Old Herborn University Seminar Monograph

20. INFLAMMATION AND INFECTION
THE GOLDEN TRIANGLE:
FOOD - MICROFLORA - HOST DEFENCE

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John J. Cebra
1934 – 2005

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1934-2005

John was a Founder of the Old Herborn University Seminars and the International Study Group on New Antimicrobial Strategies (ISGNAS). Indeed I owe my membership of these bodies to John's suggestion a few years ago. I remember him with great fondness and much respect for his scientific achievements which greatly influenced me in my own career in mucosal immunology.

John Cebra was the Annenberg Professor of the Natural Sciences and also Professor of Biology at the University of Pennsylvania when he died. He had occupied the position of Chair, Department of Biology at that university from 1979-1983 and before that was in the Department of Biology at Johns Hopkins University. He received his AB in chemistry and graduated Summa Cum Laude in 1955, his PhD at the Rockefeller University where his advisor was Merrill Chase in 1960 and then did a post-doc with Ephraim Katchalsky at the Weizmann in Israel. He subsequently worked twice with Rodney Porter in Mill Hill in London. For five years he was Associate Professor at the University of Florida in Gainesville where he began his stellar career in basic immunology. Everyone quotes his 1971 paper with Susan Craig as a landmark in understanding that the Peyer's patches were the source of IgA committed plasma cells. John's contribution to the field of science is both deep and broad at the same time. He produced some 165 original publications, was mentor to more than 30 graduate students and even more post-doctoral fellows, and received numerous awards from professional societies around the globe. The most recent honors were from the American Association of Immunologists for excellence in mentoring "in recognition of exemplary career contributions to a future generation of scientists" in 2005, followed very closely by the First Lifetime Achievement Award from the Society of Mucosal Immunology shortly before he died.

John Cebra's contributions to mucosal immunology, as mentioned before were striking, both for their innovative use of technology and also their imagination and insight. He was the first to explore and characterize the cellular basis for IgA synthesis and secretion by mucosal tissues. He was also the first to define the specific role of commensal microorganisms in the development of the immunological repertoire, including both B and T cells. Many of the people whom he trained have gone on to develop their own careers, many of which have taken form as a result of their experiences with John.

John was extraordinarily well loved by his trainees and peers alike. He had a sense of humor and also purpose, and he and his long-time companion, wife and supporter Ethel, worked hard for the good of scientific entente in the days when this was extremely difficult for scientists behind the iron curtain. Both he and Ethel were ports of call which were always safe, respectful and hospitable to all who wished to visit, interact and learn from his scientific team.

Despite his enormous achievements and the respect which they commanded, John's work was not always popular. Many times he acknowledged that his single minded commitment to maintaining a germ-free facility at a time when none

other existed in the United States, and the field had been in essence abandoned by his scientific colleagues, nevertheless was one whose time would return. He lived just long enough to begin to see this prediction come true. John, with Ethel, was an inveterate traveler, hiker and bird watcher and they traveled all over the world together enjoying the landscapes and the people who in turn enjoyed them and were charmed by them at the same time, as we all have been. John's lectures were always stuffed with data and complex but lucid arguments, making particular points. He never, in my experience, kept to time and was always surprised when the time allotted for his talk was up. He never slacked at any scientific meeting he attended, and took voluminous notes and always offered critical comments to anything others presented. He was a true pioneer and leader, characterized by openness and humility, and his sense of humor never left him. He made enormous contributions and those of us who were privileged to know him will never forget these or our experiences of our interactions with him. But it is also true that those who follow and read the history of immunology will also note his scientific contributions and inevitably accord them the respect that they deserve.

We miss him greatly, and as a consequence also his wife and companion Ethel, who was ever with him, and we extend our thanks for a lifetime of service and interaction both scientifically and socially, and our condolences to Ethel and his remaining family.

Valete John

John Bienenstock

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NUTRITION, INFECTION AND CHRONIC TROPICAL ENTEROPATHY IN AFRICAN INFANTS

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INTRODUCTION

Under optimal conditions the interactions between food and the gut microflora act in synergy to aid host defences and modulate systemic immunity hence creating 'The Golden Triangle' that is the theme for this symposium. But when this symbiosis breaks down the interactions can become malignant and might better be described as a 'black triangle'. This struggle between the human host and pathogenic enteric organisms has been the norm over most of human evolution and remains a major challenge for the majority of the world's infants raised, as they are, in unhygienic conditions. This paper uses our research from a rural population in The Gambia as a case study to describe the struggles between the human host and its pathogens under such conditions.

GROWTH FAILURE

The typical pattern of infant growth in rural Gambia is illustrated in Figure 1. Babies are typically born small but then show rapid catch-up growth during the first 3 months of life when fully breast-fed and generally free from infections. This catch-up is so successful that at 3 months of age the population average is close to western growth norms. Thereafter there is a precipitate deterioration in growth so that by the end of infancy the population average Z-score is close to –2.0 for weight, -1.2 for length and –2.3 for head circumference. This represents the average growth pattern across all calendar months and is strongly modulated by seasonal variation (*McGregor* et al., 1961). During the rainy season (July-October) growth virtually stops in many infants as a consequence of a sharp increase in infectious diseases including diarrhoea (*Rowland* et al., 1977), and a deterioration in maternal care practices due to the fact that mothers have to work long hours in the fields and frequently leave their infants with a young nursemaid or a grandmother who is no longer able to work. The effects of this are illustrated in Figure 2, which shows the seasonal variation in the number of severely malnourished infants referred by paediatricians to a local therapeutic feeding centre.

The issue of when to start introducing complementary foods has been described as the 'weanling's dilemma' by *Rowland* and colleagues (1978). Weaning foods in sub-Saharan Africa typically have a very low energy and nutrient density, and frequently have

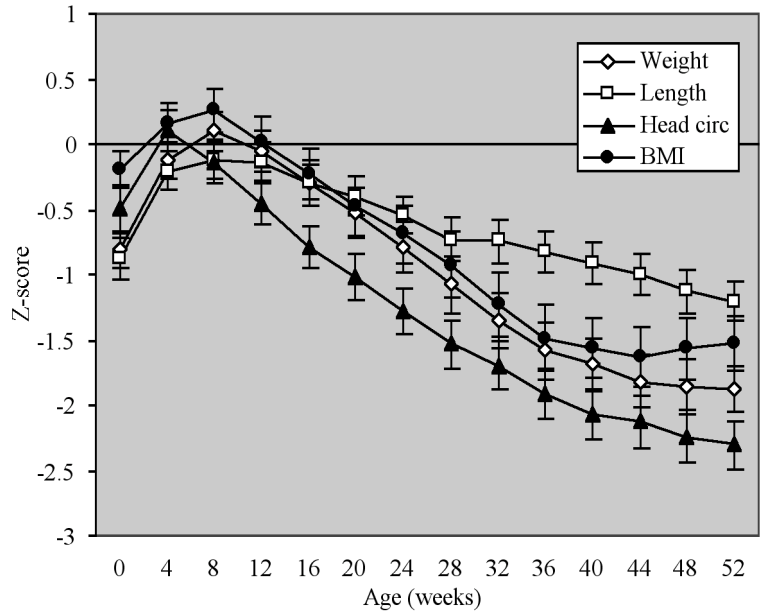

Figure 1: Early growth faltering in Gambian infants. Data from 138 Gambian infants assessed longitudinally and expressed as Z-scores relative to the UK 1990 standards. Reproduced with permission from *Collinson* et al. (2005).

high levels of bacterial contamination (*Barrell* and *Rowland*, 1979). The dilemma therefore is that if mothers introduce weaning foods too early they risk causing diarrhoea and inhibiting their own lactational performance, but if they introduce them too late their infant's energy needs may have started to exceed their milk energy supply. This dilemma is faced by all mothers but is much more acute in poor communities with few facilities for hygienic food preparation where weaning foods are likely to be contaminated. The hazards associated with this transition frequently result in the initiation of a vicious cycle of malnutrition and chronic intestinal infections as illustrated in Figure 3.

GASTROINTESTINAL INFECTIONS

Various bacterial and viral pathogens have been implicated as etiologic agents for diarrhoea in The Gambia (*Lloyd-Evan*s et al., 1983; *Sullivan* et al., 1990, 1991a; *Rowland* et al., 1980; *Billingham*, 1981; *Goh Rowland* et al., 1985). Some have been found to be significantly associated with diarrhoea while others have been seen to be equally prevalent in asymptomatic children. Bacterial contamination of the jejunum was predominant in a small series of malnourished children with diarrhoea (*Heyworth* and *Brown* 1975).

The role of giardiasis has been investigated in a study in urban Gambian children and was found more commonly in control stools rather than those of diarrheic stools (*Goh Rowland* et al., 1985). However, the prevalence was significantly higher in children with chronic diarrhoea and malnutri-

Figure 2: Seasonal pattern of severe malnutrition reflected in the monthly admissions to a therapeutic feeding centre.

tion compared to healthy controls (45% vs. 12%), but not when compared with marasmic controls (27%) (*Sullivan* et al., 1991a). In a longitudinal community study on giardiasis (measured by serology) and weight gain of rural Gambian infants, elevated titres of *Giardia*-specific IgM antibodies were associated with decreased weight gain in the 2 week period prior to serological conversion (*Lunn* et al., 1999). High *Giardia*-specific IgM was also associated with elevated intestinal permeability values and decreased

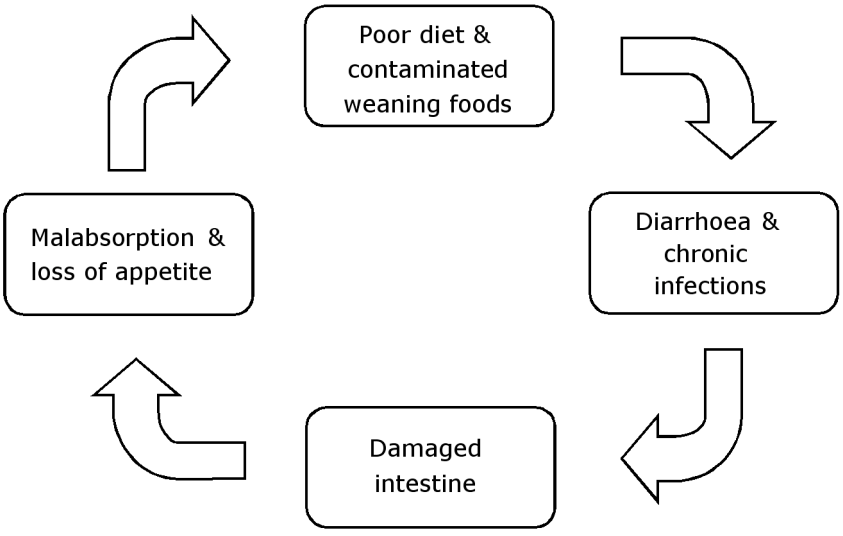

Figure 3: The vicious cycle of malnutrition and chronic infection typical of poor communities in developing countries.

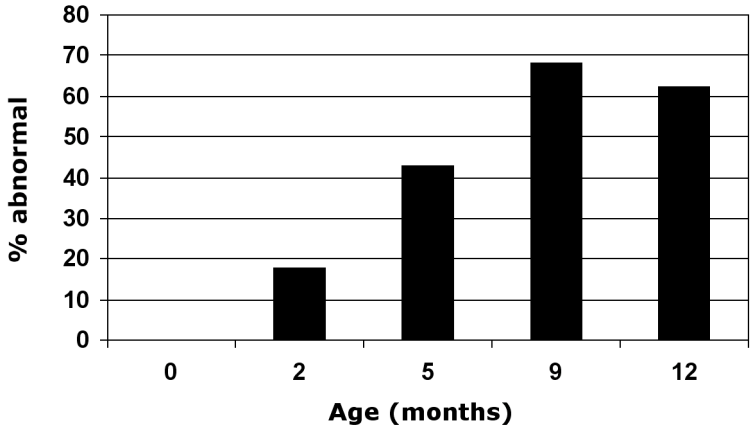

Figure 4: Raised inflammatory α-1 acid glycoprotein in rural Gambian infants. (Data from 197 infants studied longitudinally (*Darboe* et al., unpublished). The normal cut-off is ≤ 1g/l.)

mannitol absorption (*Lunn* et al., 1999). However, the mean IgM titres per child over the entire study period did not predict differences in long-term growth or intestinal permeability. In a community-based study, it was also shown that although intestinal inflammation (as measured by faecal neopterin) was inversely associated with growth, the presence of giardiasis was neither associated with poor growth or poor intestinal permeability (*Campbell* et al., 2004). It appears that in the Gambian setting, giardiasis is more prevalent in chronic diarrhoea and malnutrition, but its role in modulating the acute growth of infants seems to be less clear.

A study on *Helicobacter pylori* infection on severely malnourished Gambian children was probably the first study in African children (*Sullivan* et al., 1990). This showed that close to half the children aged between 40 to 60 months had serologic evidence for infection. Half of the children with chronic diarrhoea and malnutrition were positive as compared to a quarter of healthy controls and undernourished children. In a later study using the ^{13}C-urea breath test, it was shown that acquisition of *H. pylori* infection may occur before 3 months of age as 20% of the 3-month-old infants were positive (*Thomas* et al., 1999). An analysis of longitudinal growth data and serial breath tests demonstrated that children who acquired *H. pylori* earlier ended up shorter, lighter and thinner than their uninfected peers (*Dale* et al., 1998). It has been proposed that early *H. pylori* causes a transient hypochlorhydria and thereby increases the likelihood of enteric infection thus compromising intestinal function and nutrition. *H. pylori* infection may serve to reduce the mucosal defences and allow further colonisation of the small intestine with pathogens (*Dale* et al., 1998). It is noteworthy that studies in developed countries have shown an association between *H. pylori* infection and increased intestinal permeability (*Borch* et al., 1998, *Di Leo* et al., 2005).

Figures 4 and 5 illustrate very recent data on abnormal levels of α-1 acid glycoprotein (used as a general marker of infection) and *H pylori* infection among 197 infants studied longitudinally, and demonstrate that the pathologies described by earlier workers,

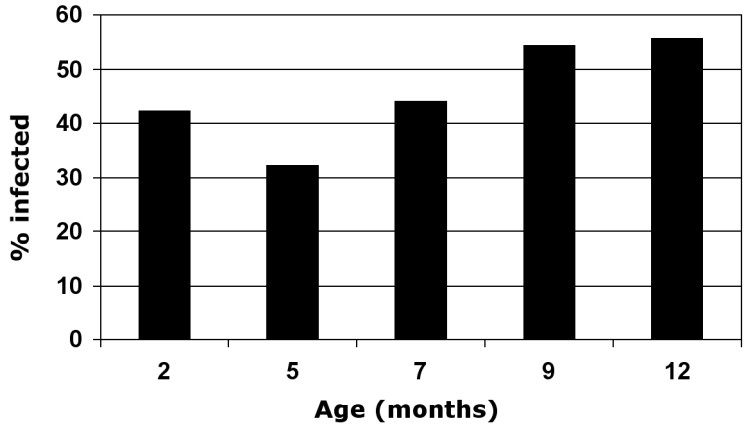

Figure 5: Proportion of rural Gambian infants with *Helicobacter pylori* infection assessed by the urea breath test. Data from 197 infants studied longitudinally (*Darboe* et al.,, unpublished).

as summarised above, are still highly prevalent despite considerable improvements in health care and major reductions in mortality (*Rayco Solon* et al., 2004).

CHRONIC ENVIRONMENTAL ENTEROPATHY

Persistent gastroenteropathy, as characterized histologically by small-intestine mucosal villous shortening and broadening, crypt hyperplasia, increased crypt depth, and lymphocyte infiltration into the lamina propria and epithelium (*Sullivan* et al., 1991b, 1992; *Campbell* et al., 2003, *Solon* et al., 2006), is a feature of many Gambian children. Previous research has established this inflammatory condition to be strongly associated with growth failure. First described in 1962, persistent enteropathy was found to affect individuals throughout the tropics, in Africa, Asia, South America and the Caribbean (*Thomas* et al., 1976, *Brusner* et al., 1987). For this reason it acquired the name 'tropical enteropathy'. The condition was particularly observed in those living in less developed, or more contaminated, environments of the tropics. It was later shown that people living in temperate areas may develop similar histological and functional changes if living in environments with similarly high levels of microbiological pathogens. For these reasons the expression 'chronic environmental enteropathy' is now accepted as a more accurate description of the condition than 'tropical enteropathy'.

Associated functional changes include subclinical malabsorption of fat and an increased mucosal permeability. The latter is demonstrated by markedly and consistently raised lactulose:mannitol (L:M) ratios in the dual-sugar permeability test (DSPT) towards later infancy (see Figure 6). Raised L:M ratios have also been described in children in several other parts of the developing world. The DSPT assesses both gut integrity and absorptive capacity, and has been used in numerous studies characterising the aetiology of growth failure in The Gambia (*Lunn* et al., 1981) and elsewhere (*Goto* et al., 1999, 2002).

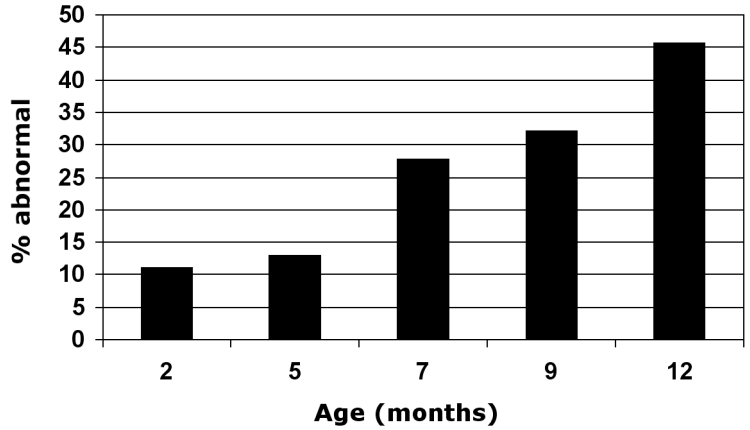

Figure 6: Proportion of rural Gambian infants with abnormal gut permeability assessed by the lactulose:mannitol test. Data from 197 infants studied longitudinally (*Darboe* et al., unpublished). The normal cut-off for the lactulose:mannitol test was set at 0.3.

Two sets of immunohistologic studies have been performed in The Gambia. These are consistent with past biopsy studies in marasmus and kwashiorkor done in other developing countries over the past four decades; in particular they describe a wide spectrum of crypt hyperplasia and villous atrophy across cases. In addition, immunohistology revealed intraepithelial lymphocyte infiltrates in the surface villi and crypts (*Sullivan* et al., 1991b), predominantly of the CD8+ phenotype. Follow-up studies on rehabilitated children showed that with treatment, the crypt cell compartment increased in size, but there was no corresponding increase in epithelial volume (*Sullivan* et al., 1992). T-cells are known to play an important role in other inflammatory enteropathies such as Crohn's disease and coeliac disease (*MacDonald* et al., 1999). The most recent biopsies done in The Gambia (*Campbell* et al., 2003) have demonstrated a generalised cellular hyper-responsiveness and a cytokine profile biased towards proinflammatory cytokines. The intestinal infiltrate was dominated by T Cells (CD3), γδ T cells, activated T cells (CD25), activated cytotoxic T cells (perforin+) and elevated levels of TNFα, IFNγ, and TGFβ, but not IL-10. TGFβ:TNFα and TGFβ:IFNγ ratios were higher with better nutritional status. L:M ratios were increased (more gut damage) in children with more T cells and activated cytotoxic T cells, and decreased with more B cells (CD19).

The immunohistological studies contradict the commonly held belief that malnutrition is associated with an immunosuppressed state, and instead suggest that both lymphocyte activation and ineffective enterocyte development play a significant role in chronic environmental enteropathy and malnutrition. These findings support the view that malnutrition is not necessarily accompanied by severe T-lymphocyte deficiency, and that T-lymphocyte dysregulation may be present (*Morgan*, 1997).

THERAPEUTIC INTERVENTIONS AIMED AT TREATING CHRONIC ENVIRONMENTAL ENTEROPATHY

Under optimal conditions affected children who develop severe malnutrition will be admitted to a specialist treatment centre and treated according to the formalised WHO guidelines for the Treatment of the Severely Malnourished Child (*WHO*, 1999). If implemented properly such therapy can greatly reduce the normally high levels of mortality associated with severe malnutrition (*Ashworth* et al., 2004). But as with many clinical syndromes the greatest burden of disease is associated with less severe forms since they affect a much larger proportion of children. In The Gambia we have tested a number of preventive and therapeutic population-based interventions aimed at reducing the enteropathy and hence at preventing the widespread growth faltering. These have included supervised protein-energy supplementation between 3-6 months, and interventions with glutamine, zinc, early high-dose vitamin A, probiotics and multiple micronutrients. To date none of these have been successful and we will shortly be embarking on a randomised controlled trial of long-chain N3 polyunsaturated fatty acids (PUFAs) administered daily to infants 3-9 months of age with the aim of suppressing the persistent and inappropriately vigorous inflammatory response within the mucosal epithelium.

Ultimately it is recognised that a whole package of interventions including clean water supplies, better hygienic conditions and maternal education will be required in order to fully rectify the growth failure and the attendant developmental disadvantages suffered by so many infants in the developing world.

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$CD4^+CD25^+$ REGULATORY T CELLS

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SUMMARY

During the past decade $CD4^+CD25^+$ regulatory T cells have emerged as key players in the development of tolerance to auto-antigens as well as to foreign antigens. This review aims to recapitulate some of the current understandings about the phenotype and function of these regulatory T cells and describe some recent findings on their interaction with bacterial antigens.

INTRODUCTION

The main role of the immune system is to protect the individual from pathogens and one of its fundamental qualities is the ability to distinguish between self and non-self, and between antigens encountered in harmful and non-harmful contexts. In the thymus potentially self-reactive T cells are deleted, resulting in the generation of a peripheral T cell repertoire that is largely self-tolerant. In spite of this, some self-reactive T cells are present in most individuals. Nevertheless, autoimmune diseases only occur infrequently, which suggests that auto-reactive T cells are controlled in the periphery. Peripheral tolerance is sustained by several mechanisms such as deletion, anergy and ignorance (*Mackay*, 2000; *Klein* and *Kyewski*, 2000). In addition there is compelling evidence for the existence of more "active" mechanisms of tolerance that operate through the generation of immune-regulating T cells. Several types of regulatory T cells have been described including, γδ T cells, NKT cells, $CD8^+$ and $CD4^+$ T cells (*Bach*, 2003). $CD4^+$ regulatory T cells can be further divided into induced regulatory cells that secrete IL-10 and TGF-β such as Tr1 cells (*Levings* et al., 2002) and Th3 cells (*Weiner*, 2001) and the so called naturally occurring $CD4^+CD25^+$ regulatory T cell ($CD25^+$Treg), which is the focus of this review.

REVIEW

T cell mediated suppression of autoimmune disease was first described by Nishizuka and Sakakura more than 30 years ago (*Nishizuka* and *Sakakura*, 1969). They discovered that thymectomy on day 3 of life (d3Tx) results in organ-specific autoimmunity. However, disease did not develop in mice thymectomized as early as day 2 or as late as day 7 of life. Further studies

showed that d3Tx animals could be rescued from disease if reconstituted with thymocytes or splenocytes from a normal adult animal but not from an adult animal that had been d3Tx (*Kojima* et al., 1976). This indicated that murine T cells that exit the thymus before day 3 of life are qualitatively different from cells that emigrate later on. However, the cells responsible for the inhibition of autoimmune disease were not discovered until the mid-1990s when Sakaguchi and co-workers identified a sub-population of $CD4^+$ T cells expressing the IL-2 receptor α-subunit (CD25) (*Sakaguchi* et al., 1995). Depletion of $CD25^+$ T cells from adult splenocytes followed by transfer of $CD25^-$ T cells to immune-deficient hosts resulted in a similar spectrum of organ-specific autoimmune diseases as d3Tx. Indeed, the frequency and severity of autoimmune disease correlated with the degree of CD25+ Treg depletion (*Ono* et al., 2006). In addition, co-transfer of $CD25^+$ T cells prevented induction of autoimmunity in the cell-transfer model as well as in the d3Tx model (*Suri-Payer* et al., 1998). Due to intense research during the past decade, $CD4^+CD25^+$ regulatory T cells ($CD25^+$Treg) has emerged as a central T cell population for preserving peripheral tolerance, not only to auto-antigens but also to foreign antigens, in mice as well as in humans (*McHugh* et al., 2002; *Viglietta* et al., 2004).

Antigen specificity

Both murine and human $CD25^+$Treg have as diverse TCR repertoires as $CD4^+CD25^-$ T cells as judged by the expression of various TCR α/βgene segments (*Takahashi* et al., 1998; *Kasow* et al., 2004; *Taams* et al., 2002). This suggests that $CD25^+$Treg are capable of responding to a wide spectrum of antigens. Whether they are biased towards responding to self-antigens or are as broad in their repertoire as $CD25^-$ T cells is not known. A very recent study on murine $CD25^+$Treg indicate that the TCR repertoire of $CD25^+$Treg and $CD25^-$ effector T cells although being similarly diverse, recognize only partly overlapping antigens. Furthermore, particular $CD25^+$Treg have substantially higher avidity for MHC class II bound peptides from peripheral self than the $CD25^-$ T cells (*Hsieh* et al., 2004). Additionally, a few studies of human $CD25^+$Treg have shown that they suppress proliferation and cytokine production to both self-antigens and foreign antigens, including MOG, hHSP60, *Helicobacter pylori* antigens, beta-lactoglobulin (beta-LG) and pollen extract *in vitro* (*Wing* et al., 2003; *Lundgren* et al., 2003; *Taams* et al., 2002, *Tiemessen* et al., 2002; *Ling* et al., 2004; *Grindebacke* et al., 2004). This exemplifies the diversity of the $CD25^+$Treg pool in un-manipulated individuals. Indeed, if the primary function of $CD25^+$Treg is to prevent the activation of potentially hazardous T cells then it would be an advantage to have a similarly composed TCR repertoire as the potentially reactive $CD25^-$ T cells.

Phenotype of CD25+ regulatory T cells

Naturally occurring regulatory T cells express CD25 constitutively and this marker has proven to be very useful for isolation of these cells in mice. However, CD25 is not an optimal marker as it is up-regulated upon activation of T cells. This is especially apparent when investigating human $CD25^+$Treg. In the naïve mouse, $CD4^+CD25^+$ T cells are seen as a distinct population of cells easily distinguished from $CD4^+CD25^-$ T cells that comprise between 5-10% of peripheral $CD4^+$ T cells. The isolation of murine

$CD25^+$Treg is therefore fairly straightforward unless the animal is suffering from ongoing inflammation. Among human $CD4^+$ T cells, approximately 30% express CD25. The majority of these cells express CD25 with low to intermediate intensity ($CD25^{int}$) and only between 1-3% of the $CD4^+$ T cells express CD25 with high intensity ($CD25^{high}$) (*Wing* et al., 2002). *In vitro* studies of sorted $CD25^{int}$ and $CD25^{high}$ cells have shown that it is the $CD25^{high}$ population that functions as suppressor cells (*Baecher-Allan* et al., 2001). Consequently, $CD25^{int}$ cells are most likely memory cells with CD25 expression resulting from encounter with foreign antigens. The continuous expression pattern of CD25 on $CD4^+$ T cells from adult peripheral blood has made the isolation of human $CD25^+$Treg with high purity difficult. It should also be noted that there has been several reports on $CD4^+CD25^-$ cells with regulatory properties (reviewed in *Curotto de Lafaille* and *Lafaille*, 2002). This implies that naturally occurring $CD4^+$ regulatory T cells are not necessarily confined to the $CD4^+CD25^+$ T cell population.

In mice surface expression of markers other than CD25 have been useful in isolating $CD4^+$ regulatory T cells, including CD45RB, CD38 and CD62L (*Powrie* et al., 1996; *Read* et al., 1998; *Lepault* and *Gagnerault*, 2000). Notably, *in vitro* all freshly isolated murine $CD25^+$ T cells suppressed the proliferation of $CD4^+CD25^-$ T cells irrespectively of their expression of these markers (*Thornton* et al., 2000). However this does not exclude that the suppressive property *in vivo* could be affected. Indeed, $CD4^+CD25^+CD62L^+$ but not $CD4^+CD25^+CD62L^-$ splenocytes were shown to delay the onset of diabetes in NOD mice (*Szanya* et al., 2002). However, these markers do not necessarily identify human $CD25^+$Treg. We found that $CD25^{high}$Treg in adult blood expressed intracellular cytotoxic T lymphocyte associated antigen-4 (CTLA-4/CD152) and CD122, while $CD25^-$ and $CD25^{int}$ T cells were negative for CTLA-4 and expressed low levels of CD122. Furthermore, similar $CD25^+$ T cells were identified in cord blood and in thymus (*Wing* et al., 2002). In accordance, CTLA-4 is a marker that is constitutively expressed in naïve animals only by CD25+ T cells (*Sakaguchi* et al., 1995; *Takahashi* et al 2000). In addition, $CD25^+$Treg from adults displayed a memory phenotype as they were $CD45RA^-RO^+$, $CD45RB^{low}$ and expressed both CD62L and CD38 with low intensity and this phenotype was largely shared with the $CD25^{int}$ T cells. In contrast, $CD25^+$Treg derived from cord blood had a naive phenotype and were mainly $CD45RA^+RO^-$ as were the $CD25^-$ T cells in cord blood (*Wing* et al., 2002). Notably, *Jonuleit* et al. (2001) showed that in adult peripheral blood only the $CD25^+CD45RO^+$ cells have suppressive ability. However, we found that cord blood $CD25^+$Treg, which are mainly $CD45RA^+$, were able to suppress proliferation induced by anti-CD3Ab (*Wing* et al., 2003). Furthermore, $CD45RA^+CD25^+$ T cells expressed two-fold higher levels of *FOXP3* mRNA than $CD45RA^-CD25^+$ T cells did. This suggests that expression of CD45RO is an indicator of antigen experience and of limited use for identification of $CD25^+$Treg in humans.

