Old Herborn University Seminar Monograph

16. HOST MICROFLORA CROSSTALK

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Old Herborn University Seminar Monograph 16

ISBN 3-923022-27-1
ISSN 1431-6579

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Germany
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THE GUT IMMUNE SYSTEM AND THE MUCOSAL BACTERIA

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SUMMARY

The gut associated immune system harbours the vast majority of all lymphoid cells in the human body. This corresponds to the fact that most antigens reach us via the gut. The mucosa of the upper airways and the gastro-intestinal tracts also host a rich normal bacterial flora, which serves as a stimulus to the immune system. There are many indications that the normal intestinal microflora affects the way in which other antigens, e.g. food antigens and other innocuous antigens, are handled. Thus, alterations in intestinal colonisation pattern might have predisposed for allergies and other hypersensitivity reactions. In this review, the immune system of the gut and its relation to the normal intestinal microflora will be discussed.

IgA

The lamina propria is seeded with as many as 10^{10} antibody producing cells/m of small intestine. Most of these are plasma cells that produce dimeric IgA, i.e. two IgA monomers held together by the polypeptide “joining chain” (*Brandtzaeg*, 1994). These IgA dimers bind to secretory component, also termed “polymeric immunoglobulin receptor”, a trans-membrane protein that is exposed on the basolateral aspect of intestinal epithelial cells. The entire complex is transported through the epithelium to the lumenal side, where the transmembrane part of secretory component is cleaved off. The largest part of secretory component remains bound to IgA, together forming the secretory IgA complex (*Mestecky* et al., 1999).

The secretory IgA molecule is specially designed to afford protection on surfaces populated by microbes. It is hydrophilic and highly resistant to proteolysis, much due to its rich substitution with carbohydrate chains (*Brown* et al., 1970; *Underdown* and *Dorrington*, 1974). Secretory IgA efficiently prevents microbial attachment to host mucosal structures (*Freter*, 1969; *Williams* and *Gibbons*, 1972; *Svanborg-Edén* and *Svennerholm*, 1978) and, hence, strongly reduces translocation (*Albanese* et al., 1994; *Maxson* et al., 1995; *Dickinson* et al., 1998). Secretory IgA does not activate complement (*Russell* et al., 1997) and bacteria coated by secretory IgA are not killed. In contrast, IgA can counteract the inflammatory potential of IgG or IgM antibodies that are bound to the same target.

It is known that many of the commensal microbes in the large intestine are coated by IgA, which does not seem to affect them negatively (*van Saene* and *van der Waaij*, 1979; *van der Waaij*, 1996). In fact, IgA coating might even be advantageous to commensal bacteria. The carbohydrate chains of secretory IgA function as receptors for the mannose-specific adhesin of type 1 fim-

briae, the most common adhesin of *E. coli* and other enterobacteria (*Wold* et al., 1990). A complete lack of IgA in serum and secretions constitutes is the most common primary immunodeficiency, afflicting some 1/500 individuals, two thirds of whom are healthy. We have investigated the effect of absence of IgA in secretions on the commensal *E. coli* flora. In fact, individuals who lack IgA have a lower proportion of type 1-fimbriated *E. coli*, compared to age-matched controls, and the *E. coli* strains retrieved from IgA-deficient individuals also expressed less of the mannose-specific adhesin compared to *E. coli* from the control individuals (*Friman* et al., 1996; *Friman* et al., 2002). This suggests that the interaction between bacteria and secretory IgA is advantageous to the bacteria.

T CELLS

T cells are found in two compartments in the intestinal mucosa: in the lamina propria and between the epithelial cells (so-called intra-epithelial lymphocytes).

The T cells found in the villus lamina propria are mainly CD4-positive and display signs of activation (*deMaria* et al., 1993; *Schieferdecker* et al., 1992). They do not respond with proliferation to mitogens and other stimuli, indicating that they are terminally differentiated. They secrete cytokines spontaneously, especially interferon-γ (*Hauer* et al., 1997, 1998). It appears as the micromilieu in the mucosa favours development of a certain T cell phenotype. Transgenic T cells with the same antigen specificity produce predominantly IL-2 in the spleen and Peyer's patches, but IFN-γ or IL-10 in the gut *lamina propria* (*Saparov* et al., 1997). The function of *lamina propria* T cells under physiologic conditions is unknown.

The intra-epithelial cells are mainly of the CD8+ phenotype. They carry either the αβ or γδ type of antigen receptor. The exact function of the intraepithelial cells is unknown, but they can synthesise IL-2 and IFN-γ (*Lundqvist* et al., 1996) and lyse virus-infected cells (*Cebra* et al., 1989). Their T cell receptors are oligoclonal suggesting that the entire population derives from a limited set of T cell clones (*Blumberg* et al., 1993).

INDUCTION OF MUCOSAL IMMUNE RESPONSES

Intestinal immune responses are induced in the Peyer's patches, which are mucosal lymphoid nodules situated in the wall of the small intestine (*Craig* and *Cebra*, 1971). The patches are covered by a specialised epithelium, the follicle-associated epithelium. This epithelium contains specialised epithelial cells, termed M-cells, which are specialised in transporting material from the lumen into the patches, without degrading it. M-cells lack brush borders and the enzymatic machinery of the absorptive epithelial cell (*Neutra* et al., 1996). In the patches, particulate and soluble antigens are degraded and presented by macrophages and dendritic cells. T cells and B cells with the appropriate specificities proliferate, mature and leave the patches via the efferent lymph. After circulating in the blood for a few days, they return to the intestine, but not to the

Peyer's patches, but to the *lamina propria* of the intestine, and, to a lesser extent, to other mucosa. This process is termed "homing" (*Craig* and *Cebra*, 1971).

IMPORTANCE OF GUT FLORA ON THE SPECIFIC IMMUNE SYSTEM

The majority of all lymphoid cells in the gut are there because of the normal intestinal microflora. Germfree animals have only one tenth as many IgA-producing cells and T cells in the intestinal *lamina propria* as conventional animals (*Crabbé* et al., 1968, 1970; *Hashimoto* et al., 1978).

Mucosa not regularly colonised by microbes, for example the respiratory and urinary tracts, have comparatively more IgG in their secretions compared to the upper respiratory and gastrointestinal tracts (*Reynolds*, 1988; *Svanborg Edén* et al., 1985). IgG activates complement, thereby lysing bacteria but also eliciting inflammation. Thus, the continuous presence of a normal intestinal microflora in the upper respiratory tract and gut seems to promote development of immune effector functions that are non-inflammatogenic and mainly prevent too close contact between the commensal microbes and the host. How this regulation occurs is not known. In mice, it appears as if switch from IgM can occur in the lamina propria in the absence of T cell help (*Fagarasan* et al., 2001), but whether this is true in humans is unclear (*Brandtzaeg* et al., 2001).

When germfree animals are colonised by a normal flora, lymph nodes and Peyer's patches increase in weight and germinal centres develop, the serum IgG concentration rises and antibodies appear towards the colonising microorganisms (*Carter* and *Pollard*, 1971). These antibodies may often cross-react with other bacteria, and non-bacterial structures. For example, the "natural antibodies" directed against blood group antigens, that were thought by Landsteiner to occur spontaneously as part of the normal physiologic development, have been shown to result from immunisation by gut microbes in the normal flora (*Wiener*, 1951; *Springer* and *Horton*, 1969; *Scheffel* and *Kim*, 1979).

Both live and dead bacteria can induce mucosal immune responses, but live, colonising bacteria are better than dead ones, supposedly because more antigen is delivered to the immune system with a bacteria which replicates in the intestinal tract (*Hohmann* et al., 1979). Provided, however, that high enough doses were given over long enough periods of time, killed *E. coli* or *Bacteroides* can induce equally large amounts of IgA-containing plasma cells as colonisation by live bacteria of the same species (*Moreau* et al., 1978). The best inducers of antibody responses are bacteria, which are able to invade the mucosa, because larger doses of bacterial antigens will come into contact with the immune system (*Hohmann* et al., 1979). This raises the question whether bacteria in the intestinal lumen which are not coated by IgA avoid this type of immune response simply because they are not able to translocate and, hence, they may not be "seen" by the immune system. Anaerobes usually do not translocate (*Berg*, 1983), while live facultative bacteria can regularly be found in the mesenteric lymph nodes provided that their population levels in the large intestinal microflora reaches a certain level (*Wells* et al., 1987; *Berg* et

al., 1988; *Herías* et al., 1995, 1997).

Moreau and co-workers attempted to determine whether certain types of intestinal bacteria were better than others in triggering the mucosal immune system. She colonised germfree mice with a range of Gram-positive and Gram-negative bacteria and measured the density of IgA-containing plasma cells in the intestinal lamina propria. The best inducers of IgA plasma cells were *E. coli* and *Bacteroides*, while all tested Gram-positive species were inferior (*Moreau* et al., 1978). Quite to the contrary, Cebra and co-workers found most IgA to be produced in response to colonisation by Gram-positive *Listeria monocytogenes* or segmented filamentous bacteria, while the Gram-negative *Morganella morganii*, *Ochrobacterium atrophii* and *Helicobacter muridium* all gave less IgA stimulation (*Cebra*, 1999 and personal communication).

Intraepithelial cells carrying the αβ type of receptor increase in numbers in response to bacterial colonisation, while the γδ type does not (*Kawaguchi* et al., 1993). However, the γδ type of lymphocytes produces IFN-γ in response to lumenal bacteria, which in turn upregulates the expression of MHC class II molecules on intestinal epithelial cells (*Matsumoto* et al., 1999). The oligoclonality of intraepithelial cells does, however, not seem to be determined by distinct bacterial antigens, since both germfree and conventional mice exhibit such restricted clonality (*Regnault* et al., 1996).

People ingesting probiotic bacteria exhibit activation of cell-mediated immune effector functions, such as enhanced phagocytosis and secretion of IFN-γ by blood lymphocytes and IFN-α by blood mononuclear cells (*Wold*, 2001). In addition, immunoglobulin-producing cells with specificity against the administered strain appear in the blood (*Wold*, 2001).

THE TRANSIENT NATURE OF THE RESPONSE TO GUT BACTERIA

When a bacterial strain successfully colonises the intestine and reaches numbers high enough to permit translocation, germinal centres are formed in the Peyer's patches, B cells committed to IgA production seed the mucosa and secretory IgA is produced into the intestinal lumen. However, this immune response is self-limiting, in that the secretory IgA so produced, coats the bacteria in the intestinal lumen, preventing further translocation and, hence, stimulation of the gut lymphoid tissue (*Shroff* et al, 1995). Despite the continued presence of the microbe in the gut flora, there will be no, or only minimal, further stimulation of the gut associated lymphoid tissue (*Shroff* et al, 1995).

Because of this phenomenon, a persistent activation of the mucosal immune system requires a high turnover of bacterial strains in the microflora. In accordance, Pakistani infants, who are colonised by a never-ending succession of new enterobacteria (*Adlerberth* et al., 1991, 1998) have higher secretory IgA levels in their saliva, and higher anti-*E. coli* antibody levels than Swedish infants of the same age (*Mellander* et al., 1995). Bottle-fed infants, who have a more varied and less stable microflora than breast-fed infants and probably encounter more translocated bacteria, display signs of increased immune responsiveness (*Wold* and *Adlerberth*, 2000).

IMMUNE RESPONSE TO FOOD PROTEINS

Food antigens provide little stimulation to the immune apparatus. Although an estimated 0.01 to 0.1% of ingested food proteins are taken up into the circulation in an intact, theoretically fully immunogenic form (*Husby* et al., 1985a), the immune response to food proteins in humans is limited to low levels of serum antibodies of the IgG4 and IgG2 subclasses (*Husby* et al., 1985b). These subclasses are poor in fixing complement and interacting with phagocytes. Development of antibody responses dominated by IgG1 and IgG3, antibody isotypes with strong inflammatogenic properties, may result in food intolerance reactions (*Saalman* et al., 1995, 2001).

Accordingly, serum IgA and IgG are only slightly increased in germfree mice fed a commercial rat diet compared with those fed an "antigen-free" extensively hydrolysed liquid diet, but much lower than in conventional mice (*Hashimoto* et al., 1978). One must also bear in mind that even the sterilised feed given to germfree animals is contaminated by endotoxin and other bacterial components (*Midtvedt* and *Gustafsson*, 1981), which may contribute to this low-grade immune stimulation. Rats fed very high doses of protein antigens form serum IgG antibodies, but no secretory IgA response (*Peri* et al., 1982; *Wold* et al., 1987, 1989). Hence, food proteins lack the features that enable strong immune responses to develop. When a plasmid encoding production of the food protein ovalbumin was cloned into *E. coli* and this strain is used to colonise germfree rats, secretory IgA antibodies against ovalbumin were produced (*Dahlgren* et al., 1991).

ORAL TOLERANCE

Exposure of the mucosal immune system to food antigens normally results in development of specific immunological tolerance to these proteins. This means that if the food antigen is later administered systemically, it will evoke less of an immune response than it would in an individual who had not been fed the protein. Thus, local swelling will not be elicited by intradermal injection of the antigen (so called delayed-type hypersensitivity, which is a sign of the presence of memory T cells to the antigen), and T cells taken from blood or lymph nodes will fail to proliferate when stimulated with the same antigen *in vitro*. IgE-mediated hypersensitivity reactions do not develop. There may also be a weaker and/or more short-lived serum IgG antibody response to the antigen after systemic administration than in non-fed individuals. The original observations of oral tolerance were made in guinea pigs (*Wells*, 1911; *Chase*, 1946), but most of the work on tolerance has thereafter been done in rats and mice (*Thomas* and *Parrot*, 1974; *Hanson* et al., 1977). More recently it was also shown that humans who ingest KLH develop T cell tolerance to this protein, although the antibody response was intact (*Husby* et al., 1994). Oral tolerance may be seen as a way to economise the resources of the immune system by avoiding to react to a wealth of innocuous antigens entering via the mucosal membranes. Oral tolerance also protects us from dangerous inflammatogenic responses that would destroy mucosal architecture and func-

tion. Allergies and hypersensitivity reactions may be seen as a failure of the individual to develop and/or maintain tolerance to environmental, innocuous antigens.

MECHANISMS FOR ORAL TOLERANCE

Despite decades of research, it is still very unclear where and how oral tolerance is induced and by which mechanisms immune responses are suppressed in the tolerant animal or human being (*Smith* et al., 2000). Oral tolerance may operate through at least two different mechanisms: Anergy and suppressor (regulatory) T cells.

Anergy means that T helper cells are paralysed when they encounter their antigen on an antigen-presenting cell which does not simultaneously deliver the activating signals that the T cell needs in order to proliferate and mature to an effector cell. These so-called costimulatory signals may be T cell activating cytokines (for example IL-1 and IL-12), but also a direct binding between the antigen-presenting cell and the T cell via so called accessory molecules. Both T cell activating cytokines and accessory molecules are produced when antigen-presenting cells are exposed to microbial products. Thereby, all antigens in or on microbes will be presented in a highly immunogenic fashion. Food proteins, on the other hand, lack the capacity to elicit such signals in the antigen-presenting cell, because they possess no "danger signals". T cells that encounter their antigens in the absence of activating signals during antigen-presentation may be paralysed or even receive a death signal.

Suppressor, or regulatory, T cells are formed somewhere in the gut-associated immune system. They are antigen-specific and become activated by feeding the specific antigen. However, instead of helping other T cells, they will suppress their function by mechanisms yet to be defined – secretion of IL-10 and/or TGF-β, as well as contact-mediated mechanisms have been described (*Smith* et al., 2000). Suppressor cells induced in an animal fed a protein antigen may be transferred into a naïve recipient and suppress immune responses in this animal. Nothing is known about the requirements for inducing T-suppressor cells, but one might speculate that the gut mucosa provides a suitable environment for the maturation of T-suppressor cells.

A very interesting new model suggests that the intestinal epithelial cell plays a key role in oral tolerance (*Karlsson* et al, 2001). It has been known for a long time that serum from an animal fed a tolerogenic dose of a protein antigen can be transferred to a naïve animal, which will become tolerant to that protein without having eaten it. The serum factor appears a few hours after feeding. Telemo has suggested that the serum factor consist of membrane fragments, so called "tolerosomes" produced by the intestinal epithelial cell. According to this model, intact protein is taken up by intestinal epithelial cells, processed to peptide fragments and loaded onto MHC class II molecules within the epithelial cell. Membrane vesicles are then budded off from the baso-lateral facet of the enterocyte which on their surface carry MHC class II molecules with loaded peptides. These membrane vesicles may disperse themselves in the whole body via the lymph and blood stream and merge with membranes of antigen-presenting cells in the mucosa, liver, or lymph nodes. Supposedly, these vesicles contain information that ensures that the antigen is presented in a non-immunogenic, tolerogenic, fashion.

In addition to the above two mechanisms, antigen non-specific anti-inflammatory signals may down-regulate immune effector functions. For example, whether a delayed-type hypersensitivity reaction develops or not depends not only on the presence of memory T cells, but also on the local conditions in the skin where the antigen is injected. In the presence of anti-inflammatory cytokines or other mediators, recruitment of T cells to the site of antigen deposit may be counteracted. Many individuals, especially those living in developing countries with a high infectious burden, have high levels of specific IgE antibodies to environmental antigens, such as mites, and mount a wheal-and-flare reaction if mite antigen is injected in the skin. However, they do not have clinical symptoms of allergy, probably due to the existence of active anti-inflammatory mechanisms (*Yazdanbakhsh* et al, 2002).

THE NORMAL MICROFLORA AND ORAL TOLERANCE

Oral tolerance is more short-lived in germfree compared to conventional animals (*Moreau* and *Courthier*, 1988). In conventional animals, administration of cholera toxin or *E. coli* heat labile toxin breaks oral tolerance to food antigens (*Elson* and *Ealding*, 1984; *Gaboreau-Routhiau* and *Moreau*, 1996). Thus, it is clear that bacteria or their products profoundly interfere with responses to food and other environmental antigens. It is possible that the gut microenvironment, especially in the presence of the correct intestinal microflora, provides a milieu where it is difficult to activate T cells because the antigen-presenting cells in the gut do provide much of co-stimulatory signals. This might have to do with antigen processing. Accordingly, antigen presenting cells from germfree mice are stronger stimulators of naïve T cells than antigen presenting cells from conventional animals, and the greatest T cell activation is seen when the antigen presenting cells derive from germfree animals which are fed an antigen free liquid sterile diet (*Hooper* et al., 1995). A range of products secreted by macrophages in response to bacterial products have been shown to decrease the T cell stimulating capacity of dendritic cells, for example the cytokines TNF-α (*Holt* et al., 1993) and IL-10 (*Koch* et al., 1996), the prostaglandin E_2 (*Chouiab* et al., 1985), and nitrous oxide (*Holt* et al., 1993). Thus, animals from which alveolar macrophages have been removed display greatly enhanced immune responses to inhaled antigens (*Holt* et al., 1993). Similarly, depletion of macrophages from a preparation of dendritic cells from gut lamina propria also enhances their antigen presenting ability (*Pavli* et al., 1990).

INFLUENCE OF THE COMMENSAL FLORA ON INNATE IMMUNITY

Antigen presentation represents the crossroads between the ancient innate and the more modern acquired immune system. The innate immune system reacts in a fixed fashion to certain molecular constellations that present "danger" to the host, i.e. molecular patterns that are only found in prokaryotes. The acquired immune system can be activated by any structure, because the

variable regions of antibodies and T cell receptors have endless variability. However, because antigen-presenting cells, which are monocytes, macrophages or dendritic cells, belong to the innate immune system, they have kept the tendency to become activated by microbial products. When they digest microbes and simultaneously present the antigens contained therein to T cells, they will convey activating signals to the T cells. In this way, bacteria function as their own adjuvans.

