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DIETARY COMPONENTS: EFFECTS ON MUCOSAL AND SYSTEMIC IMMUNITY

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SUMMARY

It is a major function of the immune system to control and down-regulate reactivity to food components. An important mechanism is to develop specific immunological tolerance to foods. This seems to mainly occur through mucosal membranes and via several mechanisms like suppressor cells and anergy in and elimination of specific lymphocyte clones.

Deficiencies of nutrients is a large global problem. Undernutrition is usually not a direct cause of death in spite of its many untoward effects on mucosal and systemic immunity, but is a contributing factor adding to the risk of dying from infections. Undernutrition and frequent infections are most often consequences of the pathology of poverty. Prevention of infections and food supplementation are both required and can save the lives of millions of children every year, which as a paradox contributes to decreased population growth. This is due to the fact that a lower infant mortality is usually followed by a decreased birth rate.

INTRODUCTION

Central tolerance in the thymus limits the number of auto-aggressive T cell clones. Mucosal tolerance develops to diminish or avoid unnecessary inflammatogenic immune reactivity to antigenic material in our foods and our normal microbial flora on mucosal membranes, especially in the gut. What determines whether immunological tolerance or immune reactivity is to occur against e.g. foods at the mucosal level is not well understood.

FOOD ANTIGENS AND THE MUCOSAL AND SYSTEMIC IMMUNE SYSTEM

Most new-borns who are fed formula or meet food antigens from the mother's diet in her milk will not react clinically to these food antigens. Still it has been shown that there is an antibody response to e.g. cow's milk proteins during the first year of life which slowly decreases (*Lippard* et al., 1936). This response often includes IgE antibodies as well (*Hattevig* et al., 1984). Normally, however, this response does not cause any untoward effects and van-

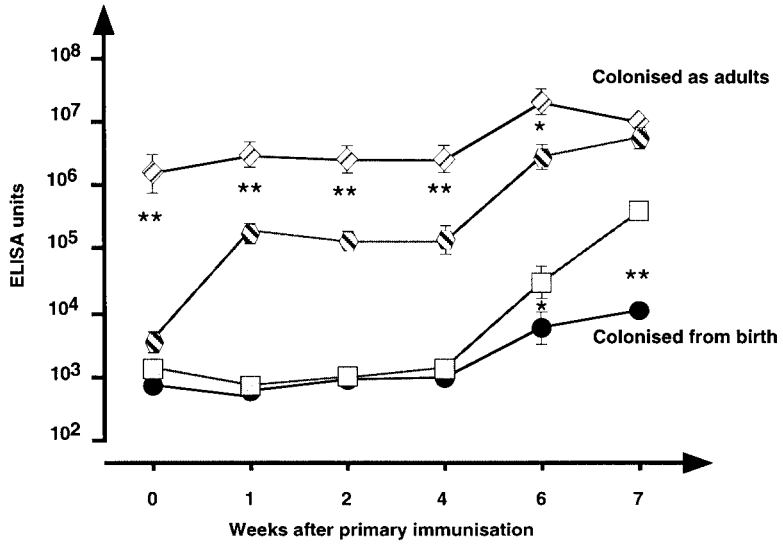

Figure 1: Serum IgG anti-LPS antibodies in young and adult colonised animals. The rats were subcutaneously immunised with a mixture of killed *E. coli* 06:K13 and Freund's complete adjuvant in the hind leg six weeks (week 0 in the figure) after the colonisation, and booster immunised 6 after the primary immunisation. The p-values (Mann-Whitney U test) are obtained by comparison with the age matched non-colonised rats, * = $p<0.05$, **= $p<0.01$.

ishes. A further increase, or remaining levels of IgE antibodies are mainly found in atopic children, many of whom later develop allergic reactions. This would suggest that mucosal tolerance normally develops early in life.

Two studies suggest that the secretory IgA antibodies to cow's milk proteins in the mothers' milk can affect the breastfed infants reactivity: the higher the levels of breastmilk antibodies to cow's milk the smaller the risk to develop cow's milk allergy (*Machtinger* and *Moss*, 1986; *Casimir* et al., 1989). It is not clear whether breastfeeding can enhance development of tolerance.