A number of studies have identified additional markers that are expressed at higher levels on $CD25^+$Treg compared to $CD25^-$ T cells (reviewed in *Curotto de Lafaille* and *Lafaille*, 2002). Some of these markers are inhibitory costimulatory receptors like PD1 or members of the tumour necrosis factor receptor (TNFR) superfamily, such as

GITR (glucocorticoid-induced TNFR-related protein), OX40, 4-1BB and TNFRII. Yet others are chemokine receptors, Toll-like receptors or homing receptors such as CD103 (αEβ7 integrin) and the recently discovered neuropilin (Nrp1), which is involved in axon guidance, angiogenesis and T cell activation (*Bruder* et al., 2004). However, the majority of these markers should be used with caution since most surface markers, although expressed on $CD25^+$Treg are up-regulated also on $CD25^-$ T cells after stimulation. In addition, several of these markers were identified on $CD25^+$ T cells isolated from mice and the expression has been difficult to confirm in humans. Currently, none of these molecules have proven to be fully responsible for the suppressive function of $CD25^+$Treg and questions regarding their functional significance remain.

Recent studies have revealed the gene *Foxp3* to be central in the development and function of $CD25^+$Treg (*Sakaguchi*, 2004). The importance of *Foxp3* was discovered when the underlying defect in scurfy mice was investigated. Scurfy mice suffer from a spontaneous X-linked mutation, which leads to fatal lympho-proliferative disease associated with multi-organ infiltrates and early death by 3-4 weeks of age in hemizygous males (*Godfrey* et al., 1991). Lately, the genetic defect in scurfy mice has been identified as a mutation in *Foxp3* (forkhead box p3), a gene coding for a member of the forkhead/winged-helix family of transcriptional regulators (*Brunkow* et al., 2001). *FOXP3*, the human orthologue of the murine *Foxp3*, has been found to be mutated in patients suffering from IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), a severe and fatal autoimmune/allergic syndrome, which reminds to great extent of the condition of scurfy mice. The majority of the mutations in the human patients have been found to be located to the forkhead region, showing the importance of this domain in the function of *FOXP3* (*Gambineri* et al., 2003).

The fact that scurfy mice are hyperresponsive to TCR stimulation and that over-expression of *Foxp3* in cells induce poor proliferation and limited production of IL-2 prompted the investigation of the relationship between *Foxp3* expression and $CD25^+$ Treg (*Hori* et al., 2003). These studies showed that both mRNA and protein levels of *Foxp3* were confined to the $CD4^+CD25^+$ subset (*Hori* et al., 2003; *Fontenot* et al., 2003; *Khattri* et al., 2003). In mice Foxp3 mRNA is detected in peripheral $CD4^+CD25^+$ T cells and in $CD25^+CD4^+CD8^-$ thymocytes, whereas other thymocytes/T cells or B cells do not express *Foxp3*. In contrast to the previously mentioned markers for $CD25^+$Treg, *Foxp3* was not induced in T cells after TCR stimulation (*Hori* et al., 2003). Importantly, retroviral transduction of both murine and human naïve $CD25^-$ T cells with *Foxp3* converts them to regulatory cells with similar functional characteristics as naturally occurring $CD25^+$Treg. Interestingly, as a result of the transduction, cell-surface molecules associated with $CD25^+$Treg were upregulated, including CD25, CTLA-4, GITR and CD103 (*Hori* et al., 2003; *Yagi* et al., 2004). Also, adoptive transfer of wild type $CD25^+CD4^+$ T cells but not $CD25^-CD4^+$ T cells rescued scurfy mice from disease, which shows that *Foxp3* is required for the development of $CD25^+$Treg (*Fontenot* et al., 2003). Investigations of *FOXP3* in humans have shown that it is expressed by all CD4+ $CD25^{high}$ T cells, a minority of the CD4+ $CD25^{int}$ T cells and of the CD4+$CD25^{neg}$ T cells, but not by CD8+ T cells (*Gavin* et al., 2006).

$CD4^+CD25^+$*FOXP3*$^+$ T cells have been identified in thymus as well as in adult peripheral blood and cord blood (*Yagi* et al., 2004; *Walker* et al 2003; *Cosmi* et al., 2003; *Godfrey* et al., 2005; *Takahata* et al., 2004). In contrast to the murine studies, human $CD4^+CD25^-$ T cells have been reported to express *FOXP3* and to acquire suppressive ability after stimulation *in vitro* with plate-bound anti-CD3 and anti-CD28 mAbs (*Walker* et al., 2003). $CD4^+CD25^-$ T cells in human intestinal lamina propria were reported to contain *FOXP3*$^+$ cells (*Makita* et al., 2004). These cells were anergic upon TCR stimulation *in vitro* but did not display suppressive function when cultured in a one to one ratio with responder T cells, which possibly was due to a low frequency of *FOXP3*$^+$ cells. Of note, TGF-β, which is abundant in the intestine, has been shown to induce *FOXP3* expression in both murine and human $CD4^+CD25^-$ T cells (*Chen* et al., 2003; *Fantini* et al., 2004). Conclusively, co-stimulation with TGF-β might be a key for development and maintenance of peripheral regulatory T cells (*Marie* et al., 2005) Overall, *Foxp3* plays a vital part in the generation $CD25^+$Treg and is the most specific marker currently available. Since Foxp3 is a nuclear protein, it is of limited value as a tool for isolation of $CD25^+$Treg *ex vivo,* why we are still confined to the surface molecule CD25 that shows the best correlation to the expression of FOXP3.

Activation of CD25+ regulatory T cells and target cells

Freshly isolated $CD25^+$Treg are not able to suppress T cell responses and only exert inhibitory function after stimulation via the TCR. Antigen-specific as well as polyclonal TCR stimulation activates $CD25^+$Treg and induces suppressive function *in vitro*, whereas irrelevant antigens do not (*Takahashi* et al., 1998; *Thornton* et al 1998). $CD25^+$Treg are very sensitive to stimulation with antigen and are suppressive at antigen doses 10-100 times lower than those needed to activate $CD25^-$ T cells (*Takahashi* et al., 1998). They have a diverse TCR repertoire and are therefore able to respond to many different antigens, including food, microbial, allo- and auto-antigens (*Wing* et al., 2003; *Sakaguchi* et al., 2004; *Lundgren* et al., 2003; *Dieckmann* et al., 2001). In contrast to the lack of proliferation *in vitro*, $CD25^+$Treg actively proliferate to antigen stimulation *in vivo* in non-lymphopenic normal hosts and persist without antigen stimulation for long periods of time (*Gavin* et al., 2002; *Fisson* et al., 2003; *Walker* et al., 2003; *Klein* et al., 2003). Therefore, the anergic behaviour *in vitro* is probably due to lack of appropriate stimulation by for example IL-2.

$CD25^+$Treg have been most thoroughly studied with regard to their effects on T cells. Once $CD25^+$Treg have been activated with specific antigen and IL-2, they inhibit the IL-2 transcription by their target cells and suppress both $CD4^+$ and $CD8^+$ T cell responses of proliferation and cytokine production in an antigen non-specific manner (*Thornton* et al., 2000; *Takahashi* et al., 1998; *Thornton* and *Shevach*, 1998; *Piccirillo* and *Shevach*, 2001). $CD25^+$Treg have also been found to down-regulate the expression of co-stimulatory molecules and reduce the stimulatory capacity of both human and murine DC (*Cederbom* et al., 2000; *Misra* et al., 2004; *Serra* et al., 2003). Others have reported that $CD25^+$Treg act directly on the target cells since suppression can be detected using *in vitro* culture systems devoid of APC (*Thornton* and *Shevach*, 2000; *Piccirillo* and *Shevach*, 2001; *Ng* et al.,

2001). This does, however, not exclude that $CD25^+$ Treg influence the stimulatory capacity of APC *in vivo* and it was recently shown using two-photon laser scanning microscopy of lymph nodes *in vivo* that CD25+ Tregs interacted with antigen-presenting DCs which resulted in prevention of T cells priming (*Tang* et al., 2006). $CD25^+$ Treg also suppress B cells directly and in addition both natural killer T cells (NKT) and natural killer (NK) cell functions have been reported to be down-regulated by $CD25^+$Treg (*Lim* et al., 2005; *Azuma* et al., 2003; *Tronkowski* et al., 2004).

The role of IL-2

A distinctive feature of $CD25^+$Treg *in vitro* is that they are hypo-responsive to stimulation and do not proliferate and produce either no or low levels of cytokines (*Sakaguchi* et al., 2001; *Shevach*, 2002). Accordingly, they are dependent upon the cytokines that the effector cells produce. IL-2 seems to be particularly important since mice deficient for IL-2, IL-2Rα or IL-2Rβ have very few or no $CD25^+$Treg and prematurely succumb to severe lymphoproliferative and autoimmune syndromes. Administration of IL-2 or transfer of IL-2 producing cells to IL-2 deficient animals restores the production of $CD25^+$Treg and lymphoid homeostasis. Further, thymic expression of IL-2Rβ in the thymus of IL-2Rβ$^{-/-}$ mice restores the production of $CD25^+$Treg and prevents lympho-proliferation and lethal autoimmunity (*Malek* and *Bayer*, 2004). This indicates that IL-2 has an important role in the generation of $CD25^+$Treg in the thymus and is crucial for peripheral homeostatic maintenance (*Setoguchi* et al., 2005). Studies of $CD25^+$Treg activation *in vitro* have shown that IL-2 is also needed for induction of suppressive ability. Murine $CD4^+CD25^+$ T cells cultured with plate-bound CD3 Ab in absence of IL-2 resulted in both poor recovery and suppressive ability (*Thornton* et al., 2004). More importantly, the addition of anti-IL-2 completely abrogated the suppressive effect of $CD25^+$Treg on IL-2 mRNA transcription.

The role of CTLA-4

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4; CD152) is a CD28 homologue, which also binds to CD80/86. CTLA-4 is induced upon T cell activation and then functions as a negative regulator of activation. Interestingly, the only cells in naïve animals or human cord blood that express CTLA-4 in the absence of activation are $CD4^+CD25^+$ T cells (*Wing* et al., 2002; *Takahashi* et al., 2000). This raises questions regarding the role of CTLA-4 for the inhibitory mechanism and induction of suppressive capability in $CD25^+$Treg. The addition of CTLA-4 Ab or Fab fragments to *in vitro* co-cultures of murine $CD4^+CD25^-$ and $CD25^+$ T cells neutralizes the inhibitory effect (*Takahashi* et al., 2000) and administration of CTLA-4 mAb abolished the protective ability of $CD25^+$Treg in the murine inflammatory bowel disease (IBD) model (*Read* et al., 2002). With regard to human *in vitro* studies it was shown that suppression by $CD4^+CD25^+$CTLA-4^+ T cells was partly inhibited by blocking CTLA-4 (*Birebent* et al., 2004). However, the majority of investigations have not been able to establish a role for CTLA-4 in the suppressive function (*Baecher-Allan* et al., 2001; *Jonuleit* et al., 2001; *Levings* et al., 2001). CTLA-$4^{-/-}$ mice develop a fatal lymphoproliferative disease but $CD25^+$Treg development and homeostasis appear normal and $CD25^+$Treg displayed un-compromised suppressive ability *in vitro*.

Suppressive mechanisms *in vitro*

The mechanism of suppression by $CD25^+$ Treg is poorly understood. The majority of murine and human *in vitro* studies have concluded that $CD25^+$ Treg mediate suppression by a yet unknown cell-contact dependent mechanism, which is cytokine independent. Suppression cannot be abrogated by neutralizing IL-4, IL-10 or TGF-β and $CD25^+$Treg cultured with $CD25^-$ T cells in a transwell system are unable to suppress the proliferation of the responder cells (*Jonuleit* et al., 2001; *Takahashi* et al., 1998; *Thornton* et al., 1998). Interestingly, human $CD25^+$Treg fixed with paraformaldehyde remained suppressive as long as they had been activated before fixation (*Dieckmann* et al., 2002; *Jonuleit* et al., 2002). Collectively, this suggests the involvement of a surface-bound molecule that is upregulated on $CD25^+$Treg upon activation and mediates a suppressive signal to the responder cell. However, no such agent has yet been identified even though CTLA-4 has been proposed as a candidate (see previous paragraph). Another suggested mechanism of cell-contact dependent suppression is by TGF-β bound to the cell surface of $CD25^+$ Treg (*Nakamura* et al., 2001; Nakamura et al 2004). These findings have been corroborated by some, as at least suppression by human thymic $CD25^+$ Treg seem to be partly dependent on TGF-β *in vitro* (*Annunziato* et al., 2002). In contrast, others have had difficulties reproducing the results by Nakamura and co-workers (*Piccirillo* et al., 2002). The potential role of TGF-β remains controversial as $CD25^+$Treg from TGF-β1 deficient mice suppress $CD25^-$ T cells *in vitro* (*Piccirillo* et al., 2002), while adoptive transfer of TGF-β1 deficient $CD25^+$Treg did not protect recipients from colitis in the SCID transfer model *in vivo* (*Nakamura* et al., 2004). These results suggest that TGF-β produced by $CD25^+$ Treg is of particular importance in regulation of intestinal inflammation.

Suppressive mechanisms *in vivo*

There is a marked contrast with regard to the importance of immunosuppressive cytokines *in vivo* as compared to $CD25^+$Treg suppression *in vitro* and several cytokines have been implicated as mediators of inhibition. In the murine model of IBD, neutralizing Ab to IL-10 or TGF-β were shown to abolish the protective effect of the $CD4^+CD45RB^{low}$ cells (*Powrie* et al., 1996; *Asseman* et al., 1999). Similar results have been obtained using adoptive transfer of $IL\text{-}10^{-/-}$ $CD25^+$Treg. In contrast, IL-10 deficient $CD25^+$Treg are able to inhibit development of gastritis (*Suri-Payer* et al., 2001). One important difference between autoimmune gastritis and IBD is the requirement for intestinal bacteria for induction of IBD, as transfer of $CD25^-$ T cells to germ-free mice does not result in disease (*Singh* et al., 2001). *In vivo* it is likely that cell-contact dependent suppression by $CD25^+$Treg is needed but, during inflammation in the intestine, IL-10 and TGF-β are also required to control the response. Still, in conditions less dependent on bacterial presence, for instance in autoimmune thyroiditis, protection from disease is reversed by neutralizing antibodies to IL-4 and TGF-β (*Seddon* et al., 1999). Similarly, the protection of NOD mice by transferred $CD4^+CD25^+CD62L^+$ is abrogated after treatment with anti-TGF-β Ab (*Lepault* et al., 2000). Overall these data indicate that more than one mechanism of $CD25^+$Treg suppression is operating *in vivo*. One possibility is that there are different subsets of $CD25^+$Treg that either suppress by cell-contact dependent mechanisms or via production of cytokines. Alter-

natively, one $CD25^+$Treg might suppress by more than one mechanism depending on the local environment.

The possibility for a third mechanism of action was raised by two groups who simultaneously showed that human $CD25^+$Treg are able to induce suppressive properties in $CD4^+CD25^-$ T cells when cultured *in vitro*. This "infectious tolerance" rendered the $CD25^-$ T cells anergic and they subsequently started to produce TGF-β (*Jonuleit* et al., 2002) or IL-10 (*Dieckmann* et al., 2002). The primary culture of $CD25^+$Treg together with $CD25^-$ T cells required cell contact for induction of anergy. However, when the anergized T cells were transferred to fresh cultures they were shown to suppress naïve T cells in a cytokine dependent and cell-contact independent manner. This mechanism of infectious tolerance could clarify the discrepancy in the *in vivo* data and might also explain how a small population of cells can regulate a much larger population of responder T cells *in vivo*.

Microbial stimulation

For the immune response to clear microbes, the suppressive effect of the CD25+ Treg must be inhibited. One possibility is to make the effector cells refractory to suppression, which occurs when the stimulatory signal is strong and leads to maturation of DC (*George* et al., 2003). Recent reports show that $CD25^+$ Treg express several members of the Toll-like receptor (TLR) family, such as TLR2, TLR5, and TLR8 and signalling through these receptors is linked to modulation of function of the regulatory cells (*Peng* et al., 2005; *Sutmuller* et al., 2006; *Crellin* et al., 2005). Signalling through TLR-4 has also been proposed to have a similar role but later studies have not been able to replicate these findings (*Caramalho* et al., 2003; *Peng* et al., 2005; *Sutmuller* et al., 2006; *Crellin* et al., 2005). TLRs recognize certain components, so called pathogen-associated molecular patterns that are shared by most microbes, and also certain endogenous molecules that are released during inflammation. Thus, TLR stimulation of $CD25^+$ Tregs may induce extensive proliferation of the regulatory cells, enhanced their survival and suppressive capacity and help to downregulate the immune response. Several epidemiological studies have shown a correlation between improved hygienic conditions and the development of IBD, allergies and autoimmune diseases (reviewed in *Bach* et al., 2002). It is possible that a reduced exposure to microbes affects the development of tolerance and also the homeostasis of the $CD25^+$ Treg pool.

CD25+ regulatory T cells and chronic infection

$CD25^+$ Treg have been shown to interfere with viral as well as bacterial and parasitic infections (*Rouse* and *Suvas*, 2004). Adoptive transfer of $CD25^+$Treg prevents lethal pneumonia in recombinant-activating gene-2 deficient mice infected with *Pneumocystis carinii*, but at the expense of deficient protective response and microbial clearance (*Hori* et al., 2002). Similarly, $CD25^+$ Treg were shown to suppress Th1 responses in mice infected with *Helicobacter pylori*, thereby limiting the mucosal inflammation but with a higher bacterial load as a result (*Raghavan* et al., 2003). Furthermore, $CD25^+$ Treg prevent sterilizing immunity to *Leishmania* infection (*Belkaid* et al., 2002) and the persistence of low numbers of microbes proved essential for the development of T cell memory and prevention of reinfection. This indicates that the prevention of complete eradication of microbes may sometimes be beneficial for the host. Few

attempts have been made to study $CD25^+$Treg and infection in humans. However, it was shown that $CD25^+$Treg from carriers of *Helicobacter pylori* suppressed responses to *H. pylori* antigens *in vitro* (*Lundgren* et al., 2003) and that *H. pylori*-infected individuals have increased frequencies of $CD25^{high}$ T cells in both the stomach and the duodenal mucosa relative to healthy controls (*Lundgren* et al., 2005). Together this indicates that $CD25^+$ Treg may actively inhibit the eradication of the bacteria which contributes to the persistence of infection.

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GLUTEN AND CHRONIC DISEASES: INFLAMMATORY ACTIVITY OF GLUTEN

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SUMMARY

Initial events as well as effector mechanisms involved in most inflammatory and autoimmune diseases remain largely unknown. We suppose that dysfunction of the innate and adaptive immune system associated with mucosae, i.e. with the largest body surfaces representing an interface between the organism and environment could lead to the impairment of mucosal barrier function followed by the development of localised or systemic inflammatory and autoimmune processes.

Celiac disease (CD) is a frequent chronic autoimmune disorder affecting small bowel and developing in genetically susceptible individuals because of intolerance to wheat gluten. Beside gut mucosa celiac disease affects also a number of other organs: extra-intestinal symptoms are pronounced mainly in adult celiac patients. Various immunologically mediated chronic diseases were demonstrated to be associated with celiac disease. Interestingly, in a subset of non-celiac patients suffering from various chronic diseases introduction of gluten free diet was shown to improve the clinical symptoms.

Animal models are helping to elucidate the aetiology and pathogenic mechanisms of human diseases. We have developed a rat model of gluten-induced enteropathy by repeated intragastric application of gliadin starting at birth. Using this model, we demonstrated a protective effect of breast-feeding in the development of gluten-induced enteropathy. Epidermal growth factor (EGF) as one of the important components of the milk was shown to be responsible for the protective effect against the development of severe forms of gluten induced enteropathy.

We have shown that wheat gliadin and its peptic fragments have the unique ability (in contrast to other food proteins) to activate mouse macrophages and human monocytes to the production of pro-inflammatory cytokines through NF kappa B signalling pathway. Human monocyte-derived dendritic cells were demonstrated to upregulate maturation markers and to increase the production of chemokines and cytokines when cultivated with the peptic digest of gliadin (but not with other tested food proteins).

Our results suggest that activation of innate immunity cells by some food proteins (e.g. gliadin) or commensal bacteria components could lead to mucosal inflammation and participate in the impairment of intestinal mucosal barrier, which consequently leads to the development of inflammatory and autoimmune diseases.

THE ROLE OF THE MUCOSAL IMMUNE SYSTEM AND THE MUCOSAL BARRIER IN THE DEVELOPMENT OF INFLAMMATORY AND AUTOIMMUNE DISEASES

Body surfaces covered by epithelial cells are immediately after birth coming into contact with a number of microoganisms and foreign substances. While the surface of the skin (about 2 m^2) is protected mechanically by several epithelial layers, surfaces of the gastrointestinal, respiratory and urogenital tracts (about 300 m^2) are mostly covered with a single-layered epithelium and to resist the invasion of microorganisms they require extensive protection: this is represented by a complex of mechanical and chemical mechanisms responsible for degradation and removal of heterogeneous substances and by highly effective innate and highly specific immune systems. However, the interface between the organism and the outside world is also the site of exchange of nutrients, export of products and waste components; mucosae must therefore be selectively permeable and at the same time, they must constitute a barrier equipped with local defence mechanisms against environmental threats (e.g. invading pathogens). The mucosal immune system evolved mechanisms discriminating between harmless antigens from food and microflora and dangerous antigens. Characteristic features of mucosal immunity distinguishing it from systemic immunity are: strongly developed mechanisms of innate defence, the existence of characteristic populations of unique types of lymphocytes and their products, colonization of the mucosa and exocrine glands by cells originating from the mucosal organized tissues ("common mucosal system"), transport of polymeric immunoglobulins through epithelial cells into secretions (sIgA) and preferential induction of inhibitory mechanisms directed against mucosal non-dangerous antigens ("oral, mucosal tolerance"). Innate mucosal immune system is represented by cells (epithelial cells, macrophages, dendritic cells, mast cells and other cells) and their humoral products (e.g. antimicrobial proteins and peptides). Basic functions of the mucosal immune system are protection against pathogenic microorganisms and prevention of penetration of immunogenic components from mucosal surfaces into the internal environment of the organism (barrier and anti-infectious functions). Another important function is induction of unresponsiveness of the systemic immunity to antigens present on mucosal surfaces ("oral, mucosal tolerance") and maintenance of the homeostasis on mucosal surfaces (immunoregulatory function) (*Mestecky* et al., 1995; *Ogra* et al., 1999; *Tlaskalová-Hogenová* et al., 2002; *Mestecky* et al., 2005).

The basic mechanism of mucosal immunity is innate, natural immunity represented by processes that protect the host immediately, within the first minutes and hours, of exposure to infection. It is of interest that these de-

fence mechanisms of vertebrates are implemented by structurally related effector molecules present in plants and insects, which do not possess higher, specialised forms of adaptive immunity. A characteristic, although not yet clearly defined, feature of innate immunity is an ability to distinguish between potentially pathogenic microbial components and harmless antigens by ''Pattern Recognition Receptors (PRRs)''. An example of these molecules is the so-called Toll like receptors (TLRs) enabling mammalian cells to recognise conserved characteristic molecules present on microorganisms and representing so called Pathogen Associated Molecular Patterns (PAMP) (*Medzitov* and *Janeway,* 2000; *Akira,* 2001). As these molecules, e.g. lipopolysaccharides, peptidoglycans and others are present also on commensal bacteria it seems more precise to call them Microbe Associated Molecular Pattern (MAMP). In mammals, PRRs are present on macrophages, neutrophils, dendritic cells and other cells belonging to innate immune system. It was demonstrated that recognition of microbes activates Nf kappa B signalling pathway, triggering in this way cytokine production, and upregulation of co-stimulatory molecules on antigen presenting cells leading to activation of T cells (*Tlaskalová-Hogenová* et al., 2005a).

In addition to well-known humoral components of innate immunity (humoral forms of PRRs) present on mucosal surfaces such as complement, lysozyme, lactoferrin, mannan binding protein and others, recently described factors have been the subject of intensive study. An important component of non-specific mechanisms are antimicrobial peptides widely distributed throughout plant and animal kingdoms. Various antibiotic peptides, defensins, were found in epithelial cells (e.g. in apical granules of Paneth epithelial cells) (*Bevins,* 1999). Innate immunity is closely linked to adaptive, acquired immunity represented by secretory immunoglobulins and epithelial compartment containing intraepithelial lymphocytes and lamina propria lymphocytes.

Starting from first hours after delivery from the sterile uterine environment, microorganisms colonize most of the mucosal surfaces and skin. The number of autochthonous bacteria (10^{14}) exceeds the number of cells forming the human body. The highest numbers of commensal bacteria exhibiting enormous diversity are found in distal parts of the gut; their identification and characterization is however hampered by the fact that many intestinal bacteria are not cultivable. The highly protective colonization of the mucosal surfaces by commensals has an important stimulatory effect on innate and adaptive immunity, metabolic processes (e.g. nutrition) and other host activities. Using gnotobiotic animal models (animals reared in germfree conditions) we and others demonstrated that components of intestinal microflora play a crucial role during early postnatal development of the immune system and cause "physiological inflammation" of the gut (*Tlaskalová-Hogenová* et al., 1971; *Tlaskalová-Hogenová* et al., 1983; *Tlaskalová-Hogenová*, 1997; *Štěpánková* et al., 1998; *Hooper* and *Gordon*, 2001; *Cebra* et al., 2005). However, under specific conditions commensal bacteria could participate in the development of intestinal inflammation (*Singh* et al., 2001; *Tlaskalová-Hogenová* et al., 2004; *Tlaskalová-Hogenová*, 2005b; *Cebra* et al., 2005).

The epithelium of most mucosal surfaces consists of a layer of interconnected, polarised epithelial cells separated by a basal membrane from the

connective and supporting tissue surrounding various types of cells present in the lamina propria. The epithelial layer is reinforced by tight junctions present in paracellular spaces of epithelial cells and forming an interconnected network. Tight junctions were found to act as a dynamic and strictly regulated port of entry that opens and closes in response to various signals (e.g. cytokines) originating in the lumen, lamina propria and epithelium. The molecules forming tight junctions (zonulins, occludins, claudins) are connected to the cytoskeleton of epithelial cells (*Fasano,* 2001). Mucosal barrier function is greatly influenced by the products of the nervous system (neurotransmitters) (*Mestecky* et al., 2005).

Initial events leading to the development of chronic inflammatory and autoimmune disease have not yet been elucidated. We suppose that dysfunction of the immune system associated with the gut and other mucosal surfaces, i.e. with the largest and most critical area of the body, which is in permanent contact with the environment and with large numbers of living bacteria and their cytokine inducing components, is a prerequisite for impairment of physiologically developing, regulatory mechanisms. Numerous chronic diseases may occur as a result of disturbances of mucosal barrier function or of changes in mechanisms regulating mucosal immunity (*Singh* et al., 2001). This may involve infectious diseases, inflammatory diseases and autoimmune diseases developing either in their initial phase or throughout on mucosal surfaces (*Tlaskalová-Hogenová* et al*.,* 1998, 2002).

The main characteristics of chronic, "idiopathic", inflammatory and autoimmune diseases are tissue destruction and functional impairment as a consequence of immunologically mediated mechanisms, which are principally the same as those functioning against dangerous (pathogenic) infections. One of the most attractive explanations for inflammatory and autoimmune phenomena has always centred on various infections as possible natural events capable of initiating the process in genetically predisposed individuals (*Tlaskalová*, 1997). There are various mechanisms by which infectious components are supposed to trigger inflammatory and autoimmune processes (*Tlaskalová-Hogenová* et al., 2002, 2004). However nutritional components could be involved in pathogenic processes as well. From empiric experience it is known that various kinds of diet could influence the clinical outcome of chronic diseases. Celiac disease belongs to autoimmune disorders caused by dietary component – gluten.