There is no question that the normal intestinal microflora substantially influences the entire innate immune system (*Wold* and *Adlerberth*, 2000). Peritoneal macrophages from conventional animals have increased levels of cyclic AMP and lysosomal enzymes, phagocytose more avidly, secrete more of oxygen radicals, and display enhanced cytotoxic activity, compared to macrophages obtained from germfree animals (*Meltzer*, 1976; *Johnson* and *Balish*, 1980; *Podroprigora* et al., 1980; *Morland* and *Midtvedt*, 1984; *Mitsuyama* et al., 1986). Spleen and bone marrow macrophages from conventional animals produce more IL-1, IL-6 and TNF-α when stimulated by LPS than macrophages from germfree animals (*Nicaise* et al., 1993, 1995). Mono-colonisation of germfree animals with *E. coli*, but not bifidobacteria, was reported to prime their macrophages for such cytokine production (*Nicaise* et al., 1993).

The long ranging effects of the microflora on sites which will not be colonised by bacteria, such as the sterile peritoneal cavity, spleen or bone marrow, can be explained by the fact that bacterial products are taken up by mucosal macrophages, which then leave the mucosa carrying their microbial components with them. Bacterial LPS may persist within macrophages in a bioactive form for very long periods of time (*Duncan* and *Morrison*, 1984). Peptidoglycans from bacterial cell walls, probably deriving from the intestinal microflora, have been detected inside macrophages in the red pulp of the spleen in rats and humans (*Kool* et al., 1994; *Hoijer* et al., 1995). Breakdown products of peptidoglycans have been detected in the urine of healthy people, indicating a constant uptake, degradation and excretion of bacteria or their components from the intestine (*Johanssen* and *Kreuger*, 1988).

The pyrogenic and sleep-inducing properties of microbial break-down products (*Johanssen* et al., 1991; *Martin* et al., 1984) may account for the fact that germfree animals have lower body temperature than conventional ones, and are more difficult to anaesthetise (*Midtvedt*, personal communication).

DIFFERENT BACTERIA ELICIT DIFFERENT INNATE RESPONSES

We have recently found that Gram-positive and Gram-negative bacteria affect human monocytes very differently. Whereas Gram-positive bacteria stimulate secretion of very large quantities of IL-12 from human monocytes, this is not seen with Gram-negative bacteria, which instead stimulate production of large amounts of IL-10 (*Hessle* et al., 2000). These two cytokines have largely opposing properties. IL-12 is a T cell activating cytokine, which stimulates cell-mediated effector functions, such as production of IFN-γ in T cells and NK cells. IL-10 instead dampens T cell activation and IFN-γ production and reduces antigen-presentation.

Further, the two groups of bacteria elicit different patterns of pro-inflammatory cytokines and inflammatory mediators in human monocytes (*Hessle* et al., in manuscript). Gram-positive bacteria induce more TNF-α than Gram-negative bacteria, whereas the latter induce more of IL-6, IL-8 and PGE2. Probably, the two response patterns are optimally suited to facilitate the killing and removal of the two types of bacteria. Gram-positive bacteria, with their very thick and sturdy cell wall, may not be digested efficiently enough by unprimed monocytes/macrophages. If the phagocyte is primed by TNF-α and IFN-γ, their lytic capacity is enhanced. Gram-negative bacteria may preferentially require soluble factors (antibody and complement) for their elimination. Antibodies are produced by plasma cells that mature under the influence of IL-4, IL-6 and IL-10, the two latter being preferentially induced by Gram-negative bacteria. Complement factors are acute phase proteins, whose synthesis is stimulated above all by IL-6. Lastly, the strong PGE2 response seen to Gram-negative, but not Gram-positive, bacteria may facilitate leakage of plasma proteins, including antibody and complement, out of the microvasculature.

Gram-positive and Gram-negative bacteria may, considering the above indications, affect antigen presentation rather differently. Whereas Gram-positive bacteria induce IL-12 production in antigen-presenting cells that will enhance so called Th1 reactions, the large amounts of PGE2, and the comparatively low amounts of IL-12, produced in monocytes stimulated by Gram-negative bacteria would instead favour differentiation of the T cells into the Th2 pathway (*Hessle* et al., in manuscript).

ARE WE INTOLERANT BECAUSE OF A FAULTY INTESTINAL MICROFLORA?

There are several diseases that increase in societies where the standard of living and level of hygiene are high. This has been most clearly demonstrated for allergies (*Williams* et al., 1994; *von Mutius*, 1994), but is also seen with inflammatory bowel disease (*Langholtz* et al., 1991; *Munkholm* et al., 1992), and perhaps some autoimmune disorders which appear at an earlier age today (*Pundziute-Lycka* et al, 2002). These diseases have in common an uncontrolled damaging immune response, which can be seen as a lack of tolerance.

Allergies are strongly linked to excessively hygienic life-styles, such as those characterising modern Western societies. Good housing standard, small families (*Strachan* et al., 1989, *Strachan*, 2000) and absence of infections (*Matricardi* et al., 1997, 2000) have all been linked to high risk of developing allergies, while exposure to early day-care (*Kramer* et al., 1999), to pets (*Hesselmar* et al., 1999), or to a life-stock farm environment (*Braun-Farlander* et al., 1999) all protect against allergy development.

The hygienic life-style of modern society has also led to a change in the intestinal colonisation pattern in infancy. Infants born in Sweden are later colonised by *E. coli* and other enterobacteria than infants born in Pakistan are, and they have a lower strain turnover in their microflora over the first 6 months of life (*Adlerberth* et al., 1991, 1998). Colonisation by enterococci and lactobacilli are also delayed in Western, compared to African, infants (*Bennet* et al., 1991). This has led to the hypothe-

sis that a defect development of the normal intestinal microflora in infancy is the cause of the allergy epidemics in the Western world (*Wold*, 1988).

The present composition of the normal flora may not be adequate in promoting the induction of oral tolerance, through a number of potential mechanisms:

1) A delayed colonisation with certain key bacterial species might deprive the developing immune system of certain necessary signals,
2) Other bacteria that can expand in the microflora in the absence of certain bacteria, may provide the wrong signals to the immune system, preventing oral tolerance to develop against innocuous antigens,
3) The low overall antigenic stimulation afforded by an abnormally stable intestinal microflora may prevent sufficient T cell activation to generate suppressor T cells.

We have recently observed that in infants born in Sweden in the late 1990s, staphylococci have become the major colonisers of the newborn infants. This includes not only coagulase-negative staphylococci, but also *S. aureus* (*Lindberg* et al, 2000). The emergence of staphylococci as major intestinal colonisers suggests that there is reduced colonisation resistance afforded by the intestinal ecosystem, perhaps as a function of a poorly developed anaerobic flora. A low exposure of the Swedish infant to faecal bacteria is suggested by a delayed acquisition of *E. coli* and a low *E. coli* strain turn-over (*Nowrouzian* et al, 2002). *E. coli* is a bacterial species that is only found in the intestinal tract of man and animals and its presence is a sign of faecal contamination. Since it has no other reservoir in nature, its spread is greatly hampered by hygiene.

Thus, we have observed both a change in which bacterial groups that are most numerous in the microflora, and the turnover rate of strains in the intestinal microflora of infants. Interestingly, infant who have low secretory IgA levels in saliva during their first months of life are at increased risk of developing atopy (*Payette* et al., 1977; *van Asperen* et al., 1985; *Neffen* et al., 1986). It remains to be discovered whether the shift in microbial colonisation pattern that seems to have occurred as a result of a highly hygienic lifestyle, is the cause of the allergy epidemic characterising affluent societies.

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DEFENSINS AND DEFENSIN-LIKE MOLECULES: ANTIBACTERIAL MODE OF ACTION

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SUMMARY

Antimicrobial peptides are important in the innate immunity and defence mechanisms of all organisms. Several models have been proposed in order to explain their antibacterial mode of action. Most antimicrobial peptides are amphipatic and cationic, and thus an effect on the cytoplasmic membrane of susceptible bacteria has been postulated as the main mode of action. The peptides may either form a channel, hereby inducing leakage of cytoplasmic content, or the peptide may induce permeability changes in a detergent-like manner. Both modes of action may lead to the death of the bacterial cell. Intracellular targets have also been identified for some antimicrobial peptides, and include binding to macromolecules, inhibition of macromolecular biosynthesis, and inhibition of bacterial enzymes. Some peptides have also been shown to have more than one target. This review addresses the models describing the antibacterial mode of action of human defensins present in the gut. In addition, the antibacterial mode of action of related antimicrobial peptides is discussed.

INTRODUCTION

The microbial load in the intestines of mammals is enormous (*Moore* and *Holdeman*, 1974). Some of these microbes are involved in the digestion and uptake of nutrients, and hence benefits the host. The presence of pathogenic bacteria may however not benefit the host, and several mechanisms are involved in the protection of the intestine from these bacteria. The mechanisms include the presence of a normal bacterial flora, volatile fatty acids, peristaltic movements, mucus, shedding of intestinal cells, and the presence of secretory IgA antibodies (*Mahida* et al., 1997; *Israel* and *Walker*, 1988). In addition, a rapid, non-oxidative, no-memory first-line defence system, the innate immunity system, involving peptides and proteins with antimicrobial activity protect the host against possible pathogenic bacteria. Such antimicrobial peptides are present in the gastrointestinal tract across phyla. Magainins are found in the stomach and intestine of the African frog *Xenopus laevis* (*Zasloff*, 1992; *Reilly* et al., 1994), and the midgut of some insects contains cells that produce antimicrobial peptides (*Nicolas* et al., 1996). Cecropin P1, an antimicrobial peptide related to the cecropins found in insects, has been isolated from the por-

cine proximal small intestine (*Lee* et al., 1989).

In the human digestive tract, many proteins and peptides with antimicrobial activity are present (summarised by *Lehrer*, 2001). Some peptides are confined to the epithelial cells and protect them from invasion by microbes (e.g. β-defensins, hCAP18/LL-37), thus creating a barrier against microbes. Others enter the digestive system through salivary glands (e.g. histatins), from the Paneth cells in the small intestine (e.g. α-defensins), and from pancreas (e.g. β-defensins). Further, some antimicrobial proteins and peptides, i.e. lactoferrin and lactoferricin, can enter the GI-tract either through food (*Kuwata* et al., 1998, 2001) or from endogenous sources (*Kayazawa* et al., 2002). Antimicrobial peptides are also found in the gut due to their presence in migrating polymorphonuclear cells (*Handy* et al., 1995).

The defensins

The defensins comprise the largest group of mammalian peptides (*Risso*, 2000), and are present throughout the digestive tract in all mammals, including humans (Table 1). There are two subfamilies of human defensins: (i) α-defensins and (ii) β-defensins, differing from each other in the position of the cysteine residues and in the bridge formation. The mature α-defensins comprises 29-35 amino acids (*Lehrer* et al., 1993), and the β-defensins 34-42 residues (*Selsted* et al., 1993). In their mature form, all defensins share a similar structural conformation; they are all β-sheets, cycled and stabilised by three disulphide-bridges (*Risso*, 2000).

Antimicrobial spectrum and activity

Although the antimicrobial peptides in the GI-tract possess several similarities, their antimicrobial properties are distinct (Tables 2, 3 and 4). Most peptides are active against both Gram-negative and Gram-positive bacteria, some also against fungi and protozoa, while others are also active against viruses and mycobacterium. The minimal inhibitory concentrations of the peptides are in the range of 0.1-100 μg/ml. They show synergistic activity between themselves and with other host defence molecules such as lactoferrin and lysozyme (*Bals* et al., 1998a, 1998b; *Nagaoka* et al, 2000; *Singh* et al., 2000; *Garcia* et al., 2001a).

Several inhibitors of antimicrobial activity have been described and inhibition of activity by NaCl has been implicated in cystic fibrosis (*Smith* et al., 1996; *Goldman* et al., 1997). HBD-3 is the only β-defensin that is salt-insensitive (*Harder* et al., 2001). The inhibition by NaCl is also dependent on the microbe, as high NaCl concentrations inhibits the activity of HNP-1 against Gram-positive and Gram-negative bacteria, but have no effect on the activity of HNP-1 against mycobacterium or Herpes simplex virus-1 (*Daher* et al., 1986; *Miyasaki* et al., 1990; *Ogata* et al., 1992; *Miyakawa* et al., 1996). For LL-37, NaCl inhibits the activity against methicillin resistant *S. aureus*, but does not influence the activity against vancomycin resistant *Enterococcus faecium* (*Turner* et al., 1998). Divalent cations such as Ca^{2+} and Mg^{2+}, serum and albumin have also been reported to inhibit the activity of some peptides (see Tables 2, 3 and 4).

The other GI-tract peptides covered in this review, lactoferricin B, magainin 2, cecropin P1 and LL-37, also show a broad spectrum of activity covering Gram-positive and Gram-negative bacteria, fungi, viruses, and protozoa (Table 2). Among these peptides, lactoferricin B is the only peptide with antiviral activity (*Andersen* et al., 2001).

GI-tract peptides are thus active against a wide range of Gram-positive and Gram-negative bacteria, as well as fungi, protozoa, viruses, and mycobacterium.

DISCUSSION

Mode of action of antimicrobial peptides

Due to the amphipatic, cationic structure of most antimicrobial peptides, an effect on the cytoplasmic membrane of susceptible bacteria has been postulated as the main mode of action (*Ganz* and *Lehrer*, 1998). After an initial interaction between peptide and bacterial cell surface, the peptide will traverse to the outer leaflet of the cytoplasmic membrane and cause an increased permeability, which eventually leads to cell death.

In Gram-negative bacteria, the antibacterial peptides are thought to cross the outer membrane through a mechanism called "self-promoted-uptake" (*Hancock* and *Bell*, 1988). Divalent cations in the LPS are replaced by the peptide, causing an increased permeability of the outer membrane, which allows more peptide molecules to cross the outer barrier. For Gram-positive bacteria, the initial interaction is shown to be with the (L)TA (*Vorland* et al., 1999), yet there are no good explanation for the subsequent crossing of the thick peptidoglycan layer present in Gram-positive organisms.

Several models have been proposed in order to explain the effect antimicrobial peptides have on the cytoplasmic membrane (Table 5). In general, the peptides may act by destabilising and hereby permeabilising the membrane, or by forming distinct pores/channels in the membrane. For the former effect, the most known models include the formation of a peptide carpet (*Gazit* et al., 1995) and thinning of the membrane (*Ludtke* et al., 1995; *Berneche* et al., 1998; *Heller* et al., 2000). For pore-forming peptides, the models include the barrel-stave model (*Shai*, 1999; *Bechinger*, 1999), the wormhole model (*Matsuzaki* et al., 1996; *Ludtke* et al., 1996), and the two-state model (*Huang*, 2000). Dependent upon the character of the pore, the formation of pores may lead to leakage of ions and cytoplasmic content, influx of water, or both.

Despite the focus on bacterial membranes as targets for antimicrobial peptides, several antimicrobial peptides have been shown to have intracellular targets. These include binding to DNA, RNA and/or proteins (*Park* et al., 1998; *Otvos* et al., 2000; *Kragol* et al., 2001), inhibition of macromolecular biosynthesis (*Boman* et al., 1993; *Subbalakshmi* and *Sitaram*, 1998; *Castle* et al., 1999; *Patrzykat* et al., 2002) and inhibition of bacterial enzymes (*Nishikata* et al., 1991; *Couto* et al., 1993; *Andreu* and *Rivas*, 1998).

Mode of action of defensins

Several lines of evidence argue for a hypothesis involving the cytoplasmic membrane as the bactericidal target for defensins:

(i) Defensins (HNP-1) sequentially permeabilise the outer and inner membrane of *E. coli* (*Lehrer* et al., 1989),
(ii) Defensins (HNP-1) form voltage-dependent channels in artificial membranes (*Kagan* et al., 1990),
(iii) Defensins induce leakage of cytoplasmic content (*Lehrer* et al., 1989; *Cociancich* et al., 1993),
(iv) Defensins induce leakage of vesicle content from negatively charged liposomes (*Wimley* et al., 1994),

(v) Defensins (HNP 1-3) are active against enveloped viruses, but not against non-enveloped viruses (*Daher* et al., 1986),

(vi) The effect of defensins is abolished by membrane-depolarising agents (*Lehrer* et al., 1988), and

(vii) Metabolically active microbes are more susceptible to human α-defensins than resting microbes (*Lehrer* et al., 1989).

However, NMR studies have shown that it is not possible to use the same model to describe the mode of action for all defensins (*Hoover* et al., 2001). HNP-3 dimers cannot be modelled using HBD-2 monomers, and HBD-1 monomers cannot be arranged into HBD-2 or HNP-3 type dimers. Hence, the exact mechanism of all defensins is not known, and there are currently two models describing the mode of action of defensins. One model describes the formation of multimeric pores in the cytoplasmic membrane (*Wimley* et al., 1994), and the other involves non-specific electrostatic interactions between negatively charged moieties in the membranes and the positive charges of the side chains of defensin molecules (*Hill* et al., 1991). Both mechanisms may lead to permeability changes, cell rupture/lysis and death.

The multimeric pore

Wimley et al. (1994) have published the results of an extensive study performed on HNP-2. HNP-2 binds to negatively charged vesicles through electrostatic interactions, induce fusion of the outer monolayer of vesicles, and cause leakage of vesicle content through pores with a maximum diameter of approximately 25 Å. The authors further present a multimeric model of such a pore made from HNP-2 molecules, based upon the crystal structure of defensins showing dimers with the form of a basket (*Hill* et al., 1991). This basket has a hydrophobic bottom and a polar top. The model pore is composed of 6 defensin dimers arranged with the polar basket tops lining a ~20 Å pore. The hydrophobic basket bottom face outwards towards the bilayer of the membrane. This channel allows the leakage of rather large molecules (up to ~4,400 Da).

Non-specific electrostatic interactions

Aley et al. (1994) reports of cell aggregation and dramatic changes in morphology of *Giardia lamblia* trophozoites after exposure to HNP-1. The mode of action was interpreted to involve binding and lysis, an event that appeared to involve charge interactions. Further, the high-resolution crystal structure of HBD-2 show that peptide monomers are capable of forming an octameric structure with a uniform positively charged outer surface (*Hoover* et al., 2000). However, the structural and electrostatic properties of the HBD-2 octamer support an electrostatic charge-based mechanism of membrane permeabilisation by beta-defensins, rather than a mechanism based on formation of bilayer-spanning pores.

Electrostatic interactions may lead to cell death through a detergent like effect, where the formation of a carpet of peptide molecules in the membrane results in membrane disruption at a critical ratio of lipid:peptide (*Shai*, 1999). The interactions may also cause separation of the polar lipid head groups of the phospholipids in the cytoplasmic membrane, as they are pushed aside by the hydrophobic residues of the membrane associated peptide molecules (*Ludtke* et al., 1995). As a result, gaps will be formed between the head groups, inducing physical stress on the bacterial cytoplasmic membrane, and result in the collapse of the membrane.