Cow's milk allergy is common in early childhood, but some 80-90% spontaneously loose this hyperreactivity by the age of 2-3 years, which could be interpreted as development of immunological tolerance. In the so called "allergy march" allergic children start with food allergy and a few years later they become allergic to inhalant allergens developing hayfever and asthma. Does this indicate that tolerance develops more efficiently in the gut than via the mucosa of the respiratory tract?

In fact we do not know very well how oral tolerance develops. It seems that the intestinal flora may be important, since it is more difficult to induce tolerance in germfree than conventional animals (*Wannemuehler* et al., 1982). There are also observations suggesting that whereas LPS given parenterally breaks tolerance, it enhances development of tolerance if given in the gut (*Khoury* et al., 1990; *Gaboriau-Routhiau* and *Moreau*, 1996).

Our recent studies show that animals colonised early in life with a transgenic *E. coli* producing ovalbumin (OvA) develop tolerance both to a T cell independent antigen, LPS, and T cell dependent antigens, type 1 pili and OvA. This was seen by decreased levels of specific antibodies to LPS, OvA and type 1 pili and a lower delayed type hypersensitiv-

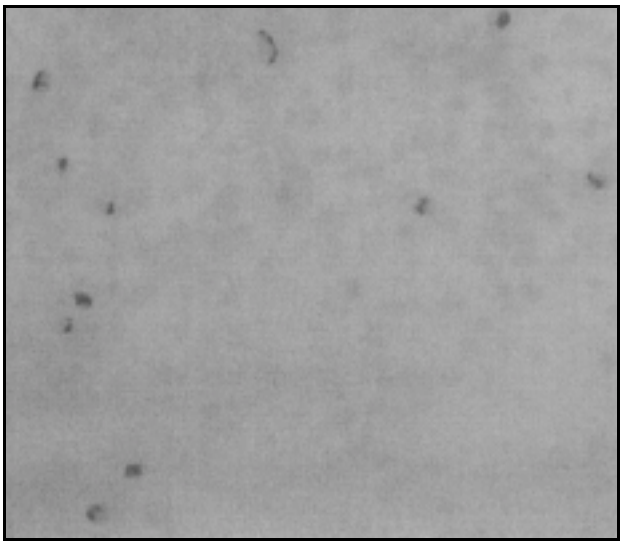

Figure 2: TGF-β positive cells (stained dark) in the T-cell area of the draining lymph node in a young colonised rat. The rats were subcutaneously immunised with a mixture of killed *E. coli* 06:K13 and Freund's complete adjuvant in the hind leg six weeks after the colonisation, and booster immunised 6 weeks later. No TGF-β positive cells were found in non-colonised rats (not shown).

ity reaction (OvA) compared to a non colonised control group (Figure 1). The reverse was seen in adult colonised animals. Further, TGF-β positive cells were found in the draining lymph nodes in the young colonised and tolerised animals after immunisation with dead bacteria (Figure 2). This indicates that the tolerance against bacterial components is fully or partially mediated by active suppression (*Karlsson* et al., 1997a).

Our information about how immune responses or mucosal tolerance develops in the intestinal mucosa is also limited. It has been demonstrated that dendritic cells from the intestinal mucosa can present antigens to naive T cells (*Liu* and *MacPherson*, 1993; *Liu* and *MacPherson*, 1995). The epithelial cells have been proposed to be antigen presenting, but this is still an open question. Our work with rat epithelial cells shows that MHC class II molecules are not found on their surface, but in vacuoles in the cytoplasm. The lamina propria contains a network of dendritic cells which may well be the central antigen presenting cells (APCs), possibly after having met the MHC class II from the epithelial cells in complex with peptides from e.g. food proteins taken up by the epithelial cells. The dendritic cells could present antigen to the local memory $CD4^+$ T cells located centrally in the villi, and/or migrate to the mesenteric lymph nodes and there appear as APCs for presentation to naive T cells. As these APCs normally lack the ”danger” signals the activated T cells turn into regulatory T_H3 cells producing TGF-β upon restimulation.