PARTICIPATION OF GLUTEN IN THE PATHOGENETIC MECHANISM OF CELIAC DISEASE AND VARIOUS CHRONIC DISEASES

Celiac disease is a disorder characterized by gluten-dependent enteropathy. Small intestinal mucosal villous atrophy with hyperplasia of the crypts, abnormal surface epithelium and increased inflammatory cell infiltration are regularly found in biopsy specimens taken from jejunum or duodenum of patients with active disease. The features of the mucosal lesions suggest that wheat gluten and other prolamins (secalins in rye and hordeins in barley) lead to aberrant, pathologically increased immune response in geneti-

cally predisposed individuals. Celiac disease is a disorder strongly associated with HLA-DQ2 expressed in more than 90% of patients, a small number of patients express HLA-DQ8. Intolerance to gluten seems to be caused by break down of oral tolerance to dietary gluten leading to the development of autoimmune responses by not yet well characterized mechanism. Increased activity of gliadin specific lamina propria CD4+T cells producing Th0 Th1 cytokines and cytotoxic intraepithelial CD8+ T cells expressing NK receptors are supposed to be involved in the pathogenic mechanism (*Brandtzaeg*, 2006; *Stepniak* and *Koning,* 2006). The increased level of antibodies to gliadin in sera of patients is regularly accompanied by the presence of auto-antibodies. We have shown that auto-antibody to calreticulin present in the sera of patients is the consequence of molecular mimicry between gliadin and this auto-antigen (*Krupičková* et al., 1999). Endomysial auto-antibodies of IgA isotype have for years been used as a specific and sensitive serological marker for celiac disease (*Ascher* et al., 1996; *Dewar* and *Ciclitira*, 2005). Recently the molecular nature of the target of anti-endomysial antibodies was identified as type 2 tissue transglutaminase (*Dieterich* et al., 1997). Serological IgA positivity for human transglutaminase seems to be nowadays the best serological marker for celiac disease. However, the definitive diagnosis of the disease is obtained by performing a small intestinal biopsy looking for characteristic pathological changes (*Dewar* and *Ciclitira*, 2005). In spite of the progress in diagnostic possibilities the diagnosis of celiac disease is still challenging due to the great variability of clinical presentations. The classical symptoms like diarrhoea, abdominal pain and weight loss with nutritional deficiencies (iron, folate, calcium) are seen less often, and are present mainly in infants. Unfortunately, most adult patients have either silent or atypical presentations, thus escaping diagnosis for several years. In patients with an atypical form of celiac disease clinical presentations are characterized by the presence of extra-intestinal manifestations including anaemia, osteopaenia, infertility, psychiatric and neurological abnormalities, hyposplenism and gastrointestinal malignancies, especially lymphomas (*Collin* et al., 1994; *Tlaskalová-Hogenová* et al., 1999; *Schuppan* et al., 2005). This is the reason why celiac disease remains undiagnosed and diagnosed and treated patients represent only the "tip of the iceberg" from the population of celiac patients (prevalence is estimated to be 0.5-1%). Although there are attempts to develop novel therapeutic options based on enzymatic digestion of "toxic" gluten peptides, the life-lasting gluten free diet is still the only treatment (*Gianfrani* et al., 2006).

Diverse inflammatory and autoimmune diseases are frequently associated with celiac disease and untreated celiac patients also carry an increased risk of various complications involving anaemia, infertility, osteoporosis and gastrointestinal malignancies. Disorders frequently associated with celiac disease are: endocrinological diseases (e.g. type 1 diabetes, thyroiditis), connective tissue diseases, liver diseases (e.g. primary biliary cirrhosis), Down syndrome, and disorders of the nervous system encompassing epilepsy, ataxia, peripheral neuropathies and other diseases (*Colin* et al., 1994; *Pynone* et al., 2004; *Szodoray* et al., 2004; *Dewar and Ciclitira*, 2005; *Abenavoli* et al., 2006). Beside dermatitis herpetiformis, which is considered a skin form of celiac disease, the most pronounced association of CD is with autoimmune diabetes, where the incidence of CD

among diabetic patients was described to occur in 5-10 % of various populations (*Barera* et al., 2002; *Ashabani* et al., 2003; *Kučera* et al., 2003). Our finding of beneficial effect of gluten free diet in spontaneously diabetic NOD mice suggests that gluten could be involved in the development of this disease (*Funda* et al., 1999). The frequent association of autoimmune diseases with celiac disease led to recommendation for regular serological screening of celiac disease in the risk groups of patients suffering from type 1 diabetes and other diseases associated with celiac disease (*Barera* et al., 2002).

The most interesting part of this story is represented by some reports suggesting that in non-celiac patients suffering from various chronic diseases gluten free diet improved clinical symptoms. Skin diseases: part of patients with psoriasis was described to benefit from introduction of gluten free diet: positive response to dietary regimen was observed in patients who had higher concentrations of anti-gliadin antibodies (about 10% of psoriatic patients), and a similar finding was described in patients with urticaria (*Wolters*, 2005). Neurological syndromes: some patients suffering from cryptogenic ataxia and peripheral neuropathies have been reported to respond to gluten restriction (*Hadjivassiliou* et al., 1998, 2003). In a subset of patients suffering from schizophrenia a reduction of schizophrenic symptoms after initiation of gluten withdrawal has been noted in a variety of the studies (*Kalaydijian* et al., 2006). Similarly, in rheumatic arthritis the vegan diet with exclusion of gluten led in a subset of patients with anti-gliadin positive antibodies to clinical improvement (*Kjeldsen-Krag*, 2000; *Paimela* et al., 1995; *Hafstrom* et al., 2001). It seems that higher concentrations of antibodies directed to food antigens could suggest that the gut barrier function is impaired (*Hafstrom* et al., 2001; *Kučera* et al., 2003). Underlying mechanisms of these effects are not yet elucidated. It could be speculated that part of the patients have impaired gut barrier function; gluten could therefore pass into circulation in an incompletely digested form and in this way activate the innate and adaptive immunity (*Drago* et al., 2006). Installation of gluten free diet then represents removal of dietary stimulus activating under specific conditions the immune system.

ANIMAL MODELS OF CELIAC DISEASE

Animal models of human diseases are helping to elucidate the pathogenic mechanisms of the diseases and to develop new preventive and therapeutic approaches. Several groups of researches tried to develop an experimental model for celiac disease and to induce celiac- like lesions by administrating gluten to various strains of mice and rats. No conspicuous changes were observed on intestinal mucosa of adult animals after gliadin application (*Troncone* and *Ferguson,* 1991). We have found that repeated intra-gastric application of gliadin to conventionally reared rats of AVN strain induced profound jejunal changes, provided that gliadin was administered immediately after birth (*Štěpánková* et al., 1989). Components of intestinal microflora induce a major stimulatory effect on mucosal immune system especially during early postnatal development. We have therefore used defined gnotobiotic conditions, which made it possible to differentiate the effects of micro-

flora and dietary antigens (*Štěpánková* et al., 1996). On analyzing the changes induced by gliadin application we found that repeated gliadin application led to shortening of the jejunal villi, crypt hyperplasia, increased number of mitoses in the crypt epithelium, increased number of inflammatory cells in gut mucosa and an increased level of IgA anti-gliadin antibodies in intestinal washings and in the sera of experimental rats. Interestingly, we have found that intra-gastric gliadin application into germfree rat pups increased the number of intra-epithelial lymphocytes and had similar effects on intra-epithelial lymphocyte subpopulation as colonization with microflora (*Štěpánková* et al., 1996). Transfer of intra-epithelial lymphocytes separated from gliadin treated rats into the intestinal loops of untreated germfree rats caused damage in the recipient gut enterocytes - disruption of tight junctions was observed on enterocytes of intestinal loops. Moreover, lymphocytes from gliadin treated germfree rats penetrated through the intestinal wall and were detected in lamina propria, while lymphocytes from control germfree rats remained in the lumen. Control lymphocytes did not cause structural damage to the epithelium of intestinal loops (*Štěpánková* et al., 1996). Together with morphological changes of enterocytes, the brush border enzymatic activities have been changed (*Kozáková* et al., 1998, 2000). Interestingly, when we used various neurological and behavioural tests to assess the changes caused by gliadin treatment, higher emotionality of gliadin treated rats was found in an open field test (*Castany* et al., 1995).

The use of the described rat model of gluten-induced enteropathy demonstrated a protective effect of breast-feeding. Suckling animals given gliadin never displayed the flat mucosa characteristic of destructive celiac disease. A significant relationship is generally assumed to exist between suckling and the occurrence of the celiac disease, which appears after introducing gluten into the diet (*Ivarsson* et al., 2002). The basis of this protective effect of maternal milk against the deleterious influence of gluten remains unclear. Maternal milk contains proteins, saccharides, fat and a number of biologically active components like hormones, immunoglobulins, cytokines and growth factors. In rats´ maternal milk, epidermal growth factor (EGF) plays an important role in the process of regeneration and proliferation of jejunal enterocytes. We studied its potential protective effect on the damage of intestinal mucosa by gliadin in a model system (*Štěpánková* et al., 2003). Enteropathy was induced by repeated intra-gastric gliadin application in inbred rat pups of AVN strain, delivered by Caesarean section, breast fed or hand-fed with a milk formula. All experimental groups were treated with interferon gamma, administered intraperitoneally after birth. One group of hand-fed pups received EGF continuously in the diet. Gliadin in interferon gamma treated formula-fed rat pups showed villous atrophy, increased numbers of inflammatory cells and damage to epithelial tight junctions and enterocyte brush border. Addition of EGF to the diet protected the rats against pathological mucosal changes and prevented celiac crisis. The role of EGF and other regulatory peptides in the development of gluten mucosal impairment is not yet fully understood and needs further study (*Štěpánková* et al., 2003).

ACTIVATION OF HUMAN INNATE IMMUNITY CELLS BY GLIADIN

It seems that the unique structure of gliadin and its fragments could be responsible for the involvement of innate immunity mechanisms in the pathogenic mechanism of this disease (*Novák* et al., 2002; *Schuppan* et al., 2003; *Maiuri* et al., 2003; *Zanoni* et al., 2006). In our previous experiments we have demonstrated that, in contrast to other dietary proteins tested, gliadin stimulated IFN gamma treated mouse peritoneal macrophages for NO production and secretion of cytokines (TNF alpha, Il-10, RANTES) (*Tučková* et al., 2002). Recently we found that cultivation of cells of human monocytic cell line THP1 with the peptic digest of gliadin leads to the production of IL8 and TNF alpha and that the production is augmented by pre-treatment of cells with IFN gamma or its addition to the culture. Ovalbumin and soya protein or their peptic digests had no effect on IL-8 and TNF alpha production either when applied alone or in combination with IFN gamma. The participation of nuclear factor- kappa B (NF kappa B) in stimulatory effect of gliadin digest on monocytes was documented by a marked increase of the DNA binding activities of NF kappa B subunits p50 and p65. Moreover, the use of NF kappa B inhibitors sulfasalazine , PDTC and TPCK led to the detection of an inhibition of p65 and p50 subunits binding (*Jelínková* et al., 2004).

The effect of a peptic digest of gliadin on the maturation of human monocyte-derived dendritic cells was studied by Palová-Jelínková et al. (2005). In contrast to other tested food proteins (ovalbumin, soya protein) gliadin led to increased expression of costimulatory molecules - maturation markers (CD 80, CD 83, CD 86 and HLADR molecules) and increased secretion on chemokines and cytokines (IL-06. IL-08, IL-10, ATN ALFA, growth related oncogine, NCP-1, NCP-2, macrophage damaged chemokine and RANTES). Moreover, gliadin-induced phosphorylation of members of three MAPK families ERK1/2, JNK, and p38 MAPK was demonstrated. Gliadin treatment also resulted in increased NF-kappa B/DNA binding activity of p50 and p65 subunits (*Palová-Jelínková* et al., 2005). It seems that gliadin can contribute to phenotypic and functional maturation of dendritic cells, which is followed by efficient processing and presentation of gliadin peptides to specific T lymphocytes and in this way the activation of innate immune system can facilitate the development of the disease.

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IMMUNOMODULATION BY DIETARY MUSHROOM COMPOUNDS

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SUMMARY

Mushrooms are long used food supplements that display profound immunomodulatory activities, due to the presence of both polysaccharides (β-glucans) and proteins (FIP). These compounds work either alone or in concert and both target different aspects of the immune system. While β-glucans are considered to bind to innate receptors and activate innate cells (dendritic cells and monocytes/macrophages), immunomodulatory proteins will be taken up, processed and presented on antigen-presenting cells to $CD4^+$ Th cells in the context of MHC class II molecules. The outcome is different and results in the activation of both innate and antigen-specific adaptive immune reactivity. The combined presence in a whole extracts is therefore expected to be of more use than either active compound alone. Here we will describe some of the immunomodulatory activities present in such extracts from edible mushrooms.

INTRODUCTION

Immunomodulation

Immunomodulation is the manipulation of the immune system by augmenting or decreasing the magnitude of the immune responsiveness. The augmentation of the immune response is known as immunostimulation or immunopotentiation, while suppression of immune responsiveness is called immunosuppression. The necessity of suppression of the function of the immune system is well recognized in the areas of transplantation and immunopathological disorders like autoimmunity. Conversely, augmentation of the immune response has been a target for increasing the host's resistance to infections and diseases. Specific immunomodulation is limited to a single antigen such as a vaccine and thus immunopotentiation is used for the development of resistance against particular diseases. Non-specific immunomodulation implies for a more generalized change in the immune responsiveness leading to altered host reactivity to many different antigens. Prevention and treatment of diseases are generally achieved by a wide range of antibacterial, antiviral, antiparasitic and antifungal agents and vaccines. The impact of chemotherapy and vaccination on many complex diseases, however, has reached a plateau and if further progress is to be made, different strategies have to be developed. Immunomodulation is one of the most important alternatives in order to control

diseases associated with the environment with additional advantages of amplifying specific responses to vaccines still needed to control severe, life-threatening diseases. The immunomodulation offers the additional benefit of reversing immunosuppression caused by various cancers, stress, infection, food-related problems, reproductive problems, and chronic inflammatory conditions.

Diet

A well-balanced diet is beneficial for a good immune function. The minerals zinc, copper, iron and selenium and the vitamins A, C and E have been established to be essential for a normal immune response (*Cunningham-Rundles* et al., 2005). Recently, food ingredients, such as fish oil, are being studied for potential immunomodulatory properties, and functional foods that influence the immune function are being developed, including pre- and probiotics (*Field* and *Schley*, 2004; *Salminen* et al., 1998).

It may be less well known that mushrooms have immunomodulating properties as well (*Wasser*, 2002). Some 140,000 species exist on earth of which some 14,000 species are described. Generally, mushrooms contain by weight approximately 90% water, 10-40% protein, 3-28% carbohydrate, 2-8% fat, 3-32% fibre and 8-10% ash (*Breene*, 1990). For millennia, they have been valued as tasteful foods and as medicinal substances. The knowledge and practice of the medicinal use of mushrooms originates from traditional eastern medicine and in Japan, China, Korea, and other Asian countries modern clinical practice still utilises mushroom-derived preparations. Over the past three decades scientific research has been performed on the medicinal properties of a growing number of mushroom species. Mushrooms are claimed to exhibit antiviral, antibacterial, cholesterol-lowering, blood pressure-lowering and hypoglycaemic effects. Mushroom products are commercially available to contribute to the treatment of infectious diseases, cardiovascular disorders, diabetics, and to prevent various diseases (*Wasser* and *Weis*, 1999; *Zaidman* et al., 2005).

Several major substances with immunomodulatory and/or anti-tumour activity have been isolated from mushrooms. These include mainly polysaccharides (in particular β-D-glucans), polysaccharopeptides (PSP), polysaccharide proteins, and proteins. Furthermore, other bioactive substances, including triterpenes, lipids, and phenols, have been identified and characterized in mushrooms with proven medicinal properties. The major immunomodulating effects of these active substances derived from mushrooms include mitogenicity and activation of immune cells, such as haematopoietic stem cells, lymphocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells, resulting in the production of cytokines. The therapeutic effects of mushrooms, such as anticancer activity, suppression of autoimmune diseases, and allergy have been associated in many cases with their immunomodulating effects.

The most extensively studied application of medicinal mushrooms is their anti-tumour activity. Polysaccharides, mostly β-glucans, have been established to be one of the most potent anti-tumour-active compounds (*Borchers* et al., 1999, 2004). While most regular chemotherapeutic agents are based on direct cytotoxicity to cells, β-glucans mediate their anti-tumour activity by stimulating the immune system (*Brown* and *Gordon*, 2003). Unfortunately, regular cancer therapy often has many serious side effects and can be unsuccessful. In addition, infectious diseases

become increasingly difficult to treat, as pathogens are becoming resistant to many antibiotics (*Sheldon*, 2005). β-glucans may contribute to the solution of these problems by stimulating the immune system to enhance anti-tumour and anti-infective responses.

IMMUNOREGULATION AND CHRONIC INFLAMMATORY IMMUNE-MEDIATED DISEASES

Properties of many diseases, particularly systemic auto-immune diseases characterised by persistent inflammation, strongly support the involvement of helper T-lymphocytes (*Abbas* and *Lichtman*, 2005). For example, pathogenic auto-antibody responses generally are of high affinity IgG class, after having undergone affinity maturation, which requires helper T cells. The protein antigens to which many auto-antibodies are directed generally require T cell help. Many of the successful therapies, e.g. cyclosporin A, act primary on T cells. Besides roles as helpers, T cells may directly provoke cellular injury during inflammatory phases of the disease process. T cells, and in particular $CD4^+$ helper T cells produces effector molecules, called cytokines, upon activation. A multiplicity of cytokine abnormalities has been associated with various auto-immune and immune-mediated diseases. It is thus becoming common practice to analyse the role of helper T cells and the cytokines they produce in studying the immunpathological basis of particular diseases, to aid in the unambiguous diagnosis of the disease, to rationally design T cell and/or cytokine based immunotherapy protocols, and to provide parameters to monitor the efficacy of treatment.

Naive $CD4^+$ helper T cells (Th) develop into functionally mature effector cells upon stimulation with relevant antigenic peptides presented by major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APC). Based on the characteristic set of cytokines produced, Th cells are commonly segregated into at least two different subpopulations: Th1 cells producing exclusively interleukin-2 (IL-2), interferon-gamma (IFN-γ) and lymphotoxin (O'Garra and Arai, 2000). Th2 cells on the other hand, produce IL-4, IL-5, IL-6, IL-10 and IL-13. These Th1 and Th2 subsets appear to be extremes in cytokine production profiles and within these polarised subsets, individual Th cells exhibit differential rather than co-ordinated cytokine gene expression. The Th-1 and Th-2 subsets appear to cross-regulate each other's cytokine production profiles, mainly through IFN-γ and IL-10. From this concept it was rationalised that disturbances in the balance of between these two subsets may result in different clinical manifestations. IL-12 is a dominant factor promoting Th1 differentiation, and is produced by dendritic cells and macrophages. Moreover, IL-12 induces IFN-γ production by T cells and NK cells. It was reported that IL-18 acts as synergistically with IL-12 to induce Th1 development while polarisation of Th2 cells is critically dependent on the presence of IL-4 produced by Th cells, basophiles and mast cells. APC-derived IL-6 has also been shown to induce small amounts of IL-4 in developing Th cells. IL-10 and APC-derived prostaglandin E_2 (PGE_2) inhibit IL-12 production and Th1 priming (see Figure 1).

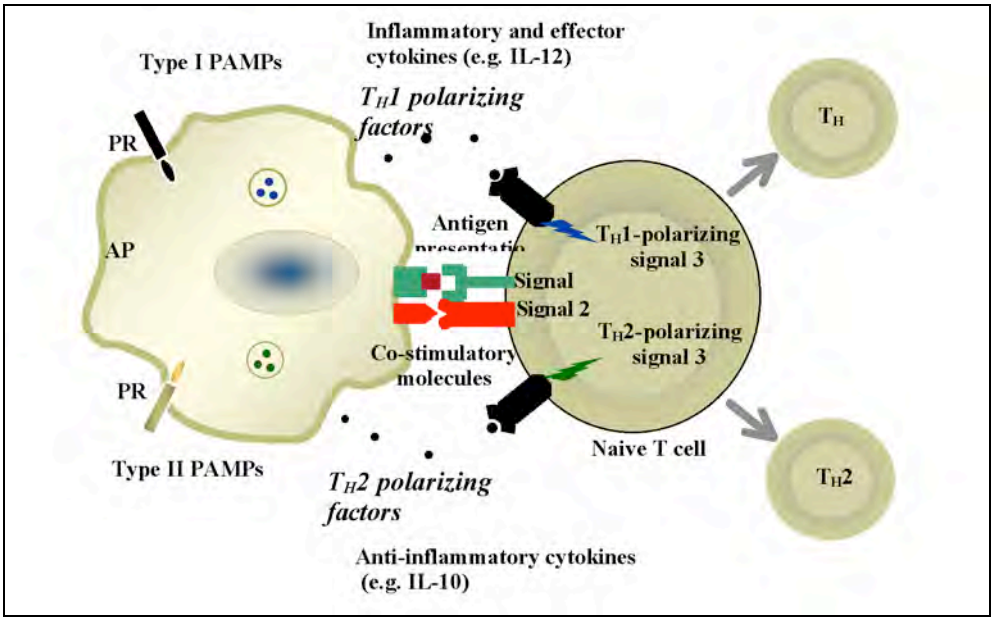

Figure 1: Cells and cytokines involved in Th1 or Th2 responses (Adapted from *O'Garra* and *Arai*, 2000).

Self-tolerance is induced in the T cell recognition repertoire by clonal deletion, anergy or silencing in the thymus. These processes are, however, not complete and thus potentially autoreactive T cell can escape into the periphery where such T cells are continuously suppressed by cells that act functionally as suppressor cells. These cells, able to suppress other cells by cell surface mediated activity and/or production of suppressive cytokines, like IL-10 and (transforming growth factor) TGF-ß are now called regulatory cells. Regulatory T cells mediate active suppression of various immune responses. These T cells comprise classical Th2 cells, capable of inhibiting Th1 responses, but also alternative T cell populations. Two main populations are distinguished based on molecular and cellular differences: naturally occurring $CD4^+CD25^+$ naturally occurring regulatory T cells (Treg) that activate the Foxp3 transcription factor and that primarily detectable in the periphery. Alternatively, antigen-induced regulatory T cell populations (Tr, Th3) were identified based on their high secrewtion of IL-10 and TFG-ß and their relation with tolerance induction on mucosal surfaces in the gastro-intestinal and the respiratory system. The mechanism of peripheral tolerance has been focused mainly on the suppression of classical cell-mediated (IFN-γ producing) Th1 responses and in animal (models of) diseases based on excessive activity of Th1 cells. It is now clear that such tolerance induction is also active in humoral type 2 responses. One of the primary mechanisms of tolerance induction is via secretion of immunosuppressive cytokines, like IL-10, IL-4 and TGF-ß. As mentioned before, regulatory T cells have been isolated from *in vitro* cultures, which appeared to produce low levels of IL-2, no IL-4, but high levels of IL-10 and TGF-ß. This demonstrates the importance of cytokines in regulating and dampening

the immune response. It will thus be of crucial importance to determine whether the immunomodulating capacity of herbal and fungal polysaccharides in many diseases act via induction of these regulatory T cell subsets.

Recent progress has provoked a breakthrough in our understanding of basic mechanisms underlying the development of chronic inflammatory immune-mediated diseases by showing that Tr-cells are important to sustain T-cell tolerance. This is achieved via the production of cytokines, like IL-10. Tr cells are involved in the suppression of both Th1 (auto-immune) and Th2 (allergic) diseases. The role of Tr-cells is currently a focus in allergic disease model systems, as in mice. This research will tell much on the immunological mechanisms that are at the basis of the development of allergies, and provide opportunities for effective immune therapy based on induction of Tr-cells (*Boonstra* et al., 2000; *Van der Velden* et al., 2001).

DEVELOPMENT OF ORAL TOLERANCE

Induction and/or maintenance of oral tolerance to orally ingested antigens also require microbial colonization of the alimentary tract in early life. As the intestine is the first line of defence from the environment, and must integrate complex interactions between diet, external pathogens, and local immunological and non-immunological processes, it is critical that protective immune responses are made to potential pathogens yet it is equally important that hypersensitivity reactions to dietary antigens are minimised. Thus, the gut immune system must distinguish not only between self and non-self, but also between potentially dangerous foreign antigens and common harmless foodstuffs to which it is constantly exposed. Such suppressive mechanisms to avoid local and peripheral overreaction (hypersensitivity) against innocuous substances bombarding the mucosal surfaces are referred to as 'oral tolerance' when induced via the gut against dietary antigens (*Brandtzaeg*, 2006). Similar down-regulatory mechanisms apparently operate against antigens from the commensal micro flora.

In physiological circumstances tolerance towards the indigenous intestinal microbiota is established and maintained. Different doses of orally administered antigens may induce anergy in antigen-specific T cells or may stimulate the production of cytokines, like TGF-ß, that inhibit lymphocyte proliferation, resulting in suppression of the immune response. Moreover, since TGF-ß also induces isotype switching to IgA antibody production, the mucosal immune system is further protected (*Abbas* and *Lichtman*, 2005). It is unclear why soluble proteins in large doses induce systemic T cell tolerance, whereas oral immunisation with attenuated poliovirus vaccines induces protective T-cell dependent antibody responses and long-lived memory.

Permeability of the intestinal barrier is greatly enhanced during foetal life, but gut closure starts before birth and is considered more or less complete by 33 weeks of gestation. Paracellular permeability can thereafter be increased, resulting in sensitisation to dietary antigens, large enough to be presented by DC to T-cells and resulting in a pro-inflammatory response. Pro-inflammatory cytokines, like IFN-γ and TNF-α induce paracellular leakiness, while IL-10 and TGF-ß promote tight junc-

tion formation and particularly these cytokines play a central role in oral tolerance (*Adams* et al., 1993; *Planchon* et al., 1994).

Besides, there is evidence indicating that IL-10, a tolerogenic cytokine, is produced in response to microbial stimuli: Mice with defective IL-10 production infected with *Helicobacter hepaticus* developed Th1-type intestinal inflammation, whereas normal mice produced IL-10 and remained health. Interestingly, IL-10 deficient mice have decreased levels of resident lactobacilli in the neonatal period, and normalising the amount of these bacteria in the colon prevented the development of intestinal inflammation. In humans, carriers of *H. pylori* infected individuals posses Treg that can *in vitro* suppress responses of antigen-specifically stimulated T cells. *In vivo* it was suggested that the induction of *H. pylori* specific Treg cells suppress protective antigen-specific responses and contribute to the persistence of the infection (*Penner* et al., 2005).

IMMUNOMODULATION BY MUSHROOM-DERIVED ß-GLUCANS

Structure of β-glucans

The interest in β-glucans as anti-infective and anti-tumour agents originates from the early 1900s, when an insoluble yeast (*Saccharomyces cerevisiae*) cell wall particle, named zymosan, was developed. Experiments showed that intravenous injection of zymosan could stimulate the immune system. Later β-glucan was identified as the biologically active constituent. β-glucans are a heterogeneous group of glucose polymers, mostly consisting of a backbone of β(1→3)-linked β-D-glucopyranosyl units with β(1→6)-linked side chains of varying distribution and length (see Figure 2).