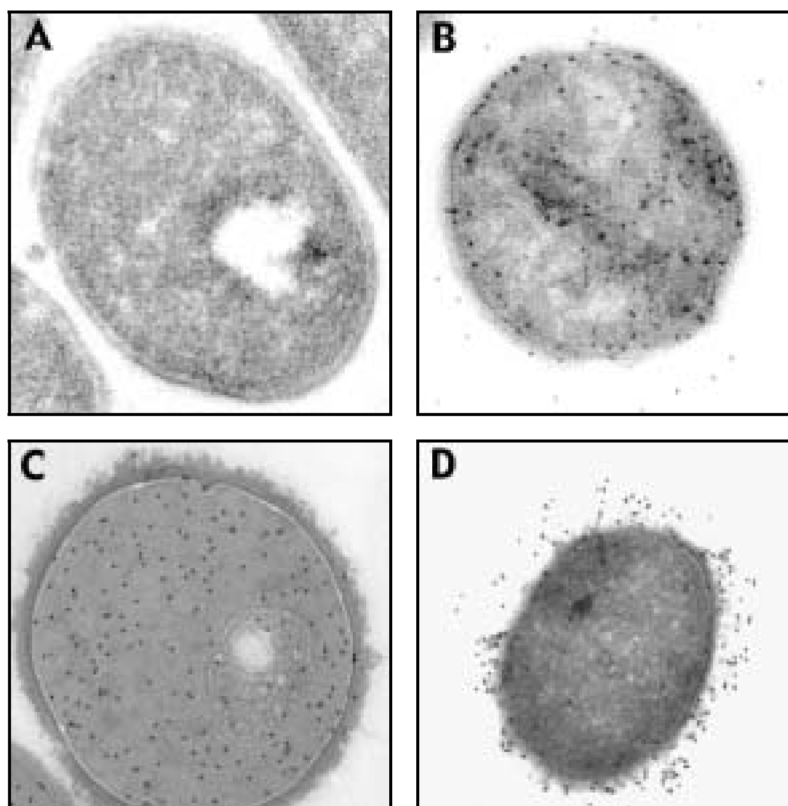

Figure 1: Electron micrographs of bacteria exposed to antimicrobial peptides, immunolabelled with polyclonal antibodies towards the respective peptide, and further visualised with gold-marked protein A. Panel A; Negative control (*E. coli* not exposed to any peptide). Panel B; *E. coli* exposed to magainin 2 for 30 minutes. Panel C; *S. aureus* exposed to lactoferricin B for one hour. Panel D; *E. coli* exposed to cecropin P1 for 30 minutes. The micrographs have previously been published by *Haukland* et al. (2001).

Targets other than the membrane

The idea of antimicrobial peptides as multi-target substances is growing. Results involving other effects than those of the cytoplasmic membrane have been published. For example, in addition to its permeabilising effects, HNP-1 also causes a reduction in bacterial macromolecular biosynthesis and a drop in the colony count (*Lehrer* et al., 1989). Further, the magainins have been extensively studied as pore-forming peptides (*Matsuzaki*, 1998), and several models have been used to explain the interaction between magainin and the cytoplasmic membrane (see Table 5). Despite these effects, *Haukland* et al. (2001) have shown that magainin 2 are capable of residing in the bacterial cytoplasm (Figure 1b). Cecropin P1 does not exhibit this feature, and are confined to the bacterial cell wall (Figure 1d), consistent with the carpet model proposed for the mode of action of this peptide (*Gazit* et al., 1995).

While proposing the model of the multimeric pore, *Wimley* et al. (1994) also points out that the actual *in vivo* mechanism for cell leakage may involve

at least three steps. These included the initial interaction of monomeric defensins and cell surface through electrostatic interactions, the oligomerisation of defensins, and at last pore formation. Translocation of the peptide via pore formation is possible as a fourth step, allowing the peptide to interfere with any intracellular process.

Evidence for the translocation of several antimicrobial peptides are accumulating, and involve magainin 2 (*Haukland* et al., 2001), lactoferricin B (*Haukland* et al., 2001), and buforin (*Park* et al., 1998). For defensins, *Sharma* and *Khuller* (2001) showed that HNP-1 is an efficient inhibitor of DNA-synthesis in *Mycobacterium tuberculosis*. They suggest that the cytoplasmic membrane is the primary target for HNP-1. Binding to this target causes permeabilising of the membrane, and thus enhanced access to the secondary, intracellular target.

Lichtenstein (1991) has also made the proposal of two targets for defensins. Working on tumour cells, they report that initial effects on the plasma membrane were not sufficient for subsequent lysis. A second phase was required which involved the continued presence of defensin. They conclude that there is two phases of interaction between defensins and tumour cells, where the initial effect is on the cell membrane, and the second phase is mediated intracellularly by defensin internalised through a permeabilised membrane. A two-phased bactericidal activity is also proposed for HBD-2 and *E. coli* (*Tomita* et al., 2000). Lactoferricin B also interacts with membranes (*Ulvatne* et al. 2001), and can be traced into the cytoplasm at sub-inhibitory concentrations (Figure 1c) (*Haukland* et al., 2001). Unpublished results show that lactoferricin B have an effect of macromolecular biosynthesis (*Ulvatne* et al., in prep.).

CONCLUSION

At this point, there is no doubt that most antimicrobial peptides, including the defensins, are membrane active molecules. Through their interaction with the cytoplasmic membrane, they may cause severe damage to the bacterial cell and cell death. It is likely that bacteria may compensate for the formation of pores/channels in the membranes, while a detergent like effect is irreversible since it involves a complete rupture of the bacterial integrity. The bacterial cell is not a closed system, and the cell is in some kind of equilibrium with its surroundings through sensing systems. Transport of nutrients, waste products, and other extracellular products are constantly crossing the cytoplasmic membrane, and some of this transport happens through pores. An efficient killing by pores must therefore be swift, rapid and sudden, to ensure that the bacteria do not initiate a defence response. Antimicrobial peptides may, by utilising another secondary target, be even more efficient in the battle against pathogenic bacteria.

Due to the fact that most antimicrobial peptides also exhibit other effects, the *in vivo* effect and exact mode of action of antimicrobial peptides are hard to elucidate (*Scott* and *Hancock*, 2000). The other effects involve interactions with host cells to stimulate gene-expression from genes encoding transcription factors, chemokines, chemokine receptors, integrins etc, products that also are part of the innate immunity (*Hancock* and *Rozek*, 2002). Antimicrobial peptides are therefore multi-functioning ef-

fector molecules involved in the delicate balance between microbes and host, and their *in vivo* role must be regarded as the whole interplay between the different functions the peptides may have. Therefore, further studies on defensins and other antimicrobial peptides must be performed in order to understand the *in vivo* antibacterial mode of action.

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DEFENSINS AND BACTERIA, A QUESTION OF "LIVE OR LET DIE"?

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SUMMARY

Antibacterial peptides have been found in many organs of the body. Defensins are a family of cationic antimicrobial peptides that are found in mammals, insects and plants. The innate immune system consists of the body's own defence mechanisms which can be activated upon exposure to foreign microorganisms without earlier exposure or priming.

One important part of this system seems to be the production of endogenous antimicrobial peptides. Such peptides, e.g., produced in the gastrointestinal tract of humans and animals have been suggested to modulate the acquirement of a bacterial microflora and play an important part in to protect against infections in general.

Antimicrobial peptides have broad-spectrum antibiotic activity against e.g. Gram-positive and Gram-negative bacteria, mycobacteria, fungi, parasites and viruses.

Antimicrobial peptides seem to be less prone to be, or to induce resistance in bacteria, which make them an interesting alternative in the treatment of infections with multiresistant bacteria.

There seem to be hope for the emergence of effective synthetic antimicrobial peptides as drugs for human use in the near future.

INTRODUCTION

Defensins are a family of cationic antimicrobial peptides that are found in mammals, insects and plants (*Del Pero* et al., 2002*; Hancock* and *Diamond*, 2000; *Hancock*, 2001; *Schutte* et al., 2002; *Zasloff*, 2002a) and as a component in venoms (*Corzo* et al., 2001). The defensins are members of the inborn, innate immune system (*Axelsson* and *Mahida*, 2000; *Fellerman* and *Stange*, 2001; *Parkin* and *Cohen*, 2001). The innate immune system consists of the body's own defence mechanisms (*Medzhitov*, 2000) which can be activated upon exposure to foreign microorganisms without earlier exposure or priming (*Boman*, 1995; *Zasloff*, 2002a).

Much of the early knowledge of antimicrobial peptides was gained from studies of the fly *Drosophila* that has been utilised as a model for genetic studies. *Drosophila* lacks adaptive immune system an relies on the inborn, innate, immune system and have been used as a model for the elucidation of the different pathways of the innate immunity (*Hoffman* and *Reichhart*, 2002) especially the Toll-pathway (*Schwartz*, 2002) and the importance of NFκB transactivators (*Luster*, 2002; *Mahida* and *Johal*, 2001).

One important part of this system seems to be the production of endogenous antimicrobial peptides (*Boman*, 1995; *Ganz*, 1994). Such peptides, e.g., produced in the gastrointestinal tract of humans and animals (*Schonwetter*, 1995), have been suggested to modulate the acquirement of a bacterial microflora in neonates (*Sepp*, 1998) and play an important part in the protection against infections in general. Antimicrobial peptides have broad-spectrum antibiotic activity against e.g. Gram-positive and Gram-negative bacteria, mycobacteria, fungi, parasites and viruses (*Boman,* 1995).

Antimicrobial peptides have been shown to be produced by many organs and cells through the body (*Boman*, 1995; *Ganz*, 2000) but this paper will concentrate on the innate immunity of the gastrointestinal tract since it is exposed to an enormous load of microorganisms, some causing disease but others are necessary for our well-being and antimicrobial peptides seem to play an important control function on these microorganisms.

INNATE IMMUNITY OF THE GASTROINTESTINAL TRACT

The innate immunity of the gastrointestinal system is in place when we are born (*Bry* et al., 1994) and will play an important role in defending the body against unwanted microbes, but also in the acquirement and maintenance of a normal and healthy microflora, microbiota (*Bevins* et al., 1999; *Boman*, 2000; *Hooper* and *Gordon*, 2001).

The first sentinels at the gate to our gastrointestinal system are found in the oral cavity. Here we find that mucus is produced, layering the physical border consisting of epithelial cells. This mucus layer is found throughout the gastrointestinal system and has many functions such as being a physical barrier, protecting the epithelial cells, helping in eliminating and transporting unwanted substances out of the body, and also harbouring endogenous protecting bioactive molecules (*Deplanke* and *Gaskins*, 2001).

The antimicrobial peptides are also at place already in the oral cavity (*Bevins*, et al. 1999) and also in the airways (*Moser,* et al. 2002).

The stomach functions as a physical gatekeeper and a producer of chemicals, which will stop and eliminate many microbes to further enter the small and large intestines.

The small intestine harbours a relative small number of bacteria compared to the large intestine (*Hooper* and *Gordon*, 2001; *Skar et* al., 1986, 1989). The large intestine, which includes the caecum and colon, is the part of the gastrointestinal tract where a large number of bacteria, as well as many different species are found (*Cunliffe* et al., 2001). These bacteria can be commensals, invading pathogenic bacteria or, as recently suggested, opportunistic semi-pathogens (*Gillespie*, 2002; *Medzhitov* and *Janeway,* 2002). One important cell type producing antimicrobial peptides and other antimicrobial substances is the Paneth cell (*Porter* et al., 2002; *Zasloff*, 2002b). Apart from antimicrobial peptides there are several other defence mechanisms in the small intestine (Figure 1).

There are, for example, antimicrobial phospholipase A2, lysozyme and trypsin. The trypsin seems to have multiple functions since recently it was shown that trypsin is the enzyme that activate HD-5 (*Gosh* et al., 2002), one of the enzymes that are able to activate prodefensins. Matrilysin has been advocated as another activating enzyme (*Wilson* et

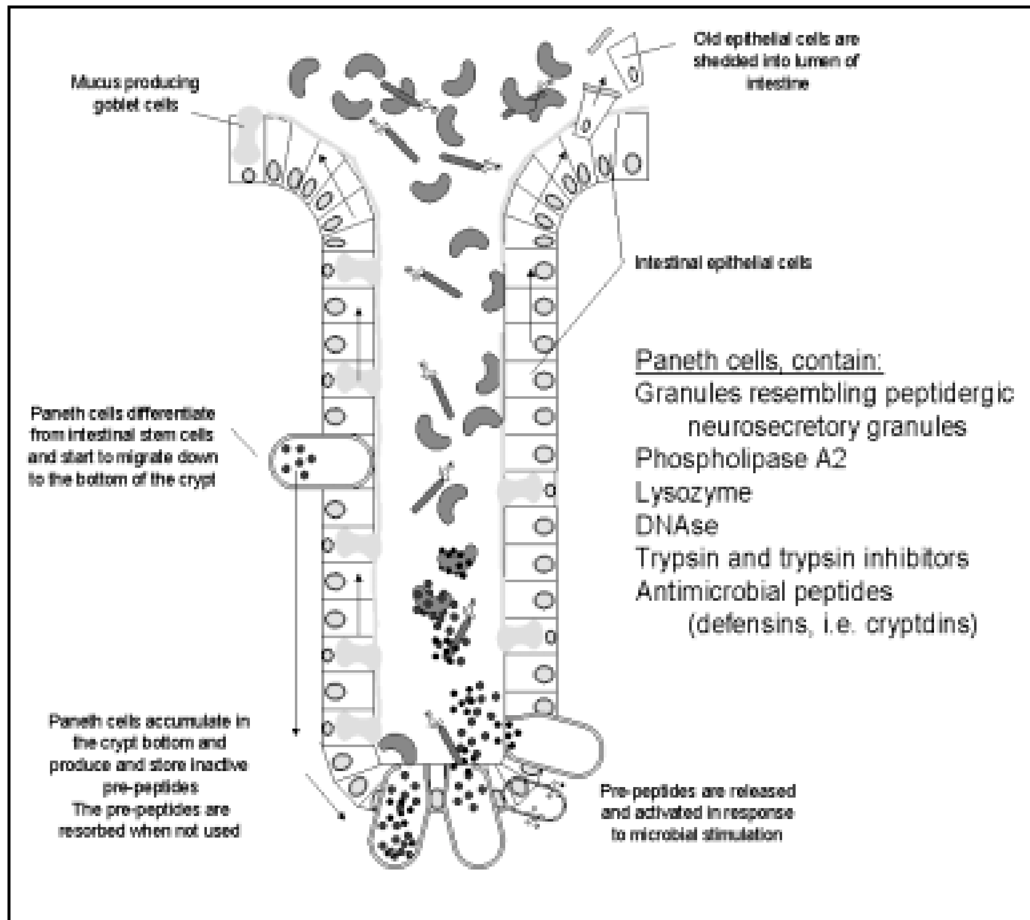

Figure 1: Defence mechanisms in the small intestine.

al., 1999), however this enzyme could not be detected in germfree animals (*López-Boado* et al., 2000) while others have shown actual activation of prodefensins to mature bioactive antimicrobial peptides in germfree animals (*Axelsson* et al., 1999; *Pütsep* et al. 2000).

ANTIMICROBIAL PEPTIDES, MOLECULAR PROPERTIES AND MODE OF ACTION

The antimicrobial effect of antimicrobial peptides on bacteria is commonly tested and expressed as for conventional antibiotics. The effective concentration range lies in the micro- to nanomolar range (*Zasloff*, 2002a). The exact mechanism by which antimicrobial peptides kill microbes is not known. Several models have been proposed and generally some kind of permeabilisation of the bacterial membrane is depicted (*van 't Hof* et al., 2001). This is accomplished by utilising electrostatic binding and the different hy-

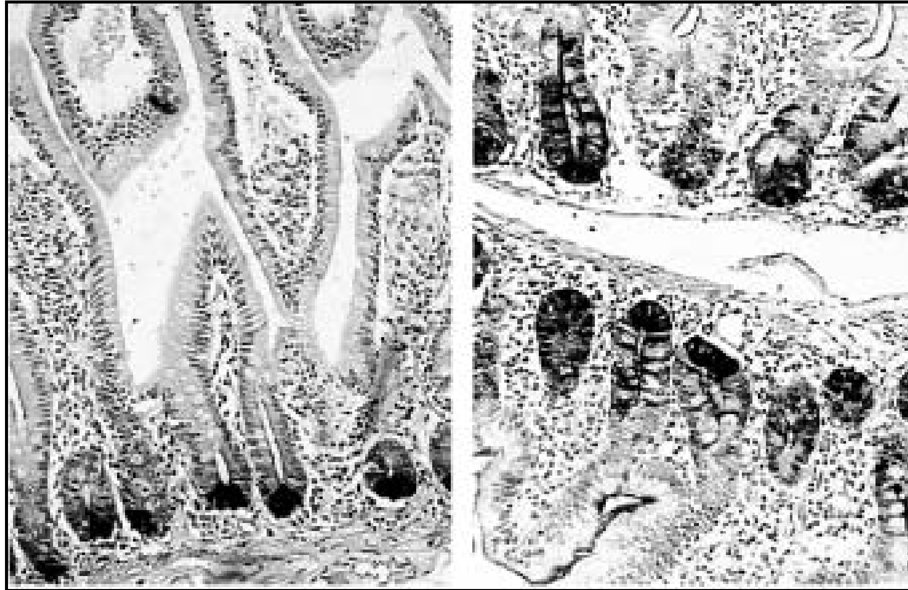

Figure 2: Sections of small intestine stained with antibody to HD-5. Left: Positive Paneth cells in the very bottom of the crypt. Right: Positive cells are found higher up in the crypt and also positive material secreted into the lumen of the crypt.

drophilic and hydrophobic properties of the peptide (*van 't Hof* et al., 2001, *Zasloff*, 2002a). Interactions with lipids have also been advocated and could play in concert with scavenger receptors (*Peiser* et al., 2002). Certain antimicrobial peptides, lactoferricin B and magainin 2, have also been found to cross over the bacterial membrane into the cytoplasm (*Haukland* et al., 2001).

The range of microbes that are sensitive to the antimicrobial peptides is quite broad and the term broad-spectrum antibiotic applies to many of the peptides (*Periathamby* and *Dento*, 2002; *Porter* et al., 1997). Some strains show resistance but this is due to the membrane structure as such (*Zasloff*, 2002a) and acquired resistance has been postulated as unlikely to occur (*Peschel*, 2002).

SMALL INTESTINAL MICROBIAL PEPTIDES

Antimicrobial peptides seem to be important to maintain a relatively microbe-free small intestine (*Ganz*, 2000; *Ouelette* et al., 2000; *Ouelette* and *Bevins*, 2001). There is differential presence of antimicrobial peptides throughout the gastrointestinal channel (*Frye* et al, 2000). In mouse small intestine, the nematode *Trichinella spiralis* induces atrophy of the villi, hyperplasia of the crypts of Lieberkühn and of the mucus-producing goblet cells (*Kamal* et al., 2001). This infection also lead to an increase of Paneth cell-number, a more widespread presence of Paneth cells and intermediate cells expressing mouse antimicrobial α-defensins, cryptdins (*Ayabe* et al., 2002a). The modulation of the mouse cryptdins has been shown to be dependent of Ca^{2+}-activated potassium channels (*Ayabe* et al., 2002b).

A similar effect on Paneth cells has been seen in humans after Roux-en-y Gastric bypass surgery (*Sundbom* et al., 2002). This is a standard surgical procedure for morbid obesity where food and oral-nasal-pharyngeal secretion pass directly into the small bowel without passing through the acid environment of the normal stomach. Immunostaining of human intestinal antimicrobial α-defensins, defensin-5 (HD-5), showed an up-regulation of the antibacterial peptide in the Paneth cells and a spread of anti-defensin positive material upwards in the crypt wall and also a release inside the crypt lumen (Figure 2).

Since the acid environment of the stomach is by-passed, there is a possibility for microbes to invade the otherwise protected small intestine. This could lead to overgrowth of bacteria in these patients. However, these patients display an almost normal microflora, which could be the result of an activation of anti-microbial peptides in response to an increased load of ingested bacteria.