As mentioned above it might be that the MHC class II molecule-containing vacuoles in the gut epithelium are of importance for the development of oral tolerance. Rats start to express MHC class II vacuoles in the gut epithelium at four weeks of age. This coincides in time with the possibility to induce oral tolerance with active supression against fed soluble protein antigens indicating a re-

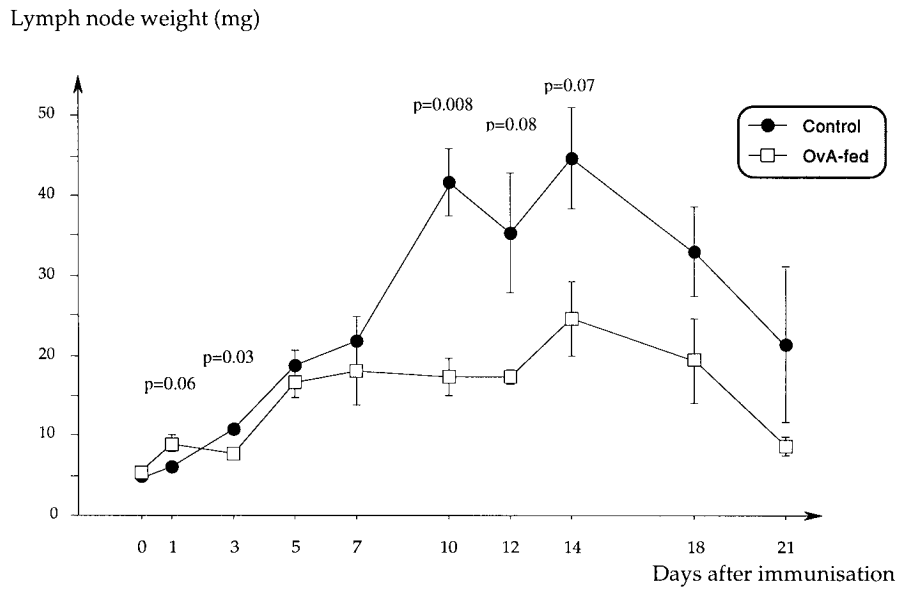

Figure 3: Rats made tolerant to OvA, and control rats, were immunised s.c. in the hind leg with a mixture of OvA and HSA in Freund's complete adjuvant. At different intervals after challenge, the draining lymph nodes were removed, and weighed. Three animals from each group was sacrificed at every time-point. The results are shown as mean ± SEM; three experiments with similar results have been performed.

quirement for MHC class II expression by enterocytes for the induction of oral tolerance (*Miller* et al., 1994; *Karlsson* et al., 1995). A fully developed intestinal proteolytic system also seems to be required to generate the tolerogenic peptides from the fed proteins (*Whitacre* et al., 1991; *Hanson* et al., 1993a; *Hanson* et al., 1993b). It is interesting to note as well that both bacterial colonisation of germfree rats and treatment with LPS dramatically increase the expression of MHC class II molecules particularly in the epithelium, but also in the lamina propria dendritic cell compartment.

It is likely, but not definitely demonstrated, that the intraepithelial lymphocytes have a regulatory role for immune reactivity in the intestinal mucosa, possibly related also to the development of tolerance.

SENSITISATION OR TOLERANCE TO OVALBUMIN (OvA) IN THE GUT OF RATS

It is quite easy and efficient to induce tolerance in rats by feeding them OvA. After immunisation and colonisation with *E. coli* genetically manipulated to produce OvA the gut mucosa of the tolerised animals contain few mast cells coated with IgE, few eosinophils and the goblet cells are not activated and emptied. Centrally in the villi these tolerant animals have a $CD4^+$ T cell population carrying CD25, the IL-2R α-chain (*Dahlman-Höglund* et al., 1996), which might be responsible for the down regulation of the immune response to OvA. Such cells were not found in the non tolerant animals.