These polysaccharides are major cell wall structural components in fungi and are found in plants and some bacteria as well. As they are not present in animals, they are recognized by the innate immune system and are considered to be classic pathogen-associated molecular patterns (PAMP). β-glucans mostly show a triple-strand right winding helix structure. Various β-glucans have been isolated from diverse mushroom species showing different immunological activity, which could be correlated with their solubility in water, molecular weight, conformation (tertiary structure) and degree of branching. Lentinan (*Chihara* et al., 1970; *Yap* et al., 2001) is isolated from the fruiting body of *Lentinus edodes* (shiitake mushroom) and consists of five β(1→3)-β-D-glucopyranosyl units in a linear linkage and two β(1→6)-linked side chains (degree of branching: 0.4). The molecular weight is about 4-8 x 10^5 g/mol. Grifolan has a degree of branching of 0.33 and a molecular weight of approximately 5 x 10^6g/mol. The compound is isolated from the liquid-cultured mycelium of *Grifola frondosa* (maitake mushroom). Another β-glucan has been extracted from this mushroom: Maitake D-fraction (*Kodama* et al., 2003). This is a high molecular weight polysaccharide. In contrast with other β-glucans, which have a β(1→3) main chain, D-fraction consists of a β(1→6) main chain with β(1→3) branches. Schizophyllan is obtained from a culture medium of *Schizophyllum commune* (split gill fungus) (*Hashimoto* et al., 1991). Its branching rate is 0.33 and the molecular weight is approximately 4.5 x 10^5 g/mol. SSG is a compound isolated from the culture filtrate of *Sclerotinia*

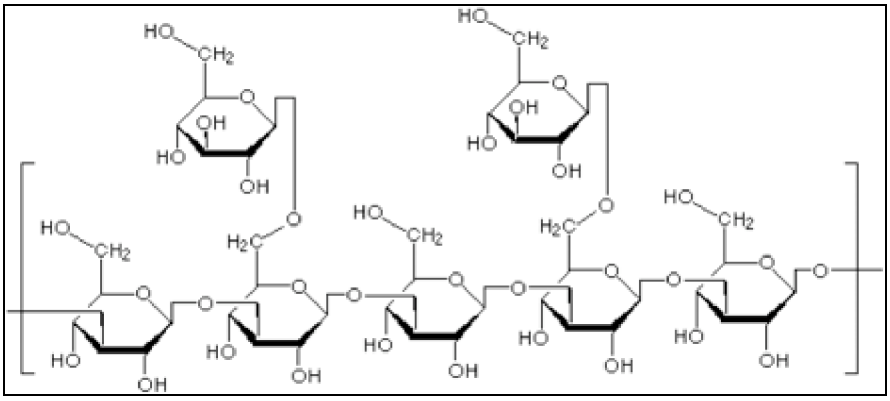

Figure 2: Chemical structure of the branched β-glucan lentinan.

sclerotiorum (white mould) and has a branching rate of 0.5 and a molecular weight of approximately 2 x 10^6 g/mol. As this field of research is growing new mushroom species are being investigated for their immunostimulating glucans, including *Agaricus blazei*, *Sparassis crispa*, *Ganoderma lucidum*, *Pleurotus ostreatus*, and *Sclerotium glucanicum* (*Brown* and *Gordon*, 2003; *De Ruiter* et al., 1992; *Karacsonyi* and *Kuniak*, 1994; *Kulicke* and *Lettau*, 1997; *Dong* et al., 2002). Several animal studies were performed on the use of β-glucans in foods to modulate natural disease resistance (*Charampopoulos* et al., 2002; *Chesterman* et al., 1981; *Gibson* and *Roberfroid*, 1995) and to study the influence of β-glucans to reproductive and pregnancy-related problems (*Cozens* et al., 1981a; 1981b; 1981c).

Effects of β-glucans on the immune system

Particular mushroom β-glucans have an immunomodulatory and anti-tumour effects (*Bohn* and *BeMiller*, 1995). These substances are regarded as biological response modifiers. This basically means that they cause no harm and place no additional stress on the body; they help the body to adapt to various environmental and biological stresses; and they exert a non-specific action on the body, supporting some or all of the major systems, including nervous, hormonal, and immune systems, as well as regulatory functions.

Three β-glucans are used as biological response modifiers: Lentinan from *Lentinus edodes,* D-fraction from *Grifola frondosa,* and schizophyllan from *Schizophyllum commune*. Lentinan and schizophyllan are approved in Japan for clinical use to improve the immunity of cancer patients. β-glucans are used as a mild and non-invasive form of treatment of cancer and other diseases, and in the prevention of metastasis spread of tumours. In cancer treatment they can be used as a co-treatment in conjunction with other forms of therapy, such as chemotherapy and surgery.

The anti-tumour action of β-glucans is largely mediated via activation of the immune response; the polysaccharides do not attack cancer cells directly (*Arinaga* et al., 1992a; 1992b; *Baba* et al., 1986; *Chihara* et al., 1970; *Maeda* et al., 1988; *Minato* et al., 1999; *Ng* et al., 2002; *Oka* et al., 1992, 1996; *Ooi* et al., 2000; *Sudate* al., 1996; *Taguchi* et al., 1980, 1987; *Usui* et al., 1983; *Wada* et al., 1987; *Wasser*, 2002; *Zaidman* et al., 2005; *Zhang* et al., 2002, 2005). Experiments showed that the anti-tu-

mour effect was lost in neonatal thymectomised mice or after administration of anti-lymphocyte serum. These results suggest that the anti-tumour action is T-lymphocyte dependent. In addition, macrophages play an important role, because the anti-tumour activity can be inhibited by pre-treatment with anti-macrophage agents. The production of various cytokines is induced by β-glucans, which results in the proliferation, maturation, and differentiation of immune cells, such as NK cells and T-lymphocytes.

Besides anti-tumour activity, many β-glucans are thought to possess anti-infective activity against various bacterial, viral, and parasitic infections (*Irinoda* et al., 1992). For example, schizophyllan demonstrated protective effects against *Staphylococcus aureus,* and *Escherichia coli* infections in mice. SSG reduced the number of live intracellular *Mycobacterium tuberculosis* in macrophage cultures when it was incubated together with the bacteria (*Markova* et al., 2003; *Hetland* et al., 2002).

Little is known about the biological effects of β-glucans after oral administration (*Rice* et al, 2005). Oral administration of glucan phosphate led to a significant, but modest, increase in the serum level of the pro-inflammatory cytokine IL-12. No changes were observed in serum levels of other tested cytokines. Oral administration of glucan phosphate to mice one day before challenge with *Staphylococcus aureus* or *Candida albicans* led to increased long-term survival.

The precise mechanism which mediates the effects of β-glucans on the immune system is not totally clarified yet. However, research is starting to shed some light on the cellular receptors and molecular mechanisms involved (*Brown* et al., 2003; *Falch* et al., 2000; *Hamano* et al., 1999; *Hamuro* et al., 1974; *Kataoka* et al., 2002; *Kerekgyarto* et al., 1996; *Kodama* et al., 2003; *Liu* et al., 1999; *Masihi* et al., 1997; *Murata* et al., 2002; *Oka* et al., 1992, 1996; *Ooi* and *Liu*, 2000). The immune response triggered by β-glucans was primarily designed for the control of fungal pathogens. The immune response to pathogens relies upon the cooperation between the innate and the adaptive immune system. Innate immunity is the first line of defence against infections. Cells of the innate immune system recognise structures that are characteristic for microbes, such as lipopolysaccharides in Gram-negative bacteria and β-glucans in fungi. The receptors for these structures, called pattern recognition receptors (PRR), include scavenger, lipopolysaccharide, mannose, β-glucan, and Toll-like receptors (TLR). Innate immunity to fungi is mainly mediated by neutrophils and macrophages. They liberate fungicidal substances, like reactive oxygen intermediates and lysosomal enzymes, and phagocytose fungi for intracellular killing. Helper T cells and cytotoxic T cells cooperate in the adaptive immune response against fungal infections.

Role of β-glucan receptors

To date, four different β-glucan receptors have been identified: Complement receptor type 3 (CR3), lactosylceramide, scavenger receptors and Dectin-1. They have been reported on monocytes, macrophages, neutrophils, eosinophils, natural killer (NK) cells, certain lymphocytes, as well as on non-immune cells including endothelial cells, alveolar epithelial cells, and fibroblasts.

CR3 is a heterodimeric integrin receptor, consisting of CD11b and CD18 chains and is expressed on monocytes, neutrophils, NK cells, and selected lymphocytes. It functions as a phagocytic receptor for iC3b-opsonized par-

ticles (iC3b is an inactive complement protein), including opsonised particulate glucans, and it possesses a lectin domain, which recognises a variety of β-glucans directly. As leukocytes lacking CR3 can still bind and respond normally to β-glucans, the receptor does not seem to be indispensable. On the other hand, CR3-dependent cytotoxicity to tumour cells is stimulated by β-glucan priming: Experiments have shown that incubation of NK cells or neutrophils with small soluble β-glucans primed the CR3-receptor to enhance the cytotoxicity against iC3b-opsonised target cells that were otherwise resistant to CR3-mediated cytotoxicity. In addition, it plays a role in the recruitment of leukocytes to sites of inflammation by binding to adhesion molecules on endothelial cells.

Lactosylceramide is a glycosphingolipid present in the plasma membranes of many cells. It has been suggested that the interaction of β-glucans with this receptor can induce macrophage inflammatory protein-2 and the activation of the nuclear transcription factor NF-κB and can enhance the neutrophil oxidative burst and antimicrobial functions. The mechanisms are still unknown. There are indications that macrophage scavenger receptors can recognise β-glucans as well.

Dectin-1, a receptor first discovered in mice, seems to have an important role in mediating the biological response to β-glucans (*Gantner* et al., 2003; *Herre* et al., 2004). It is a transmembrane receptor with an immunoreceptor tyrosine-based activation (ITAM) motif in its cytoplasmic tail. Upon β-glucan binding to the extracellular side of Dectin-1 at the cell surface, the ITAM motif becomes phosphorylated, generating a signal which induces phagocytosis and the respiratory burst (the production of reactive oxygen intermediates). It is present on monocytes, macrophages, and neutrophils, and at lower levels on dendritic cells and a subpopulation of splenic T lymphocytes. Human Dectin-1 differs from its murine counterpart in that it is alternatively spliced, in a cell-specific manner, giving rise to several isoforms of which only two are functional. They are sometimes referred to as the β-glucan receptor and are similar in structure and function to murine Dectin-1. Dectin-1 also recognises an endogenous ligand on activated T cells in a β-glucan independent manner and may act as a T cell co-stimulatory molecule. Zymosan, a particle from yeast cell wall which consists of a variety of compounds, including β-glucans, mannans, mannoproteins and chitin, was found to interact with Toll-like receptors (TLR). Both TLR2 and TLR6 are required for activation of NF-κB in macrophages and dendritic cells, leading to the production of the pro-inflammatory cytokines TNF-α and IL-12 in response to zymosan. The adaptor protein MyD88 mediates the intracellular signalling to NF-κB. The binding of β-glucans to Dectin-1 alone does not stimulate the production of TNF-α and IL-12. However, Dectin-1 enhances the production of these pro-inflammatory cytokines when TLR2 is stimulated. TLR2 was found not to recognise β-glucans, but some other component of zymosan. This suggests that the strong pro-inflammatory activities reported for β-glucans in many studies, might be partly caused by unidentified TLR2 triggering contaminants in impure extracts. This hypothesis is supported by the results of a study performed by *Kataoka* et al. (2002) who found that branched (1→3)β-glucans, like lentinan and schizophyllan, could not stimulate NF-κB activity in macrophages. Taken together, for the activation of NFκB and the induction of a pro-inflammatory

response Dectin-1 needs to cooperate with TLR-2. The activation of Dectin-1 alone by β-glucans is sufficient to stimulate the respiratory burst and phagocytosis (see Figure 2). It seems that TLR2 does not recognise β-glucans but other compounds present in zymosan

Recently, new insights in the role of Dectin-1 in the response to β-glucans were provided by *Rogers* et al. (2005) and by *Palma* et al. (2006). Mouse B cell hybridoma cells (which normally cannot bind zymosan) were transduced with Dectin-1. These Dectin-1 transduced cells were now able to bind β-glucans of zymosan and produced IL-2 and IL-10. This shows that cytokine production is also occurring independent of TLR signalling. The same research group clarified part of the signalling pathway of Dectin-1. They discovered that a kinase called Syk was recruited upon β-glucan binding to Dectin-1. They also found that single phosphorylation of the membrane-proximal tyrosine (position 15) in the ITAM-motif is sufficient to recruit Syk and to couple to downstream IL-2 and IL-10 responses. Experiments were performed with Syk-deficient dendritic cells from chimaeric mice and it was found that these cells were completely unable to produce IL-2 or IL-10 in response to zymosan but could still bind zymosan and produce normal levels of IL-12 and IL-6. It can be concluded that Syk is required for zymosan-induced IL-2 and IL-10 production, but not for IL-12 synthesis, which could be signalled via the TLR2 pathway. IL-10 induction can occur independently of TLR signalling; IL-2 production is enhanced by TLR signalling (see Figure 3).

The importance of the Dectin-1/Syk pathway lies in the biological function of IL-2 and IL-10 during an infection. IL-2 and IL-10 stimulate the development of regulatory T cells thereby limiting immunopathology to a local infection, allowing persistence of immunity and resistance to re-infection, and maintaining immunological memory. In addition, IL-10 acts on activated macrophages to terminate their inflammatory responses and returning the immune system to its resting state. The Decin-1/Syk pathway could therefore be exploited therapeutically in allergy, autoimmunity, and graft rejection. Normally, resistance to yeast infections is characterised by a strong T helper1-type response, in which IL-12 and TNF-α play an important role. In this way the infection can be cleared effectively.

In summary, it seems that β-glucans induce the production of IL-2 and IL-10 and the development of regulatory T cells via the Dectin-1/Syk pathway, while other compounds present in zymosan (and probably other fungal compounds) stimulate a pro-inflammatory T helper1-type response with production of IL-12 and TNF-α. Dectin-1 is responsible for phagocytosis and induction of the respiratory burst as well. Furthermore, CR3, lactosylceramide, and scavenger receptors mediate the stimulatory effects of β-glucans on the immune system. It should be noted that the amount or influence of the cytokines produced via the Dectin-1/Syk pathway might be small, because in animal and *in vitro* experiments a pro-inflammatory cytokine pattern is usually found, in which IL-12 and TNF-α predominate, at least for lentinan. This may be explained by the influence of other β-glucan receptors or by contaminants binding to Toll-like receptors.

Oral delivery and gastro-intestinal absorption

To understand if and how oral delivery of β-glucans can have a biologi

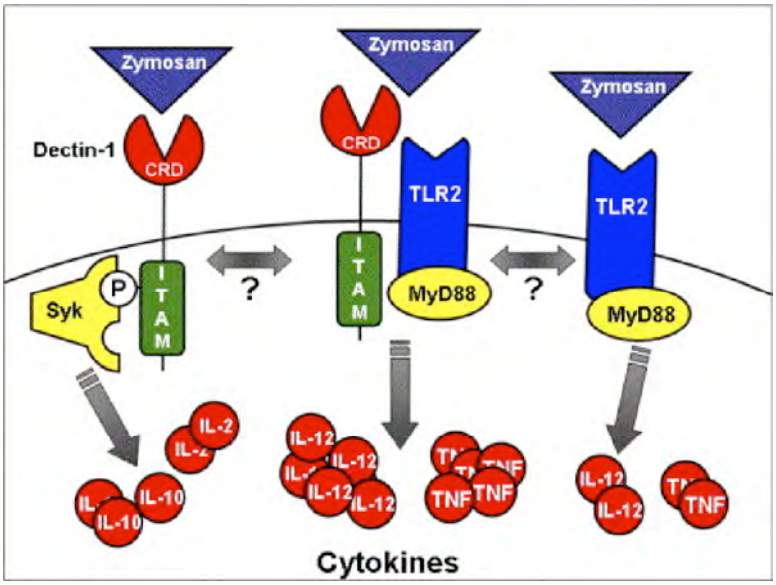

Figure 3: Zymosan can interact with Dectin-1 leading to production of IL-2 and IL-10. Triggering of TLR2 induces IL-12 and TNF-□ production and cooperative signalling of Dectin-1 with TLR2 augments this (From *Rogers* et al., 2005).

cal effect it is important to know if they are absorbed in the gastrointestinal tract, as humans cannot digest them. *Rice* et al. (2004, 2005) examined the absorption, pharmacokinetics, and biological effects of the three water-soluble β-glucans glucan phosphate, laminarin and scleroglucan that were administered by oral gavages to rodents. Laminarin (molecular weight: 7.7 $x10^3$; degree of branching (DB): 1/10; single helix) and scleroglucan (MW: 1.02 $x10^6$; DB: 1/3; triple helix) both have a backbone of (1→3)-β-D-glucopyranosyl units and β(1→6)-linked side chains and mainly scleroglucan is comparable to the mushroom β-glucans described above. Oral administration produced measurable plasma levels of the three β-glucans. Laminarin showed two peak plasma levels: one at 3 hours and one at 12 hours. Scleroglucan plasma levels peaked twice as well: at 15 minutes and at 3 hours. It is interesting that scleroglucan, the largest β-glucan, was absorbed most rapidly. The maximum plasma level was 115 ng/ml for laminarin and 355 ng/ml for scleroglucan when rats were given an oral dose of 1 mg/kg. It is noteworthy that most *in vitro* studies are performed with higher concentrations of β-glucans (approximately 5-500 µg/ml), so it is questionable if the low plasma levels after oral administration are sufficient for immunostimulation. In contrast, *Tani* et al. (1992) performed an *in vitro* study with macrophages and NK cells and stated that 25 to100 ng/ml was the optimal concentration of lentinan to improve cytotoxicity. The bioavailability of laminarin and scleroglucan was 4.9 and 4.0%, respectively. A water-insoluble, particulate glucan preparation was not detected in plasma. A possible explanation is that particles are phagocytosed and transported by macrophages.

Fluorescently labelled β-glucans were used to determine which cells in the gastrointestinal tract could bind them. It was demonstrated that gut-as-

sociated lymphoid tissue (GALT) cells, isolated from Peyer's patches, can bind β-glucans. Macrophages showed an increase in Dectin-1 expression and dendritic cells increased their TLR2 expression. In an experiment with intestinal epithelial cells it appeared that only a subpopulation (10%) of these cells incorporated β-glucan. These cells were found not to express Dectin-1 receptor, so uptake of β-glucans by this cell type could not have been mediated by this receptor in contrast to GALT cells. *Hashimoto* et al. (1991) suggested that high molecular weight β-glucans may be taken up by microfold cells in the intestine, where they interact with the GALT. It is possible that the subpopulation of epithelial cells in Rice's experiment consists of microfold cells. The data support an active uptake mechanism for β-glucans.

A different explanation might be a prebiotic effect of β-glucans on the gut flora. A prebiotic is a food ingredient that is not hydrolysed by the human digestive enzymes in the upper gastrointestinal tract and beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health. Oat β-glucan has been reported to selectively support the growth of lactobacilli and bifidobacteria in rat experiments and in *in vitro* studies. To date, it is not known if mushroom β-glucans could have a similar effect. In addition, there could be an effect of contaminating substances like proteins or fats in impure lentinan extracts.

It should be noted here, that the choice to use laminarin and glucan phosphate for these experiment by *Rice* et al. (2004,2005) is unusual, because laminarin is generally considered inactive and the biological effects of glucan phosphate are not clear. Taken together, these experiments show that orally administered β-glucans interact with a variety of gastrointestinal cells, enter the systemic circulation, and persist in the plasma up to 24 hours.

Distribution, metabolism, and excretion

Suda et al. (1996) studied the distribution and metabolism of i.p. administered, labelled SSG (MW: $2x10^6$) in mice. Following administration, the concentration of SSG was first high in peritoneal exudate and blood, but these concentrations lowered sharply in 48 hours. In contrast, concentrations rose mainly in liver and spleen, and a slight increase was found in the kidney, intestine and faeces. After one month about 30% of administered SSG was present in the liver and about 10% in the spleen.

Because of the absence of (1→3)-β-D-glucanase in mammals, these glucans are thought to be metabolised by oxidative degradation by macrophages (*Miura* et al., 1995, 1996). However, the majority of SSG in liver and spleen was recovered from the non-cellular fraction and not from macrophages. These results suggest that β-glucans are not easily taken up by macrophages to degrade and exclude them from the body. Even five weeks after administration, the metabolites of SSG extracted from the liver of the mice retained significant anti-tumour effects. Nevertheless, the molecular weight of SSG in the liver was lowered with time, and it became about $1x10^5$ g/mol. It was suggested that the degree of branching decreased as well. In contrast, no significant change was observed in the molecular weight and degree of branching of SSG in the spleen.

The elimination of β-glucans from the blood is quite slow (*Sortwell* et al., 1981). After a single dose (intravenous: 1 mg/kg) administered to rats, the elimination half-life was 2.6 hours for

laminarin and 3.1 hours for scleroglucan. The administration (i.p.) of multiple doses of β-glucans (0.25 mg/week) to mice led to saturation of the liver and spleen and circulation of glucan in the blood, as blood glucan concentrations were high all the time.

Not much is known about the clearance of β-glucans from the whole body. Lower molecular weight β-glucans (such as glucan phosphate, MW: 1.3 x 10^5) are possibly cleared by glomerular filtration in the kidney. High molecular weight glucans (such as lentinan) are retained mainly in the liver and degraded by liver macrophages, the Kupffer cells, which may take several weeks.

EFFECTS OF LENTINAN ON THE IMMUNE SYSTEM

The immunomodulatory effects and the applications of lentinan are various. Lentinan is well known for its anti-tumour effect, and recently the anti-infective properties are being appreciated as well. Lentinan influences many components of the immune system: Several cells as well as molecules. *Chihara* et al., (1969) reported the anti-tumour activity of lentinan after conducting an experiment with Sarcoma 180 transplanted in CD-1/ICD mice. These results were confirmed by other research groups, like *Baba* et al (1986), who found a rapid decrease in the number of tumour cells from sarcoma 180 tumours in ICR mice and an accumulation of polymorphonuclear leukocytes (now called neutrophils) in the tumour after i.p. lentinan injection. Later, lentinan also showed anti-tumour activity to syngeneic and autochthonous tumours. Tumour regressions of up to 100% were reported in experimental animals in different studies. Clinical studies with lentinan in cancer therapy followed, although very few are placebo-controlled and double-blind, and showed that lentinan was particularly effective in patients with gastric and colorectal cancer. A follow-up, randomised controlled study in patients with advanced and recurrent stomach cancers showed a prolongation of life. The results of clinical studies also gave indications that lentinan treatment increased NK and LAK cell activity and induced IL-1 and TNF-α production by human monocytes/macrophages. LAK cells (lymphokine activated killer cells) are IL-2 activated NK cells. There are also indications that lentinan could prevent chemical and viral oncogenesis. Furthermore, i.p. injection of lentinan in mice induced vascular dilatation and haemorrhage dose-dependently. This response is probably mediated by T cells and macrophages and correlates well with anti-tumour activity. Recently, many other possible applications for lentinan besides tumour suppression have been investigated. Lentinan may be useful in boosting the immune response against various infections. Lentinan has shown antiviral (e.g. human immunodeficiency virus, *Herpes simplex*), antibacterial (e.g. *Mycobacterium tuberculosis, Listeria monocytogenes*), antiparasitic (*Schistosoma* spp.), and antifungal effects (*Candida albicans*). *Irinoda* et al. (1992) treated mice with lentinan (intranasal or intravenous) before giving them an aerosol of virulent influenza virus. Significant protection was achieved by administrating 200 μg of lentinan intranasally.

The protective effects of lentinan against *Mycobacterium tuberculosis* infection were studied by *Markova* et al. (2003) in *in vitro* and *in vivo* mouse

models. The administration of lentinan before infection at a dose of 1 mg/kg three times at 2-day intervals could reduce mycobacterial infection. Peritoneal macrophages from animals treated with lentinan were greatly stimulated. *Wierzbicki* et al. (2002) studied the effect of the addition of lentinan to an orally administered vaccine against human immunodeficiency virus (HIV) envelope glycoprotein. Lentinan was found to increase envelope glycoprotein-specific T-helper 1 type cytokine production (IL-2 and IFN-γ) and cytotoxic T-lymphocyte activities but had no effect on humoral responses.

Influence at the cellular level

Lentinan exerts its influence on different cells of the immune system. An *in vitro* assay showed that phagocytosis by mouse macrophages was enhanced. In addition, *in vivo* lentinan administration to mice led to a higher number of macrophages, a higher percentage of activated macrophages, and enhanced antibody-dependent macrophage-mediated cytotoxicity compared with controls. In tumour-bearing mice the cells responsible for the anti-tumour activity of lentinan were studied. T cells played a role in the specific cytotoxicity and NK cells contributed to the a-specific cytotoxicity to tumours (*Borchers* et al., 1999).

In an *in vitro* experiment human peripheral blood mononuclear cells were cultured with lentinan (25 to 1000 ng/ml). Cytotoxicity of macrophages and NK cells was increased. The optimal concentration of lentinan was from 25 to 100 ng/ml, which is equivalent to the plasma concentration obtained after clinical doses of this agent (*Tani* et al., 1992).

In 15 patients with gastric carcinoma, peripheral blood mononuclear cells were obtained before and 3, 5, and 7 days after lentinan administration (2 mg i.v.). The ability to generate lymphokine activated killer (LAK) cell activity, tested by *in vitro* activation of blood cells with IL-2 was significantly augmented 5 days after lentinan injection, when compared with before administration. LAK cells are IL-2 activated NK cells. NK cell activity was significantly enhanced after seven days. The conclusions of this study can be questioned, because it was performed without control patients receiving a placebo, and the data of only 9 of 15 patients are presented without an explanation for the missing data (*Arinaga* et al., 1992).

In an *in vivo* study tumour-bearing mice received chemotherapy with or without lentinan (0.1 mg/day i.p.). Additional lentinan treatment induced increased intra tumour $CD86^+$ dendritic cell (DC) infiltration and splenic DCs were more potent stimulators of T cell proliferation. In addition, the activity of splenic cytotoxic T cells was increased. Furthermore, the survival period of the mice treated with lentinan was significantly longer than that of mice treated with chemotherapy alone. It should be noted here, that the immunohistochemical staining for CD86 is not very specific for DCs, as other cells also possess CD86 (*Musiake* et al., 2005).

The effect of lentinan on B cells, neutrophils, eosinophils, basophils and mast cells is not clarified yet. However, it is known that neutrophils can bind lentinan. Monocytes bind lentinan stronger, but lymphocytes bind lentinan only minimally. This suggests that the effect of lentinan on lymphocytes is mediated by the stimuli of DCs and monocytes/macrophages, such as cytokine production. Lentinan is able to restore the suppressed activity of helper T-cells to their normal state in tumour-bearing hosts, leading to restoration of humoral immune responses. In addi-

tion, lentinan promotes a skewing of the Th1/Th2 balance towards Th1 (*Oka* et al., 1996).

Influence on Th1/Th2 balance

A very important feature of lentinan is that it affects cytokine production. Lentinan influences the cytokine production of different cells, including macrophages and T helper cells. Activated macrophages produce cytokines which have a role in inducing inflammatory reactions and in stimulating T cells. In this way T helper cells are stimulated to differentiate, and their cytokines activate macrophages and B cells.

$CD4^+$ T cells can differentiate into different subsets, of which T helper 1 (Th1) and T helper 2 (Th2) cells are very important (see Figure 1). The two subsets are distinguished by the cytokines they produce: IFN-γ is the signature of Th1 cells; and IL-4, IL-5, IL-10, and IL-13 are produced by Th2 cells. These cytokines determine the effector functions and promote growth or differentiation of their own respective subset. The development of Th1 cells is induced by IL-12 produced by activated macrophages and dendritic cells and is antagonised by IL-4 and by IL-10. IL-4 favours induction of Th2 cells. Th1 cells are important for the intracellular destruction of phagocytosed pathogens, including bacteria, parasites, yeasts and viruses and the elimination of cancer cells. IFN-γ acts on macrophages to stimulate phagocytosis and killing of pathogens and on B-lymphocytes to produce opsonising antibodies. Th1 cells also produce TNF-α, which activates neutrophils and stimulates inflammation, and IL-2 which acts as an autocrine growth factor. The principal function of Th2 cells is in eradicating helminths and other extracellular parasites by activating mast cells and eosinophils, and stimulating IgE-production. If uncontrolled, Th1 cells can mediate immunopathology and autoimmune diseases. Overactivation of Th2 cells can lead to allergic manifestations.

Murata et al. (2002) found that lentinan administered i.p. to C57BL/6 and DBA/2 mice could skew the T-helper response toward Th1. Peritoneal macrophages isolated from lentinan-treated animals could produce more nitric oxide and IL-12 in response to stimulation, while the production of IL-6, IL-10, and Prostaglandin E2 (PGE_2) was decreased. PGE_2 is known to function as an endogenous immunosuppressive mediator. Nitric oxide is considered an effector molecule of cytotoxic macrophages against tumours. The lowered amount of IL-6 contributes to the inhibition of Th2 induction. IL-12 strongly stimulates Th1 development and the lowering of IL-10 neutralises the inhibition of activation of Th1 cells by IL-10. To study the effect on the Th cells, lentinan (5 mg/kg) or saline (0.5 ml) was injected i.p. on days 1, 3 and 5 and spleens were harvested the day after the final injection. The culture supernatants of purified splenic $CD4^+$T cells stimulated for 24 h with coated anti-CD3 antibody were analysed for IFN-γ and IL-4. IFN-γ was significantly increased, but IL-4 levels did not change significantly. This indicates a polarisation toward Th1, which can be related to the cytokine pattern induced in macrophages. Skewing of Th1/Th2 balance to Th1 favours cellular immune responses against tumours and intracellular pathogens.

Experiments performed with other cell types also give indications for a Th1 polarisation. *Liu* et al. (1999) administered lentinan i.p. to mice and the cytokine gene expression levels of IL-1α, IL-1β, TNF-α, IFN-γ, and monocyte colony stimulating factor (M-CSF) were analysed in peritoneal exudate

cells (PECs) and splenocytes. The biological effects of IL-1 are similar to those of TNF: they both induce inflammation. The expression of all five cytokines was up-regulated in PECs of treated mice; in splenocytes only IL-1α expression was not up-regulated. This cytokine pattern was also found in a study by *Arinaga* et al. (1992a,b), in which patients with gastric carcinoma received intravenous administration of lentinan. The ability of monocytes to produce IL-1α, IL-1β, and TNF-α *in vitro* in response to LPS stimulation was significantly augmented as compared with before treatment. It should be noted that this study was not placebo controlled.