HD-5 is normally stored in precursor form and is activated upon stimulation, as discussed above, by bacteria but also by inflammation (*Axelsso*n, 1999; *Cunliffe* et al., 2001). However, in inflammatory bowel disease the epithelial barrier is defect and the intestinal tissue is exposed to bacteria and bacterial products which could be part of the mechanism of this activation. Similar changes in Paneth cell distribution as in the patients having gastric by-pass surgery could also be detected in patients with active inflammatory bowel diseases pointing to that similar mechanisms could be at play, possibly involving bacterial interference with the mucosa (*Cunliffe* et al., 2002)

This precursor form can be found in both individuals having a normal microflora or being exposed to bacterial products, and in germfree animals. It has been shown that germfree mice, which are bred for many generations in an sterile environment after they have been born under aseptic conditions and then maintained germfree, generate the same products from enteric prodefensins (*Pütsep* et al., 2000). So, animals which are naive to microbes have precursor forms of antimicrobial peptides which can be activated and momentarily exert their antimicrobial functions (*Ayabe* et al., 2002c). However, there is a possibility that these animals are exposed to bacterial products which have been left unaffected by sterilisation in their animal feed. For example there can be bacterial LPS originating in the raw material or from the manufacturing process.

Experiments which compare germfree animals with animals having a conventional commensal microflora or with animals mono-associated with a specific bacterial strain or species, can give valuable information of the interplay between intestinal microflora and the individual or animal (*Pütsep* et al, 2000). The intestinal microflora has profound effects on the development and maintenance of a healthy intestinal mucosa (*Falk* et al., 1998). In the newborn there is a succession of microbial habitants building up this "normal" flora. Starting with bacteria acquired from the mother during labour, neonates acquire for example *Clostridium spp.* and *Bifidobacterium spp.* and the resultant flora is in part determined by environmental factors such as food and eating habits, country of living, sociological factors and level of sanitation (*Falk* et al., 1998). Postnatal studies have shown that the Paneth cells are found early and differentiate to mature cells around postnatal day 14-28 (*Bry* et al., 1994).

PHARMACEUTICAL APPLICATIONS

During the last years there has been a mounting problem with bacterial strains that have become resistant to commonly used antibiotics. Some strains have even acquired resistance to the antibiotics that are used as a last means to treat life-threatening infections. Biotechnological companies have seen the potential in using antimicrobial peptides for treatment of multi-resistant bacteria. For example, Magainin Pharmaceuticals Inc., Philadelphia, USA, was founded in 1997 with one of the early peptide researchers, Dr. Michael Zasloff, as Executive Vice President. Clinical testing has been done of some investigational drug but no real break-through has been seen so far. One problem has been the handling and administration of the drug, another problem, the very high cost of manufacturing synthetic antimicrobial peptides.

One approach has been to develop manufacturing processes to construct the complex structures of biologically effective antimicrobial peptides. Recently, reports of successful synthesis of bioactive polymers displaying antimicrobial activity have been publicised. One approach has been to use amphiphilic acrylamide polymers resembling some of the properties of the β-peptide class. These acrylamide polymers showed a bactericidal activity against *E. coli* and minimal inhibitory concentration (MIC) values for the Gram-negative *Klebsiella pneumoniae*, ampicillin and streptomycin-resistant *E. coli*, and the Gram-positive *Bacillus subtilis* were established (*Tew* et al., 2002). The most effective polymer was additionally tested with good results against ampicillin and streptomycin-resistant *E. coli* and tetracycline resistant *Salmonella typhimurium*. The ability to interact and disrupt phospholipid bi-layers was also confirmed (*Tew* et al., 2002).

Another approach was used by self-assembly of amino acids in synthetic membranes into tubular structures (*Fernandez-Lopez* et al., 2001). Antibiotic activity was established against several bacteria including methicillin resistant *S. aureus* (MRSA) and the membrane disrupting ability was confirmed in a membrane depolarisation assay (*Fernandez-Lopez* et al., 2001).

Another antimicrobial peptide has been identified in the mouse, cathelin-related antimicrobial peptide, CRAMP (*Gallo* et al., 1997). Analogues of this structure have now been designed and shown to have strong antibacterial activity, but without the endogenous CRAMPs haemolytic properties (*Shin* et al., 2000).

With these developments there seem to be hope for the emergence of effective synthetic antimicrobial peptides for human use in the near future. These developments give the possibility to synthesise peptides that are suitable for production at an industrial scale and at reasonable prices. However, clinical testing is now needed to find out whether they have adverse effects that make them not suitable for use in man or animals.

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PHENOTYPIC EXPRESSIONS IN THE SMALL INTESTINE

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INTRODUCTION

Every man and animal is born germfree, i.e., without any microorganisms, and the colonisation of all surfaces such as the respiratory, urinary and alimentary tract starts immediately after birth. Initially, when space is not limited, bacteria with a high multiplication rate may dominate, but as the number of bacteria increases and accessible nutrient pools becomes limited, habitats will be filled up by more specialised bacterial species and the complexity of the flora increases.

When trying to investigate the composition of samples from any site the alimentary tract other than the mouth or lower part of large intestine from volunteers, it is difficult to obtain proper samples. Endoscopic sampling for microbiological evaluations has both advantages and disadvantages, and some investigators have also pointed on some difficulties in the sampling depending on from where it is taken in the stool specimens. Porcine faecal material was investigated with regard to microbes present in materials from the inner and the outer content, and differences were found due to atmospheric conditions. Another problem arises for instance due to storage and/or freezing and transport conditions from the sampling to analysis – these variables will also influence the final findings (*Rall* et al., 1970). Some of these problems can however be overcome today by the use of polymerase chain reaction and direct sequencing of 16S ribosomal DNA analysis of the flora. However, there are still limitations and the detection level is approximately the same as in previously well established microbiological evaluations. A complementary method is to evaluate what the flora has done, i.e., to evaluate the products – the outcome - of the crosstalk between the host and its microflora. Using this technique, substances produced in, e.g., the small intestine can be detected in faecal samples and thus reflect microbiological products from areas normally difficult to obtain samples from.

Comparisons of conventionally raised (Conv) organisms and germfree (GF) counterparts have revealed series of anatomic, biochemical, immunological and physiological phenotypes, collectively known as Microflora-Associated Characteristics or MACs. When the functionally active flora is absent, as in GF animals, healthy new-borns and sometimes in relation to antimicrobial treatment, a MAC is termed GAC (Germfree Animal Characteristic) (*Midtvedt* et al., 1985). Some phenotypic expressions are presented in Table 1. In the following, some of these phenotypic expressions, occurring in the small intestine, will be presented and discussed.

Table 1: Some anatomical structures, physiological and biochemical functions influenced by the microflora, and microorganisms involved (Modified from *Midtvedt*, 1999)

Parameter	MAC[1]	GAC[2]	Microorganism
Anatomical/Physiological			
Caecum size (rodents)	Normal	Enlarged	Partly known
Cell kinetics	Normal	Slower	Unknown
Colloid osmotic pressure	Normal	Increased	Unknown
Electro-potential Eh, mV	Low (<-100)	High (>-100)	Unknown
Intestinal wall	Thick	Thin	Unknown
Migration motor complexes	Normal	Fewer	Unknown
Osmolality	Normal	Reduced	Unknown
Oxygen tension	Low	High	Several species
Production of peptides	Normal	Altered	Unknown
Biochemical			
β-aspartylglycine	Absent	Present	Species in concert
β-glucuronidase	Low activity	High activity	Several species
Bile acid metabolism	Deconjugation	No deconjugation	Many species
	Dehydrogenation	No dehydrogenation	Many species
	Dehydroxylation	No dehydroxylation	A few species
Bilirubin metabolism	Deconjugation	Little deconjugation	Many species
	Urobilinogen	No urobilinogen	A few species
Cholesterol metabolism	Coprostanol	No coprostanol	A few species
Faecal tryptic activity	Little or absent	High activity	A few species
Intestinal gasses	Carbon dioxide	Some CO_2	Many species
	Hydrogen	No hydrogen	Some species
	Methane	No methane	A few species
Mucin	Degradation	No degradation	Several species
Short-chain fatty acids	Large amounts	Far less	Many species
	Several acids	Few acids	

[1] Microflora-Associated Characteristic; [2] Germfree Animal Characteristic.

CELL KINETICS

Since long time it has been assumed that the rate of crypt epithelial cell proliferation in the intestine represents a major defence mechanism against invading intestinal microorganisms. The cell renewal system involves proliferation of undifferentiated epithelial cells followed by differentiation and migration from the site of production to the functional site and finally elimination from the mucosa. Under normal conditions in mice, the cell turn over rate has been estimated to be about 10^8 cells per day (*Hageman* et al., 1970). By introducing strict standardisation techniques, we have been able to show that there are different phenotypic expressions in different compartments of the intestine in GF and Conv rats and mice with regard to age, gender and microbial status. In these studies, also diet and fasting time was standardised. Cell kinetic and morphological parameters reflecting the crosstalk between the host and its flora was investigated (*Banasaz* et al., 2000, 2001).

We found a great similarity between the mitotic index in young and old rats and mice, being high in the upper part of the small intestine and lower in the

colon, and we also found higher mitotic indexes in males as compared to females. A second similarity was that the crypt/villus ratio was on the same level throughout the small intestine. Thus, we found that in those areas, where the number of microbes was high, microbes triggered the mitotic index. We also found that, when establishing a microbial strain as a mono-contaminant, an immediate triggering of the mitotic index was seen, irrespectively of whether the mono-contaminant was a probiotic microbe (*Banasaz* et al., 2002), a pathogen or a commensal microbe (*Banasaz*, 2002).

When a toxin producing *Clostridium difficile* strain was mono-inoculated into young rats, there was initially an increase of the mitotic index. However, within some few days there was a marked decrease of the mitotic index and also occurrence of some epithelial border disruptions without leading to any disease or other signs of discomfort in the animals was seen. Lack of disease signs does not exclude that *Clostridium difficile* is a pathogenic microbe. By opening up for other microbes to cause disease, as we observed some few patchy morphological alterations in the intestinal mucosa of the rats, the disease signs could be caused by secondary infections. This study needs to be expanded to animals harbouring a normal flora.

INTESTINAL MOTILITY

It is well known that several microbial species may cause increased intestinal motility, expressed as cramps and diarrhoea, due to many different mechanisms. GF animals are known for having far less spontaneous muscular contractions than their Conv counterparts. Already in the sixties, a slower transit time in GF animals was reported (*Abrams* and *Bishop*, 1967; *Gustafsson* and *Norman*, 1969). Obviously, the intestinal flora plays an important role related to intestinal motility and transit time – some species influencing the small intestine (*Salmonella* spp.) and others the large intestine (*Shigella* spp.).

The microbes responsible for inducing motility, contractions and increased transit time, found under normal physiological conditions, are not known. However, intestinal movements are of a paramount importance for the regulatory and protective role of the microflora, and for the host (*Midtvedt*, 1989).

SHORT CHAIN FATTY ACIDS

The intestinal microflora ferments the dietary and endogenous large carbohydrates into mono- and di-saccharides in the small intestine and these appear to be the main contributors to the energy requirement in mammals after an anaerobic fermentation. The origin of intestinal short chain fatty acids has been substantiated in studies of GF and Conv rats and mice, and the faecal content of these acids are representing the net sum of production, absorption and secretion of the acids throughout the whole intestinal tract.

In the mouth of man, there are quite high amounts of these acids present, representing products of an anaerobic metabolism in the gingival pockets. In the stomach and upper small intestine, the amount of these acids is quite low

under normal, healthy conditions – in contrary to, e.g., patients with microbial small intestinal bacterial overgrowth. Findings from these patients indicate that there are overgrowth symptoms - the patients seem to have a colon-like flora in the lower small intestine, and the main part of these acids are produced by the altered flora in the jejunum (*Høverstad* et al., 1885). As an example can be mentioned that in 6 healthy volunteers, the short chain fatty acid content in saliva was quite high (2780-9940 µmol/l), decreased successively to 185-1470 µmol/l in jejunal juice, and the main part was acetic acid (approximately 85%), propionic acid accounted for almost 11%, and less than 2% of iso- and n-butyric and i-valeric acid (*Høverstad* et al., 1984). A very similar relative distribution was earlier found in the saliva, gastric juice and duodenal aspirates, however, essential different from the faeces content. This depends on the fact that, as the number of microbes increases in the lower small intestine and in the colon, also the short chain fatty acid content is altered in the composition, and totally 24-243 mmol/kg faeces – with significantly higher amounts in men as compared to women, have been found (*Siigur* et al., 1994).

INTESTINAL TRYPTIC ACTIVITY

Trypsin is chosen as a model substance for studying endogenous derived digestive enzymes. The precursor – trypsinogen - is excreted from the pancreas and activated in the upper part of the small intestine, mainly by brush border enzymes.

In total, faecal tryptic activity involves the net sum of processes such as secretion of trypsinogen from the pancreas, activation of the pro-enzyme in the small intestine by enterokinase and presence of host-, microbial-, and diet-derived compounds that inactivate or otherwise degrades the trypsin molecule during the passage through the intestine. In GF rats, tryptic activity is detected in the upper small intestine already at two days of age, and as the animals grow older, tryptic activity is detected in increasing amounts all the way down of the intestine and in faecal samples, where high amounts of the enzyme activity is detected. This in contrast to what has been found in materials from Conv animals, which are more or less devoid of tryptic activity in the lower intestine.

It is also shown that new-born children excrete faeces/meconium without any tryptic activity, although immunological studies detect the molecule – not yet activated (*Norin*, 1985). Thereafter, the enzyme activity increases successively during the first year of life followed by a decrease down to adult values some years later (*Norin* et al., 1985).

In Conv animals it is found that the enzymatic activity disappears mainly in the caecum. Only very seldom, a low level of tryptic activity is detected in the lower intestine and in faecal samples from rats and mice. It is found that the amount of this enzyme activity is variable depending on species investigated and on which diet the animals is given. Horses and pigs show the same low levels of faecal tryptic activity (*Collinder* et al., 2000, 2002), as found in e.g. rats, mice and man.

Obviously, intestinal microbes are responsible for the inactivation of trypsin, and at least one human strain of *Bacteroides distasonis* (*Ramare* et al., 1996) has been isolated and found ca-

pable to inactivate pancreas derived trypsin in both rats and mice. Previously, in Crohn´s disease patients, there were found high levels of tryptic activity in faecal samples as compared to samples from healthy volunteers (*van der Merwe* and *Mol*, 1982), and intestinal tryptic activity could possibly contribute to the pathological symptoms observed, mainly in the lower intestine of these patients. These microbes are also found to be influenced by several antimicrobial drugs, when given to rats (*Norin*, 1997).

BILIRUBIN AND UROBILINS

The bile pigments consist partly of bilirubin, a toxic and water insoluble end-product after catabolism of haemoglobin and some other haem-containing substances. The reduction of bilirubin to urobilins by intestinal microbes represents one natural detoxification process of toxic intestinal substances such as xenobiotics, drugs, hormones and certain dyes. Bilirubin as well as other toxic substances is conjugated in the liver with glucuronate to a less toxic and water-soluble molecule, which are secreted with the bile into the intestine.

In the intestine, the bilirubin conjugates are de-conjugated by ß-glucuronidases and further transformed to series of metabolites, most often termed urobilins. Most of the ß-glucuronidases are derived from the microbes, only a minor part is produced by the host. This detoxification process will be discussed in a deeper setting later during this meeting. Enhancement of microbial conversion of bilirubin to urobilins decreases the intestinal concentration of bilirubin, which is a potential risk factor for hyperbilirubinaemia, which can lead to extrapyramidal disturbances, hearing loss, delay in motor development and less often, also to intellectual deficits (*Saxerholt*, 1990; *Vitek* et al., 2000).

CONCLUDING REMARKS

The normal intestinal flora plays an important regulatory and protective role in all organisms, but external disturbances could be harmful for the host and its flora. It is, e.g., known that antimicrobial treatments causing marked alterations of the flora-composition could induce altered intestinal functions. Supplementation of the intestinal microflora with live microbial species, used for many years to protect and maintain the balance in the intestine, could eventually also be a potential risk factor. Introduction of live microbes when e.g., the intestinal flora is still not fully settled or otherwise strongly disturbed, could alter the succession of the establishment of the normal flora. One could thus speculate that, when the intestinal flora is immature and continuously developing, an altered succession of the "normal" establishment pattern of the flora could cause unexpected consequences related to functions both in the small and in the large intestine.

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MICROBIAL P450: DOES IT EXIST, AND WHAT CAN IT MEAN?

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SUMMARY

P450 enzymes play important physiological and patho-physiological roles in the complex interplay between a host and his intestinal microflora. The need for more information is underlined.

NOMENCLATURE

The cytochrome P450s (CYP) constitute a superfamily of haem-thiolate enzymes and their Fe-carbon complexes show an absorption spectrum with a maximum near 450. There are now over 1000 different P450s that have been identified from the whole biological kingdom. Due to this large number a standardised nomenclature has been developed (*Nelson* et al., 1996). In short, the P450 superfamily is subdivided into families. An individual P450 within a family are defined as having less than 40% sequence identity with a P450 in another family. The families are divided into sub-families and enzymes within a sub-family are more than 55% identical in sequence. A P450 enzyme is designated by the root symbol "CYP" (describing cytochrome and P450), an Arabic number denoting the family, a letter designating the subfamily and a Arabic numerals representing individual enzymes. It should be kept in mind that this system is based only on sequence similarity among the P450s and - unfortunately does not indicate the function(s) of individual P450s.

P450s AND METABOLISM OF XENOBIOTICS

Nowadays, it is generally recognised that multicellular organisms, including humans, are continuously exposed to foreign chemicals, collectively known as xenobiotics. They are found in our environment and include a vast range of compounds such as drugs, industrial chemical, pollutants, pesticides, plant products, alkaloids and toxins. Most of them are rather lipophilic, and due to their lipophilicity, many xenobiotics can be - and are - absorbed through our surfaces (intestine, lungs, and the skin). Also due to their lipophilicity – if the xenobiotics are not metabolised in the body - they will be concentrated in the tissue and sooner ore later they might be toxic for the host. Therefore, in order to be eliminated, many xenobiotics have to be converted into more water-soluble compounds, thereby influencing upon their excretion in the urine or faeces.

The enzymes catalysing these reactions, i.e. the xenobiotic-metabolising enzymes, are – for convenient reasons - often divided into two groups refereed

Table 1: Major reactions and groups of enzymes in xenobiotic biotransformation

Reaction	Type of enzyme
Phase I	
	Cytochrome P450
	Alcohol dehydrogenase
	Aldehyde dehydrogenase
Oxidation	Xanthine oxidase
	Monoamino oxidase
	Flavin mono-oxidase
Reduction	Quinone reduction
	Reductive dehalogenation (P450)
Hydrolysis	Epoxide hydrolase
Phase II	
Glucuronide conjugation	UDP-glucuronosyltransferase
Glutathione conjugation	Glutathione S-transferase
Sulphate conjugation	Sulphotransferase
Acetylation	N-acetyltransferase
Methylation	Methyltransferase

to as phase I and phase II (Table 1) As summarised by *McLellan* (2000), enzymes involved in phase I reactions, most of which represent P450s, expose or introduce a function group (-OH, -NH_2, -SH or -COOH) on the compounds by oxidation, reduction or hydrolysis reactions among others. In general, these alterations increase hydrophilicity to a minor extent. It is evident from Table 1 that phase II mainly involves conjugation of the compound, with molecules such as gluthatione, glucuronic acid, sulphate, taurine, glycine and other amino acids.