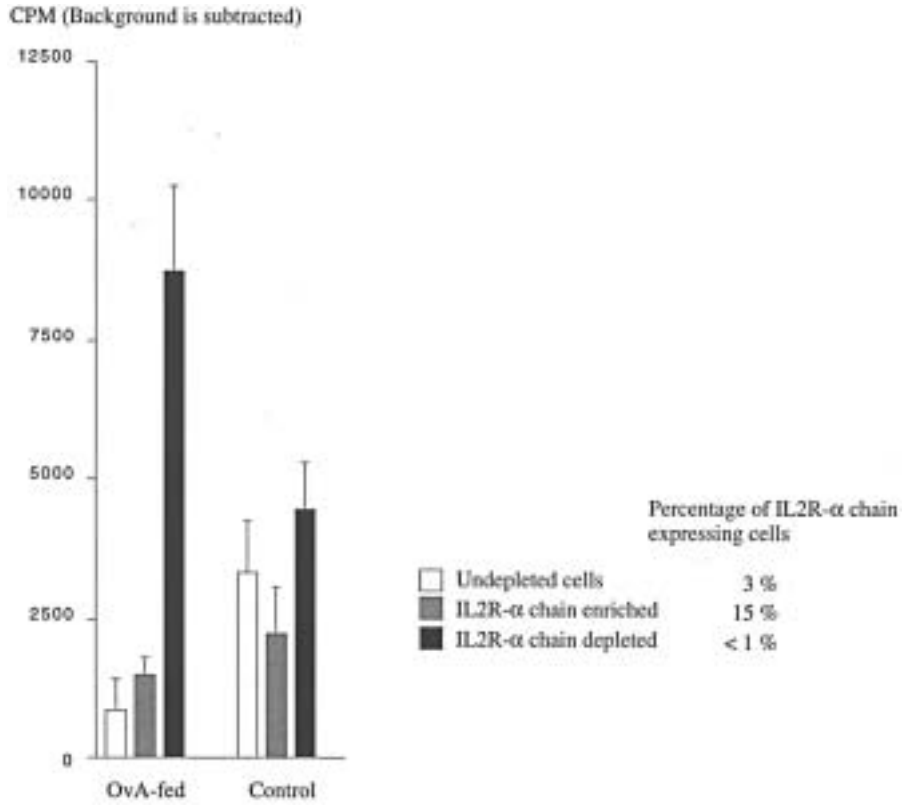

Figure 4: Proloferation of MLN-cells after OvA stimulation. 10 weeks old rats were given ovalbumin (OvA)-containing pellets for two weeks; control rats were given ordinary pellets throughout the experiment. Two weeks after the removal of the OvA-pellets all rats were immunised perorally with 20 μg Cholera toxin, 20 mg OvA and 20 mg Human serum albumin in 2 ml PBS. The rats were subsequently immunised another two times in the same way, with a 5-day interval (in total 3 times in 15 days). One day after the last immunisation, the rats were sacrificed, and the mesenteric lymph nodes were taken out. The lymph node cells from each group of rats (three rats per group) were pooled, and either enriched for or depleted of CD25-expressing (IL2-Roc chain-expressing) cells, using superparamagnetic beads (MACS). The cell populations were stimulated with OvA *in vitro* for 4 days, and the proliferation was measured.

$CD25^+$ cells isolated with magnetic beads from draining lymph nodes in the tolerised animals are shown to suppress T cell reactivity to OvA *in vitro*, as well as B cell responsiveness measured as IgE and IgG antibody production to OvA upon transfer (*Lundin* et al., 1997a) (Figure 3). That these cells are generally present was evident from the fact that after immunisation in the food pad with OvA together with an unrelated antigen in Freunds complete adjuvant the OvA tolerant animals showed no swelling of the draining popliteal lymph nodes despite the use of such a very strong adjuvant (*Lundin* et al., 1997b) (Figure 4). Furthermore, TGF-β production was more frequently detected in the lymph nodes from the tolerised than from the sensitised animals (*Karlsson* et al., 1995; *Lundin* et al., 1997b).