Although most studies show a similar cytokine profile, the production of these molecules seems to be dependent on the genotype of the host and the specific health/disease status. *Kerekgyarto* et al. (1996) found that the cytotoxic activity and TNF secretion of murine macrophages was elevated by lentinan when applied *in vitro* or *in vivo*. The effectiveness of lentinan to induce these responses was highly influenced by the genotype of the host. In the experiment by Irinoda with mice infected with influenza virus (described above), TNF production could not be detected. The amount of IL-6 produced differed between uninfected and infected mice: in uninfected mice, IL-6 was higher in lentinan treated mice than in untreated mice; in infected mice, lentinan treated mice first showed higher IL-6 levels, later much lower levels than untreated mice. IL-6 has diverse actions including the stimulation of the synthesis of acute-phase proteins by the liver and the proliferation of antibody-producing cells.

A remarkable cytokine pattern was observed by *Masihi* et al. (1997) in their experiment with bacillus Calmette-Guerin (BCG)-primed mice. BCG is widely used as a vaccine against tuberculosis. It has also been recognized as an immune modulator and it induces local inflammation. Mice were pre-treated with lentinan and LPS was used to stimulate cytokine production. Lentinan induced an inhibition of up to 82% of TNF, a moderate reduction of 25% of IL-lβ, and no significant differences in IL-6 or IL-10 levels, and a marked depression of chemiluminescence (respiratory burst) activity.

IMMUNE ACTIVITIES OF FUNGAL POLYSACCHARIDES

Mushrooms are abundant sources of a wide range of useful natural products (*Zaidman* et al., 2005; *Borchers* et al., 2004; *Wasser*, 2002; *Ooi* and *Lui*, 2000). Medicinal properties have been attributed to mushrooms for thousands of years, particularly in traditional Chinese and Japanese medicine.

Mushrooms have recently attracted much attention on account of their *in vivo* and *in vitro* immunomodulatory activity (*Shamtsyan* et al., 2004), which has been demonstrated for many mushrooms, including extracts and isolated compounds from the fruiting body, spores, mycelia, and culture medium of various mushrooms (*Kodama* et al., 2003, 2005). The major immunomodulating effects of these active substances include mitogenicity, stimulation of haematopoietic stem cells and activation of immune effector cells, such as helper T cells, cytotoxic T cells, macrophages, dendritic cells, endothelial cells, neutrophils, monocytes, and NK cells (*Lull Nogiera* et al., 2005).

One of the approaches to evaluate potential immunomodulating activity is the assessment of the capacity of ex-

tracts or pure compounds to influence the production of cytokines by immune cells. Cytokines are soluble glycoproteins which are critically involved in the immune response. The functions of these proteins are diverse and include roles in normal humoral and T cell-mediated immune response. Various pathologic conditions are accompanied by changes in cytokine levels and by disturbances in the cytokine-mediated interplay between innate and acquired immune responses (*Stanilova* et al., 2005).

For the last years a few studies have reported the immunomodulating activity of mushrooms in a human peripheral blood mononuclear cells (PBMC) assay. PBMC represent a heterogeneous population of immune cells (B cells, T cells, and various granulocytes) that arise from pluripotent haematopoietic stem cells in the bone marrow. PBMC account for cellular and humoral immune responses; some PBMC (B and T cells) have the inherent ability to proliferate rapidly after antigenic and mitogenic stimulation (*Zempleni* and *Mock*, 2000).

The immune activities of mushroom derived polysaccharides are well documented and are considered to have function of promoting activities of antigen non-specific immune NK cells that are able to rapidly and effectively kill cancerous cells; placing a premium on the production of interferons that effectively prevent virus reproduction; increasing the activities of complement C_3 that enhance animals' disease resistance; increasing the number and activities of the phagocytes (neutrophilic granulocytes) that release H_2O_2 dissolving cancerous cells; protecting the normal cells and preventing the reduction of leucocytes (*Wargovich* et al., 2001). Therefore, mushroom polysaccharides can play an important role in the health care of human. *Berovič* et al. (2003) studied the effects of extra- and intra-cellular polysaccharides isolated from mycelia of *Ganoderma lucidum* on the induction of IFN-γ and TNF-α, synthesis in primary cultures of human peripheral blood mononuclear cells (PBMC) isolated from a buffycoat. They found that the TNF-α inducing activity was comparable with romurtide, which has been used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy. *Jin* et al. (2003) treated PBMC with PG101, a water-soluble extract from cultured mycelia of *Lentinus lepideus*. PG101 increased levels of TNF-α, IL-1β, IL-10, and IL-12 by 100- to 1000-fold, whereas GM-CSF and IL-18 were activated by an order of magnitude. On the contrary, IFN-γ and IL-4 were not affected. Considering the type of affected cytokines, it is possible that PG101 could be used to enhance the immune system in immunosuppressed or immunocompromised individuals or to control the haematopoiesis of specific cell types or lineages.

In brief, as immunodulaters, polysaccharides affect the growth of immune organs (bursa, thymus, and spleen), activities of immune cells (granulocyte, monocyte and macrophage), functions of both cellular immunity and humoral immunity as well as cytokines and complement system (*Yuan* and *Shi*, 2000). The effects of herb polysaccharides on immune system are summarized below according to *Xue* and *Meng* (1996):

- Stimulating growth of immune organs: The organs of the immune system are concerned with the growth, development, and deployment of lymphocytes. Herb polysaccharides, e.g. *Isatidis radix*, *Ligustri fructus*, *Polypori scierotium*, *Astragali membranacea radix, Tremella fuciformis, Cistanchea*

herba and *Cordyceps* polysaccharide increase the weights of immune organs.

- Promoting activities of immune cells: Herb polysaccharides, e.g. *Isatidis radix*, *Codongopsis radix,*, *Tremella fuciformis, Bupleuri radix*, *Angelicae sinensis radix*, *Astragali membranacea radix* and *Polypori scierotium* polysaccharide, increase the number and activities of many interdependent cell types such as T, B lymphocytes, macrophage, NK cells that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumour cells.
- Enhancing functions of cellular immunity: Herb polysaccharides, e.g. *Isatidis radix, Tremella fuciformis*, *Astragali membranacea radix, Lycii fructus* and *Angelicae sinensis radix* polysaccharide, increased spleen and thymus index, rate of T-lymphocyte transformation and proliferation as well as production of IL-2, while decreased prohibition effects of serum, macrophage and suppressor T cell populations on T-cells' function and against deformation and necrosis of lymphocytes in spleen, thymus and lymph nodes.
- Promoting humoral immunity: Herb polysaccharides, e.g. *Eucommiae cortex*, *Cordyceps*, *Codongopsis radix*, *Astragali membranacea radix, Tremella fuciformis* and *Atractylodis macrocephalae rhizoma* polysaccharide, enhanced humoral immune response by increasing spleen and serum antibody production, antibody titres and plague forming cells (PFC).
- Inducing cytokine production and complement: Cytokines are central molecules that control host immune response to infectious agents. Cytokines, which are produced and secreted by activated T-cells and NK cells activated by antigens, are responsible for clonal T-cell proliferation and antibody production of B-cells, proliferation and activity of macrophages, and NK cells. Herb polysaccharides (e.g. *Phytolaccae radix*, *Ginseng radix*, *Tremella fuciformis*, *Poriae albae sclerotium* and *Eleutherococci radix* polysaccharide) increased cytokine production such as interferons (IFN-α, IFN-β and IFN-γ) and interleukin (IL-2), TGF-β, and TNF-α.

Cell wall fragments of higher plants and yeasts, such as from the brewing industry, are known to have an impact on the immune system and to be able to stimulate innate immunity. Such preparations are already applied in the feed industry, for instance in shrimp and broiler feed (*Thanardkit* et al., 2002; *Von Wettstein* et al., 2000).

From preliminary immuno-assays, it has become clear that mushroom extracts show superior immune-modulating properties when compared to for instance β-glucans form beer yeast or from herbs. Striking differences between fungal and yeast cell walls may exist, as fungi are reputated for their high content in amino-glucans (*Hamuro* et al., 1974; *de Ruiter* et al., 1992; *Usui* et al., 1983; *Karácsonyi* and *Kuniak*, 1994; *Dong* et al., 2002; *Wasser*, 2002). This phenomenon is unexplained yet, i.e. it is not known what e.g. the differences in chemical structure between cell wall components from fungi resp. yeast are in relation to immune modulation, or whether fungi produce additional biologically active immune-modulating substances. Modification of polysaccharide fragments may increase their activity (*Zhan* and *Cheung*, 2002).

FUNGAL IMMUNOMODULATORY PEPTIDES

Proteins and peptides from mushrooms are also known to activate macrophages. An ubiquitin-like peptide isolated from fruiting bodies of the mushroom *Agrocybe cylindracea* enhanced NO production in murine peritoneal macrophages with potency comparable to that of LPS. Two lectins isolated from the mushroom *Tricholoma mongolicum* (TML-1 and TML-2) stimulated the production of nitrite ions and TNF-α by macrophages in normal and tumour-bearing mice.

Vvo, a fungal immunomodulatory protein (FIP) purified from the edible mushroom, *Volvariella volvacea*, induced most Th1-specific cytokines (IL-2, IFN-γ, and LT) and one TH2-specific cytokine (IL-4) within 4 hours in mouse spleen cells. This result indicates that Vvo principally acts on Th1 cells and to a lesser extent on Th2 cells in the early event of activation. It is known that IL-4 acts on B cells to induce activation and differentiation, leading in particular to the production of IgE. The lower effect of Vvo compared with other FIPs on the prevention of systemic anaphylaxis may be attributed to the elevated expression of IL-4. Fve, a FIP isolated from the fruiting body of *Flammulina velutipes*, selectively stimulates a Th1 response in hPBMCs (*Hsu* et al., 1997, 2003). Recently *Hsieh* et al. (2003) have characterized the immunomodulatory effects of Fve in more detail and investigated the prophylactic use of Fve via the oral route in a murine model of food allergy. They have demonstrated that oral administration of Fve during allergen sensitization could induce a Th1-predominant allergen-specific immune response in mice and protect the mice from systemic anaphylaxis-like symptoms after subsequent oral challenge with the same allergen. It is worth pointing out that Fve could be administered orally and retain its activity, while most protein drugs cannot. This characteristic greatly promotes the potential of immunoprophylactic use of Fve. *Liu* et al (1999) have demonstrated the efficacy of local nasal immunotherapy (LNIT) for group 2 allergen of house dust mite *Dermatophagoides pteronyssinus*- (Dp2-) induced airway inflammation in mice, using Dp2 peptide and Fve or LZ-8, a FIP isolated from *G. lucidum*.

In contrast to the polysaccharide metabolites of edible mushrooms, only little is known about the effect of their proteins on the immune system. *Hsieh* et al. (2003) studied at the possibilities of therapy for food allergy using an edible-mushroom derived protein. They used a Fungal Immunomodulatory Protein (FIP) isolated from the commonly eaten mushroom *Flammulina velutipes*, called FIP-*fve*. It was demonstrated that oral administration of FIP-*fve* during allergen sensitization could induce a Th1- predominant allergen specific immune response in mice which protected the mice from systemic anaphylaxis-like symptoms after subsequent oral challenge with the same allergen. They concluded that the FIP-*fve* could activate T cells and selectively stimulate a Th1 response. In addition they demonstrated that the suppression of allergen-specific IgE response may play a crucial role in this protection. FIP-*fve* might either activate primed type-1 T cells or drive naive T cells to a type-1 phenotype. This strategy is convenient in practice and may have the potential to be used clinically in young children for the prevention of allergic diseases, but the optimal dosage, efficiency, and adverse effects in humans should be determined. It is well established that many

mushrooms-extracted compounds are commonly used as immunomodulators or as biological response modifiers. The basic strategy underlying immunomodulation is to identify aspects of the responses that can be enhanced or suppressed in such a way as to augment or complement a desired immune response (*Wasser*, 2002; *Zaidman* et al., 2005). Indications for the latter are for instance the identification of an immune-modulating protein (Fip-*vvo*) from *Volvariella volvacea* (*Hsu* et al., 1997).

Utilization of a T cell polarization pulse, followed by comparison of relative cytokine production by intracellular cytokine staining under polarized conditions allows *ex vivo* assessment of the T cell polarization state *in vivo* (*Cameron* et al., 2005). Since strong and clear T cell polarization typically takes place in chronic disease states or exposure to (potentially toxic) agents, this technique provides an assessment of the current direction of polarization the T cells are heading towards during acute responses, on a population basis via relative cytokine production, and on a cell-by-cell basis via intracellular cytokine staining (ICS). Most importantly, the comparison of cytokine production by enzyme-linked immunosorbent assay must be made on a relative scale, as outlined since the potency of the key Th1 and Th2 cytokines, IFN-γ and IL-4 respectively, may not necessarily be equal. Similar relative comparisons have been made during micro-array analysis of Th1 and Th2 gene transcripts. Additionally, all polarizations must be compared to the results obtained from CD4+ T cells isolated from healthy controls polarised under identical conditions. The rationale for this is apparent when looking at the data, because some individuals are naturally Th2 biased in their cytokine profile, while others are naturally Th1 biased. The addition of ICS to this method most importantly allows for further assessment of the T helper population in experimental groups, to determine if a mixed cytokine profile detected via ELISA and comparison of relative cytokine production truly represents an undifferentiated Th0 response of naive cells, or rather if it is indicative of a heterogeneous population of simultaneously differentiating Th1 and Th2 cells. Additionally, it can allow for further characterization of the cytokine-producing subsets.

CONCLUSIONS

A wide variety of immune interactions can be identified by which components present in mushroom extracts can exert their immunomodulatory activity. Some of these interactions are preferred by β-glucan, particularly those associated with TLR interactions on innate immune cells and resulting in the induction of pro-inflammatory cytokine production. Others, like FIP will be presented by APC to T-cells and (in)directly modulate Th cell differentiation. By integrating the various mechanisms exploited by these mushrooms predictions can be made for their immunomodulating activity *in vivo*. Figure 4 summarizes these known and suggested activities of the various mushrooms used for dietary immune intervention in humans. From these known interactions *in vitro* extrapolations to the potential use of such extracts for the treatment of human diseases can be inferred and increasing research is performed according to this paradigm.

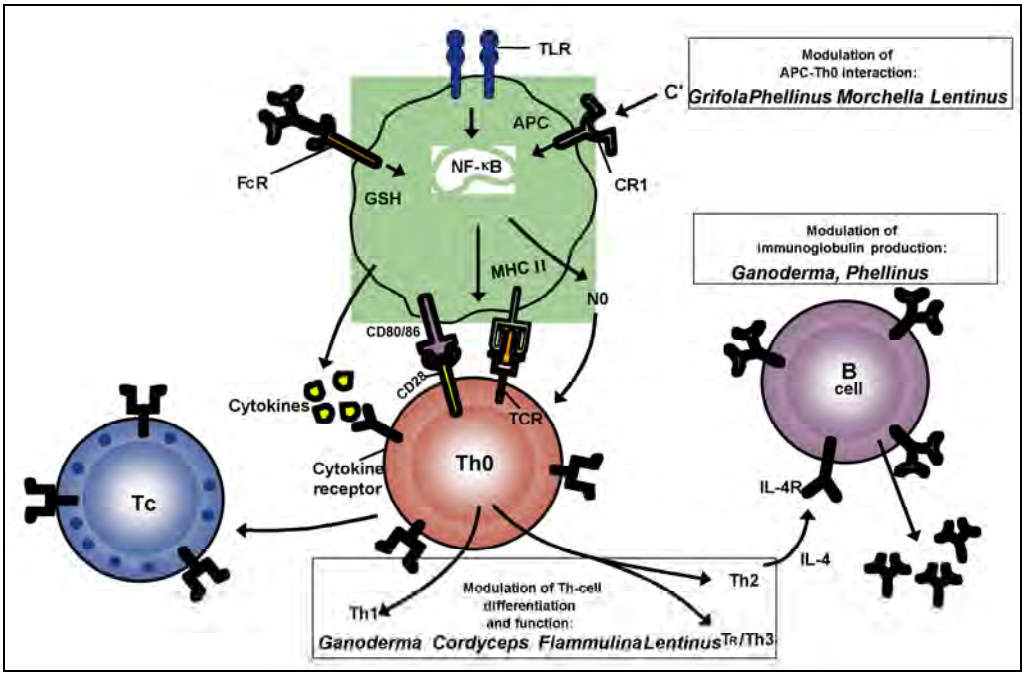

Figure 4: Summary of published interactions of isolated components or extracts of various edible mushrooms on the induction of immune responses. From: *Lull Nogiera* et al., 2005.

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CURCUMIN AS A DIETARY MODULATOR OF INFLAMMATION

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AN EPIDEMIC OF CHRONIC DISEASES

World Health organization estimates that 46 % of global disease burden and 59 % of global mortality is due to chronic diseases; 35 million individuals die each year from chronic diseases, and the numbers are steadily increasing (*World Health Organisation*, 2003). Accumulating evidence supports the association of chronic diseases to modern life style, stress, lack of exercise, abuse of tobacco and alcohol, and to the transition from natural unprocessed foods to processed, calorie-condensed and heat-treated foods. There is a strong association between chronic disease and reduced intake of plant fibres, plant antioxidants and increased consumption of industrially produced and processed dairy products, refined sugars and starch products. The per capita consumption of refined sugar has increased from about 0.5 kg per person and year in 1850 to almost 50 kg/person/year in the year 2000 and the per cow milk production from 2 to 50 litres/day. Dairy products, especially milk (mostly from pregnant cows) are rich in pro-inflammatory molecules: Hormones such as estrogens and growth factors such as IGF-1. Consumption of bovine milk has also been shown to release inflammatory mediators, increase intestinal permeability and induce leakage of larger molecules such as albumin and hyaluronan into the body. Heating up milk (pasteurization), and especially production of and storage of milk powder, produces large amounts of advanced glycation products (AGEs) and advanced lipoxidation products (ALEs) (*Baptista* and *Carvalho*, 2004), known as potent inducers of inflammation. This information is especially important as many foods such as ice cream, enteral nutrition solutions and baby formulas are based on milk powder. Bread, especially from gluten-containing grains, is also rich in molecules with documented pro-inflammatory effects, and bread crusts often used experimentally to induce inflammation. See further *Bengmark* (2004, 2005, 2006).

Despite some breath-taking advances in medico-pharmaceutical and surgical treatment medical and surgical emergencies, as well as advanced medical and surgical treatments, are still affected by an by unacceptably high morbidity and mortality. Sepsis is the most common medical and surgical complication, estimated only in the US to annually affect as many as 751,000 (*Arias* and *Smith*, 2003; *Angus* et al., 2001), and cause death of approximately 215,000 patients (29%) (*Angus* et a., 2001), which makes sepsis the tenth most common cause of death in the country. It is especially alarming that both morbidity and mortality in critical illness, especially when septic, is fast increasing and has done so for several decades. With a documented 1.5 % rate of increase per year it might double within the coming 50 to 60 years.

PLANT-DERIVED PROTECTION

Common to those suffering from chronic disease as well as critical illness is that they suffer an increased degree of inflammation, most likely due to their Western lifestyle. We are increasingly aware that plant-derived substances, often referred to as chemo-preventive agents, have an important role to play in control of inflammation. These substances are not only inexpensive, they are also easy available, and have no or limited toxicity. Among these numerous chemo-preventive agents are a whole series of phenolic and other compounds believed to reduced speed of aging and prevent degenerative malfunctions of organs, among them various curcumenoids found in turmeric curry foods and, thousands more of hitherto less or unexplored substances. However, this review will mainly focus on curcumin and its effects.

Polyphenols have in recent few years received an increasing attention for their strong chemo-preventive ability. Curcumin and many other plant-derived substances are increasingly regarded as shields against disease. Curcumin is the most explored of the so-called curmenoids, a family of chemo-preventive substances present in the spice turmeric. Although the substance has been known for some time, it is in the most recent years that the interest has exploded, much in parallel with increasing concern for severe side effects of synthetic COX-2 inhibitors, marketed by pharmaceutical industry. Most of the reported curcumin studies in the literature are experimental and few clinical studies are this far presented.

CURCUMIN – AN ANTIOXIDANT AND INHIBITOR OF NF-κB, COX-2, LOX AND INOS

NF-κB plays a critical role in several signal transduction pathways involved in chronic inflammatory diseases (*Bernes* and *Karin*, 1997) such as asthma and arthritis and various cancers (*Amit* and *Ben-Nereiah*, 2003). Activation of NF-κB is linked with apoptotic cell death; either promoting or inhibiting apoptosis, depending on cell type and condition. The expression of several genes such as cyclo-oxygenase-2 (COX-2), matrix mettaloproteinase-9 (MMP-9), inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF), interleukin-8 (IL-8), eotaxin, cell surface adhesion molecules and anti-apoptotic proteins are regulated by NF-κB (*Pahl*, 1999). COX-2 is inducible and barely detectable under normal physiological conditions, but is rapidly, but transiently, induced as an early response to pro-inflammatory mediators and mitogenic stimuli including cytokines, endotoxins, growth factors, oncogenes and phorbol esters. COX-2 synthesizes series-2 prostaglandins (PGE2, PGF2-α), which contribute to inflammation, swelling and pain. PGE2 promotes production of IL-10, a potent immunosuppressive cytokine produced especially by lymphocytes and macrophages, and suppression of IL-12 (*Stolina* et al., 2000). Inducible nitric oxid synthase (iNOS), activated by NF-κB is another enzyme that plays pivotal role in mediating inflammation, especially as it acts in synergy COX-2.

TURMERIC – APPROVED AS FOOD ADDITIVE

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenol) - 1,6- heptadiene - 3,5 - dione), a polyphenol rich in the dietary spice turmeric, is received from dried rhizozomes of the perennial herb *Curcuma longa* Linn, a member of the ginger family. Turmeric is mainly known for it's excellent ability preserve food and is approved as food additive in most Western countries. It is produced in several Asian and South-American countries. Only in India are about 500,000 metric tonnes produced each year, of which about half is exported. It has in addition to extensive use as food additive, for generations also been used in traditional medicine for treatment of various external or internal inflammatory conditions such as arthritis, colitis and hepatitis.

The molecule of curcumin resembles ubiquinols and other phenols known to possess strong antioxidant activities. Its bio-availability on oral supplementation is low, but can be improved by dissolution in ambivalent solvents (glycerol, ethanol, DMSO) (*Sharma* et al., 2001). It is also reported to be dramatically elevated by co-ingestion of peperine (a component of pepper), demonstrated both in experimental animals and humans (*Shoba* et al., 1998). Several studies has demonstrated that curcumin is a-toxic, also in very high doses (*Bravani Shankar* et al., 1980; *Chainani*, 2003). Treatment of humans during three months with 8000 mg curcumin per day showed no side effects (*Chainani*, 2003). It is estimated that adult Indians consume daily 80-200 mg curcumin per day (*Grant* and *Schneider*, 2000). A common therapeutic dose is 400-600 mg curcumin three times daily corresponding to up to 60 g fresh turmeric root or about 15 g turmeric powder. The content of curcumin in turmeric is usually 4-5 %.

CURCUMIN - EFFECTIVE AGAINST STRESS-INDUCED OVERINFLAMMATION

Curcumin is not only an inexpensive a-toxic and potent COX-2 and iNOS inhibitor (*Suhr* et al., 2001), it is also a potent inducer of heat shock proteins (HSPs) and potential cytoprotector (*Dunsmore* et al., 2001; *Chang*, 2001). Curcumin does not only inhibit COX-2, it also inhibits lipo-oxygenases (LOX) and leukotreines such as LBT4 and 5HETE (*Wallace*, 2002), especially when bound to phosphatidylcholine micelles (*Began* et al., 1999). It is also reported to inhibit cytochrome P450 iso-enzymes and thereby activation of carcinogens (*Thapliyal* and *Maru*, 2001). Curcumin has the ability to intercept and neutralize potent pro-oxidants and carcinogens, both ROS (superoxide, peroxyl, hydroxyl radicals) and NOS (nitric oxide, peroxynitrite) (*Jovanovic* et al., 2001). It is also a potent inhibitor of TGF-β and fibrogenesis (*Gaedeke* et al., 2004), which is one of the reasons, why it can be expected to have positive effects in diseases such as kidney fibrosis, lung fibrosis, liver cirrhosis and Crohn's disease and in prevention of formation of tissue adhesions (*Srinisan* et al., 2004). Curcumin is suggested to be especially effective in Th1-mediated immune diseases as it effectively inhibits Th1 cytokine profile in $CD4^+$ T cells by interleukin-12 production (*Kang* et al., 1999).

Many medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another, and interac-

tions between herbs and drugs, even if structurally unrelated, may increase or decrease the pharmacological and toxicological effects of either component (*Fugh-Berman*, 2000; *Groten* et al., 2000). It is suggested that curcumin may increase the bioavailability of vitamins such as vitamin E and also decrease cholesterol, as curcumin in experimental studies significantly raises the concentration of α-tocopherol in lung tissues and decreases plasma cholesterol (*Kamal-Eldin* et al., 2000). Polyphenols, isothiocyanates such as curcumin and flavonoids such as resveratrol, are all made accessible for absorption into the intestinal epithelial cells and the rest of the body by digestion/fermentation in the intestine by microbial flora (*Shapiro* et al., 1998).

CURCUMIN IN ACUTE AND CHRONIC DISEASES

Atherosclerosis

Oxidation of low-density lipoproteins (LDL) is suggested to play a pivotal role in the development of arteriosclerosis, and LDL oxidation products toxic to various types of cells including endothelial cells. Curcumin has a strong capacity to prevent lipid peroxidation, stabilize cellular membranes, inhibit proliferation of vascular smooth muscle cells, and inhibit platelet aggregation; all important ingredients in the pathogenesis of arteriosclerosis. Curcumin was found to be the most effective, when the ability to inhibit the initiation and propagation phases of LDL oxidation of a defined antioxidant butylated hydroxy anisole (BHA), curcumin, quercetin, capsaisin were compared, and quercetin the least (*Naidu* and *Thippeswamy*, 2002). Supply of curcumin, but also capsaicin and garlic (allecin) to rats fed of a cholesterol-enriched diet prevented both increase in membrane cholesterol and increased fragility of the erythrocytes (*Kempaiah* and *Srinivasan*, 2002). Significant prevention of early atherosclerotic lesions in thoracic and abdominal aorta are observed in rabbits fed an atherogenic diet for thirty days, accompanied by significant increases in plasma concentrations of co-enzyme Q, retinol and α-tocopherol and reductions in LDL conjugated dienes and in TBARS (thiobarbituric acid-reactive substances, an expression of ongoing oxidation) (*Quiles* et al., 2002).

Cancer

Cancer is a group of more than 100 different diseases, which manifest itself in uncontrolled cellular reproduction, tissue invasion and distant metastases (*Levi* et al., 2001). Behind the development of these diseases are most often exposure to carcinogens, which produce genetic damage and irreversible mutations, if not repaired. During the last fifty years attempts have been made to find or produce substances that could prevent these processes, so called chemopreventive agents. Cancers are generally less frequent in the developing world, which has been associated both with less exposure to environmental carcinogens and to a richer supply of natural chemopreventive agents. The incidence per 100,000 population is in the USA considerably higher for the following diseases compared to India: prostatic cancer (23x), melanoma skin cancer (male 14x, female 9x), colorectal cancer (male 11x, female 10x), endometrial, cancer (9x), lung cancer (male 7x, female 17x), bladder cancer (male 7x, female 8x) breast cancer (5x), renal cancer (male 9x, female 12x) (*Sinha* et al., 2003). These differences are for some diseases

such as breast cancer and prostatic cancer even greater when compared to China. The consumption of saturated fat and sugary foods is much less in the Asian countries, but equally important, the consumption of plants with high content of chemopreventive substances is significantly higher in these countries. As an example, the consumption of curcumin has for centuries been about 100 mg/day in these Asian countries (*Choudhuri* e al., 2002). Curcumin induces *in vitro* apoptosis of various tumour cell lines: Breast cancer cells (*Choudhuri* e al., 2002; *Shao* et al., 2002), lung cancer cells (*Pillai* et al., 2004), human melanoma cells (*Zheng* et al., 2004), human myeloma cells (*Han* et al., 2002), human leukaemia cell lines (*Bharti* et al., 2004), human neuroblastoma cells (*Liontas* and *Yeger*, 2004), oral cancer cells (*Elattar* and *Virji*, 2000), and prostatic cancer cells (*Mukhopadhyay* et al., 2001; *Nakamura* et al., 2002; *Hour* et al., 2002; *Deeb* et al., 2004). Curcumin has in experimental models also demonstrated ability to inhibit intra-hepatic metastases (*Ohadshi* et al., 2003). Few *in vivo* experimental studies and no clinical controlled trials are this far concluded. However, a recent phase I study reported histologic improvement of pre-cancerous lesions in 1 out of 2 patients with recently resected bladder cancer, 2 out of 7 patients of oral leucoplakia, 1 out of 6 patients of intestinal metaplasia of the stomach, and 2 out of 6 patients with Bowen's disease (*Cheng* et al., 2001). However, the main purpose of the study was to document that curcumin is not toxic to humans when taken by mouth for 3 months in a dose of up to 8,000 mg/day.