It has to be kept in mind that the biotransformations included in phase I may change the pharmacokinetic behaviour of many drugs. In fact, some drugs have to undergo biotransformation before exerting their effects. On the other side, however, biotransformation included in phase I may convert many xenobiotics to more reactive electrophilic metabolites that can form protein and DNA adducts, thereby exerting their toxic or tumourigenic effect (*Nebert* et al., 1996) It should also be kept in mind that important co-factors for phase II reactions are functional groups that are either present on the xenobiotics or have been introduced during phase I reaction. Thus, phase II biotransformation may or may not be preceded by phase I biotransformation

P450s AND EVOLUTION

It is generally believed that P450s are very old enzyme, probably occurring before the divergence of prokaryote and eukaryote. A early eukaryotic mitochondrial P450 (influencing upon the metabolism of cholesterol to steroids) is found in both plants and animals, indicated that animals diverged from plants around 1400 millions years ago (giving rise to P450s localised in the mitochon-

Table 2: Major human P450 families and primary function(s)

Family	Catalytic function
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic catabolism
CYP3	Xenobiotic catabolism
CYP4	Fatty acid hydroxylation
CYP5	Thromboxane A2 synthase
CYP7A	Cholesterol 7-alpha-hydroxylase
CYP8A	Prostacyclin synthase
CYP8B	Sterol 12-alpha-hydroxylase
CYP11A1	Cholesterol side-chain cleavage
CYP11B1	Steroid 11-beta-hydroxylase
CYP11B2	Aldosterone synthase
CYP21	Steroid 21-hydroxylase
CYP27A1	Sterol 27-hydroxylase
CYP46	Cholesterol 24-hydroxylase

dria and endoplasmatic reticulum. A major evolutionary steps seems to have taken place around 900 millions years ago, resulting in one lineage continuing as endogenous P450s and the other began a new function, i.e. xenobiotic metabolism (*Nelson* and *Strobel*, 1997).
In this respect, it is a fascinating theory that the xenobiotic-metabolising enzymes have involved due to a continuous interaction or "evolutionary fight" between plants and animals (*Gonzales* and *Nebert*, 1990; *Nebert*, 1997). Plants are continuously evolving biosynthetic pathways in order to synthesise secondary metabolites for their reproductive cycles and to defend themselves from insect and animal predators (*Schuler*, 1996). Going back in history, it seems reasonable to assume that when animals started to consume plants, the plants responded by evolving new genes to synthesise toxic metabolites. In order to defend themselves from these plant toxins, animals developed new enzymes to cope with these new plant toxins (*Gonzales* and *Nebert*, 1990). Thus, the P450s have – and have played – a crucial role in the ecological balance in Mother Nature.

In this respect, it is neither surprising that many prokaryotes may contain P450s nor that many of our currently used drugs, often derived from natural plant metabolites, are metabolised by the P450 superfamily of enzymes.

XENOBIOTIC METABOLISM AND SUBSTRATE SPECIFICITY

As shown in Table 2, the CYP families 1,2 and 3 are primarily associated with xenobiotic metabolism. Most of the enzymes belonging to these 3 families have an extremely broad substrate specificity. It has also to be mentioned that many substances are metabolised – to varying degrees – by several different P450s and that one single enzyme can metabolise numerous, structurally diverse chemicals. Taken together, the enzymes, including in these three families have a collective capacity to metabolise – most often detoxify - an

enormous number of substances that we may be exposed to.

It should be mentioned that, in general, enzymes belonging to other families have a higher degree of substrate specificity and they are usually acting more upon endogenous compounds than xenobiotics. On the other hand, enzymes belonging to the three first families may also metabolise some endogenous compounds, as steroids.

LOCALISATION OF P450s

By far, liver is the main site of expression of xenobiotic-metabolising P450s. However, some of them may be found in extrahepatic tissue. It is known that that extrahepatic tissue may contribute to the xenobiotic-metabolising capacity of the body. In turn, this might results in a high local turnover of a drug, thereby influencing upon the local effect of the drug. Such extra-hepatic metabolism might even compensate to some extent for reduced hepatic elimination in cases of severe liver cirrhosis (*Krishna* and *Klotz*, 1994). This can be exemplified as follows. The CYP3A enzymes are involved in the metabolism of around 50% of clinically useful agents and have a very wide substrate specificity. CYP3A4is one of the major enzymes within this family. It is found at relatively high levels in enterocytes in the small intestine. Similar to what found in the liver, it can be induced by rifampin (*Kolars* et al., 1994).

P450s AND MICROORGANISMS

Over the years, it has been found that cytochrome P450s are not uncommon in prokaryotes and it has been found to be present in a number of bacterial strains (*Fulco*, 1991; *Nelson* et al., 1996). The huge enzymatic capacity of the intestinal flora also indicate metabolic reactions similar to those carried out by mammalian P450s It is indeed well known that the intestinal microflora is able to carry out enzymatic reactions involved in phase II, and previous investigations also indicate that the flora may (*Bakke* and *Midtvedt*, 1970) – but not always (*Borud* et al., 1971, 1973), play a role in phase I type of reactions. Comparative studies in germfree and conventional animals have shown that presence of an intestinal microflora induce and/or repress certain isoforms of hepatic P450s (*Nugon-Baudon* et al., 1998). However, the mechanism(s) behind these modulating effects of the intestinal microflora are not well understood. Additionally, it should be mentioned that several microbial enzymes might act upon metabolites formed during phase II reactions. Indeed, deconjugation of bile acids (*Midtvedt*, 1974) and steroid are solely a bacterial event and so is nearly also deconjugation of glucuronides (*Roed* and *Midtvedt*, 1977) and some drugs (*Peppercorn* and *Goldman*, 1972)

P450s AND SPECIFIC MEMBERS OF THE INTESTINAL FLORA

This possible enzyme-modulating effect of the intestinal microflora was the background for a resent study concerning presence of P450s in 18 bacterial strains, selected among the major group of species known to be present in the

human intestinal microflora (*John* et al., 2001). As summarised by the authors, "the amino acid identity, Southern blot and CO difference spectrum data all suggest the presence of a cytochrome P450-like gene in *Eubacterium aerofaciens*". They claim that *Eubacterium* might be found in the human intestine in a density of more than log 10 organisms per ml of content, and that their findings, "demonstrating the presence of cytochrome P450 or P450-like proteins in microflora will help us to gain a better understanding of the role specific microbes, like *E. aerofaciens*, may play in metabolising xenobiotics. In addition, it will allow us to understand its influence on hepatic cytochrome P450 expression and its overall association with tumour suppression and/or formation."

Surely, work like this should be extended. It goes without saying that if probiotics express P450 activity, they may influence upon the metabolism – and efficacy - of several drugs. It is indeed easy to predict that intake of some probiotics may influence upon the efficacy of contraceptives. A scenario that should be taken into consideration is that a long-term intake of a probiotic in a child might influence upon the development of the normal spectrum of P450s in the liver. To the best of my knowledge, investigations along these lines are not included in any of the long-term studies of intake of probiotics in cohorts of children. Additionally, in groups of individuals with an increased number of microbes in the small intestine (elderly people, patients using antacida, etc.), a microbial metabolism of drugs might easily take place before they are absorbed.

On the other hand, however, by accepting that microbes may have the possibility of exerting these types of enzymatic reactions it should be a future goal to select specific bacterial strains with specific action(s) on the (pro)drug given, thereby creating an increased local concentration of active drug. A more distant goal might be to profiling the levels of P450 iso-enzymes in the liver. The answers of the initial questions are: Microbial P450 does exist, but we do not know what it really means.

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A LINK BETWEEN MUCOSAL REGULATORY LYMPHOCYTES AND CHILDHOOD FOOD ALLERGY

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INTRODUCTION

There has been important recent advance in understanding basic concepts of intestinal food allergy, and the role of infectious challenge in the prevention of allergy. There has also been increasing appreciation of the role of non-IgE-mediated pathology, and the basic concepts of food allergy have broadened as the mechanisms of oral tolerance have been unravelled. It has been suggested that the traditional emphasis on IgE-mediated allergy has become less appropriate, as evidence mounts that the role of IgE may be one of modulation of the response to sensitising antigen, rather than as a prime mediator of sensitisation itself (*Walker-Smith* and *Murch*, 1999).

IgE responses to dietary antigens do occur in children in the tropics, but without consequent disease in most children. In allergic children of the developed world, children may manifest immediate and obvious reactions or a complex of delayed symptoms including diet-responsive eczema and a marked disturbance of intestinal motility (*Murch*, 2000).

THE INCREASE IN THE INCIDENCE OF FOOD ALLERGIES

Food allergies are not alone in showing increased incidence. It is well-recognised that there has been substantial increase in incidence of all types of childhood allergy. It is not simply a matter of incidence. Previously rare allergies, such as to peanuts, have become common (*Ewan*, 1996; *Hourihane*, 1997). Thus, in addition to advance in the scientific basis of allergic sensitisation, there has been recognition of novel patterns of food allergic disease in children. Marked increase in the incidence of food allergies of all kinds has occurred. In addition, multiple food allergies and sensitisation of exclusively breast-fed infants to maternal dietary antigens have become commonplace (*Walker-Smith* and *Murch*, 1999; *Murch*, 2000).

DOES IgE OR IgA DETERMINE SENSITISATION?

Genetic predisposition is clearly important in allergy, and candidate genes for allergy susceptibility have been identified, most concerned with IgE generation (*Cookson*, 1999). A population study from Iceland however demonstrated that an IgA concentration in the lower quartile of the normal range was more strongly predictive of allergic disease than elevated IgE (*Ludviksson*, 1993). This concords with Soothill's early report (*Soothill*, 1976) of transient IgA deficiency of infancy in the pathogenesis of allergic sensitisation. It is

probably not coincidental that the cytokine most centrally involved in isotype shift to IgA is transforming growth factor-β (TGF-β), a molecule now recognised as central in oral tolerance mechanisms.

The role of infectious exposures in the generation of TGF-β responses will be discussed later. However it is striking that early-life IgA concentrations, including in cord blood, are elevated in infants born in the developing world compared to developed world infants (*El Seed* and *Dafallah*, 1983). There is as yet little study of specific placental mechanisms regulating cord blood IgA, but breast milk cytokine concentrations may differ in atopic and non-atopic mothers (*Jones* and *Warner*, 2000). It will be intriguing to compare breast milk cytokines in developing and developed-world mothers.

GENETIC PREDISPOSITION TO FOOD ALLERGY

While a history of other atopic diseases is common in food allergic individuals, the major genetic studies have so far been only been carried out in classic atopy, and thus may not be a true representation of susceptibility for food allergy itself. There are regions on chromosomes 2q, 5q, 6q, 12q and 13q that are consistently linked with atopic disorders, with candidate genes including the IL-1 cluster, the IL-4/IL-9/IL-13 cluster, the Major Histocompatibility Complex and the interferon-γ gene (*Rosenwasser*, 1997; *Cookson*, 1999). Several susceptibility regions are shared with chronic inflammatory bowel disease, where tolerance is lost to the enteric flora rather than dietary antigen.

Epidemiological studies suggest a role for T cell responses and MHC type in food allergy, with regional variation in patterns of sensitisation despite broadly similar antigen exposures (*Hill* et al., 1999). Although peanut hypersensitivity is common in Indonesia, it is uncommon in Malaysia, Japan and the Philippines.

REGULATION OF IgE RESPONSES

While non-IgE-mediated food allergy may be the most frequent cause of chronic symptoms, IgE-mediated mechanisms account for the majority of immediate hypersensitive reactions to foods. Transient IgE responses to foods are found in many normal children and these may thus not be clinically relevant (*Sigurs* et al., 1994). However exaggerated IgE responses are clearly important in severe food allergies and anaphylaxis.

Isotype shift to IgE is regulated by products of Th1 and Th2 T cells (reviewed by *Corry* and *Kheradmand*, 1999). Th1 cytokines (particularly IFN-γ and IL-2) limit IgE production, as do Th1-associated cytokines such as IL-12 and Il-18. By contrast, Th2 cytokines, particularly IL-4 and IL-13, directly promote IgE synthesis. This Th1 effect may partly explain the protection against allergy provided by childhood within the developing world, but do not encompass the role of Th2 responses against helminths (*Yazdanbakhsh* et al., 2002).

Class-switching to IgE in response to IL-4 and IL-13, whose receptors share a common α chain (IL-4Rα), is mediated by a signal cascade involving Stat-6 (signal transducer and activator of

transcription-6), and gain of function mutations in this pathway are associated with both murine and human allergic sensitisation (*Shimoda* et al., 1996; *Hershey* et al., 1997). There is evidence of compartmentalised IgE responses within both the intestine and lung, with transportation of mucosally-produced IgE into the gut lumen or airway by a IL-4-dependent mechanism distinct from the poly-immunoglobulin transporter that mediates IgA secretion (*Ramaswamy* et al., 1994).

MULTIPLE FOOD INTOLERANCE, FOOD-ALLERGIC DYSMOTILITY AND THE EOSINOPHIL RESPONSE

In addition to clear increase in IgE-mediated and non-IgE-mediated food allergies, a remarkable alteration in disease presentation has been noted in several countries, where increasing numbers of infants are now sensitising to multiple antigens despite exclusive breastfeeding, often within the first weeks of life (*Hill* et al., 1999; *Walker-Smith* and *Murch*, 1999; *Murch*, 2000). This was rare a generation ago and is still almost unknown in the developing world. These children represent a major clinical challenge, and extensive dietary exclusions are often required. There is a clear association with eczema, food-allergic colitis or enteropathy, and these infants demonstrate a prominent disruption of intestinal motility. This pattern of disease suggests a primary failure to establish basic oral tolerance mechanisms, rather than the loss of previously acquired tolerance of classic food allergy.

There is now increasing evidence that both gastro-oesophageal reflux and constipation may be features of the food-allergic dysmotility syndrome, which is characterised by local eosinophilic infiltration. Epithelial expression of the eosinophil chemokine eotaxin appears to distinguish allergy-associated gastro-oesophageal reflux from primary mechanical reflux in infants (*Butt*, 2002). Eotaxin-deficient mice are protected from the dysmotility associated with mucosal allergy, suggesting that this is an important local response (*Hogan* et al., 2001).

DEMOGRAPHICS OF ALLERGIC SENSITISATION: THE ROLE OF ENTERIC CHALLENGES

Improvement in social conditions in an individual country appears to cause rapid increase in childhood allergies. Thus former East Germany, Estonia and Singapore have seen an increased incidence of allergic diseases of all kinds (*Goh* et al., 1996; *von Mutius* et al., 1998). The particular exposures that reduce risk of sensitisation appear to be gastro-enterological rather than respiratory. Serology performed in Italian military recruits demonstrated that past exposure to food-borne and oro-faecal pathogens in childhood was associated with a reduced risk of allergic sensitisation (*Matricardi* et al., 2000). Both rural upbringing and exposure to animals offers protection against later allergy in both developed-world and developing-world children (*Braun-Farländer* et al., 1999; *Lewis*, 2000).

There may thus be an important role for early environmental exposures in the determination of immune tolerance,

modulating the effects of genetic predisposition. Interest now centres on the specific links between the innate immune system and bacterial exposures in early life (Table 1). There is increasing evidence to suggest an obligatory role for the gut flora and probably also a maturational role for bacterial pathogens in the establishment of immune tolerance (*Fearon* and *Locksley*, 1996; *Sudo* et al., 1997; *Rook* and *Stanford*, 1998; *Sebra*, 1999).

MUCOSAL CHALLENGES IN CHILDREN BORN IN DEVELOPING COUNTRIES

Most children born within the tropics have evidence of enteropathy, and mucosal biopsies would usually be considered abnormal by UK standards. Our study of regulatory lymphocyte responses in the mucosa of developing world children has been based on biopsies obtained at the MRC Unit at Keneba in the Gambia. Gambian children show a pattern of growth faltering typical of deprived areas of the developing world, with UK normal growth velocity for the first 4 months, prior to weaning, followed by decline against UK centiles. At age 2 the mean weight-for-age lies 2SD below UK standards (z-score –2). Previous studies from Keneba confirmed biochemical and dietary deficiencies in these infants. Despite massive dietary supplementation (twice recommended values for energy, $2^1/_2$ times for protein), there was some short-term catch-up growth in malnourished children following gastroenteritis, which reversed as soon as the child was discharged (*Rowland* et al., 1981; *Sullivan* et al., 1992). Because of the failure of dietary intervention to restore growth, other factors have been studied. The most important is small bowel enteropathy, with particular evidence of a role for excess paracellular permeability on lactulose:mannitol (L:M) dual sugar permeability testing (*Lunn* et al., 1991). Over a 1-year period, increased L:M ratio accounted for 40% of growth faltering in Gambian children. Infection alone accounts for a minority of cases: Bacterial pathogens were isolated in <12% in one Keneba study and viruses detected more frequently in non-diarrhoeal controls (*Rowland* et al., 1978). Small bowel bacterial overgrowth and *Giardia lamblia* infection are found in >80% of rural Gambian infants, but neither correlate with growth or gut permeability (*Lunn* et al., 1999).

It is noteworthy that infant mortality rates in Gambia 2002 (c. 100/1000) are similar to those that were seen in London, Paris or New York 1902, where gastro-enteritis and wasting was also the major cause of infant death in underprivileged children. There is clear evidence that polymorphisms in cytokine response genes may give survival advantage in tropical children, and that these vary from country to country (e.g. high TNF producers do better against intracellular pathogens but have higher mortality from cerebral malaria). It is likely that similar selection pressures will have existed a century ago in European children, and that these may play a role in the development of atopy as infectious challenge decreases.

Table 1: Some potential interactions between innate immunity and the gut flora (After: *Murch*, 2001)

Recognition element	Distribution	Bacterial component	Effect transduced
Mannose receptor	Dendritic cells, macrophages, B cells	High-mannose carbohydrates	Increased efficiency of antigen presentation
Natural Antibody	Secreted by peritoneal and intestinal B-1 cells	Surface glycans	Modulation of T cell activation
Complement	Synergy with natural Antibody	O- and N-linked glycans	Opsonisation. Also regulates T cell activation and B cell tolerance
Toll-like Receptors TLR's 1-10 identified, most with as yet undetermined ligands	Dendritic cells, macrophages, T cells, Enterocytes	TLR2 - peptidoglycans TLR4 -LPS TLR9 - Unmethylated CpG repeats in bacterial DNA	NF-κB signaling pathway Increased surface expression of Class II MHC and co-stimulatory ligands
Mannan-binding lectin	Serum-derived. Binds to macrophages, monocytes and B cells	Carbohydrates on Gram-negative and Gram positive bacteria	Activates complement directly via serine proteases MASP-1 and MASP-2
Nod receptors	Intracellular recognition molecules in innate immune cells	Bacterial LPS	NF-κB signaling pathway
Vα24 NK T cells, Vδ1 γδ T cells	Epithelial lymphocyte subsets. Invariant T cell receptor chains	Conserved glycolipid sequences, presented by non-classical MHC (CD1d)	Modulate enterocyte responses, polarise towards Th1 and mucosal IgA production

CONTRASTING CHANGES IN EARLY-LIFE GUT FLORA IN THE DEVELOPED-WORLD CHILD

Lack of appropriate early infectious exposure has been postulated for many years as a cause of the overall increase in allergies (*Rook* and *Stanford*, 1999). These changes may be occurring from very early in life. There is evidence that the initial intestinal colonisation of the developed-world neonate has altered dramatically compared to infants born in the developing world, with reduced colonisation by previously dominant species such as *Bifidobacteria* and frequent discordance between the flora of the mother and her child (*Grutte* and

Muller-Beuthow, 1979; *Simhon* et al., 1982). These changes were not found in infants born in Nigeria, when compared to London-born infants (*Simhon* et al., 1982). Infants born by caesarean section show prolonged abnormality in composition of the intestinal flora and distinct alterations in immune function (*Gronlund* et al., 1999a,b).