Another striking finding was that in tolerised animals the suppression of immune reactivity would include also a

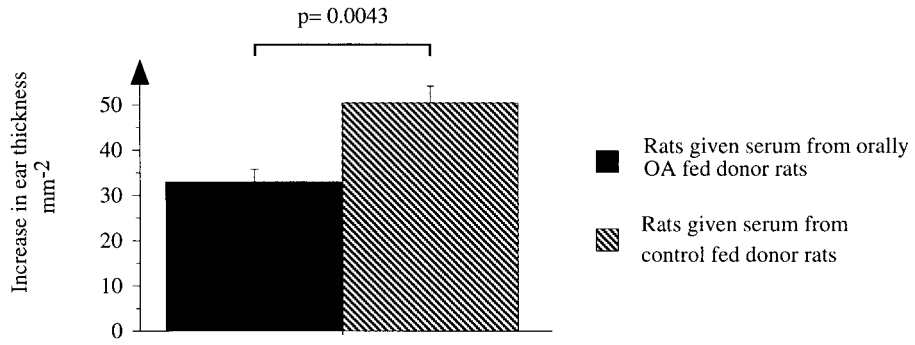

Figure 5: DTH reaction against human serum albumin (HSA) measured as increase in ear thickness 24 hours after challenge with 50 μg HSA in 20 μl PBS. All rats were subcutaneously immunised with a mixture of OvA and HSA in Freund's complete adjuvant in the hind leg two weeks prior to the DTH challenge. The p-value (Mann-Whitney U test) are obtained by comparison with rats receiving control serum.

second unrelated antigen given simultaneously, so called bystander tolerance (*Dahlman-Höglund* et al., 1995; *Lundin* et al., 1996). This is presumably due to the non-specific suppression mediated by e.g. TGF-β from the $CD4^+$ suppressor cells.

Oral tolerance may be due to suppressor cells, like those described above, as well as anergy in or deletion of antigen specific cell clones. The latter two mechanisms would obviously not result in bystander tolerance. There were also indications that the oral tolerance induced in young and older individuals may differ in that active suppression and bystander tolerance dominates in adult animals, but anergy and/or deletion in young animals. This may be due to the different levels of MHC class II expression in the gut epithelium observed in young and adult animals as discussed above (*Lundin* et al., 1996).

We have also observed that tolerance to a soluble protein antigen can be transferred with a serum factor from antigen fed donors. This factor is also capable of inducing an actively suppressed immune response in the recipients as shown by bystander tolerance to an unrelated antigen, human serum albumin (*Karlsson* et al., 1997b), (Figure 5). The nature of this serum factor has not been fully described. We are currently investigating the possibility that it consists of preformed MHC class II-peptide complexes possibly emanating from the gut epithelium. Interestingly SCID mice that lack MHC class II in the epithelium are unable to produce the tolerogenic serum factor (*Furrie* et al., 1994).

THE CONSEQUENCE OF DEFICIENCY OF VARIOUS DIETARY COMPONENTS ON THE MUCOSAL AND SYSTEMIC IMMUNITY

Above has been described the consequences of the normal exposure of the immune system to dietary components.

Below will follow a description of some of the consequences for the immune system of deficiencies in various food components, like proteins and micronutrients. The clinical consequences of such undernutrition will be debated since they are not always so clear-cut,

although many forms of undernutrition may lead to various abnormalities of the immune system.

Mostly undernutrition, especially in field studies, is poorly defined. Undernutrition is often labelled as protein-calory undernutrition, PEM, but simultaneous deficiencies of various important micronutrients like vitamin A, zinc and iron are usually not determined. This makes it difficult to evaluate many earlier studies of undernutrition and the effect on the immune system.