Diabetes

Turmeric (TU, 1 g/kg body weight) or curcumin (CU, 0.08 g/kg body weight) were in a recent study supplied daily for three weeks to rats with alloxan-induced diabetes (AID) and compared to controls (CO) (*Giltay* et al., 1998). Significant improvements were observed in blood glucose (mg/dl; CO 88.3, AID 204.4, TU 142.7, CU 140.1), haemoglobin (gm/dl; CO 14.7, AID 10.8, TU 13.6, CU 13.1) and glycosylated haemoglobin (gm/dl; CO 2.8, AID 11.2, TU 9.0, CU 7.8). Significant differences were also observed in TBARS in liver tissue (nmoles/g tissue; CO 43.0, AID 54.0, TU 34.0, CU 29.0), TBARS in plasma (nmoles/ml; CO 3.8, AID 7.3, TU 5.3, CU 4.6) in glutathione in liver (μgm/mg; CO 23.4, AID 11.2, TU 16.6, CU 20.9) and glutathione in plasma (mg/dl; CO 22.4, AID 14.2, TU 18.4, CU 20.1). It was also observed that the activity of sorbitol dehydrogenase (SDH), which catalyzes the conversion of sorbitol to fructose, was significantly lowered by treatment both with turmeric and curcumin.

Gastric diseases

When the in vitro effects against 19 different *Helicobacter pylori* strains, including five cagA+ strains (cag A is the strain-specific *H. pylori* gene linked to pre-malignant and malignant lesions) were studied, both treatments were found to be equally effective as both treatments did significantly reduce growth of all the strains studied (*Mahady* et al., 2002). Subsequent studies did also demonstrate that curcumin inhibits infection and inflammation of gastric mucosal cells through the inhibition of activation of NF-κB, degradation of IκBα, NF-κB DNA binding and the activity of IκB kinases α and β. No curcumin-induced effects were observed on mitogen-activated protein kinases (MAPK), extra-cellular signal regulating kinases ½ (ERK1/2) and p38. *H. pylori*-induced mitogenic re-

sponse was completely blocked by curcumin (*Foryst-Ludwig* et al., 2004). Significant antifungal properties against various fungal, especially phytopathogenic, organisms by curcumin are also reported (*Kim* et al., 2003).

Hepatic diseases

Dietary supply of curcuminoids is also reported to increase hepatic acyl-CoA and prevent high-fat diet-induced accumulation in the liver and adipose tissues in rats (*Asai* and *Miyazawa*, 2001). Ethanol-induced steatosis is known to be further aggravated by supply of PUFA-rich vegetable oils, which has been thermally oxidized. Rats gavaged for 45 days with a diet containing 20 % ethanol and 15 % sunflower oil, heated to 180° C for 30 min (AO), showed extensive histo-pathological changes with focal and feathery degeneration, micro-necroses and extensive steatosis in the liver and extensive inflammation vessel congestion and fatty infiltration in the kidneys, changes, which largely could be prevented by simultaneous supply of curcumin (CU) or particularly photo-irradiated curcumin (PCU) e.g. curcumin kept in bright sunshine for five hours (*Rukkumani* et al., 2002). Both products were supplied in a dose of 80 mg/kg body weight. Both products did significantly inhibit elevations in alkaline phosphatases (ALP): controls (CO) 85.88, PCU 239.56, CU 177.41 and PCU 149.15 and γ-glutamyl transferase (GGT): CO 0.60, PCU 2.51, CU 1.43, PCU 1.15. Similar beneficial effects were observed on histology in various tissues and in hepatic content of cholesterol, triglycerides free fatty acids and phospholipids. Rats were in another study for four weeks fed with fish oil and ethanol (FE), which resulted in hepatic lesions consisting in fatty liver, necrosis and inflammation. Supply of curcumin in a daily dose of 75 mg/kg body weight to these rats prevented the histological lesions (*Nanji* et al., 2003). Curcumin was observed to in part to suppress NF-κB-dependent genes, to block endotoxin-mediated activation of NF-κB and to suppress the expression of cytokines, chemokines, COX-2 and iNOS in Kupffer cells. Similar effects were also observed in carbon tetrachloride-induced injuries. Pre-treatment for four days with curcumin (100 mg/kg body weight) before intraperitoneal injection of CCl_4 prevented significantly subsequent increases in TBARS: CO 274, CCl_4 556, CU 374, alanine aminotransferase (ALT): CO 46, CCL_4 182, CU 97 and aspertate aminotransferase (AST): CO 97, CCl_4 330, CU 211 and in hydroxyproline (µg/g liver tissue): CO 281, CCl_4 777, CU 373 (*Park* et al., 2000).

Intestinal diseases

Pre-treatment during 10 days with curcumin in a daily dose of 50 mg/kg body weight before induction of trinitrobenzene sulphonic acid (TNBS) colitis resulted in a significant reduction in degree of histological tissue injury, neutrophil infiltration (measured as decrease in myelo-peroxidase activity) and lipid peroxidation (measured as decrease in malondialdehyde activity) in the inflamed colon and in a decreased serine protease activity (*Ukil* et al., 2003). A significant reduction in NF-κB activation and reduced levels of NO and a marked suppression of Th1 functions: IFNγ and IL-12p40 mRNA, was also observed. Curcumin was in another similarly designed study added to the diet during five days before induction of TNBS colitis, which resulted in a significant reduction in myelo-peroxidase, and attenuation of the TNBS-induced message for IL-1β on semi-quantitative RT-PCR (*van 't Land* et al., 2004). Western blotting revealed a significant attenuation of the

activation of p38 MAPK. Curcumin was also supplied in combination with caffeic acid phenethyl ester (CAPE) to animals treated with cytostatic drugs (arabinose cytosine, Ara-C, and methotrexate, MTX) (*van 't Land* et al., 2004). The treatment did not only inhibit the NF-κB induced mucosal barrier injury but was also shown to increase the *in vitro* susceptibility of the non-transformed small intestinal rat epithelial cell, IEC-6, to the cytostatic agents.

Neurodegenerative diseases

A growing body of evidence implicates free radical toxicity, radical induced mutations and oxidative enzyme impairment and mitochondrial dysfunction in neurodegenerative diseases (NDD). Significant oxidative damage is observed all NDDs, which in the case of Alzheimer disease (AD) leads to extra-cellular deposition of β-amyloid (Aβ) as senile plaques. Non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen has proven effective to prevent progress of AD in animal models (*Lim* et al., 2000), but gastrointestinal and occasional liver and kidney toxicity induced by inhibition of COX-1 precludes widespread chronic use of the drug (*Björkman*, 1998). Use of antioxidants such as vitamin E (α-tocopherol) has proven rather unsuccessful even when high doses were used (*Sano* et al., 1997). Vitamin E, α-tocopherol, is in contrast to γ-tocopherol a poor scavenger of nitric oxide (NO) based free radicals. Curcumin is a several times more potent scavenger than vitamin E (*Zhao* et al., 1989), and in addition also a specific scavenger of NO-based radicals (*Sreejavan* and *Rao*, 1997). When tried in a transgenic mouse model of AD did a modest dose (24 mg/kg body weight), but not a > 30 times higher dose (750 mg/kg body weight) of curcumin significantly reduce oxidative damage and amyloid pathology (*Lim* et al., 2001). Similar observations, reductions in both Aβ deposits and in memory deficits are also made in Sprague Dawley rats (*Frautschy* et al., 2001). The age-adjusted prevalence of both AD (*Ganguli* et al., 2000) and Parkinson´s disease (PD) is in India, with its significantly higher intake of turmeric, much lower than in Western countries, especially the USA (*Muthane* et al., 1998). However, the preventive effects of consumption of turmeric can also be achieved with other polyphenol-rich fruits and vegetables if consumed in enough quantities. Blueberries, strawberries and spinach in doses of 18.6, 14.8 and 9.1 gm of dried extract/kg body weight were demonstrated effective in reversing age-related deficits in both neuronal and behavioural parameters (*Joseph* et al., 1999). A study from 1999 is of special interest: Rats on chronic ethanol supply were randomized to 80 mg/kg body weight of curcumin (CU) or control (CO) and compared to non-intoxicated normal rats (NI) (*Rajakrishnan* et al., 1999). The degree of histo-pathological changes, the levels of TBARS (NI 1.29, CU 2.41, CO 2.98), cholesterol (NI 1531.9, CU 1658.2, CO 2031.1), phospholipids (NI 1845.5, CU 2011.5, CO 2795.1), and free fatty acids (NI 26.7, CU 39.9, CO 53.1) in brain tissue were significantly improved after curcumin treatment.

Ocular diseases

Cataract, an opacity of the eye lens, is the leading cause of blindness worldwide, responsible for blindness of almost 20 million in the world (*Thylefors*, 1998). Nutritional deficiencies, especially lack of consumption of enough antioxidants, diabetes, excessive sunlight, smoking and other environmental factors are known to in-

crease the risk of cataracts (*Ughade* et al., 1998). The age-adjusted prevalence of cataract in India is, however, three times that of the United States (*Brian* and *Taylor*, 2001). Despite that have three different experimental studies reported significant preventive effects of curcumin against cataracts induced by naphthalene (*Pandya* et al., 2000), galactose (*Suryanarayana* et al., 2003), and selenium (*Padmaja* and *Raju*, 2004).

Pancreatic diseases

The effect of curcumin to reduce the damage to pancreas was studied in two different models; cerulein-induced and ethanol & CCK-induced pancreatitis (*Gukocvsky* et al., 2003). Curcumin was administered intravenously in parallel with induction of pancreatitis. A total of 200 mg/kg body weight was administered during the treatment period of six hours. Curcumin treatment reduced significantly histological injuries, the acinar cell vacuolization and neutrophil infiltration of the pancreatic tissue, the intra-pancreatic activation of trypsin, the hyper-amylasaemia and hyper-lipasaemia, and the pancreatic activation of NF-κB, IκB degradation, activation of activator protein (AP)-1and various inflammatory molecules such as IL-6, TNF-α, chemokine KC, iNOS and acidic ribosomal phosphoprotein (ARP). Curcumin did in both models also significantly stimulate pancreatic activation of caspase-3.

Respiratory diseases

As mentioned above, curcumin is a potent inhibitor of TGF-β and fibrogenesis (*Srinisan* et al., 2004), and suggested to have positive effects in fibrotic diseases in kidneys, liver, intestine (Crohn's Disease), body cavities (prevention of fibrous adhesions) (*Chang*, 2001) and on conditions with lung fibrosis, including cystic fibrosis. The latter is of special interest as it has been especially linked to glutathione deficiency. The effect of curcumin against amiodarone-induced lung fibrosis was recently studied in rats (*Punithavatihi* et al., 2003). Significant inhibition of LDH activity, infiltration of neutrophils, eosinophils and macrophages in lung tissue, LPS-stimulated TNF-α release, phorbole myristate acetate (PMA)-stimulated superoxide generation, myeloperoxidase (MPO) activity, TGF- β1 activity, lung hydroxyproline content and expression of type I collagen and c-Jun protein were observed when curcumin was supplemented in a dosis of 200 mg/kg body weight in parallel with intra-tracheal instillation of 6.25 mg/kg body weight of amiodarone. Curcumin exhibits structural similarities to isoflavonoid compounds that seem to bind directly to the CFTR protein and alter its channel properties (*Illek* et al., 2000). *Egan* et al. (2004), who had previously observed that curcumin inhibits a calcium pump in endoplasmic reticulum, thought that that reducing the calcium levels might liberate the mutant CFTR and increase its odds of reaching the cell surface- see also *Zeitlin* (2004). The ΔF508 mutation, the most common cause of cystic fibrosis, will induce a misprocess in the endoplasmatic reticulum of a mutant cystic fibrosis trans-membrane conductance regulator (CFTR) gene. A dramatic increase in survival rate and in normal cAMP-mediated chloride transport across nasal and gastrointestinal epithelia was observed in gene-targeted mice homozygous for the ΔF508 when supplemented curcumin (*Illek* et al., 2000). No human studies are yet reported and it too early to know if this treatment will be able to halt or reverse the decline in lung function also in patients with cystic fibrosis. An eventual anti-asthmatic effect of curcumin was re-

cently tested in guinea-pigs sensitized with ovalbumin and significant reductions observed both in airway constriction and in airway hyper-reactivity to histamine (*Ram* et al., 2003).

Tobacco/cigarette smoke (CS)-induced injuries

CS is suggested to cause 20 % of all deaths and ~30 % of all deaths from cancer. CS contains thousands of compounds of which about hundred are known carcinogens, co-carcinogens, mutagens and/or tumour promoters. Each puff of smoke contains over 10 trillion free radicals. Antioxidant levels in blood are also significantly reduced in smokers. Activation of NF-κB has been implicated in chemical carcinogenesis and tumourigenesis through activation of several genes such as COX-2, iNOS, matrix metalloproteinase (MMP)-9, IL-8, cell surface adhesion molecules anti-apoptotic protein and others. A recent study reports that curcumin abrogates the activation of NF-κB, which correlates with down-regulation of COX-2, MMP-9 and cyclin D1 in human lung epithelial cells (*Shishodia* et al., 2003).

CONCLUSIONS

All chronic diseases are in a way related, they develop all as a result of a prolonged and exaggerated inflammation (*Bengmark*, 2004). Their development can most likely be prevented or at least delayed by extensive consumption of anti-oxidants such as curcumin. It is important to remember, that it is almost exclusively through microbial fermentation of the different plants that bioactive anti-oxidants are released and absorbed. Clearly flora and supplied lactic acid bacteria/probiotics play an important role. It is therefore unfortunate that both size and diversity of flora is impaired and intake of probiotic bacteria significantly reduced among Westerners. For example, reduction in total numbers and diversity of flora is also associated with certain chronic diseases such as IBD (*Ott* et al., 2004). A study from 1983 demonstrated that *Lactobacillus plantarum,* a strong fibre fermentor, is found in only 25 % of omnivorous Americans and in about 2/3 of vegetarian Americans (*Finegold* et al., 1983). Great differences in volume and diversity of flora have also been observed between different human cultures. It is reported that Scandinavian children have compared to Pakistani children a much reduced flora (*Adlerberth* et al., 1991). Astronauts, who return from space flights have during the flight lost most of their commensal flora including *lactobacillus* species such as *Lactobacillus plantarum* (lost to almost 100%), *Lactobacillus casei* (lost to almost 100%), *Lactobacillus fermentum* (reduced by 43%), *Lactobacillus acidophilus* (reduced by 27%), *Lactobacillus salivarius* (reduced by 22%) and *Lactobacillus brevis* (reduced by 12%) (*Lencner* et al., 1984), changes most likely attributed to poor eating (dried food, no fresh fruits and vegetables) and a much reduced intake of plant fibres and natural antioxidants, to the mental and physical stress and eventually also to the lack of physical exercise. Many individuals in Western Societies exhibit a type of “astronaut-like lifestyle” with unsatisfactory consumption of fresh fruits, vegetables, too much stress and no or little outdoor/sport activities. Furthermore flora seems not to tolerate exposure to chemicals including pharmaceuticals. This is also demonstrated in critically

ill, who most often have lost their entire *Lactobacillus* flora (*Knight* et al., 2004). A recent Scandinavian study suggest that fibre-fermenting LAB such as *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus paracasei* ssp. *paracasei,* present in all humans with a rural lifestyle, are only found 52%, 26% and 17% respectively of persons with a more urban Western type lifestyle (*Ahrné* et al., 1998). These LAB are present in all with more rural lifestyle. The lack of these LAB is probably negative as these LAB are unique in their ability to ferment important fibres such as inulin and phlein, otherwise resistant to fermentation by most *Lactobacillus* species (*Müller* and *Lier*, 1994), and superior to other *Lactobacillus* in their ability to eliminate pathogenic microorganisms such as *Clostridium difficile* (*Naaber* et al., 2004).

To use medicinal plants and their active components is becoming an increasingly attractive approach for the treatment of various inflammatory disorders among patients unresponsive or unwilling to take standard medicines. Food derivates have the advantage of being relatively non-toxic. This is certainly so for turmeric and curcumin. If one chooses to supply it with the fibre e.g. as turmeric, additional supplementation with probiotic bacteria will most likely enhance the efficacy of treatment.

Increasing evidence suggest that saturated fat in the diet increases and plant fibre intake reduces the inflammatory reaction in the body (*King* et al., 2003). A high fat/low fibre diet is clearly associated with chronic diseases (*Campbell* and *Junchi*, 1994), and fruit and vegetable intake with reduction in incidence of chronic diseases (*Knekt* et al., 2002). Focus is increasingly turning from fibre per se to active ingredients in the plant fibres such as curcumin in turmeric.

Not only turmeric and curcumin, but also numerous other plants contain compounds that reduce inflammation and protects against disease. Among them are several thousands of plant-derived chemo-preventive agents, polyphenols and many other, most often unexplored, substances, which seem to have potential to reduce inflammation, speed of aging, prevent degenerative malfunctions of organs and development of chronic diseases. Among them isothiocyanates in cruciferous vegetables, anthocyanins and hydroxycinnamic acids in cherries, epigallocatechin-3-gallate (EGCG) in green tee, chlorogenic acid and caffeic acid in coffee beans and also in virgin tobacco leaves, capsaicin in hot chilli peppers, chalcones in apples, euginol in cloves, gallic acid in rhubarb, hisperitin in citrus fruits, naringenin in citrus fruits, kaempferol in white cabbage, myricetin in berries, rutin and quercetin in apples and onions, resveratrol and other procyanidin dimers in red wine and virgin peanuts, various curcumenoids, the main yellow pigments in turmeric curry foods, and daidzein and genistein from the soy bean. These compounds have all slightly different functions and seem to complement each other well.

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PERINATAL PROGRAMMING OF ESSENTIAL FATTY ACIDS MODULATES IMMUNITY IN RATS

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SUMMARY

Recent studies have shown that besides the eicosanoids modulating the immunological response, also the peroxisome proliferator-activated receptor gamma (PPARγ) and its ligands can modulate both B and T lymphocytes and the dendritic cells. Fatty acids are potent ligands to PPARs and some of the effects might therefore be mediated via the PPAR systems and NF-κB pathways (*Appel* et al., 2005; *Jakobsen* et al., 2006). However, fatty acids can also influence dendritic cells independently of activation (*Zeyda* et al., 2005). Further studies have to consider the influence on gene expression and the modulation by different fatty acids, probably including the balance not only between the essential fatty acids, but also the balance to saturated and monounsaturated fatty acids. We are probably just in the beginning to understand the complex balance of the immune system with possible early programming, which might even include epigenetic aspects.

Our results indicate important physiological changes by fatty acids in the development of oral tolerance. Leptin might be involved in this development but also fatty acid products, the eicosanoids are probably important. PPARs, which also are influenced by fatty acids and other ligands, can further modulate the immune response via or independently of NF-κB. It is obvious that in animals, the composition of the maternal diet has direct and long-term influence on the immune system and it would not be too speculative to suggest that the complicated system also have similar impact in the humans. The complexity of the systems may explain the difficulties to get clear results in observational studies in the humans, and strongly implies that longitudinal studies from pregnancy on are urgently needed.

INTRODUCTION

There has been an increase of allergy in the Western world during the latest decades and much focus has been directed to both the "hygiene" hypothesis and diet (*Guarner* et al., 2006; *van Schayck* and *Knottnerus*, 2004; *Renz* et al., 2006; *Duchen* and *Björksten*, 2001). Several studies have reported abnormal fatty acid pattern in serum in patients with allergy but the results have not been uniform and a causal relation between an abnormal lipid

metabolism and allergy has remained puzzling (*Duchen* and *Björksten*, 2001; *Laitinen* et al., 2006a; *Oddy* et al., 2004). Despite that it is well known that bacterial growth can be modulated by fatty acids, there have been little interest to combine these hypotheses.

FATTY ACIDS AS MODULATORS OF IMMUNE SYSTEM

A possible link between allergy and abnormal fatty acid pattern is the fact that the relation between different fatty acids in diet has changed markedly during the latest decades (*Simopoulos*, 1999; *Sanders*, 2000; *Kris-Etherton* et al., 2000). By the general recommendation to decrease the intake of saturated fat and recommend increase of polyunsaturated fat, a marked displacement has taken place; essential fatty acids of the n-6 series have increased several fold but those of the n-3 series have remained low and even decreased (*Ailhaud* et al., 2006). Partly also this has occurred smouldering for the man in general, because the changed feeding of animals from natural sources to special forage have also markedly decreased the n-3 fatty acids in diary products and in the meat (*Ailhaud* et al., 2006; *Raes* et al., 2004). All these changes are reflected in the breast milk, which shows different ratios between the n-6 and n-3 fatty acids depending on mothers dietary intake, with big differences for different districts and countries and also by time in the Western countries, reflecting the general development (*Ailhaud* et al., 2006; *Chen* et al., 1997; *Fidler* and *Koletzko*, 2000). From this point of view, it is not surprising that investigations of breast milk in relation to allergy have given controversial results (*Laitinen* et al., 2006a,b; *Black* and *Sharpe*, 1997; *van Gool* et al., 2004).
Our interest in this subject is related to two factors; first that the n-6 and n-3 fatty acids have strong effect on immunity, like for instance different effects on Th1 and Th2 responses and influence on the maturation of dendritic cells (*Lee* et al., 2003; *Harizi* and *Gualde*, 2005; *Gosset* et al., 2005; *Zhang* et al., 2005), and second that essential fatty acids can influence gene expression (*Clarke* et al., 2002) and therefore might be especially interesting in the context of programming, i.e. the influence during early development of the foetus on diseases in adult life (*Lucas*, 1998).

Essential fatty acids contain unsaturated bindings starting at the carbons in position 3 (n-3) or 6 (n-6) from the methyl end of the fatty acid molecule. These fatty acids have to be supplied by food because they cannot be synthesised by the mammalians. Introduction of double bonds in other positions can be performed in the body, and occur both in the essential fatty acids and in fatty acids we can synthesize (Figure 1). There is a competition between enzymes to prolong the fatty acid molecules (elongases) and to increase the unsaturation (desaturases) between the essential fatty acid series and the newly synthesised fatty acids, and this competition is influenced by the access of substrate, i.e. it matters in which relation the diet supply the body with different kind of food and fat. Since the very long polyunsaturated fatty acids are important for many functions, like the long fatty acids from the n-3 series, the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for the brain and nervous system and the long fatty acid product of the n-6 series, arachidonic acid (ARA) for several meta-

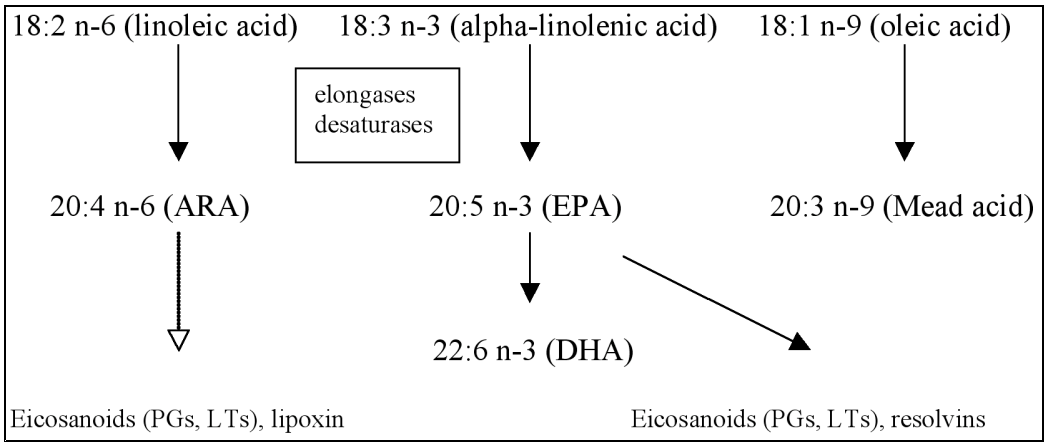

Figure 1: The major fatty acids of the n-6, n-3 and the endogenously (n-9) synthezised series. In deficiency of the essential fatty acids, especially the n-6 series, mead acid is compensatory increased and can therefore be used as an index of the essential fatty acid deficiency. The eicosanoids of ARA are inflammatory and those of EPA, lipoxins and resolvins are anti-inflammatory.

bolic processes and working as the substrate for very important eicosanoids, like PGE_2 and the cysteinyl-leukotrienes (Figure 1).

DEVELOPMENT OF ORAL TOLERANCE

The knowledge about the induction or breakdown of oral tolerance in the neonatal period is incomplete. In the development of oral tolerance the antigen-presenting cells, especially the dendritic cells, play a central role in the induction of adaptive immunity. Dendritic cells are activated by cytokines and eicosanoids and the balance between saturated and polyunsaturated fatty acids can reciprocally modulate the function of the dendritic cells through Toll-like receptors (*Weatherill* et al., 2005). Active suppression is important for and dependent on the induction and the maintenance of the regulatory suppressor cells (Treg/Th3) and their presence in the lymph nodes of the intestinal wall. These cells are triggered by a specific antigen and responsible for the release of the antigen-non-specific suppressive cytokine TGF-β (*Lundin* et al., 1999). As a consequence immune responses to other antigens in the close vicinity are decreased. If immunological tolerance is not developed, the immune response may lead to allergic sensitisation to food allergens. The dose and nature of antigen, as well as the maturation of the immune system, and the permeability of the intestinal wall are all factors of importance if an antigen induces tolerance or priming (*Strobel*, 2001). For the newborn most antigens are presented via the food, usually the breast milk, and the content of polyunsaturated fatty acids is dependent on the mother's diet. That means that the maternal diet has the potential to influence the development of immunological tolerance to food antigens.

Dietary polyunsaturated fatty acids have been shown to influence the tolerance induction in adult animals, and to influence the Th1 and Th2 responses to ovalbumin (*Cinader* et al., 1983; *Harbige* and *Fisher*, 2001).

EXPERIMENTAL PROGRAMMING OF ORAL TOLERANCE BY EFA

We have focused our interest on the possible potency of essential fatty acids to programme for diseases in the adult animal, and have been especially interested if they can influence the later development of oral tolerance, important in the context of allergy. Besides these studies, which will be reviewed in the following, we have also investigated whether the essential fatty acids might programme for bone mass (*Korotkova* et al, 2004a, 2005a) and the metabolic syndrome (*Korotkova* et al, 2005b). We have used two models, one providing the pregnant and lactating animal with essential fatty acid deficient diet (**A**) and one where the animals were provided three different diets, one rich in n-3 fatty acids (ratio n-6/n-3 of 0.4), one "control" diet with a so called balanced ratio of n-6/n-3 fatty acids (ratio n-6/n-3 of 9) and one with extremely high amount of n-6 fatty acids in relation to n-3 (corresponding ratio of 210) (**B**).

All diets had the same composition except that the quality of fat differed. Fat constituted 7 percent of all diets and in the diet free of essential fatty acids; the animals were given saturated fat except for trace of monounsaturated fat. In the diets with different ratios of essential fatty acids, 21-32 % of the fatty acids were monounsaturated and 13-20% was saturated fatty acids. The diet were introduced from the 10^{th} day of gestation and kept during lactation and till 7 weeks of age. The rats were subsequently exposed to ovalbumin either as pups via the milk at postnatal days 10-16, or as adults via the drinking water at 7-9 weeks of age.

A. For control one group of rats received the essential fatty acid deficient diet only as adults, from week 7 of age (31). They were immunized at 11 weeks with ovalbumin and delayed type of hypersensitivity (DTH) was tested at 13 weeks of age. Oral exposure of ovalbumin lead to suppression of the DTH response and also depressed response by IgG antibodies in both the animals deficient of essential fatty acids and the control group (31). The tolerance to ovalbumin was accompanied by reduction of DTH and IgG antibody responses to an unrelated antigen, human serum albumin, due to bystander suppression. Thus oral tolerance was developed and maintained by an active suppression mechanism in the adult animals of both the dietary groups.

In the adult offspring of the dams fed the deficient diet during the last half of pregnancy and during lactation, neonatal antigen exposure via the milk resulted in suppression of the serum antibody levels and DTH response against ovalbumin indicating induction of oral tolerance (31). In contrast, ovalbumin exposure of the dams fed the control diet did not result in suppressed ovalbumin responses of their offspring. Also the IgG and IgM antibody responses were only depressed in the animals on the deficient diet. Higher expression of TGF-β mRNA in the draining lymph nodes suggested that these effects were mediated via Treg cells. In the intestinal cells the essential fatty acid pattern was markedly abnormal at the time of weaning in the deficient animals and the CD8+ T-cells were increased. The expression of MHC class II was not different between the groups. Hence, these data indicate that the dietary content of polyunsaturated fatty acids was one factor of importance for the induction or failure of oral tolerance.