We examined the hypothesis that changes in infant handling practices at the time of initial gut colonisation may be important, by study of the 1970 UK national birth cohort, where every infant born in one week in April 1970 has been followed long-term. Those infants who spent the first night away from their mother in the communal nursery had a significantly increased incidence of hayfever at age 26, suggesting that increased exposures to non-familial microorganisms or reduced colonisation by family microorganisms, may be associated with later allergic disease (*Montgomery* et al., 2000). In addition, early analysis suggests that this is a risk factor for inflammatory bowel disease, but not diabetes mellitus (unpublished data). Further support that early-life colonisation is a determinant of later sensitisation has been provided by studies of gut flora in Estonian and Swedish children, and allergic children from either country showed reduced lactobacilli and anaerobes but higher numbers of coliforms and *Staphylococcus aureus* (*Björksten* et al., 1999, 2001). These changes are detectable very early in life, before the development of clinical allergies.

Neonatal administration of probiotics to infants at risk of later allergies induced a remarkable reduction in later eczema (*Kalliomaki* et al., 2001). However it was notable that systemic IgE responses were unaffected, arguing for compartmentalisation of mucosal and cutaneous responses (*Murch*, 2001).

THE DEVELOPMENT OF ENTERIC TOLERANCE

Food allergy requires breakdown of oral tolerance, in which systemic immunological tolerance to an antigen is induced by its ingestion. The molecular mechanisms of oral tolerance to dietary antigens have recently been partially elucidated. The dose of ingested antigen is important in determining the means by which tolerance is maintained (*Weiner*, 1997; *Strobel* and *Mowat*, 1998; *Strober* and *Kelsall*, 1998). Tolerance for high doses of antigen occurs through anergy of potentially responsive T cells. This may reflect antigen presentation by gut epithelium in the absence of co-stimulatory ligands, or active suppression of the lymphocytes by suppressor cells or regulatory cytokines (*Mayer*, 2000). These pathways may be abrogated by breakdown of the epithelial barrier and presentation of dietary antigen by activated antigen presenting cells, as seen in infant sensitisation to cow's milk formula following gastro-enteritis. High dose dietary antigen induces apoptosis of antigen-specific lymphocytes within Peyer's patches in mice, but this has not yet been demonstrated in man (*Chen* et al., 1995). By contrast, tolerance to low-dose antigen is an activation-dependent process, where antigen-specific regulatory lymphocytes producing transforming growth factor-β (TGF-β) are generated (T_H3 cells). This activation-dependent response to low doses may be more difficult to establish in infancy than high dose tolerance, which is mediated by T cell anergy, as neonatal lymphocytes are relatively difficult to activate. Indeed, oral administration of low-dose myelin basic protein sensitised

neonatal rats, while similar amounts induced protective tolerance in adults (*Miller* et al., 1994).

Both T_H3 cells and IL-10 producing lymphocytes (Tr1 cells) suppress immune reactivity within the intestine by a process termed “bystander tolerance“, in which they home to the intestinal mucosa and release TGF-β or IL-10 upon encountering antigen, thus suppress potential reactivity of all surrounding lymphocytes (*Groux* and *Powrie*, 1999). If this process breaks down, immunological tolerance may be lost and allergy or gut inflammation the consequence.

MECHANISMS OF ORAL TOLERANCE: A CENTRAL ROLE FOR NF-κB

The final pathway in the generation of tolorogenic lymphocytes appears to be shared with Th1 responses, with the nuclear transcription factor NF-κB in a pivotal role. Impaired NF-κB responses are clearly linked to mucosal sensitisation, to both the intestinal flora and to dietary antigen in experimental models, and indeed human IBD.

Spontaneous IBD develops in response to the normal flora in CE3Hej mice, who are genetically deficient in the LPD-sensing Toll-like receptor Tlr4 (*Poltorak* et al., 1998). Targeted deletion of NF-κB sub-units in murine knockouts also induces intestinal inflammation. In man, the primary genetic association in Crohn’s disease is a loss-of-function mutation in Nod2, an intracellular LPS sensor which induces a NF-κB response to bacterial responses (*Ogura* et al., 2001). More profound multi-system inflammation occurs in children with mutations in the NEMO molecule (IKKγ), which is an important regulator of NF-κB function (*Courtois* et al., 2001). Thus the paradox is seen that a sub-optimal inflammatory response to the normal flora leads to an exaggerated pro-inflammatory response.

Similar mechanisms appear to apply in the generation of tolerance to dietary antigen. Again, blockade of a sufficiently pro-inflammatory response prevents the normal establishment and maintenance of tolerance to dietary antigen. In study of transgenic mice, whose only T cells recognised a class II MHC-restricted peptide in hen egg lysozyme, *Newberry* and colleagues (1999) demonstrated an obligatory role for physiological inflammation in the establishment of oral tolerance. These mice could only respond to their luminal flora through innate immune cells, but were fully tolerant to dietary hen egg lysozyme in normal conditions. When mucosal production of prostaglandin E2 (PgE2) was prevented by cyclo-oxygenase 2 (COX-2) antagonists, tolerance was abrogated and enteropathy developed in response to antigen ingestion. Thus PgE2 appears to play a fundamental role prevention of immune reactivity to dietary antigens. Constitutive production of PgE2 occurs in lamina propria macrophages in response to the enteric flora, and it functions as a potent inducer of IL-10 production in lymphocytes (*Newberry* et al., 1999). In turn, IL-10 is critical in the generation of regulatory lymphocytes, probably through facilitating generation of TGF-β producing cells (*Groux* and *Powrie*, 1999). There is also clear evidence that mucosal inflammation induces a compensatory TGF-β response (*Xian* et al., 1999).

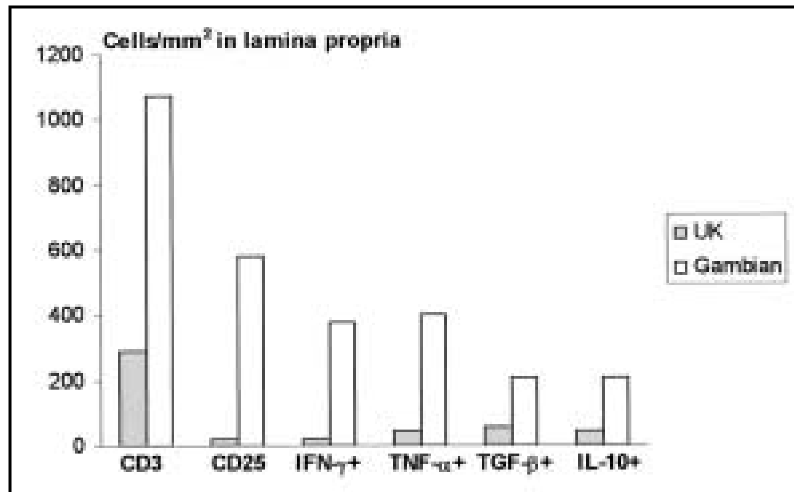

Figure. Mean density of lymphocyte populations in the duodenal lamina propria of well-grown UK and Gambian infants. (Data from *Campbell* et al., 2003).

IS FOOD ALLERGY RELATED TO DEFECTIVE GENERATION OF REGULATORY LYMPHOCYTES?

The data presented above argue for a specific role of early infectious exposures in the generation of enteric tolerance, mediated specifically through regulatory lymphocytes. They would suggest that children brought up in underdeveloped countries with low allergy would have higher numbers of regulatory lymphocytes. In addition, those children within privileged countries who develop allergies may be less efficient in generating regulatory lymphocytes than those who do not. Our data suggest that this might be so.

In our immunohistochemical analysis of Gambian children, the mucosal density of CD25+ lymphocytes was 50-200 times that seen in UK normal controls (*Campbell* et al., 2002). The density of IFN-γ and TNF-α expressing mononuclear cells was approximately 10 times that see in UK infants (Figure 1). Importantly, the density of IL-10+ and TGF-β+ lymphocytes in the Gambian children was also some 10 fold higher than in the UK controls. We noted progressive reduction of TGF-β producing lymphocytes with worsening nutritional status in these children, while Th1 responses were relatively maintained.

In studies of UK children with food allergies, using flow cytometry, immunohistochemistry and *in situ* hybridisation, we found the dominant abnormality to be failure to generate mucosa TGF-β producing lymphocytes, rather than simple deviation of Th1/Th2 responses (*Pérez-Machado* et al., 2000).

These data thus support early contention (*Murch*, 1996) that impaired generation of regulatory lymphocytes might underlie the increase in food allergies within the developed world. All the available evidence points toward a critical role for the gut flora in this process.

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SYNBIOTIC TREATMENT IN CLINICAL PRAXIS

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"Health and well-being is more than merely absence of disease"
Mark Twain

INTRODUCTION

Health and well-being seems depending on availability of some more than two million different molecules, all available in the body in rather exact amounts. Most of the molecules are supplied by foods and made available in the large intestine by fermentative actions. It is a considerable problem that the variability in the food supplied both to domestic animals and humans has dramatically decreased in the modern society. Our Palaeolithic forefathers are said to each year have consumed food from some four to five hundred plants. Modern man has reduced this to a few dozens, and furthermore, many important molecules in foods are destroyed by modern methods to store and prepare the food. The nutritional content of key farming products such as meat and milk has with modern farming methods changed dramatically. As an example, due to the limited variation of diet to domestic cows is the content of omega-3 fatty acids only about 2% in beef and milk, compared to about 30% in free-living, grass-eating and fresh-plant eating cows. It is also known that treatment of foods with high temperature not only destroys important nutrients, particularly antioxidants, but also adds to the food cancer-promoting chemicals, mutagens.

Several observations suggest that health and well-being is the result of a dynamic interplay and balance - homeostasis – between numerous processes that control energy balance, appetite, cell proliferation, repair systems, apoptosis, metabolic rate, stress response, immune response and numerous other processes on which we are depending for our well-being (*Frame* et al., 1998). The attention increasingly given to the homeostasis between omega-3 and omega-6, to the balance between pro- and anti-inflammatory cytokines, and balance in Th1 and Th2 immune response serves as examples.

FERMENTED FOOD HAS OUTSTANDING QUALITY

Our Palaeolithic forefathers used fermentation as their main method to prepare and store food. This method, unfortunately today abandoned in developed countries, but still in use in most developing countries is superior to modern technologies as it not only maintains the content of important nutrients, especially antioxidants, but also sometimes increases it. Microbial enzymes are known to release numerous nutrients from fruit and vegetable fibres

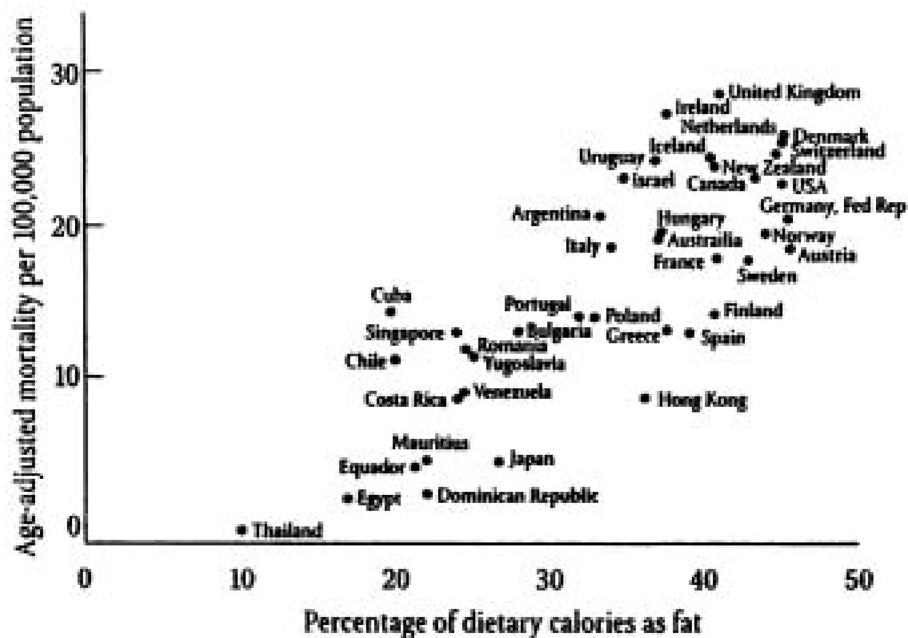

Figure 1: Correlation between percentage of calories as fat in various countries and age-adjusted mortality in breast cancer. (Reproduced with permission from: *Carroll*, 1994).

and make them accessible to the metabolism of the body. As a matter of fact the majority of the more than two million molecules constituting our body are products of microbial digestion in the lower GI tract. The complexity of functions by the flora is illustrated by the fact the intestinal microbes together contain more than 300,000 different genes, compared to the about 65,000 in the rest of the human body.

The Palaeolithic food, the food to which are genes have been adjusted during several millions of years, is said to have contained only half as much of proteins, 1/4 as much of saturated fat and 1/10 of sodium salts. Instead it contained at least 4-5 times as much of plant fibres, 10 times as much of antioxidants, fifty times as much of omega-3 fatty acids, and billion times or more of microbes. It is reasonable to assume that the human genes, adapted during million of years to the lifestyle and food habits of our prehistoric ancestors, badly tolerate the dramatic changes, especially in food habits, which have occurred, during the recent few hundred years (*Eaton* and *Konner*, 1985), and that this could be an explanation to the epidemic in chronic diseases, which has occurred during the last few decades – see further *Bengmark* (2001).

It is clear that people, who live in rural areas of developing countries, and consume large amounts of fruits, vegetables and live microbes – but also much less of animal fat have a much richer GI flora, a better immune response, a compared to Westerners reduced ability to form blood clots, and a significantly better resistance to disease. They also rarely suffer the endemic diseases so frequently observed in Western countries as demonstrated for breast cancer in Figure 1, similar associations being demonstrated for several forms of cancer such as colonic cancer and prostatic cancer, prostatic hyperplasia, diabetes, coronary heart disease, neuro-

Table 1: Probiotics-claimed molecular effects

General:	Produces nutrients and antioxidants
	Produces growth and coagulation factors
	Activates the MALT system
	Modulates Th1/Th2 response
	Promotes antioxidant actions
	Controls potentially pathogenic microorganisms (PPMs)
	Reduces production of endotoxins
	Reduces mutagenicity
Humoral:	Stimulates IgA production
	Inhibits IgE production
	Stimulates NO production
	Modulates cytokine response
Cellular:	Stimulates macrophage function
	Stimulates NK cell activity
	Promotes growth and regeneration
	Promotes apoptosis

degenerative diseases and other endemic diseases. It is not unrealistic to suggest that the numerous compounds released by microbial fermentation and absorbed by the mucosa are important contributing factors.

FLORA BOOSTS THE IMMUNE SYSTEM OF THE HOST

It is increasingly observed that the GI tract is a key organ in our immune defence. Here up to 80% of the immune cells are to be found, and here up 80% of the immunoglobulins are produced (*Brandzaeg* et al., 1989). Commensal flora, some powerful supplemented lactic acid bacteria (LAB), often referred to as probiotics, and bioactive fibres from fruit and vegetables, often called prebiotics, as well as their fermented products, synbiotics, are known to have immuno-modulatory, anti-infectious, anti-inflammatory and antioxidant effects. Table 1 summarises some the effects on the immune system described in the literature. But not all LAB and all fibres are equally effective. The LAB in various yoghurts are chosen for their palatability and have most often rather weak immuno-modulatory effects. LAB do not constitute an authentic genus, it is said that there are greater genetic differences between on LAB and another than between a fish and a human being. LAB with the strongest ability to ferment fibre are found on fruits and vegetable fibres, often semi-resistant to fermentation/digestion by microbial enzymes and found in ethnic foods such as sauerkraut and sourdough. As an example, oligofructans such as inulin and phleins, fibres rich in several fruit and vegetables, but claimed to have strong biological effects, are difficult to ferment and only a few LAB are able to do so (*Müller* and *Lier*, 1994). Only 16 of studied 712 LAB were able to ferment phlein-type fibre and only 8/712 inulin type fibre. *Lactobacillus plantarum* was clearly the most effective and only three other LAB species, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus brevis* and *Pediococcus pentosaceus*

demonstrated ability to ferment these relatively resistant fibres. *Kruszewska* et al. (2002) did in a recent study isolate no less than 180 microbial strains from growing rye. Several of these demonstrated strong bioactivity including strong adhesion to human mucus, induction of pro- and anti-inflammatory cytokines and antioxidant activity. As some of the isolated LAB showed superior bioactivity did they choose to specifically study the effects of *Leuconostoc mesenteroides* 77:1, *Lactobacillus plantarum* 2592, and Pediococcus *pentosaceus* 16:1 – one from each genus of the family of *Lactobacillus* – plus *Lactobacillus paracasei* subsp. *paracasei* 19, chosen among 355 human strains. Interestingly all the strains were able to transcribe NF-κB, to induce pro-inflammatory cytokines (IL-1β and IL-8) and anti-inflammatory (IL-10) and to produce antioxidants. In all these processes did *Lactobacillus plantarum* 2592 show superior ability compared to the others. These four LAB are together bioactive fibres (inulin, beta-glucan, resistant starch and pectin) chosen to constitute a new symbiotic composition – Synbiotic 2000 – which presently is under clinical evaluation (see further below).

PREBIOTIC FIBRES ARE ESSENTIAL

The human digestive tract is for its growth and functions much depending on supply of prebiotics. In contrast to cows milk is breast milk very rich in fibres. Apart from elephant milk, no other mammalian milk analysed till today contains as much of fibre as breast milk (*Gnoth* et al., 2000). The complex fucosylated oligosaccharides in human milk, with structural similarities to immunomodulating cell surface glycoconjugates, are supposed to protect breast-fed infants against inflammations and infections (*Gnoth* et al., 2000). In addition, these fibres are likely to function as prebiotics and stimulate growth of the non-pathogenic gut microflora in the breast-fed infants.

Fruit and vegetable fibres are known to also have strong influence on intestinal growth in most mammalians. As an example, six weeks of supply of fermentable fibres (beet pulp and oligofructose) to experimental animals increases the GI surface area by 28%, the mucosal mass by 37%, the mucosal weight by 35% and the capacity for carrier-mediated glucose uptake by 95% (*Buddington* et al., 1999). Another important function of fibre is to block receptors and prevent colonisation by potentially pathogenic microbes. For example mixing 2.5% of D-mannose into drinking water reduces significantly colonisation of newborn chicken with *Salmonella* (*Oyofo* et al., 1989).

Up to 25% of the adult population in Western countries suffer today of metabolic syndrome, a condition in which insulin resistance is a significant characteristics. However, increased intake of dietary fibre (celluloses, hemicelluloses, pectins and starches), the main substrate of SCFA production, increases insulin sensitivity in humans (*Randle* et al., 1963). Malhotra, an Indian physician, observed already in 1968, that men living in North India (Udaipur), and consuming large quantities of cellulose and vegetable fibres + live lactobacilli had a longer mean clotting time, and soft jelly-like clots compared to men in urban Madras. It has also been shown that supplement of the fibre konjac-glucomannan to baboons living on Western diet significantly lowers the plasma level of fibrinogen and of factor X (*Vorster* et al., 1985)

and that plasma viscosity and fibrinogen levels decrease significantly in diabetic children on supplementation with the fibre guar gum (*Koepp* and *Hegewisch*, 1981). It is also reported in the literature not only that the incidence of post-operative thrombosis is significantly reduced in patients on a high fibre diet (*Frohn*, 1976; *Latto*, 1976), and that supply of fibre (glucans) will significantly reduce the mortality rate in hospital infections in patients with severe trauma (*DeFillipe* et al., 1993).