EFFECTS OF UNDERNUTRITION ON THE IMMUNE SYSTEM

PEM has been reported to cause "nutritional thymectomy", as well as diminished spleen and lymph nodes. In cases of kwashiorkor (mainly protein deficiency) and marasmus (mainly energy deficiency) there is less lymphoid tissues in the tonsils, appendix and the Peyer's patches. In severe PEM $CD4^+$ T cells seem to be more reduced than $CD8^+$ cells. In experimental PEM in rodents there is impairment of mesenteric lymph node cells (*Chandra*, 1992; *Gupta*, 1993; *Keusch*, 1993; *Hanson* et al., 1998).

Proliferative responses of lymphocytes to mitogens are reduced, as are delayed type hypersensitivity reactions (DTH). B cells are mostly normal. However, in children before the age of 7 months there is panhypoimmunoglobulinaemia, but later polyclonal hyperimmunoglobulinaemia.

Undernutrition of lactating mothers decreased the S-IgA levels in milk, although within the normal range, whereas avidity of certain specific S-IgA antibodies seemed to be impaired (*Herías* et al., 1993). Severe malnutrition results in decreased secretory component (SC) and S-IgA; also S-IgA responses to measles and poliovirus vaccines are decreased (*Chandra*, 1975; *Watson* et al., 1985).

Complement levels are reduced in PEM and bacterial killing by phagocytes may be impaired. So is production of cytokines like IL-1, IL-2 and IFN-γ, as well as the responsiveness of T cells to cytokines (*Chandra*, 1992).

UNDERNUTRITION AND FREQUENT INFECTIONS

The many effects of undernutrition on the mucosal and systemic immune system should be expected to result in impaired host defence and decreased capacity to inflammatory reactivity. However, that is not so clear from clinical studies. A major reason for this is that undernutrition and frequent infections are both part of the pathology of poverty.

Early work assumed that the frequent infections were the consequences of the undernutrition and the impairment of host defence. Therefore large programmes with food supplementation were initiated in poor populations. They had very limited effects because the frequent infections continued and they are a major cause of undernutrition. This is due to the reduced appetite during infections, increased losses of nutrients due to vomiting and diarrhoea, impaired digestion and uptake of nutrients, mucosal changes including inflammation caused by released cytokines, NO and activated inflammatory cells on the vast mucosal surfaces of the respiratory tract and gut consuming much energy (*Mata*, 1978, 1992).

Mata, who did much of the classical

work in this field, showed that repeated infections were the main cause of kwashiorkor and marasmus. He also estimated that as much as 21% of yearly calories and 24% of total yearly protein was not consumed by children in Guatemala because of frequent diarrhoea (*Mata*, 1992). The effects of the repeatedly disturbed intestinal flora by all these infections are not quite clear.

On this basis it was understood that undernutrition must be fought as part of the pathology of poverty: prevention and treatment of infections, food supplementation, and education, especially health education, are all essential parts of preventive programmes.

Later it has been demonstrated that although undernutrition per se does not kill, it can add to the problem of repeated infections. Thus underweight children (<70% weight for age) had a higher risk of diarrhoea, especially of chronic diarrhoea (*Bhandari* et al., 1989). Skin test anergy (DTH) related to a higher attack rate and longer duration of diarrhoea (*Koster* et al., 1987). Although in other studies the relation between undernutrition and diarrhoea was modest (*Baqui* et al., 1993), it must be realised that so many confounding factors are at play in the situation of the children studied that it becomes very difficult to define causative relationships. Still *Pelletier* (1994) claims a definite relation between undernutrition and child mortality. Although infections are the major cause of death, undernutrition is a contributing factor. These two risk factors potentiate each other, according to Pelletier 8-10 times more than previous more conservative measures.

It must be stressed again that the undernutrition in field studies of children mostly are not well defined. *Good* and *Lorenz* (1992) could not reproduce the severe immunodeficiency caused by PEM in experimental animals. Only if there was also a zinc deficiency then cell-mediated immunity was impaired. They showed that mice on chronic calorie restriction – undernutrition without malnutrition – had a prolonged life, less cancer and autoimmune diseases (*Good* et al., 1991).