B. These data stimulated us to investigate the effects of different ratios of the n-6/n-3 fatty acids in the mater-

nal diet on the induction of the neonatal oral tolerance in the rat offspring (*Korotkova* et al., 2004c). Interestingly, the milk from the dams contained exactly the same ratio of essential fatty acids as the diet, but in serum of the pups at 3 weeks of age, the ratios were adapted, well into physiological range, being 3, 8 and 17, respectively. The serum fatty acid pattern was different between the pups reflecting the diets, as was the fatty acid composition of the adipose tissue. As described above, rat pups were exposed to ovalbumin via the milk at postnatal days 10-16. In the adult offspring from dams receiving the n-3 diet, exposure to ovalbumin via the milk resulted in lower DTH and antibody responses against both ovalbumin and human serum albumin, compared to those offspring on the same diet but not exposed to ovalbumin postnatally, indicating induction of oral tolerance. Also the antibody response followed the same pattern as the DTH reaction. Serum antibodies of the IgE class were also depressed in the n-3 animals exposed to ovalbumin. The lymph nodes draining the immunisation site were also less enlarged in the offspring exposed to ovalbumin via their dams, suggesting that in these animals the tolerance was mediated at least partly by an active suppression mechanism. In contrast, the adult offspring from dams receiving the n-6/n-3 diet did not show tolerance. Further increase of the n-6/n-3 ratio in the maternal diet was associated with induction of oral tolerance in the n-6 group of offspring. However, the bystander suppression was not observed in the offspring receiving the n-6 diet, suggesting that the oral tolerance probably was mediated by anergy in these animals. The results suggested that the ratio of the n-6/n-3 fatty acids in the maternal diet affected the mechanisms of neonatal oral tolerance and are in line with previous published data, demonstrating that dietary levels of the n-6/n-3 polyunsaturated fatty acids influence the mechanism of oral tolerance in adult mice (*Harbige* and *Fisher*, 2001).

We also investigated the mammary glands at the time of weaning (*Korotkova* et al., 2004c). There was no difference in the number of macrophages, T-cells or dendritic cells between the different dietary groups and although the number of activated dendritic cells differed numerically (3.4, 4.9 and 11.4 cells/mm^3, respectively), the differences were not statistically significant. The number of MHC class II positive cells in the mammary glands was significantly lower in the n-3 group of mothers ($p<0.05$). The results showed that the quality of fat ingested by the mother had effects on the development of immunological tolerance to dietary antigens in the offspring of the animals but the mechanisms have to be further studied. The relevance of our findings in understanding of allergy in the humans has thus to be determined.

POSSIBLE ASSOCIATED FACTORS IN PROGRAMMING OF ORAL TOLERANCE

There are many possible factors which might be involved in the development of oral tolerance. Leptin is widely distributed in the body, also with receptors in the gastrointestinal tract and in hypothalamus. It is known to regulate food intake and energy expenditure and immune responses. It is structurally similar to IL-6 cytokines and binds to receptors, which belong to the class I cytokine receptors. Leptin up-regulates monocytes/macrophage

functions (*Santos-Alvarez* et al., 1999) and modifies T cell responses with increasing Th1 (IL-2, IFN-g) and suppressing Th2 (IL-4, IL-10) cytokine production (*Lord* et al., 1998). In a recent study serum leptin in mice was increased related to enhanced metacholine responsiveness and IgE responses on sensitisation with ovalbumin (*Shore* et al., 2005). Leptin is secreted in breast milk and might play an important role in the induction and maintenance of immune and inflammatory responses, especially vital in the perinatal period. It has been shown that the quantity of dietary fat affects serum leptin levels perinatally. Increased maternal fat intake raises plasma leptin concentrations in neonatal rats and affects hypothalamus-pituitary-adrenal responsiveness in neonates and prepuberal rats (*Trottier* et al., 1998).

A. We found in our studies that the quality of the dietary fat modulated serum leptin levels in rat offspring during the suckling period, both in deficiency of the essential fatty acids and in studies of different ratios of the n-6 and n-3 series (*Korotkova* et al., 2001, 2002a,b). In the model with the essential fatty acid deficient diet, the weight of inguinal white adipose tissue depots and the serum leptin levels of the essential fatty acid deficient offspring were significantly lower than in the control pups during the whole suckling period, despite that milk leptin levels were higher in the deficient dams than in the control dams at 3 weeks of lactation. Furthermore, leptin receptor mRNA was significantly increased in mesenteric lymph nodes (*Palsdottir* and *Korotkova*, to be published), but the mRNA levels of leptin was decreased in inguinal adipose tissue compared with the control pups at 3 weeks of age (*Korotkova* et al., 2002a).

B. The different dietary ratios of n-6/n-3 fatty acids also influenced the serum leptin levels in the postnatal period (*Korotkova* et al., 2002b). Body weight, body length, inguinal fat pad weight, adipocyte size and serum leptin levels of the offspring receiving the n-3 diet were significantly lower during the whole suckling period compared with the n-6/n-3 fed offspring. The mean serum leptin levels of the n-6 offspring were between the other two groups, but not different from either group. Despite that the serum leptin levels were increased and the milk leptin content did not differ between the groups, the leptin mRNA in the adipose tissue was significantly lower in the n-6/n-3 group compared with the other two groups at 3 weeks of age (*Korotkova* et al., 2002b).

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CYSTIC FIBROSIS: FOOD, MICROFLORA AND HEALTH - THE GOLDEN TRIANGLE -

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SUMMARY

Cystic fibrosis remains a life long, life limiting disease, with median survival in Europe and the United States steadily increasing and now into the third decade. The primary cause of death remains respiratory failure brought on by the constant cycle of infection and inflammation occurring in the lungs. Improved survival is related to an aggressive three-pronged approach: Good nutrition, targeted antibiotic therapy and increasing lung clearance. Inflammation appears to play a role in the intestinal tract as well as the lungs. Correction of inflammation both in the gastrointestinal tract and the lungs may be possible with concentration on correcting micronutrient deficiencies, especially those linked to the antioxidant defence systems. However, there is still only limited evidence in this area. The environment in the gut is also affected by the frequent courses of antibiotics given to patients with cystic fibrosis and early work has started to look at correction of imbalance in flora using antibiotics and probiotics, with theoretic benefits further a field in the lung. Apart from improved body mass index and its direct effect on increased survival, the benefits of good nutrition and micronutrients run over into other areas such as bone metabolism and cognitive function. This review attempts to emphasis these important areas in the overall disease process of cystic fibrosis.

INTRODUCTION

Cystic Fibrosis is the commonest autosomal recessive condition in the European Caucasian population. In the United Kingdom (UK) there are over 7500 people with cystic fibrosis with a birth rate of 1 in 2415 (*Dodge* et al., 1997). The basic defect found is in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (*Kerem* et al., 1989). It is now recognised that there are over 1000 different mutations of this gene (*The Cystic Fibrosis Genotype-Phenotype Consortium*, 2004), with the commonest 32 mutations found in over 96% of the UK population and indeed the most prevalent of these, a deletion of phenylalanine at position 508 (ΔF508), accounting for over 74% of the deletions (*McCormick* et al., 2002).

The primary function of the CFTR is as a chloride channel, (*Linsdell*, 2006) in health it is positioned on the apical membrane of epithelial cells (*Tsui*, 1995). The subsequent imbalance in electrolytes and water flow from this defective channel is clinically relevant in several body systems: the

lung, the pancreas, the gut, the liver, the sweat glands and the vas deferens in males. All lead to important problems for the individual with cystic fibrosis, the most studied of which is the abnormality in the lungs. Here, the relentless cycle of infection and inflammation, caused by airway surface fluid dehydration and mucus plugging of airways, has a profound effect on both the morbidity and mortality associated with this disease. Over 90% of deaths in cystic fibrosis are from respiratory failure with the median age of survival in the USA being reported as 36.8 years (Cystic Fibrosis Foundation, 2005 database figures, personal correspondence).

There is extensive work on the inflammation and infection cycles within the lungs and this article will only briefly cover this before exploring the problems in the cystic fibrosis gut: inflammation, malabsorption, nutrition and the links with health.

INFLAMMATION AND THE LUNGS

Inflammation in the cystic fibrosis lung is well described (*Courtney*, 2004; *Schiotz*, 1978) with debate about whether the CFTR defect leads to inflammation even without the presence of infection. In 1995 two papers clearly demonstrated the presence of lung inflammation as early as 4 weeks of age in populations of neonatally screened infants with cystic fibrosis. In these studies markers of inflammation such as Interleukin 8 (IL-8) and neutrophils were raised, both with and without the presence of bacterial or viral infection (*Khan* et al., 1995; *Armstrong* et al., 1995). Neutrophils appear to be the predominant cell involved in the inflammation of the airway. More recently *Armstrong* et al. (2005) have again looked at bronchoalveolar lavage (BAL) fluid in infants with cystic fibrosis diagnosed by neonatal screening. They show that the increased inflammation only appears in the presence of bacterial or viral infection and that over subsequent years inflammatory response decreases in the absence of infection. This suggests that it is the infection promoting this inflammatory response, rather than an innate effect of the CFTR.

This theory has been questioned again recently. It has been known for some time that increased levels of arachidonic acid (AA) are present in the airways of patients with cystic fibrosis (*O'Driscoll* et al., 1984). AA is a protagonist in the inflammatory pathway and like other raised inflammatory markers in cystic fibrosis it has been thought to be a reactive process following infection. Work by *van Heeckeren* et al. (1997) using *Pseudomonas* beads in mouse airway suggests that there is an inflammatory response above and beyond that expected, they and others feel that CFTR is responsible for this up regulation.

It is impossible not to mention the role of *Pseudomonas aeruginosa* infection here. The cystic fibrosis lung appears particularly susceptible to infection by *Pseudomonas aeruginosa*. Work by *Davies* et al. (1997) implies that the CFTR defect itself is responsible for increased binding of *Pseudomonas aeruginosa* to the cystic fibrosis airway, they found correction of the CFTR lead to decreased *Pseudomonas aeruginosa* adherence. We know that chronic colonisation of the airway with *Pseudomonas aeruginosa* leads to a more rapid decline in lung function (*Pamukcu* et al., 1995) and the American Cystic Fibrosis Foundation Data from 2002 showed that median sur-

vival, when chronically colonised with *Pseudomonas aeruginosa,* is 10 years younger than in those patients not growing it in their sputum.

INFLAMMATION AND THE GUT

The cystic fibrosis knock out mice models are notorious for their severe and early gut involvement, making study of therapies and strategies in the lungs hard. It does, however, give us a useful starting point to look for evidence of abnormal inflammation in the gut.

Several authors have shown the presence of inflammation in the gut of cystic fibrosis knock out mice. *Norkina* et al. (2004a) found that the gut of sacrificed Peptamen fed cystic fibrosis knockout mice showed up regulation of genes governing innate immunity but not adaptive immunity. The most up regulated gene they found was RELMB/FIZZ2, associated with cell growth and inflammation. In this study, expression of genes involved in lipid metabolism was found to be down regulated, especially cytochrome P450. They authors hypothesised that the increased inflammation in the gut may cross over to the systemic circulation and cause priming of the immune system leading to the over response to subsequent infections in the lungs.

Croft and colleagues produced a triad (*Croft* et al., 1995, 1996, and *Smyth* et al., 2000) of papers looking at gut inflammation in patients with cystic fibrosis. They described a process of looking at inflammatory markers in the fluid obtained by whole gut lavage (WGL) (*O'Mahony* et al., 1990), in the same way that inflammatory markers in the fluid from BAL are examined. In the largest of their studies looking at 21 patients with cystic fibrosis and 12 controls it was evident that the cystic fibrosis patients had raised levels of proteins derived from the plasma: Albumin, and IgG. Increases in the secretory immunoglobulin, IgM and the cellular constituents, eosinophil cationic protein and neutrophil elastase were also found. A key cytokine in cystic fibrosis: IL-8 and the lesser studied IL-1β were both also found to be raised. This was a similar finding to a previous study looking at inflammatory markers (IL-8) in the faeces of patients with cystic fibrosis (*Briars* et al., 1995). Like investigators of the inflammation in the lungs, they concluded that the CFTR itself had inherent pro-inflammatory properties and was likely to be responsible for the raised gut inflammatory markers (*Smyth* et al., 2000).

More recently, a small study looking at 30 children with cystic fibrosis confirms gut inflammation in 27 of them, showing significantly raised levels of faecal nitric oxide and calprotectin (both markers of inflammation) when compared to controls. Their levels were comparable to a second control group suffering from inflammatory bowel disease (*Bruzzese* et al., 2004).

The presence of chronic inflammation in the intestine of cystic fibrosis patients (*Raia* et al., 2000) would also go some way to explaining the increase in bowel cancer found in cystic fibrosis (*Schoni* et al., 1996). Increased oxidative stress is implicated in non-cystic fibrosis bowel cancer (*Skrzydlewska* et al., 2005) and may also play a role in cystic fibrosis. Other forms of cancer are not increased, specifically lung cancer, suggesting that CFTR is not responsible for the increased risk of cancer.

ABNORMAL MICROFLORA

Infection, both acute and chronic has been shown to be responsible for the inflammation found in the cystic fibrosis lung (*Armstrong* et al., 2005). Is there evidence that there is bacterial overgrowth in the gut? An increase in small intestine bacteria by up to 40 fold in CF knockout mice (*Norkina* et al., 2004b) has been shown using PCR (16S) techniques. Subsequent treatment with broad-spectrum antibiotics (ciprofloxacin and metronidazole) decreased this bacterial load down to levels similar to control mice, decreased the expression of inflammatory genes and also led to improved growth. This group also showed that a 3-week course of antibiotic treatment decreased the amount of small intestinal mucus found in these mice (*De Lisle* et al., 2006). The improved growth (as measured by weight gain) after decreasing bowel bacterial load may have implications for patients with cystic fibrosis. However, the evidence of bacterial overgrowth in cystic fibrosis is actually quite sparse. One previous study in 54 children with cystic fibrosis examined the prevalence of bacterial overgrowth using breath tests, it showed that it is more common than in children with non-CF related bowel disease: 32% vs. 7%. It also showed slower transit times in the cystic fibrosis group as a whole and when divided into those with overgrowth and those without (*Lewindon* et al., 1998).

Reasons for this apparent bacterial overgrowth have not been studied, it could be postulated that the same mechanisms up regulating inflammation at the lung epithelium are occurring in the intestine. Why increased inflammation occurs in the lung is not that well understood (*Courtney* et al., 2004) and it is likely to be a while before an understanding of the process in the intestine becomes clear. However, some theories, especially related to micronutrient roles are discussed later.

PROBIOTICS

Imbalance of natural flora within the intestine from bacterial overgrowth (*Norkina* et al., 2004b) and the repeated use of antibiotics to treat chest exacerbations must lead to some discussion of the use of probiotics in patients with cystic fibrosis. The theory behind their use already exists in other disease models, with a reduction in antibiotic associated diarrhoea and *Clostridium difficile* diarrhoea now being confirmed by meta-analysis (*McFarland*, 2006). *C. difficile* is more prevalent in the cystic fibrosis population being reported as between 22 and 32% (*Welkon* et al., 1985; *Peach* et al., 1986). However, in these two studies its presence was reported as being asymptomatic with no associated diarrhoea.

Bruzzese's paper (*Bruzzese* et al., 2004) describing gut inflammation in cystic fibrosis is the only study also looking at probiotic use for patients. They showed a significant reduction, after using *Lactobacillus* GG, in both calprotectin (n=10) and faecal nitric oxide (n=5). Cystic fibrosis mouse work has focused on the effect of *Lactobacillus casei* on lung clearance of *Pseudomonas aeruginosa* (*Alvarez* et al., 2001) again with a significant improvement in the treated group. *Miake* et al. (1985) showed a protective effect using *Lactobacillus casei* on peritoneal cavity infection with *Pseudomonas* in non-cystic fibrosis mice. These studies give some hope for further work in this area.

NUTRITION

The interplay between the gut and the lung is very important; the majority of people with cystic fibrosis are pancreatic insufficient, requiring supplementation with pancreatic enzymes to help with absorption of food. Since *Corey* et al (1988) reported improved survival and weight of patients on a high fat, high energy diet rather than the traditional low fat diet there has been a shift to aggressive nutritional management.

Registry data from Germany (*Steinkamp* and *Wiedemann*, 2002) looking at 3298 patients over a 2-3 year time period confirms this original article. They defined patients as malnourished if they had a weight for height percentile of <90% and showed progression of the proportion of malnourished patients with advancing age. More importantly they demonstrated worsened lung function when malnourished, which was particularly evident in the adolescent age range. They were able to demonstrate an improvement in lung function of 2.1 % on gaining > 5% weight. This was a similar finding to our own data following the introduction of gastrostomy feeding in a small group. This showed an improvement in weight for height with the introduction of feeds (92% vs. 98%) and an arrest in the yearly rate of lung function decline (FEV_1 -5% vs. 4%) (*Collyer* et al., 2000). Unfortunately, the recent multicentre CALICO Trial of oral supplementation in moderately malnourished children with cystic fibrosis has not replicated these results (*Poustie* et al., 2006). They were unable to show an increase in weight in this randomized trial, so effects on lung function in relation to weight gain were not possible to determine.

Looking to the mouse model again, *van Heeckeren* et al. (2004) tried to tease out whether nutrition had a specific effect on host response to lung infections. They compared three groups of mice: Wild type, cftr knock out and gut corrected cftr knockout mice (expressing human CFTR primarily within the gut). *Pseudomonas aeruginosa*-laden agarose beads were placed in the bronchi of each group after various enriched diets and in a separate experiment after adding the essential fatty acid Docasahexaenoic acid (DHA). They found no differences in lung inflammation (post sacrifice BAL samples) or on early death within the groups, suggesting nutrition and DHA imbalances alone, are not able to explain the different inflammatory responses in CF and wild type mice.

ESSENTIAL FATTY ACIDS AND CURCUMIN

The essential fatty acid (EFA), Docasahexaenoic acid was used in van Heeckeren's study (*van Heeckeren* et al., 2004) because of several studies pointing to the imbalance of EFA in patients with cystic fibrosis (*Bhura-Bandali* et al., 2000; *Strandvik* et al., 1988; *Carlstedt-Duke* et al., 1986) and especially with the knowledge that low levels of DHA are seen in relation to the severe CFTR mutations (*Strandvik* et al., 2001). A pivotal paper reporting the correction of pancreatic duct abnormalities in knock-out cftr mice following supplementation with 40mg day of DHA has lead to increased research in this area (*Freedman* et al., 1999). Pilot data on the safety of oral DHA supplementations (*Lloyd-Still* et al., 2005) and proof of concept (*Jumpsen* et al., 2006) are now available and there are ongoing studies of supple-

Table 1: Role of vitamins in cystic fibrosis

Vitamin	Role
Vitamin A	Immunity (*Reifen*, 2002) Respiratory epithelium integrity (*Biesalski* and *Nohr*, 2003) Vision (*Fulton* et al., 1982; *Huet* et al., 1997)
Pro-vitamin A (b carotene)	Antioxidant defences (*Lepage* et al., 1996)
Vitamin D	Bone metabolism (*Haworth* et al., 2004)
Vitamin K	Bone Metabolism (*Beker* et al., 1997; *Weber*, 2001)
Vitamin E	Antioxidant defences (*Wood* et al, 2001) Neurological integrity (*Bye* et al., 1985)
Vitamin C	Antioxidant defences (*Winklhofer-Roob* et al., 1997) Immunity (*Wintergerst* et al., 2006)

mentation of DHA in newborn infants with cystic fibrosis.

Aside from the frenzied research in trying to find a cure for cystic fibrosis by gene therapy, equally vigorous attempts are being made to find substances that can correct the function of the faulty CFTR rather than replace it. There was therefore some excitement a few years ago when *Egan* and colleagues (2004) reported that a simple food substance, curcumin appeared to correct the basic defect, as demonstrated by correction of nasal potential difference in nasal epithelium in a hamster model. Sadly, this has not been replicated and doubt as to its future as a treatment is now rife (*Dragomir* et al., 2004).

ROLE OF MICRONUTRIENTS

It is well established that there are deficiencies of the fat-soluble vitamins in cystic fibrosis early in life (*Bye* et al., 1985; *Sokol* et al., 1989), this is in part due to the malabsorption of fats due to pancreatic enzyme deficiency. Routine supplementation of fat-soluble vitamins is now well established. The role of the fat-soluble vitamins in disease process is myriad and some are summarised in Table 1.

ANTIOXIDANT ROLE

The argument for oxidant antioxidant imbalance in cystic fibrosis is well documented (*Brown* and *Kelly*, 1994a). Several micronutrients play an important role in the antioxidant defences (Table 2). Evidence of decrease in these antioxidants and increased oxidative stress has been shown in several studies looking at antioxidant blood levels (*Langley* et al., 1993), breath

Table 2: Examples of links between dietary constituents and antioxidant defence

Nutrient	Role
Cu	Superoxide dismutase (SOD), caeruloplasmin
Fe	Catalase, SOD
Mg	Co-factor for multiple enzymes
Mn	SOD
Nicotinamide	DNA repair, energy source
Proteins	Amino Acids for synthesis of antioxidant enzymes, metal binding proeins, albumin
Riboflavin	Glutathione reductase
Se	Glutathione peroxidases

pentane levels (*Bilton* et al., 1991) and markers of oxidative stress in the urine (8 hydroxyguanosine) (*Brown* et al., 1995) and plasma: 8-isoprostaglandin F 2α (8-iso $PGF_{2\alpha}$) (*Collins* et al., 1999), thiobarbituric acid-reactive substances (T-bars) (*Benabdeslam* et al., 1999, *Brown* and *Kelly*, 1994b) and malondialdehyde (*Rust* et al., 1998).

Linking micronutrients to lung or general health in cystic fibrosis is hard as there is little research in this area. *Bines* et al. (2005) suggest that at 1 year follow up of a group of 39 screened infants with cystic fibrosis there was no link to increased lung inflammation (BAL inflammatory markers) in the 20 infants that had been found to have low retinol at diagnosis. Our own work suggests that there may be a link between vitamin A and lung function in a group of 78 clinically stable children with cystic fibrosis: a low vitamin A level gave an odds ratio of 5 (95% CI 1.7-14.4) to predict an FEV_1 < 50% (predicted for height and sex) (*Carr* and *McBratney*, 2000). Follow up of these children over a 3 year period suggested a continuation of this independent link in a regression model, between vitamin A and FEV_1 (*Ranganathan* et al., 2002). A note of caution, however, vitamin A is negatively correlated to markers of inflammation (CRP) and depressed levels in cystic fibrosis may just be an expression of acute inflammation in some cases (*Greer* et al., 2004).

When moving on from simple observational studies that look at associations to cause and effect studies the literature becomes thinner. A recent small double blind trial of multiple antioxidants therapy (vitamin A, E, C b-carotene and Selenium) in 46 patients with cystic fibrosis showed good increases in the measured blood levels of these antioxidants and glutathione peroxidase but failed to show any improvement in oxidative stress as measured by 8-iso $PGF_{2\alpha}$ (*Wood* et al., 2003). An earlier open label study in 12 children given β-carotene for two months showed a decrease in the lipid peroxidation marker malondialdehyde (*Lepage* et al., 1996). A short study of

vitamin E supplementation also showed an improvement in the tolerance of low density lipoprotein to oxidation (*Winklhofer-Roob* et al., 1995).

Apart from the Wood trial which showed a slight correlation between change in lung function and selenium and 8-iso $PGF_{2\alpha}$ (*Wood* et al., 2003), these studies show proof of concept rather than any other clinically relevant outcomes such as fewer chest exacerbations, improved weight or increased survival. This is again demonstrated by a recent randomised, blinded, crossover study in a group of 22 patients. They were supplemented with a micronutrient mixture containing, minerals, trace elements, vitamins, fats (linoleic acid) or with placebo, the study failed to show any effect on pulmonary function or muscle performance (*Oudshoorn* et al., 2007).

Excitingly, an old drug N-acetylcysteine (NAC) is having a resurgence and not just as a mucolytic. It is a strong antioxidant and a precursor of glutathione. Glutathione (GSH) is found in large quantities in the epithelial lining fluid (ELF) of the lung and plays an important role in antioxidant defence. It also appears that the efflux of GSH may be controlled by CFTR, giving another reason for imbalance in antioxidant defences (*Hudson*, 2004). A recent phase 1 trial in 18 patients with cystic fibrosis has shown supplementation with high oral doses of NAC decreases lung inflammation as evidenced by lower sputum elastase, and neutrophil counts (*Tirouvanziam* et al., 2006). Similarly, proof of concept has also been shown in trials of inhaled glutathione (*Bishop* et al., 2005).

OTHER EFFECTS OF MICRONUTRIENTS ON HEALTH

Moving away slightly from the antioxidant effects of micronutrients we must take note of a recent report suggesting that early deficiency of Vitamin E in a non-screened group of infants with cystic fibrosis led to impaired cognitive function at age 6-14 years when compared to their screened peers (*Koscik* et al., 2005). There are several case reports in the literature related to side effects of this and other fat soluble vitamin deficiencies in cystic fibrosis: varying from night blindness (*Huet* et al., 1997), to bleeding (*Verghese* and *Beverley*, 2003) to peripheral neuropathies (*Bye* et al., 1985).

Another important complication of cystic fibrosis is osteoporosis which tends to be preceded by osteopenia (*Elkin* et al., 2001. *Haworth* et al., 1999). This does not appear to be related to the CFTR defect but rather a side effect of having cystic fibrosis: the prevalence rises with increasing age (*Buntain* et al., 2004). Its cause is probably a combination of malabsorption of the micronutrients related to bone metabolism, especially the fat soluble vitamins D and K, but also of decreased muscle bulk, immobility and occasional use of steroids. A placebo controlled study supplementing calcium and vitamin D for a year in adult patients failed to reach statistical significance, but did show a trend towards decreasing bone turnover (*Haworth* et al., 2004). A recent article supplementing 20 children with cystic fibrosis for a year with vitamin K has shown improvement in biochemical markers of bone health, without alteration to their vitamin D intake or exercise regimens (*Nicolaidou* et al., 2006).

CFTR AND MICRONUTRIENTS

Finally, going full circle back to the CFTR, micronutrients are beginning to stir interest for their potential roles in modulation of the CFTR function. Vitamin C (ascorbic acid) which is found in the respiratory epithelial lining fluid appears to help in the CFTR regulation of chloride flow as evidenced by changes in potential difference, in both *in vivo* and *in vitro* studies (*Fischer* et al., 2004). Zinc in combination with ATP appears to help activate an alternative, calcium dependent chloride channel. In a cystic fibrosis mouse model this allowed restoration of chloride permeability across the nasal epithelia (*Zsembery* et al., 2004).

Short chain fatty acids, easily absorbable orally, are being looked at in airway cell models for their potential ability to up regulate both the CFTR and other chloride channels, some success having being shown with 2,2-dimethyl-butyrate and alpha-methylhydrocinnamic acid (*Nguyen* et al., 2006).

CONCLUSION

This review has provided a brief guide from the basic defect in cystic fibrosis, through the malfunctioning of the CFTR protein leading to increased inflammation and infection and the knock on effect this has at the all important lung and intestine surfaces. The malabsorption from lack of pancreatic enzyme leaves the majority of patients with cystic fibrosis the everyday challenge of maintaining good overall nutrition, in the knowledge that poor intake will lead to greater morbidity and likely early death. It has shown the complex interplay of CFTR, micronutrients and gut flora in the overall picture, especially in relation to lung health. Hopefully it has confirmed the need for careful thought about achieving the best dietary approach to aid in the everyday struggle to lengthen survival in cystic fibrosis.

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READDRESSING THE BALANCE: OLD FRIENDS, MICROFLORA AND IMMMUNOREGULATION

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SUMMARY

As a result of modern day living conditions, it has become critical to readdress the balance of stimuli of the immune system. To this end, a balance between effector T cells and regulatory T cell-mediated mechanisms is crucial. It is here proposed that renewed exposure to Old Friends and an appropriate intestinal microflora in the host is essential for immunoregulation. The efficient induction of an immunoregulatory network may prevent the development of diseases of immunodysregulation which are characterized by exuberant and inappropriate effector responses to harmless antigens. Vaccinations or oral supplements based on Old Friends may provide a novel therapeutic approach for the treatment of these diseases.

DISEASES OF IMMUNODYSREGULATION

Epidemiological data suggest that the incidence of diseases of immunodysregulation has increased dramatically in the last century in developed countries (*Wills-Karp*, 2001; *Bach*, 2002). These disorders are characterized by inappropriate immune responses leading to chronic inflammation. Example of immunodysregulatory diseases include allergic conditions such as allergic rhinitis and asthma, which results from inappropriate responses to harmless ubiquitous allergens; autoimmune diseases such as type I diabetes and multiple sclerosis which results from inappropriate responses to self antigens, and inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease, which are caused by inappropriate responses to the contents of the intestine. Interestingly, a rise in these conditions has yet to be reported in developing countries.

The escalation in some diseases of immunodysregulation in developed countries has been dramatic. A poignant example is asthma, which is now a common condition among children and young adults with prevalence rate reaching 17-30% in the UK, New Zealand and Australia. In contrast, prevalence remains relatively low (1-7%) in less developed countries such as in some Eastern European states, China and Indonesia (*ISAAC study*, 1998). Asthma is a multifaceted disease in which allergen exposure leads to both early and late phase airway inflammatory responses culminating in severe bronchoconstriction and airway remodelling. This disease has become of major public health importance, placing a heavy burden on both affected families and on society and adding further strain on the healthcare system.