Another group of fibres, fructo-oligosacharides (FOS), are known both to increase the numbers of certain lactic acid bacteria, particularly *Bifidobacteria,* but also to significantly decrease the number of *Enterobacteriaceae* in healthy humans. Mixing 10% of FOS in the diet to experimental animals had both protective and therapeutic effects against sodium sulphate-induced colitis (*Umemoto* et al., 1998). It is observed that supplementing patients with IBD with 30 g fructo-oligosaccharides (FOS) per day increases significantly the amount of luminal SCFAs (*Umemoto* et al., 1998). But also other fibres such as *Psyllium husk* (*Hallert* et al., 1991) and *Platago ovata* seeds (pectins) (*Fernandez-Banares* et al., 1999) are reported to have similar effects. A most recent study is of considerable interest. Germinated barley, the aleurone and scutellum fraction, of the grain is known to be rich both in hemicellulose fibres and in glutamin-rich proteins. When supplied to experimental animals did it result in dramatic improvement of induced colitis, an effect further attenuated by combining with LAB (*Fukuda* et al., 2002). Also antibiotics (vancomycin, metronidazole) did in this study significantly attenuate clinical and pathological scores – but in contrast to treatment with pre- and probiotics did treatment with antibiotics result in a significant decrease in caecal buturate levels.

Pectins are one of the several fibre groups known for their many strong bioactivities. It is a superior mucosa protectant, strong antioxidant, vehicle for transport of LAB through the GI tract and a superior substrate for bacterial fermentation. The unripe banana (green sweet banana as well as plantain) is rich in both pectin and resistant starch. 250 g/l of green banana (equivalent to two fruits) or 2 g pectin/kg food was recently tried as a supplement to rice diet in children in Bangladesh suffering from persistent diarrhoea. The amounts of and frequency of stools, the duration of diarrhoea, numbers of vomiting, and use of oral rehydration or amounts i.v. fluid solutions given were all significantly reduced in the two treatment groups (*Rabbani* et al., 2002). Recovery on third day was seen in 59% in the green banana group, in 55% in the pectin group compared to 15% in the only rice group.

PROBIOTICS IN DIARRHOEA IN CHILDREN

A larger European multi-centre trial in children one month to three years of age was undertaken: One-hundred-and-forty children were randomly allocated to oral rehydration and placebo, another 147 children to oral rehydration and daily supply of 10^{10} CFU of *Lactobacillus* GG (*Gualdalini* et al., 2000). Clinical signs of diarrhoea lasted 58.3±27.6 hours in the LAB-treated group to be compared to 71.9±35.8 hours (p=0.03) in the placebo group. Diarrhoea lasted in rotavirus-positive children treated with LAB 56.2±16.9 hours compared to 76.6±41.6 in the control group (p=0.008).

Lactobacillus GG was also tried in order to prevent diarrhoea in a placebo-controlled trial performed in 204 under-nourished Peruvian children, age 6 to 24 months (*Oberhelman* et al., 1999). The treatment was given to all children during a period of 15 months. The lactobacillus-treated children had fewer episodes if diarrhoea (5.21 episodes/child and year compared to 6.02 in the placebo group, p=0.028). The therapeutic gain, as pointed out by *du Pont* (1999) and others, must be regarded as modest. Most likely use of other and more potent LAB, or combinations of LAB, should lead to a more significant therapeutic success.

One thousand three-hundred-thirty-six new-born Columbian children with risk of developing severe diarrhoea received prophylactically during one week (or until they were discharged) a daily supply 250 million live *Lactobacillus acidophilus* and 250 million live *Bifidobacterium infantis* and the outcome compared to outcome for similar children treated during the year before (*Hoyos*, 1999): The incidence of narcotising enterocolitis was reduced by two third (18 vs. 47, p<0.0005), and by half (19 vs. 38, p<0.03) in the patients transferred from other hospitals – patients which most likely were sicker and came late under treatment. No complications could be attributed to the use of probiotics, even when given to very sick new-born children with an average weight of 2600 g (range <1000 to >4000 g), and often suffering from severe conditions such as sepsis, pneumonia or meningitis. Incidentally it was observed that the LAB-treated children suffered significantly less diaper dermatitis.

Lactobacillus GG (LGG) was also tried in order to prevent diarrhoea in a series of 202 antibiotic-treated children. Twenty-five of the placebo placebo-treated (26%) and only 7 of the LGG-treated children developed diarrhoea (*Vanderhof* et al., 1999). The mean duration of diarrhoea was 4.7 days in the LGG group vs. 5.88 days in the placebo group. Again, the efficacy of the treatment is not impressive, and as pointed out by *Saavendra* (1999), "the reduction of 1 day of two liquid stools over a 10 day period in a child might be questioned".

PROBIOTICS – AND PREBIOTICS - IN INFLAMMATORY BOWEL DISEASE (IBD)

We observed in the early nineties that humans with inflammatory bowel disease have a reduced LAB flora, but also that induced colitis in experimental animals could be significantly reduced by supply of a combination of pre- and probiotics – synbiotics (*Fabia* et al., 1993a, 1993b). Subsequently it has been convincingly demonstrated that the concentrations of endogenous *Lactobacillus* and *Bifidobacteria* are significantly reduced in patients with active Crohn´s disease, ulcerative colitis, pouchitis as well as in experimental colitis (*Favier* et al., 1997; *Sartor*, 1999). Another recent study found both quantitative and qualitative changes in the LAB flora, when studying colonic biopsies from patents with ulcerative colitis (UC) (*Pathmakanthan* et al., 1999). A significant quantitative decrease in growth of *Lactobacillus* spp. in colitis biopsies was observed, but also a reduction in total aerobic speciation: 18 subspecies being found in UC patients compared to 32 in controls. Furthermore, anaerobic speciation revealed in average 4.7 subspecies in UC patients compared to 6.7 in controls. Incidentally it was observed that *Bacteroides*

thetaiotaomicron occurred more often in UC patients: 8/10 biopsies vs. 4/10 in controls - an observations, which significance remains to be explored.

A LAB cocktail called VSL#3 consisting in four *Lactobacillus* strains, three *Bifidobacterium* strains plus *Streptococcus salivarius* ssp. *thermophilus* ($5x10^{11}$ cells/g) is presently tried quite extensively around the world. This composition is most probably chosen at random without any further documentation of the molecular/immunological effects for each of the LAB, nor any evidence of synergistic effects. When three gram a day was given during one year did 15/20 patients remain in remission, one lost to follow up and 4/20 showed signs of relapse (*Venturi* et al., 1999). VSL#3 was also tried in a small controlled study in patients with pouchitis. Only 3/20 patients had relapse of the disease when supplied with VSL#3 compared 20/20 control patients (*Gionchetti* et al., 2000). These results are most likely better than what presently can be achieved by any conventional treatment, an assumption supported by a recent systematic review of the literature suggesting that " metronidazole is an effective treatment for active chronic disease" (odds ratio 12.34) but "oral probiotic therapy with VSL#3 for maintaining remission" (odds ratio 15.33) (*Sandborn*, 1999).

Although the scientific basis for treatment of IBD seems reasonable and attractive, it must be emphasised that it is far too soon to recommend routine use of probiotics in IBD. Further studied are much warranted. The good results obtained in the two small studies cited above seem to suggest that combination of several LAB might have strong clinical effects in IBD, eventually stronger than the use of single-bacteria treatments. It is tempting to anticipate that a cocktail consisting in LAB, where each of the bacteria has been chosen with the regard to their documented metabolic and immunological effects, should eventually be even more successful. It also tempting to suggest that combination with strong bioactive fibres (prebiotics) might even more improve the efficacy of treatment. The ideal treatment remedy will probably be complex, and much remains before the most suitable prebiotics, and the most effective probiotics have been identified.

A most recent study is of considerable interest (*Swidsinsky*, 2002). These authors studied the flora in 305 IBD patients and 40 controls using the most modern techniques: Quantitative PCR, cloning, sequencing fluorescence *in situ* hybridisation and electron microscopy. They observed a high density of mucosal bacteria in sick patients, but also that the microbial "close to mucosa"-density of microbes increased progressively with increasing severity of disease. Patients with > 10,000 CFU/µl showed a pronounced "band" of bacteria attached to mucosa, and patients with >50,000 CFU/µl had also signs of inclusions of polymorphic bacteria within solitary enterocytes next to lamina propria. The authors speculate that healthy mucosa is sterile - capable of holding back faecal bacteria and prevent a close contact of the microflora to the epithelial surface. It is likely that this function can be supported by treatment with a combination of pre- and probiotics (synbiotics).

PROBIOTICS IN *HELICOBACTER PYLORI* INFECTIONS

It is now almost fifteen years since it was demonstrated that lactic acid produced by *Lactobacillus acidophilus* has the capacity to inhibit *Helicobacter pylori* (*Bhatia* et al., 1989). The antibacterial activity of seventeen strains of lac-

tobacilli against ten different strains of *H. pylori* was recently studied (*Lorca* et al., 2001). All *Lactobacillus* strains were able to inhibit *H. pylori*, but the effect was lost if pH was adjusted to 6.0. However, the effect of *Lactobacillus acidophilus* CRL 639 remained even after pH was adjusted. The effect seemed less related to pH and more to release of a proteinaceous compound, with autolysin effects.

One-hundred-and-twenty *H. pylori* patients were randomised to, in addition to a 7-day triple therapy (Rabeprozole, Chlarithromycin, Amoxicillin), receive either placebo or a lyophilised and inactivated culture of *Lactobacillus acidophilus*. The eradication rate was significantly improved by supplementation of the LAB: 52/59 patients (88%) vs. 42/58 patients (72%) (p=0.03) (*Canducci* et al., 2000). The effects of live *Lactobacillus* GG was also investigated but with less success: although the study reports improved tolerability (reduced antibiotic-induced bloating, diarrhoea and taste disturbances), no improvement in the rate of eradication was when live *Lactobacillus* GG was used (*Armuzzi*, 2001a, 2001b).

Daily oral consumption of 4x50 ml of the supernatant from a whey-based *Lactobacillus acidophilus* (La1) culture, combined with either omeprazole or placebo, was reported to show a significant reduction in breath test both with and without supply of omeprazole, immediately as well as six weeks after the treatment episode (*Michetti* et al., 1999). It should be remembered that whey is extraordinarily rich in immunologically active and anti-infectious substances such as lactoferrin, lysozym and many other antimicrobial peptides. It is thus, this far not clear whether the observed effects are due to the *Lactobacillus* used, to the whey or a combination of both.

A recent study (*Sakamoto* et al., 2001) reports considerable improvement, both in urea breath test and serum pepsinogen in 31 patients with *Helicobacter pylori infections* treated during eight weeks with *Lactobacillus Gasseri OLL 2716.*

SYNBIOTICS IN ICU PATIENTS

There are good reasons to believe that pre-, pro-, and synbiotics could dramatically change the outcome for critically ill patients, and be a good alternative to the use of antibiotics in ICU patients. It is regrettable that this far only a handful of studies have been performed in critically ill and postoperative patients, and, furthermore, and most of these studies are under publication.

Severe acute pancreatitis

Contamination of the pancreatic tissue occurs frequently in severe pancreatitis, being reported to be 24% during the first week and amounting to 72% during the third week (*Beger* et al., 1986). Pancreatic sepsis seems to be a strong determinant for complication such as multiple organ failure (MOF) and of death. It has rather recently been shown that infection of the pancreatic tissue is almost always preceded by about one week of colonisation the large intestine with non-coli Gram-negatives: *Pseudomonas*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Acinetobacter*, *Morganella*, *Serratia* or *Proteus* (*Luiten* et al., 1998). Prevention of such a colonisation could be expected to have a dramatic influence on outcome.

A prospective double-blind randomised study, comparing the influence of *Lactobacillus plantarum* 299 and oat fi-

bre with heat-killed Lactobacillus plantarum 299 and oat fibre (control) was recently performed in severe pancreatitis (*Oláh* et al., 2002). The study was designed to be concluded when repeat statistical analysis demonstrated statistically significant differences between the two study groups. This occurred after all together 45 patients had entered the study. At that time 22 patients had during seven days received treatment with live LAB and 23 with heat-killed LAB during seven days. Infected necrosis and abscesses occurred in 1/22 patients (4.5%) in the live LAB group and in 7/23 patients (30%) with heat-killed LAB. Abscesses occurred in 1/22 (4.5%) in the treatment group vs. 7/23 (30%) (p=0.023) in the control group. Although the length of stay was 13.7 days in the treatment group vs. 21.4 days in the control group, the differences had not reached statistical significance at the time when the study was interrupted. The only patient who developed sepsis in the treatment group did that after fifteen days, e.g. eight days after the treatment has been discontinued. This seems to suggest that treatment should be provided for a minimum of 14 days and most likely as long as the patients are on antibiotics or have signs of GI colonisation.

Abdominal surgery patients

A prospective randomised study compares the effect of live *Lactobacillus plantarum* 299 in a dosis of 10^9 with heat-killed *Lactobacillus plantarum* 299 in the same dose and parenteral nutrition in 3x30 patients undergoing abdominal operations such as liver resection, pancreas resection, gastric resection, colon resection and intestinal by-pass (*Rayes* et al., 2002a). The groups treated with either live or heat-killed LAB suffered less infections (3/30 in each group, e.g. 10%) compared to 9/30 (30%) in the parenteral group (p>0.001). An even larger difference was observed when the subgroup of gastric and pancreatic surgery patients was separately analysed: None of eight patients receiving live LAB group, one of eight patients (12%) receiving heat-killed LAB group and 3/6 (50%) conventionally treated with parenteral nutrition suffered infections.

Liver transplantation patients

A separate study was performed in human liver transplants by the same group of clinicians in a study with a similarly sized material of patients. Comparison was made between selective bowel decontamination (SBD) + a standard enteral formula, live *Lactobacillus plantarum* 299 + oat and inulin fibres, and heat-killed *Lactobacillus plantarum* 299 + oat and inulin fibres (*Rayes* et al., 2002b). The total amount of fibres in the two last groups was about 11 gram. The LAB were supplemented during the first five days. The sepsis rate was 48% in the selective bowel decontamination group, 34% in the group treated with heat-inactivated LAB and 13% in the group receiving live LAB. Also the mean duration of antibiotic therapy, the mean total hospital stay and the stay on ICU were shorter compared to the groups with inactivated lactobacilli and fibre or with SBD. However, the size of the patient material did not allow statistical significance to be reached.

FLORA IMPORTANT ON ALL BODY SURFACES

Not only the gastro-intestinal tract, but also all body surfaces are coated by a protective flora, essential for prevention of infection and inflammation. Second to the GI tract with its one to two kg of flora is the skin, calculated in the

adult human to be inhabited by approximately 200 gram of bacteria. Other important sites are the mouth and pharynx, the respiratory tract and the vagina, each supposed to be inhabited by approximately 20 gram of flora. Too much washing and cleaning will impair this defence and open the door for opportunistic infections. Animals have the instinct to lick their wounds and hereby provide both protective flora and growth factors, produced by the salivary glands. To apply topically lactic acid bacteria on the skin and around all penetrations of the skin by foreign materials such as tubes, drains, tracheostomies etc is receiving an increasing interest. Such a treatment could also be of potential interest for treatment of burns.

It has been observed that infants treated with probiotics suffer much less diaper dermatitis (*Hoyos*, 1999). Two recent reports suggest that consumption of LAB-containing drinks prevents formation of biofilm and removes both yeast and bacteria from silicon rubber voice prostheses (*Free* et al., 2000; *van der Mei* et al., 2000). The flora is invariably reduced at all these sites in sick and hospitalised patients due to special hygienic requirements and large supply of antibiotics and other drugs. An overflow of probiotic bacteria from the GI tract to all the other sites seems normally to occur, a function, which most likely is severely reduced in the sick. It is not unlikely that in the future a dietary supply of pre- and probiotics be complemented by spraying or applying gels of LAB on sensitive body surfaces, especially around the skin penetrations, but also by using LAB-containing aerosols to promote flora of the respiratory tract, where such a protection layer is much needed.

GUT ECOLOGY AND HEALTH – FUTURE ASPECTS

Gut ecology is of the greatest importance for maintenance of health and prevention of diseases, increasingly seen in Western countries and increasingly linked with deranged gut flora and mucosal lesions. Not only has it been observed that diseases such as rheumatoid arthritis (*Midtvedt*, 1987; *Zhang* et al., 2000; *Nieuwenhuis* et al., 2000) and atopic diseases (*Satomi*, 1966; *Rock*, 1998; *Wold*, 1998) are associated with gut flora derangements but also diseases such as autism (*Sandler* et al., 2000; *Wakefield* et al., 2000; *Furlano* et al., 2001; *Lindsey*, 2001; *Torrente* et al., 2002), graft-versus-host disease (*van Bekkum* et al., 1974; *Porrata* et al., 2001) and formation of serosal adhesion (*Bothin* et al., 2001) are intimately associated with gut flora and mucosa.

Some observations suggest that both pre- and probiotics can modify basic bodily functions such as appetite, sleep, mood and circadian rhythm, most likely through signal molecules but also through metabolites produced by microbial fermentation in the gut, known to influence lymphocyte function, production of immunoglobulins and resistance to disease - see further *Bengmark* (2002a,b).

There are also indications in the literature that intestinal microflora stimulates myo-electric activity in the intestine and hereby controls gastrointestinal motility and transit of food under digestion (*Husebye* et al., 1994). Recent studies performed in germfree animals do also suggest (*Hooper* et al., 2001) that some commensal bacteria modulates the genes involved in whole series of important intestinal functions such as nutrient absorption, mucosal barrier fortification, xenobiotic metabolism,

angiogenesis and postnatal intestinal maturation.

The dramatic change in recent years in our knowledge and understanding of the complex functions of the lower GI tract and its function has without question contributed considerably to our understanding of health and disease. During my lifetime the view on the large intestine and its functions has radically changed from being an organ mainly for re-absorption of electrolytes and water to a complex organs, which holds important keys to health and well-being. Further exploration of the large intestine and its interaction with flora has the prospect of helping us to understand and prevent a whole series of diseases including the endemic diseases so much plaguing the Western, and increasingly also the Eastern world.

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DEVELOPING AN *IN VITRO* MODEL ON THE INVESTIGATION OF THE CROSSTALK AMONG BACTERIA, ENTEROCYTES AND LEUKOCYTES NEAR THE INTESTINAL MUCOSA

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SUMMARY

In vitro models comprising human cells are potent tools to improve our understanding on the crosstalk between enterocytes and bacteria on the one hand and between leukocytes and enterocytes on the other. The present manuscript deals with the possibility to develop a co-culture model for the investigation of inflammatory processes near the intestinal mucosa. One important demand on such a model is the spatial separation of bacteria and leukocytes mimicking the morphologic conditions in the intestine. This is achieved by the separation of the apical compartment with non-pathogenic bacteria from the basolateral compartment with human leukocytes by a confluent monolayer of a differentiated cell line of intestinal origin (Caco-2).

The gut-associated lymphoid tissue (GALT) comprises Peyer's patches, mesenteric lymph nodes, intra-epithelial lymphocytes, and leukocytes of the lamina propria. The complex interaction of these four compartments cannot be simulated by an *in vitro* model, but inflammatory processes are likely to be initiated and controlled by alpha-beta T cells in the lamina propria, the action of which is likely to be simulated most closely by the present model.