MICRONUTRIENT DEFICIENCIES AND THE IMMUNE SYSTEM

Vitamin A deficiency

While about 250.000 children turn blind from xerophtalmia caused by severe vitamin A deficiency every year, some 125 million children have subclinical vitamin A deficiency. Vitamin A deficiency has numerous effects on the host defence mechanisms and it is clear that it should make a major difference if lack of this micronutrient is part of the deficiency pattern in cases of undernutrition.

Vitamin A deficiency and its effect on host defence

Vitamin A deficiency and its effect on host defence has been mainly studied in experimental animals (*Hanson* et al., 1998). The deficiency impairs serum IgM, IgG and IgE antibody responses to most antigens, but for T cell independent antigens of type 1 (*Pasatiempo* et al., 1990; *Wiedermann* et al., 1993b). The S-IgA response in the bile of rats is reduced by 90% and is linked to fewer antibody producing cells in the mesenteric lymph nodes (*Wiedermann* et al., 1993a). SC is not clearly decreased.

The vitamin A deficient rats are underweight by some 25% due to loss of appetite. However, pair-fed animals repleted with vitamin A have normal antibody responses, which is also seen in deficient rats supplemented with retinoic acid (*Wiedermann* et al., 1993a). This suggests that the immune abnormality is

really related to the vitamin A deficiency, not to the general undernutrition - PEM.

B lymphocytes from vitamin A deficient rats have recently been demonstrated to increase proliferation of activated T cells (*Bjersing* et al., 1997). T cells from vitamin A deficient rats seem to be activated as T_H1 cells, producing increased amounts of IFN-γ and IL-12, but less IL-5 and IL-6 than control animals (*Carman* and *Hayes*, 1991; *Wiedermann* et al., 1993b, 1996b). As a result B lymphocytes get less support presumably contributing to the reduced antibody production.

Delayed type hypersensitivity can be impaired, or increased under different circumstances (*Smith* and *Hayes*, 1987; *Wiedermann* et al., 1993b, 1996a). Children with vitamin A deficiency show reversible disturbances of their T cell populations with reduced $CD4^+$ and $CD45Ro^+$ T cells (*Semba* et al., 1993).

Vitamin A deficiency has effects on numerous other mechanisms which are important for host defence. NK cells are fewer and with lower cytotoxic capacity (*Zhao* et al., 1994). However, their ability to produce IFN-γ seems undisturbed. Uptake of bacteria, as well as killing by macrophages and neutrophils is impaired (*Ongsakul* et al., 1985; *Wiedermann* et al., 1995, 1996b).

Since the differentiation of mucosal epithelium, including goblet cells, are dependent on vitamin A it is obvious that the deficiency impairs the epithelial as well as the mucus barrier (*Rojanapo* et al., 1980; *De Luca* et al., 1994).

Colonising the intestine of vitamin A deficient rats with an *E. coli* of low virulence, these bacteria appeared in large numbers all through the intestinal tract (*Wiedermann* et al., 1995). These rats showed increased levels of serum antibodies to the *E. coli* 0 antigen of the isotypes IgM, IgG and IgE. But the number of IgA secreting B cells in the lamina propria were lower than in vitamin A replete rats. There was also an increased translocation of the *E. coli* to mesenteric lymph nodes, kidneys and joints, inducing arthritis.

Adherence of bacteria to intestinal and respiratory epithelium is increased in vitamin A deficient animals (*Gabriel* et al., 1990; *Schoeb* et al., 1993). This might enhance translocation. Gnotobiotic rats with vitamin A deficiency showed increased translocation to mesenteric lymph nodes after colonisation with *E. coli* strains compared to controls. The expression of P fimbriae did not contribute to the increased translocation. The translocation also occurred in spite of the fact that the vitamin A deficient rats had higher levels of serum IgG and IgM antibodies to the bacteria than the vitamin A replete controls. It might be that these antibodies are not protective, but rather inflammatogenic.