The causes responsible for these observed increases in diseases of immunodysregulation remain unclear. It has been suggested that genetic factors alone cannot be blamed for the observed rise. It is unlikely that the number of people with a strong genetic predisposition to develop these conditions have increased over the last 50 years to the extent required to explain such rise in the incidence of these disorders. Hence, environmental factors may be playing a role (*von Mutius*, 2000; *Rook* and *Rosa Brunet*, 2002; *Guarner* et al., 2006). The change in living conditions which has occurred over the last century may favour the development of these diseases in a segment of the population which are predisposed to them (*Rook* and *Rosa Brunet*, 2002). Indeed, there have been a number of changes in our living environment which have resulted in a severe reduction in the stimuli needed to correctly educate the immune system. These include a reduced exposure to helminths and to saprophytic mycobacteria, once common encounters (*Wills-Karp*, 2001; *Yazdanbakhsh* et al., 2002; *Rook* and *Rosa Brunet*, 2005a). In addition, the inappropriate use of antibiotics and changes in diets has been shown to alter the intestinal microflora and significantly affect immune responses in the host (*Noverr* and *Huffnagle*, 2004; *Rook* et al., 2004).

THE HOST IMMUNE SYSTEM

The role of the immune system is to distinguish between self, to which no response is necessary, and foreign antigens, to which a response is required and an effective defence mechanism needs to be developed. Antigen presenting cells such as dendritic cells (DC), pick up antigens, process them into immunogenic peptides and present them to naïve CD4+ T cells. Depending on the immunostimulatory molecules on the DC and the cytokine milieu at the time of activation, the naïve CD4+ T cells develop into either T-helper 1 (Th1) or T-helper 2 (Th2) cells. The Th1 and Th2 phenotypes have long been considered mutually exclusive and regulating each other (*Coffman* and *Mossman*, 1991). It is the knowledge of this reciprocal regulation which has influenced the interpretation of the epidemiological data on diseases of immunodysregulation until very recently.

It was originally thought that a lack of Th1 stimulation was responsible for the increased incidence in asthma, a Th2 cell-mediated immunopathology. This hypothesis, named the Hygiene Hypothesis was based on the observation of an inverse relation between the development of hay fever and family size. Children in large families, exposed early and more frequently to childhood infections, were less likely to develop allergic diseases (*Strachan*, 1988). This hypothesis was further supported by a number of observations which suggested lower risk of developing allergies if one was exposed to conditions favouring Th1 stimulation as it may happen when children have older siblings or attend nursery schools and are therefore at increased risk of childhood infections, when they live with a dog, when they contract infections by the oral-faecal route etc. (*von Mutius*, 2000; *Wills-Karp*, 2001). Unfortunately, this hypothesis failed to explain the increase in concomitantly rising diseases of immunodysregulation. It was the lack of Th2 stimulation which was blamed for the increase prevalence in IBD (*Elliot* et al., 2005).

To add further confusion, it was a lack of both Th1 and Th2 stimulation which was thought to play a role in the development of autoimmune conditions (*Christen* and *Herrath*, 2005).

Hence, an altered balance between Th1 and Th2 cells fails to provide an adequate explanation for the rise in diseases of immunodysregulation in developed countries. The unifying hypothesis that explains this rise is not a reduced Th1 or Th2 stimulation but rather the lack of stimuli required for the maturation of regulatory T cells (Tregs).

REGULATORY T CELLS

Tregs comprise a number of distinct CD4+ T cell populations, which are all characterized by the preferential production of the immunoregulatory cytokines IL-10 and TGF-β. These cells effectively control both Th1 and Th2 effector populations and downregulate their responses. Two major categories of CD4+ Tregs have been described so far (*Hawroylowicz*, 2005; *Stock* et al., 2006). The first are naturally occurring, are characterized by the expression of CD25 on their cell surface and are derived from the thymus. They express high levels of the transcription factor Foxp3, which is essential for their development and function. The second are called adaptive Tregs, they are antigen-specific and are induced in the periphery under particular immunological conditions.

In the context of diseases of immunodysregulation rather than a Th1/Th2 balance, the crucial factor is the balance between effector and Treg cells. In the absence of optimal levels of immunoregulation, one may develop a specific immunodysregulatory disorder based on one's Th1/Th2 bias, one's genetic background and immunological history. The critical role Tregs have in preventing the development of immunodysregulation is given additional support by the observation that a genetic defect in the transcription factor Foxp3 induces a syndrome that includes symptoms of allergic diseases, autoimmunity and IBD. Indeed, Scurfy mice and patients with IPEX/XLAAD, who have a disruption in the Foxp3 gene, develop pathologies which encompass all three diseases of immunodysregulation (*Brunkow* et al., 2001; *Wildin* et al., 2001).

So what can be done to readdress the balance between effector and Treg cells. It has been hypothesized that because of the long evolutionary association with a number of relatively harmless organisms, these are recognized by the immune system as innocuous. Hence, rather than promoting effector immune responses which not only would be useless but potentially tissue damaging for the host, they preferentially induce immune responses towards regulatory modulation. Examples of these organisms include gastrointestinal helminths, the saprophytic mycobacterium, *Mycobacterium vaccae*, and lactobacilli such as *Lactobacillus casei* and *L. reuteri*. Reports suggest that exposure to such organisms may induce unusual maturation patters in DC facilitating their ability to induce Tregs or they may induce Tregs directly (*Maizels* and *Yazdanbakhsh*, 2003; *Rook* et al., 2004, *Guarner* et al., 2006).

THE HELMINTHS

Helminths were once common parasites of man leading to chronic infection of the host. The majority of human helminthiases, barring the cases of super-infections, are relatively benign with minimal deleterious consequences. Interestingly, infected host may even be protected from subsequent challenges by the same parasite species by a mechanism referred to as concomitant immunity.

Helminth infections are associated with Th2 responses and are characterized by high levels of IL-4, IgE isotype switching, eosinophilia, goblet cells hyperplasia and mastocytosis. Although allergic diseases and helminth infection are associated with similar immunological responses, they do not appear to overlap in patients. Indeed, a number of epidemiological studies have shown that when infected with helminths, the host has a reduce risk of developing clinical symptoms of allergic diseases such as airway hyperresponsiveness, wheeze and asthma (*Yazdanbakhsh* et al., 2002). Moreover, there is a strong inverse relationship between infection with *Schistosoma spp.* and *Ascaris lumbricoides* and skin reactivity to environmental allergens (*Yazdanbakhsh* et al., 2002).

There have been a number of hypothesis put forward to explain the protective effect of helminths. It was proposed that the high polyclonal IgE levels produced during helminth infections saturate IgE receptors, FcεRI, on mast cells and blocks the binding of specific IgE to environmental allergens. This prevents degranulation and immediate hypersensitivity reactions to allergens (*Lynch* et al., 1993). Alternatively, the production of IgG4 antibodies, a Th2 dependent isotype that is characteristic of helminth infection, can inhibit IgE mediated degranulation. The hypothesis for a role for the blocking antibody IgG4 has received particular attention as patient receiving allergen immunotherapy have been shown to switch to an IgG4 phenotype (*Akdis* et al., 2006).

A hallmark feature of helminthiasis is the hyporesponsiveness that develop during chronic infections (*Pearce* and *MacDonald*, 2002). For example, hosts chronically infected with *S. mansoni* have poor antigen specific T cell responses and reduced cytokine production. The production of IL-10, TGF-β and nitric oxide has been associated with this immunosuppression, which is not limited to parasite-specific antigens but includes unrelated antigens. Indeed, the immunological responses to tetanus toxoid after tetanus vaccination in schistosome-infected patients are reduced compared to healthy controls (*Sabin* et al., 1996). It is believed that during infections, helminths induce an immunoregulatory network characterized by the induction of Tregs and the modulation of dendritic cells favouring the maturation of Tregs (*van der Kleij* et al., 2002; *Maizels* and *Yazdanbakhsh*, 2003; *Hesse* et al., 2004; *McKee* et al., 2004).

Evidence is accumulating from experimental models and from epidemiological studies confirming an immunoregulatory circuit developed during infections with helminths (*Yazdanbakhsh* et al., 2002; *Maizels* and *Yazdanbakhsh*, 2003). The ability of these parasites to downregulate host immunity thereby preventing their elimination from the host and minimizing tissue pathology can be harness to prevent diseases of immunodysregulation. The hypothesis that helminth infections downregulate allergic reactions through the action of Tregs has found experimental confir-

mation recently. In a well executed study, *Wilson* and colleagues (2005) showed that infection with the murine helminth *Heligmosomoides polygyrus* significantly reduced the inflammatory cellular infiltrate in the lungs of allergen sensitized and challenged mice. Suppression was passively transferred from infected mice to uninfected sensitized mice by a cell population containing elevated numbers of CD4+ CD25+Foxp3+ T cells. These cells were characterized by high TGF-β expression and by IL-10 production. These data support the hypothesis that helminth infections elicit Tregs able to downregulate allergen induced lung pathology *in vivo*.

THE INTESTINAL MICROFLORA

The intestinal microflora may play a critical part in the regulation of immune responses in the host.

Alteration to the microflora has been associated with a number of immunological effects (*Noverr* and *Huffnagle*, 2005; *Guarner* et al., 2006). For example, germ-free mice have few intra-epithelial T and B cells, have smaller Peyer's patches, lower levels of IgA and most significantly are unable to develop oral tolerance. In spite of these effects, until recently the role of the microflora in diseases of immunodysregulation was neglected by investigators. There is now an increasing amount of epidemiologic and clinical data to suggest that alterations to the intestinal microflora predisposes to the development of these disorders. For example, the development of allergic diseases has been shown to correlate with the use of antibiotic early in life, altered faecal microflora and dietary changes (*Noverr* and *Huffnagle*, 2004, 2005).

Bacterial colonization of the intestine starts soon upon delivery and involves a succession of microbial populations which change with the infant's diet changes and development. By adulthood, the microflora which normally comprises for the most part anaerobes (97%) and only a small percentage of aerobic/facultative anaerobes (3%) is generally stable and is composed of both permanent residents and transient colonizers, introduced with food. Whereas approximately 30-40 species predominate, 400 to 1000 may be present in the intestine including among the anaerobes *Bacteroides, Bifidobacterium, Eubacterium, Fusobacterium, Clostridium* and *Lactobacillus*, among the facultative anaerobes *Escherichia coli*, *Salmonella* spp., *Enterococcus*, *Staphylococcus* and *Streptococcus* and even some fungi such as *Candida albicans*.

Epidemiological studies have reported alterations to the microflora of allergic individuals compared to healthy controls (*Kalliomaki* and *Isolauri*, 2003). Allergic states are associated with increased levels of aerobic and lower levels of anaerobic microbes. In particular, decreased levels of lactobacilli and bifidobacteria have been reported in children with allergies and atopic eczema (*Noverr* and *Huffnagle*, 2005). In a mouse model of ovalbumin-induced allergic pulmonary inflammation, alteration to the microflora though treatment with antibiotic exacerbated the development of allergic airway responses following allergen challenge (*Novarr* et al., 2005). Treatment with antibiotics and a single oral gavage of *Candida albicans*, altered the microflora with an increased fungal component persisting for 3 weeks. Treated mice were then chal-

lenged intranasally with ovalbumin. In the absence of systemic priming, mice with altered microflora developed vigorous airway allergic responses to ovalbumin. Pulmonary eosinophilia, goblet cells metaplasia, and levels of IgE, IL-5 and IL-13 were all significantly increased. In contrast, mice with unaltered microflora did not develop an allergic response following intranasal challenge. These studies suggest that changes to the intestinal microflora can break airway tolerance to an aeroallergen (*Noverr* et al., 2005).

It has been hypothesized that the microflora plays a role in promoting the development of Tregs that can downregulate Th2 responses developed in the airways. The gut mucosa has long been considered an ideal environment for the development and maintenance of Tregs (*Coombes* et al., 2005). Antigens acquired by DC in an anti-inflammatory environment such as in a microflora-balanced healthy intestine preferentially stimulate the generation of Tregs. Indeed, exposure to specific members of the intestinal microflora such as *L. casei* and *L. reuteri* has been shown to prime DC to promote the development of Tregs. The effect of these lactobacilli on the Th-cell polarizing capacity of DC was investigated *in vitro* by stimulating naïve T cells with the superantigen *S. aureus* enterotoxin B, in the presence of DC matured with these microbes. It was shown that *L. casei* and *L. reuteri* modulate DC function and prime for induction of IL-10- producing Tregs which are able to suppress T cell proliferation (*Smits* et al., 2005). Hence, a disruption to the intestinal microflora may lead to the development of inflammatory signals so that when inhaled/swallowed allergens are acquired by DC, these cells fail to induce Tregs because of altered balance in immune stimuli (*Noverr* and *Huffnagle*, 2004, 2005).

THE SAPROPHYTIC MYCOBACTERIA: *MYCOBACTERIUM VACCAE*

Saprophytic mycobacteria comprise at least 80 species, which are ubiquitous in mud and untreated waters. Exposure to mycobacteria has been a common occurrence throughout human evolution. Evidence for this shared evolutionary past is clearly indicated by the presence of a subset of T-cell which are CD1-restricted and appear to recognize only mycobacterial lipid and glycolipid (*Dutronc* and *Porcelli*, 2002). Exposure to mycobacteria has been significantly limited in developed countries where concrete and chlorinated waters have limited bacterial contact. Indeed, whereas the majority of individuals in developing countries respond positively to skin test to soluble antigens from environmental mycobacteria, only few individuals in developed countries do (*Rook* and *Rosa Brunet*, 2002).

Because of the long evolutionary association with saprophytic mycobacteria, it has been hypothesized that they are recognized by the innate immune system of the host as harmless. Therefore rather than mounting aggressive immune responses to organisms that are either non-pathogenic or only lead to self-limiting infections, the host responds to immunostimulatory clues from these organisms by developing immunoregulation. This hypothesis has been called the "The Old Friend hypothesis" and encompasses also helminthes and lactobacilli (*Rook* et al., 2003). Triggering mechanisms that induce immunoregulation through the exposure to Old Friends may be ex-

ploited therapeutically to prevent diseases of immunodysregulation. Evidences to support this hypothesis have been collected from a number of studies. Treatment with either live or dead mycobacteria inhibits allergen induced lung pathology in experimental models of allergic inflammation (*Walker* et al., 2003).

M. vaccae, a saprophytic mycobacteria isolated from mud of lake Kyoga in Uganda, has shown particular therapeutic promise. In experiments using heat killed *M. vaccae*, subcutaneous treatment was able to induce Tregs which suppressed allergen-mediated inflammatory responses. Treatment reduced the severity of pulmonary inflammation in mice sensitized and subsequently challenged with ovalbumin. Protection was long-lasting and associated with the development of CD4+ Tregs rather than with a Th1/Th2 phenotype switch (*Zuany Amorim* et al., 2002a,b; *Adams* et al., 2004). Transfer of *M. vaccae*-induced Tregs into allergen-sensitized mice prior to allergen challenge reduced subsequent pulmonary eosinophilia in an allergen-specific manner. This inhibition was shown to reside within the $CD45RB^{low}$ CD4+ T cell subset and was dependent on IL-10 and TGF-β. Treatment with neutralizing anti-IL-10 and anti-TGF-β antibody reversed the inhibitory effect of the $CD4+CD45RB^{low}$ population.

The effects of *M. vaccae* are not limited to the induction of Tregs. *M. vaccae* was shown to act also on pulmonary APC such as CD11c+ DC. These cells have received renewed attention as they have been shown to produce IL-10, stimulate development of Tregs, mediate airway tolerance and prevent the development of airway reactivity and asthma (*Akbari* et al., 2001). Treatment with *M. vaccae* correlated with the development of CD11c+ cells which express high levels of the immunoregulatory cytokines IL-10, TGF-β and IFN-α (*Adams* et al., 2004). It is hypothesized that because the development of Tregs is likely to be dependent on the maturation state of DC, influencing development of these cells is an additional strategy for immunoregulation.

Initial clinical studies investigating the therapeutic benefits of *M. vaccae* have been promising. A single intradermal injection of *M. vaccae* was administered to atopic individuals with asthma. Whereas treatment is usually well tolerated, a small scar may develop at the site of injections in some individuals. Patients were evaluated 3 weeks after treatment. Treatment with *M. vaccae* reduced the late phase response to inhaled allergens as well as serum levels of IgE and *in vitro* production of IL-5. However, none of these effects reached statistical significance (*Camporota* et al., 2003). A larger multicentre, phase II, randomized placebo-controlled study was conducted but was severely hampered by a large placebo effect. Treatment failed to improve mean weekly asthma symptoms as recorded in the patients' diary card (primary efficacy analysis), even though there was a trend towards significance. In spite of these initial disappointing findings, exploratory analysis did reveal a significant beneficial effect of treatment. When the data were adjusted for variation between treatment groups at baseline, patients receiving two doses of *M. vaccae* 6 weeks apart showed a significant reduction in asthma symptom scores and a significant decrease in asthma exacerbations. Additional studies to ascertain the therapeutic benefit of *M. vaccae* are required.

During evolution, the most likely route of exposure to *M. vaccae* was oral. A study to determine whether oral delivery of *M. vaccae* was effective in

reducing symptoms of pulmonary allergic inflammation revealed that the therapeutic effects were retained regardless of the delivery route. The intestinal epithelium is easily penetrated by mycobacteria whose size and hydrophobicity are ideal for uptake by M cells and Peyer's patches, leading to an interaction with the mucosal immune system. Once again the beneficial effects of *M. vaccae* are not associated with the development of Th1 responses but rather with the preferential induction of immunoregulatory cytokines such as IL-10 (*Hunt* et al., 2005). It is anticipated that exposure of the gut mucosa to *M. vaccae* may further facilitate the induction of Tregs as this site as long been considered an ideal environment for the development and maintenance of Tregs. These observations raise exciting new clinical possibilities. The ease of administration and the lack of scarring may be a preferred alternative to intradermal injection of *M. vaccae*.

CONCLUSIONS

It is here proposed that the reduced exposure to Old Friends is to blame for the significant increase in prevalence of diseases of immunodysregulation that has been observed in the last few decades. Old Friends are defined as those organisms that in virtue of their long standing evolutionary association with the human host, rather than inducing effector immune responses promote the development of immunoregulation. Among these, intestinal helminths, lactobacilli and *M. vaccae* feature prominently. Experimental evidence and clinical data is accumulating in support of the hypothesis that exposure to these organisms may facilitate the induction of Tregs by influencing the maturation of DC (*Maizel* and *Yazdanbakhsh*, 2003; *Rook* et al., 2004, *Guarner* et al., 2006).

It is critical that we learn to harness the immunoregulatory potential of these Old Friends (*Rook* et al., 2003; *Rook* and *Rosa Brunet*, 2005b). In order to readdress the balance of the immune system in terms of effector and Treg cell, the reintroduction of Old Friends in our modern living conditions is advisable. Whether as vaccinations to evoke specific mechanisms of immunoregulation or as oral supplements, this novel approach may offer therapeutic potential for the treatment of diseases of immunodysregulation.

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RATIONALE FOR THE SEMINAR: FOOD, INTESTINAL IMMUNE SYSTEM AND GUT MICROBIOTA

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THE OHUS 2006 SEMINAR

Food components, the gut microflora and the gastrointestinal immune system are in a constant interaction with each other. This continuous interference is generally referred to as the golden triangle as the interaction is considered crucial for intestinal health, proper and efficient immune protection, and physical well-being. This interaction can be boosted by food components that stimulate gut immune defence, and qualitative and quantitative aspects of the gut microflora. Commonly suggested food components with immune enhancing properties are pre- and probiotics, functional foods, nutraceuticals, etc. On the other hand, improper functioning of this golden triangle can result in chronic and persistent disease. Many of such diseases have involvement of an improper functioning gut. Based on such considerations the concept of immunomodulation has become important in recent years. The underlying hypothesis is that via selected food components, the functioning of the golden triangle can be influenced, leading to a persistently better functioning gut. Therefore, immunomodulation has become a leading principle to look for food constituents having such capacities. To better understand the current insights on mechanism of action, the search for useful biomarkers to detect immunomodulatory capacity *in vitro* and *in vivo* and the rational design of food components with these properties that could be added to the diet, we have organized this OHUS 2006 seminar.

IMMUNOMODULATORY FOOD COMPONENTS AS BIOFUNCTIONAL INGREDIENTS

Biofunctional ingredients are compounds that are present in or added to foodstuffs, and that have a health-promoting effect for the human body. The mechanisms underlying these positive effects can be very diverse, but generally they are strongly related to hot-button health issues like prevention of cardiovascular diseases, cancer and cara, enhanced well-being, longevity, *etc.* Examples are isoflavones, lignans, glucosinolates, sterols, and conjugated linoleic acid. For this proposal, biofunctional ingredients are distinguished from pro- and prebiotics. Probiotics are live microorganisms that are considered beneficial for humans. Prebiotics (or non-digestible oligosaccharides) are in principle a food supply for the beneficial bacteria in the human colon. Although pro- and prebiotics also have health-promoting effects for the human body and are added to food systems, we do not consider them as biofunctional ingredients here.

People are moving away from medical solutions to nutritional and lifestyle solutions. Supplementation of biofunctional ingredients in food, or improving the wholesomeness of food

(such as dairy products, breads, beverages, snack foods, condiments), is preferred over taking pills. With the escalating costs of health care due to a rapidly aging population biofunctional ingredients are expected to gain importance. Epidemiological and experimental studies have shown a relationship between important health issues and a high consumption of particularly vegetables and fruits. In some cases, compounds contributing to the health-promoting effect have been identified. However, there is a general belief that not a single component is responsible for this effect, but that the secret is actually in the overall composition of the mixture of components. The interplay between various food components clearly deserves much more attention than it has received until now.

The genetic constitution and age of a person determines to a large extent its health and sensitivity to illness. However, little is known on which food components trigger particular processes in the human body. The complexity of "food signalling" increases tremendously by the presence of the intestinal microflora. The total combined genome size of intestinal bacteria surpasses that of humans many times, indicating the enormous potential to modulate the functionality of food components here (both positively and negatively). An example of this is the conversion of plant isoflavones to equol. Equol is a much more potent ligand for the human oestrogen receptor than plant isoflavones; its estrogenicity is similar to that of (human) estradiol. However, it is important to note that (i) this conversion takes place in only approximately 15% of the human population, and (ii) this bioactive compound can be degraded by (other) microorganisms in the colon. This means that the potential of this bioactive compound in foodstuffs is used very inefficiently. Therefore, it will be advantageous to produce such compounds on an industrial scale, and add them as an ingredient to food stuffs, so that they can be absorbed in the small intestine, before arrival in the colon. It is important to verify that the new biofunctional ingredient is compatible with the food system in which it is incorporated. Solubility, stability, bioavailability and sensoric aspects should be taken into account.

During processing and storage of food (ingredients), many chemical conversions can take place, depending on the conditions used (temperature, pH, oxygen supply, *etc.*). As a consequence, the fine structure of desirable compounds may be altered. In some cases, their functionality may be coincidentally lost. It is then necessary to adjust the processing conditions. In other cases, the compounds are deliberately altered to enhance their functionality. Often, this is done by application of commercially available enzyme preparations (*e.g.* removal of sugar residues to improve bioavailability) or functional fermentations. With respect to improving health, the latter hold an enormous potential; health-promoting substances like arachidonic acid and β-carotene can be produced very efficiently using microorganisms. Fermentation of whole foods is also used to improve digestibility and utilization. Different kinds of analytical techniques (including HPLC, mass spectroscopy, *etc.*) will be employed to monitor structural changes during processing. With respect to functional fermentations, high-throughput techniques will be used to screen for microorganisms capable of performing desirable conversions, and to identify (and ultimately improve) the key enzymes involved in these reactions. Facilities to purify large amounts of the desired compounds are available.

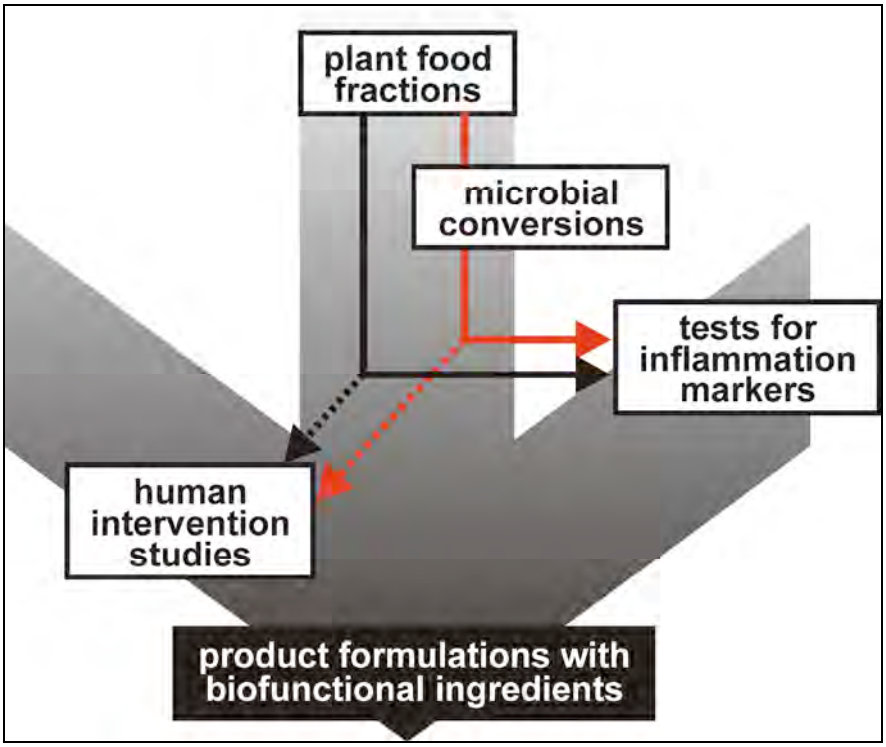

Figure 1: Schematic representation to arrive at immunomodulatory components for use in the treatment of human diseases.

Intake of vegetables and fruits is generally regarded as beneficial for human health, but the intake in Western European countries is low as compared to *e.g.* Southern Europe. Many of the Western diseases or discomforts, such as heart disease, cancer, inflammatory bowel disease, rheumatic arthritis are in one way or another related to chronic inflammation in specific target organs, like lungs (asthma), pancreas (diabetes), peripheral nervous system (multiple sclerosis), gut (morbus Crohn), skin (psoriasis), *etc.* Fruits and vegetables contain numerous beneficial components, and therefore the population in the western world is advised to increase their consumption. Over the years, the success of this advice has lost its persuasive force, and opportunities to improve health and prevent disease are sought more and more in supplementation of foods (such as dairy products, breads, beverages, snack foods, condiments) with these beneficial components as biofunctional ingredients. With the escalating costs of health care due to a rapidly aging population biofunctional ingredients are expected to gain importance. The causal factors for health-promoting effects of fruits and vegetables remain in many cases unknown, and it has been postulated that the mixture of compounds is responsible, rather than any one single component. Research strategies to identify mixtures of compounds, and to convert these to functional ingredients in product development, are currently lacking for this important group of plant foods. The intention of this project is to iden-

tify which specific compounds, or which combination of compounds, in vegetables and fruits play a protective role in persisting inflammatory reactions.

It is recognized that food components can be subjected to extensive microbial conversions during their passage through the intestinal tract. The total combined genome size of intestinal bacteria surpasses that of humans many times, underlining the enormous potential to modulate the functionality of food components. Examples showing that plant components can be converted by microbiota to more bioactive molecules are known, and their number is increasing rapidly. On the other hand, there are also examples indicating that the bioactive potential of plant components can be destroyed by intestinal bacteria. Therefore, specific focus will be on microbial conversions of plant-derived fractions, i.e. all fractions will be incubated with faecal slurries and tested for bioactivity as described for the untreated fractions.

Most research on the health-protecting properties of biofunctional ingredients is based on analyses with more or less purified compounds. However, their effectiveness in health protection is strongly dependent on so-called matrix-interactions: i.e. their interaction with the entire of other compounds in the food product and the physico-chemical environment in which they are embedded, in particular proteins, carbohydrates, fats/oils. The effect of processing conditions on the activity of biofunctional ingredients from processed food has been investigated to only a limited extent. For instance, it has been found that commercially processed seaweed had a lower antioxidant activity than fresh samples. This indicates that it is very important to investigate the remaining functionality after processing of food. Nevertheless, the number of interaction studies of biofunctional ingredients, particularly in relation to processing, is very limited. To our knowledge, no information is available on the interaction of such ingredients with components in beverages and milk products, as well as in more severe processed snacks. Another important aspect is that supplementation of food products with biofunctional ingredients should not negatively influence the sensory attributes of the products. The food industry requires insight in the so-called matrix-interaction in order to market attractive products with the appropriate biofunctional ingredients in its essential form.