact that breast-feeding provides significant protection against breast cancer is in any way is linked to the effects of HAMLET.[47] It is unknown if breast-feeding via HAMLET can protect against tumours in infants. There have been some suggestions that breast-feeding may decrease the risk of e.g. childhood leukaemia and some other tumours, but this is debated.[25, 48]

7. MATERNAL SIGNALS FROM THE MOTHER TO THE INFANT: CYTOKINES, HORMONES, GROWTH FACTORS ETC

Some studies suggest that breast-feeding may enhance vaccine responses in the offspring.[49-52] Other studies have not seen such effects.[53-57] It is not really surprising that different outcomes have been obtained. Different vaccines have been applied in different populations with varying pre-vaccination degrees of exposures and immune responses and with different length of breast-feeding. The transplacental IgG antibodies in the infant may inhibit vaccine responses relating to their levels.[58]

One explanation for a stimulatory effect of breast-feeding may be that the milk may contain anti-idiotypic antibodies against the vaccine agents as mentioned above.[42] The long-term enhancement of the IgG2 antibody response *to Haemophilus influenzae* type b (Hib) in breast-fed children may be due to the IFN-γ in the milk specifically enhancing this kind of protective antibody production. This is seen especially against bacterial polysaccharide capsules, which are important virulence factors on pneumococci, meningococci, certain *E. coli* and Hib.[59] This observation agrees with the finding that exclusive breast-feeding for >13 weeks compared with < 13 weeks resulted in enhanced protection against invasive infections with *H. influenzae* type b for up to 10 years.[60-61] In agreement with this higher levels of antibodies to Hib in preschool children related to the longer duration of breast-feeding.[62] The enhancement of the protective IgG2 anti-Hib responses correlated with the appearance of the idiotypes Id-1 and Id-2 identified for the anti-Hib, indicating a rather specific enhancing effect of breast-feeding.[63] Recent evidence suggests that breast-feeding for >90 days results in a higher proportion of children who up to 13 months of age have protective levels of antibodies to Hib and to pneumococcus 6B, compared to those breast-fed for a shorter period (Silfverdal and Ekholm, this volume abstract A62).

The higher levels of IFN-γ in breast-fed infants compared to non-breast-fed, who attract RSV infection may relate to one or some of the many signals in the milk.[64] That may also explain the higher levels of IgA and lactoferrin

in the urine of breast-fed infants and the elevated niveau of SIgA in the saliva during the first 6 months of life in breast-fed compared to non-breast-fed infants.[65] For instance the TGF-β in the milk may be responsible for this. High levels of TGF-β in the milk may also help prevent or delay allergic disease [66], possibly since this cytokine is anti-inflammatory and lower levels of TGF-β1 are related to higher IgE production against cow´s milk protein by B cells from cow's milk protein allergic children.[67] In contrast high levels of IL-4 in milk relates to higher IgE production. Furthermore TGF-β levels in the mothers´ milk were inversely related to the risk of the infant to develop wheezing.[68] This would agree with another recent observation: a significant positive relation was found in milk between TGF-β2 and the proportion of polyunsaturated fatty acids and a negative association between TGF-β2 and saturated fatty acids.[69] This again may be considered to agree with our results in the experimental model where a low ratio of *n*-6:*n*-3 fatty acids given to pregnant and lactating rats favoured the development of neonatal tolerance to a food protein in the offspring, as described earlier.[9]

Actually, many factors in milk may be linked to the risk of the offspring developing allergic disease. A recent finding suggested that the presence of subclinical mastitis relates to the risk of atopic disease and positive skin-prick-tests in babies.[70] Possibly this results from an increased exposure of the breast-fed offspring to allergens from the mother via the milk.

The fact that the thymus of fully breasted infants reaches twice the size of the thymus of non-breastfed infants illustrates another effect of signalling via the milk.[71] In fact the content of IL-7 in the milk relates to the size of the thymus.[72] This cytokine also enhances the development in the Tγδ cells, which are found as intraepithelial lymphocytes in the gut.[73-74] A larger thymus shows a higher output of T cells and a small thymus at birth predicts a higher infant mortality from infections in a study from Africa, independent of other factors known to reduce the size of the thymus, such as low birthweight and malnutrition.[75] A small thymus due to undernutrition expands on refeeding. It is not known whether IL-7 in milk is also instrumental in this effect on the thymus, but it is likely that leptin is, as detailed below.

There are numerous other cytokines in milk and also some soluble cytokine receptors, such as TNF-αR, which may modify the effects of the TNF-α in the milk.[76] The TNF-α enhances the production of the polyIgR, which transports IgA dimers into milk. The IL-6 in milk, like the TGF-β, enhances the development of B lymphocytes and also the production of α1-antitrypsin by phagocytes, possibly explaining why this protein is present in the stools of breast-fed infants.[77-78]

Human milk contains many hormones and growth factors. Some of these have effects on the immune system. A striking example of this is leptin,

which has the structure of a cytokine. Leptin is increased by the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, which is one explanation why infections usually lead to a loss of appetite.[2] Leptin stimulates the differentiation and proliferation of haematopoietic cells, increases the functions of monocytes/macrophages and influences T cell functions. Leptin also stimulates proliferation and survival of T cells in the thymus.[2] Insulin-like growth factor, epidermal growth factor and prolactin in milk seem to act as immuno modulatory components in the gut.[79] Such factors as well as the many anti-inflammatory factors demonstrated in human milk provide an interesting area where many more biologically interesting functions may be waiting to be defined.

8. MILK LYMPHOCYTES

Several experimental studies, recently summarised, suggest that there may in fact be a transfer of lymphocytes via the milk to the offspring.[80] It really seems as if there can be an uptake not only of the cells by the lymphoid system, but even a transfer of immune capacity to judge from experiments in sheep showing transfer via milk of priming of a vaccine response.[81] In humans the observation showing that the child develops tolerance to the mother's HLA, may help explain how the maternal lymphocytes may be accepted by the infant's immune system.[82]

9. LONG TERM EFFECTS OF BREAST-FEEDING ON PROTECTION AGAINST CERTAIN INFECTIONS, TUMOURS, OBESITY ETC

The previously mentioned possible effects on the immune system of the offspring via milk cytokines, growth factors, anti-antibodies, the possible transfer of immunological information via sub-cellular elements from milk lymphocytes and so on, may help explain the observations that the protection afforded by breastfeeding against certain infections may be long lasting. The first observation was made by Saarinen.[83] She noted an enhanced protection by breast-feeding against otitis media lasting for the 3 years of observation. Similar observations have since been reported for gastroenteritis, and respiratory infections for up to 7 years.[84] The improved protection by breast-feeding against invasive Hib infections was still present 10, but not 15 years later and paralleled increased levels of protective IgG2 antibodies up to school age.[61] This may be linked to the long term enhancement of the IgG2

antibody response against protective *H. influenzae* type b by breast-feeding previously mentioned.[59] One study supports the suggestion that breast-feeding resulted in enhanced protection against urinary tract infections for the first 2 years of life.[20] A protective effect against more severe manifestations of clinical measles by breast-feeding lasting up to the age of 10 years was recently proposed (Silfverdal and Montgomery, this volume, abstract A63).

Long-term protection against allergic disease has been much debated and questioned, but recent critical studies provide evidence that breast-feeding offers significant protection, although weak, against both wheezing, asthma and eczema.[85-87]

Similarly, long-term protection by breast-feeding has been claimed against obesity as recently summarised.[25] It is likely that further work on long-term effects of breast-feeding, especially on protection against infections and inflammatory and certain other diseases will provide additional interesting information.

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