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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

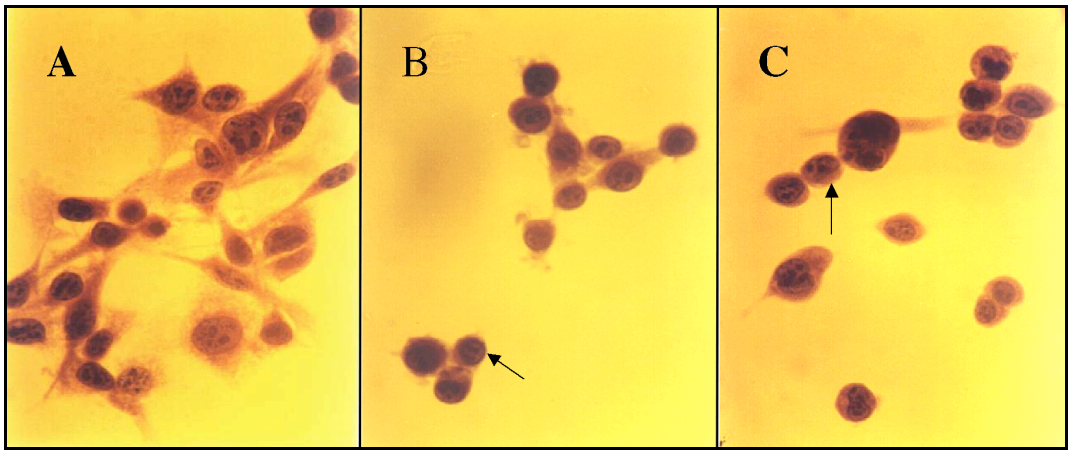

Figure 1: **A)** B16-F1 cells – control 24 h. **B)** Small groups of atypical round cells (arrow) with a rim of cytoplasm. (500 μg/ml of LF, 24 h.). **C)** Groups of hyperchromatic round cells (arow) with pyknotic nuclei (500 μg/ml of LF, 24 h.). Magnification 800x.

(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

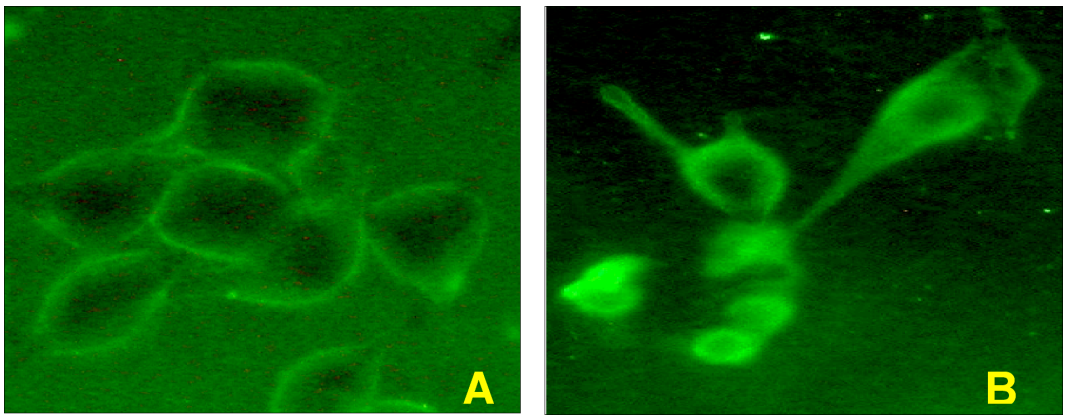

Figure 2: A) Binding of LF to B 16-F1 cells. **B)** Internalisation of LF into B 16-F1 cells.

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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