A model of arthritis caused by *Staphylococcus aureus* applied in vitamin A deficient rats showed a worse course of the arthritis (*Wiedermann* et al., 1996b). The T cell response was increased in these animals, while B cell reactivity was unchanged. Phagocytic uptake and killing by phagocytes was decreased as was complement lysis activity.

Measles as well as several other infections are well known to reduce vitamin A levels. Vitamin A supplementation should always be given to children with measles, whether in developed or developing countries.

Rotavirus infections in mice with vitamin A deficiency destroyed the villus tips and exposed the lamina propria to the small intestinal content (*Ahmed* et al., 1990). DTH of these animals was reduced and so was the antibody response to the rotavirus.

The consequences of subclinical vitamin A deficiency in man

The consequences of subclinical vitamin A deficiency in man has been

much debated. A meta-analysis of several vitamin A supplementation studies in poor communities comes to the conclusion that mortality is significantly reduced (*Fawzi* et al., 1993). Several studies find reductions of some 30 per cent (*Anonymous*, 1993; *Fawzi* et al., 1994, 1995).

However, from clinical studies it is not clear how this comes about. They do not clearly show that supplementation saves numerous children from dying in pneumonia or diarrhoea as expected to explain the 30 per cent. However, it was found that there is a reverse relationship between vitamin A deficiency measured as blood retinol and diarrhoea (*Tafesse* et al., 1996). Supplementation reduced the incidence and the severity of diarrhoea as well as mortality (*Fawzi* et al., 1995; *Ross* et al., 1995). However, other studies found no effect, or even an increase in the incidence of diarrhoea of young children during the two first weeks after supplementation (*Bloem* et al., 1990; *Stansfield* et al., 1993; *Dibley* et al., 1996).

The reason for this complex picture is not clear, but may relate to the numerous and various microbes which can cause diarrhoea, the many other factors like the mode of feeding of the child such as partial or exclusive breast feeding, the extent of microbial exposure, the likely complexity of the food deficiencies and the multiple damages on host defence caused by vitamin A deficiency.

This reasoning may hold also for the morbidity in respiratory tract infections which shows a relation to vitamin A deficiency (*Sommer* et al., 1987). However, a relation to mortality has only been suggested (*Fawzi* et al., 1994). Supplementation with vitamin A has shown a reduction in incidence in some studies, but not in others (*Bloem* et al., 1990; *Ramakrishnan* et al., 1995).

Iron deficiency

Iron deficiency, as well as overload, have been shown to cause impaired immune functions (*Bryan* and *Stone*, 1993). Iron deficiency, which is the most common micronutrient deficiency in the world, impairs macrophage function, lymphocyte blastogenesis, NK cell activity and reduces numbers of circulating T cells and thymus size (*Srikantia* et al., 1976; *Rothenbacher* and *Sherman*, 1980; *Kuvibidila* and *Wade*, 1987; *Hallquist* and *Sherman*, 1989). Iron supplementation has been claimed to decrease respiratory and gastrointestinal infections by 50 per cent in anaemic children compared to non supplemented controls (*Mackay*, 1928). Such an effect is supported by later studies (*Latham* et al., 1990; *Sherman*, 1992).

Zinc deficiency

Zinc deficiency was mentioned above as a likely important component in undernutrition, to which it may add further to by decreasing appetite (*Krebs* et al., 1984). Zinc deficiency causes splenic atrophy and decreases T cell responsiveness to antigens and mitogens (*Chandra*, 1985; *Sherman*, 1992). The deficiency also causes reduced IL-4, IFN-γ, peripheral eosinophil levels and serum IgE, as well as IgG1 (*Shi* et al., 1994).

Supplementation with zinc reduces incidence of diarrhoea in children and enhances recovery from persistent diarrhoea (*Tomkins* et al., 1993). There are other studies to support these observations (*Rosado* et al., 1997; *Ruel* et al., 1997).

Deficiencies of other micronutrients like copper, selenium, vitamin E, D, K, B1, B6 and B12 also have consequences for the immune system and it is important to study them further to define when supplementation can be helpful (*Hanson* et al., 1998).

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