Preliminary results on the kinetics of cytokines in the basolateral compartment revealed a temporary occurrence of tumour-necrosis factor alpha, a steady increase in the concentration of interleukin (IL)-8 and a stable concentration of IL-6 and IL-10 after 24 hours. Cytokines associated with the activation of Th1-cells (IL-2 and interferon gamma) occurred after 24 h and steadily increased from that time. Th2-type cytokines (IL-4, IL-5) were not detectable in the basal medium.

The present model offers chances in the investigation of drugs and food ingredients on the enhancement or impairment of inflammatory processes in the intestine avoiding differences between the immune responses of man and animals in e.g. feeding models with rodents.

INTRODUCTION

Background

To improve our understanding of mechanisms inducing processes in the intestine makes sense from different points of view. First, events leading to inflammatory bowel disease (IBD), namely Crohn's disease and ulcerative colitis, are not completely understood. Second, the need for developing new strategies to take influence on the effi-

cacy of the immune response by compounds or nutrients will increase. Especially as the percentage of elderly people increases in industrialised countries, problems associated with the impaired immune response in these people ('immunosenescence') have to be met.

The systemic immune response in terms of resistance to pathogens can highly be improved by the application of probiotic organisms. In mice, the application of *Lactobacillus casei* prior to infection with *Salmonella typhimurium* (dose: 20 x LD_{50}) prevented completely the occurrence of pathogens in the liver and spleen (*Perdigon* et al., 1991). As evident from these experiments, the effect of e.g. probiotics can be immense but further investigations are necessary to understand the mechanisms standing behind these effects. Although insights can be achieved by using experiments with animals, considerable differences can occur in parameters of immune function between animals and man (*Lebrec* et al., 1995). Therefore, *in vitro* models using cells of human origin have become more important during the last two decades, but efforts in the development of such models mimicking the immune response in the intestine were very limited so far. Furthermore, at least at the primary stage of investigating immunomodulating properties of foodstuff, screening methods will be needed enabling first statements on their effectiveness.

An important mediator of the systemic immune response is the gut-associated lymphoid tissue (GALT). This complex system, which is part of the mucosa-associated lymphoid tissue (MALT), comprises four different parts: The Peyer's patches, mesenteric lymph nodes, intra-epithelial lymphocytes (IEL), and various leukocytes located in the *lamina propria*. Despite the fact that the latter two compartments are separated only by the thin basal membrane, they have to be distinguished clearly due to their immunological functionality. Developing an *in vitro*-model for the modulation of inflammatory processes in the intestine, one has to be aware which parts of this immune system *should* and *can* be simulated.

Which part of the GALT action can be mimicked in an *in vitro* system?

The initiation of the specific immunity acquired within the GALT takes place in the Peyer's patches (PP), which can be considered as classical secondary lymphoid organs. After controlled translocation of single bacteria by specialised M-cells, interdigitating dendritic cells (IDC) present antigens to T lymphocytes and macrophages mainly to B lymphocytes. During the maturation process, the primed cells leave the PP via the mesenteric lymph nodes to relocate after maturation in the lamina propria or other sites such as the mammary gland, the lacrimal gland and beneath other mucosa surfaces of the body. The expression 'homing' termed for this process is not completely exact, as the cells never return to the site of their initiation but to places where they can fulfil their efferent immunological tasks. The main function of the cells having undergone this fate is the predominant production of IgA. Evidently, this process involving differentiation processes in other tissues than near the intestinal mucosa cannot be mimicked in an *in vitro* model dealing with the immune response in the intestine. Therefore, investigations within such a model can only focus on actions of cells other than naïve T/B cells in PP and activated memory B lymphocytes and $CD45RO^{+}/CD45RB^{low}$ lymphocytes, which have lost their ability for being stimulated in terms of acute inflammation (*Abreu-Martin* et *Targan*, 1996).

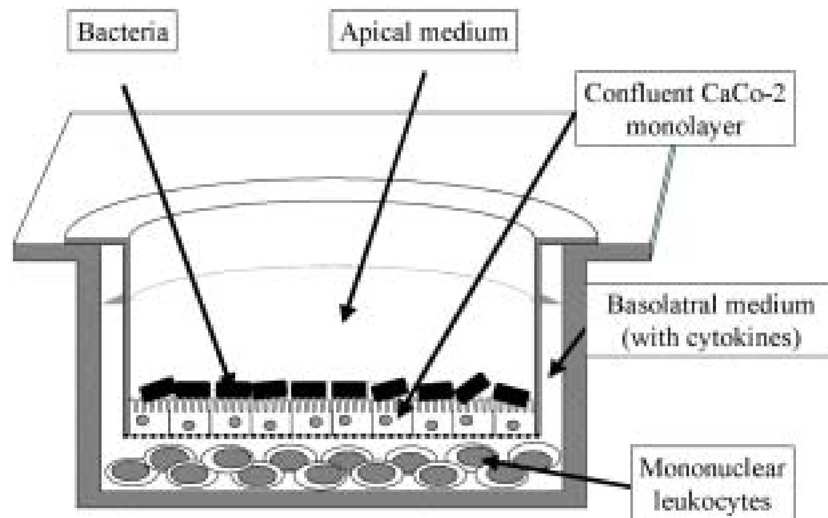

Figure 1: Arrangement of bacteria, Caco-2 cells and peripheral blood mononuclear cells (PBMC) for the investigation of immune responses near the intestinal surface

Although an important part of the GALT, intra-epithelial lymphocytes (IEL), which comprise a considerable percentage of CD8-positive cells (>80%) expressing frequently the CD8αα+ receptor (*Latthe* et al., 1994), are also considered as being practically not being able to respond to TcR stimuli (*Ebert*, 1989). The low, but quite constant percentage of γδ-TcR cells (in humans: ~10% of total T cells; *Viney* et al., 1990) seems also to be of inferior importance for intestinal inflammation, as αβ-TcR cell depleted mice failed to develop inflammation after *Salmonella* infection but not γδ-TcR cell depleted animals (*Weintraub* et al., 1997). Hence, an *in vitro* model focussing on inflammatory processes within the intestine should mainly be oriented on the interaction among naïve LPL (and macrophages), enterocytes and the intestinal flora.

Which kind of cells can be used in the co-culture model?

To achieve the best possible results in experiments concerning reproducibility and similarity to conditions in the intestine, the following demands should be met by cells representing the three parts of the system mentioned above:

Leukocytes should mainly comprise human naïve αβ-T cells and cells of the monocyte/macrophage lineage being susceptible for an inflammatory challenge. An acceptable source for these leukocytes is peripheral blood mononuclear cells (PBMC), which can easily be obtained in large amounts from cell-enriched plasma ('buffy coats').

Enterocytes are only appropriate for an *in vitro* model of intestinal inflammation if they can maintain the separation of two compartments: the 'outer' (apical) compartment with bacteria and the 'inner' (basolateral) compartment with leukocytes. This will only succeed with cells that keep their proliferation only until confluence and are able to develop a polar differentiation. Caco-2 cells meet these requirements and are able to form desmosomes, microvilli, and tight junctions (*Hidalgo* et al., 1989). Additionally, this extremely well-defined cell line (*Pinto* et al., 1989) keeps its viability also in media suitable for incubation of leukocytes such as DME medium.

The Caco-2 cells have to be kept for growth until confluence and differentiation on a semi-permeable membrane until the end of incubation to enable signalling between leukocytes and the enterocyte-like cell line by soluble factors. To guarantee that bacteria cannot transmigrate through the membrane, a pore diameter below the average size of bacteria (e.g. 0.4 μm) is suitable for this purpose.

Bacteria found in the intestine are very inhomogeneous and their composition can differ significantly between single hosts. Apart from studies where differences among specific rods are to be investigated, the application of one non-pathogenic, well-characterised bacteria species that is able to evoke an immunological response within the described system seems appropriate. The immunomodulating effect of drugs or nutrients to be investigated can than be assessed by enhancement or impairment of the immunological response to the standardised bacterial challenge. Evidently, the germs have to be impaired in their growth to avoid destruction of the Caco-2 cell monolayer. A major problem in this context is the question in how far the growth of the applied bacteria can be reduced without changing the induced immune response. Antibiotics damaging the integrity of the bacterial membrane should be avoided, as the bacterial lysate is likely to cause modifications of the induced immuno-activation. A sketch of the co-culture model is given in Figure 1.

How to measure the extent of the immune response?

Concerning the readout of the experiments, the middle-termed immune response can be assessed by measurement of cytokines in the medium. First experiments with this model by *Haller* et al. (2000) revealed that practically all cytokines are secreted into the basolateral but not the apical compartment. Out of the numerous cytokines/chemokines, those indicating acute inflammation [tumour necrosis factor alpha (TNF-α), Interleukin 1 beta (IL-1β), IL-8)], immunomodulation [IL-10, transforming growth factor beta (TGF-β), IL-6], activation of Th1 cells (IL-2, interferon gamma (IFN-γ)], and activation of Th2 cells (IL-4, IL-5, IL-13) might be of special importance for the assessment of changes in the immune response. For a profound understanding of the interaction between these cytokines and the eukaryotic cells of the system, the recording of a time kinetic of each cytokine is necessary. The involvement of the enterocyte-like cell line can be estimated by (semi-)quantitative measurement of mRNA expression for some of the cytokines mentioned above (i. E. IL-8 and TNF-α, *Haller* et al., 2000).

Further important information can be obtained by measuring the release of reactive oxygen species (ROS). The activation of phagocytotic cells, which are also part of the PBMC (monocytes, ~20-35%), results in the pentose phosphate shunt, NADPH production, and the massive release of ROS. As ROS are known as potent reagents being able to modify DNA, especially mechanisms leading to the high coincidence of chronic inflammatory bowel disease (IBD) and malignant neoplasiae in the colon might be elucidated by investigations with the present model. The release of ROS can easily be measured online by the application of chemiluminescence-enhancing techniques (*Parlesak* et al., 1998).

MATERIAL AND METHODS

Cell culture

The cell line Caco-2 was obtained from the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (Braunschweig, Germany: DSMZ No. ACC169). Cells were cultured in inserts on a semi-permeable PET membrane (Falcon, Becton-Dickinson, Le Pont De Claix, France). Inserts were placed into cavities of six-well plates (Greiner, Frickenhausen, Germany), which were filled with cell culture medium that consisted of Dulbecco's Modified Eagle Medium (DMEM) and heat-inactivated (56°C for 30 min) foetal calf serum (FCS) (Gibco BRL/Life Technologies, Karlsruhe, Germany). FCS was controlled for endotoxin content (<0.2 ng/ml: LAL Test, Chromogenix, Mölndal, Sweden). Confluency was checked by measurement of transepithelial electrical resistance (TEER; *Haller* et al., 2000) and visual control of cell layer integrity under the microscope. After complete differentiation, which lasted for further 10 days, co-incubation experiments were performed.

As a stimulus, the non-pathogenic rod *Escherichia coli* K12 was used at the stationary growth phase. Gentamicin (120 μg/ml; Gibco BRL/Life Technologies) was added to the medium to avoid uncontrolled growth that would lead to destruction of the Caco-2 cell monolayer (*Haller* et al., 2000).

Mononuclear leukocytes from peripheral venous blood (PBMC) of healthy donors (n=3) were isolated from buffy coats (cell-enriched plasma) by density gradient centrifugation (Ficoll® solution, Seromed/Biochrom, Berlin, Germany). After centrifugation, mononuclear leukocytes were taken from the interface and washed three times with cell culture medium.

Each experiment was performed as a triplicate. Incubations with non-pathogenic bacteria (*E. coli* K12, 2.0 x 10^7 CFU) were applied either apically or basolaterally with PBMC (4.0 x 10^6). The release of cytokines into the basolateral compartment was measured by commercially available ELISA kits (BD Pharmingen, Heidelberg, Germany). The resulting values were compared to the corresponding control experiments.

FIRST RESULTS AND CONCLUSION

First results from experiments with this model revealed that findings obtained in this model cannot be compared with those achieved by bacteria-leukocyte or bacteria-enterocyte co-incubations. *Haller* et al. (2000) stimulated the differentiated Caco-2 cell monolayer in the described trans-membrane system with different bacteria (*Lactobacillus johnsonii, L. sakei,* and *E. coli*) with and without leukocytes in the basolateral compartment. Messenger RNA for IL-8 and monocyte chemoattractant protein 1 (MCP-1) was expressed in Caco-2 cells only if leukocytes were present in the basal compartment and only by *E. coli* and *L. sakei* but not by *L. johnsonii*. Hence, the Caco-2 cells can distinguish between single bacteria species only if mononuclear leukocytes are present in the basolateral compartment. Further actual findings with this model demonstrated that IL-2, which cannot be produced by the enterocyte-like cell line (*Jung* et al., 1995), is produced in the basolateral compartment only if the Caco-2 cell monolayer separates bacteria and leukocytes. If bacteria have cell-cell contact to the leukocytes, practically no IL-2 production occurs within the first 68 h after challenge.

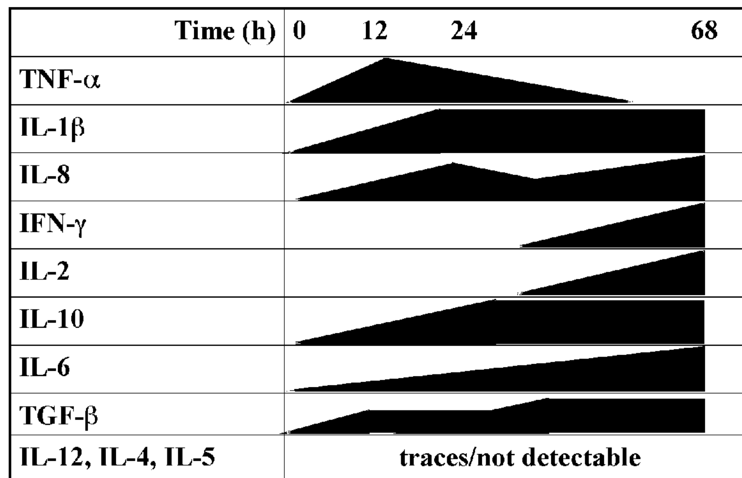

Figure 2: Kinetics of cytokine release into the basolateral compartment of the co-culture model after apical stimulation with E. coli K12 (4.0 x 10^7 bacteria); the absolute concentrations of the single cytokines are not identical.

These findings underscore the diversity of the present model compared to models where the spatial separation between bacteria and leukocytes by a cell monolayer comparable to the intestinal mucosa does not occur. Furthermore, from the results motioned above a three-step mechanism can be concluded in the activation of the eucaryotic cell 'crosstalk'. First, the leukocytes secrete unidentified soluble factors sensitising the Caco-2 cells for selective activation by bacteria. Second, leukocytes are activated by soluble factors from Caco-2 cells, enhancing the production of cytokines already produced by the enterocyte-like cell line (TNF-α, IL-8, IL-6 and others) and initiating the production of cytokines that cannot be synthesised by the Caco-2 cells (IL-2, IL-10, IFN-γ and others). In the third step, changes in the concentration of cytokines in the basolateral compartment regulate the activation status of enterocytes.

Using a non-pathogenic rod as a standard, a characteristic pattern of cytokine concentrations that depends on the incubation time will be evoked. The single cytokines differ significantly in some orders of magnitude regarding their absolute concentration. As can be expected, out of the measured cytokines (TNF-α, IL-1β, IL-12, IL-8, IL-6, IL-2, IL-10, IFN-γ, IL-4, IL-5, and TGF-β) those having a systemic effect (IL-6) or being important for chemotaxis (IL-8) contribute a major part of the produced cytokine mass (ng/ml range; >90%) while cytokines with local efficacy occur only at clearly inferior concentrations (pg/ml-range). Cytokines associated to the activation of Th2 cells (IL-4 and IL-5) were not detected in the basolateral medium at all. A schematic overview of the time kinetics of the investigated cytokines is given in Figure 2.

To further elucidate the differences of the present model as compared to models of leukocyte challenge by direct contact with bacteria, further experiments were performed. Keeping the number of leukocytes constant, the direct cell-cell challenge with bacteria (*E. coli* K12) without Caco-2 cells resulted in the production of about 12,000 to 20,000 pg/ml TNF-α after 16 h. The

presence of soluble factors produced by the differentiated Caco-2 cells reduced this production by about 45%. The spatial separation of the bacteria from the leukocytes by the semi-permeable membrane only reduced the TNF-α production by about 80%. Separation of leukocytes and bacteria by the semi-permeable membrane <u>and</u> the differentiated, confluent Caco-2 cell monolayer reduced the TNF-α synthesis to about 80 to 300 pg/ml (~1 to 2%), which was still significantly higher than the value resulting from the incubation of Caco-2 cells and leukocytes solely (2-30 pg/ml). Evidently, investigations on effects of e.g. nutrients on immunomodulation in the intestine with the present model are performed in a completely different inflammatory 'range' than those where leukocytes are challenged directly by bacteria or bacterial products.

In conclusion, from the primary experimental results obtained with the described co-culture model it can be seen suitable for the assessment of immunomodulation near the intestinal mucosa by food ingredients, drugs, and different bacteria species.

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HOW MUCOSAL IMMUNITY IS CONTROLLED BY LOCAL FACTORS

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The mucosal immune system, the body's largest immunological compartment, is constantly exposed to an enormous load of foreign antigens derived from commensal bacteria and food. Under physiologic circumstances, however, no systemic immunity can be generated at this interphase of the body with its environment. Given that the T lymphocyte population which exists in the lamina propria and organised tissues of the mucosa (LPT) is of polyclonal nature one has to assume that luminal T cells can interact with antigenic determinants present in this location. Under physiologic circumstances, however, "typical" immune responses are not generated. Since such reactions of the mucosal immune system toward luminal antigens result in inflammatory reactions as observed in various types of inflammatory bowel diseases.

An initial important clue to understanding why mucosal T cells, unlike peripheral blood or lymphnode T cells, do not respond to antigen encounter by their specific T cell receptors with systemic immune responses *in vivo* was the finding that freshly isolated LPT when compared with peripheral blood T cells (PBT) do not undergo clonal expansion/proliferation and cytokine production when stimulated *in vitro* through their CD3 antigen-receptor complex. Interestingly, no phenotypic abnormalities could be detected in LPT when analysed extensively in order to understand their lack of proliferation to T cell receptor simulation. A second set of experiments then made obvious that this fundamental observation could not be explained at the level of T cells: When LPT were mixed with peripheral blood macrophages and subsequently simulated through TCR/CD3 their proliferative behaviour was comparable to that of PBT. In a reciprocal fashion, PBT when incubated with mucosal macrophages could not respond to such stimulation. Therefore, one had to conclude that central regulators of immune responses in the gut where derived from the monocyte/macrophage lineage. A phenotypic comparison between mucosal monocytes and monocytes circulating in peripheral blood then pointed towards substantial differences between these two cell-populations in that the receptor for LPS (CD14) as well as a variety of additional adhesion molecules known to be required for T cell costimulation such as CD58 and CD54 were down-regulated on LPMO. The functional consequences of this phenotypic alteration and the question how such a particular phenotype as observed in LPMO can be generated will be dealt with below.

Earlier data had indicated that the mucosal environment produces pro-oxidative substances of as yet unknown nature. Thus, supernatants generated from mucosal cells (epithelial cells and monocytes) when added to peripheral blood mononuclear cells (PBMC) dose dependently inhibited their proliferative response to antigen receptor stimulation. If one, however, added reducing substances such as 2-mercapo-ethanol (2-ME) inhibition was abolished and proliferation was normal. This suggested that the activity of pro-oxidative

locally secreted products could be counteracted by reducing agents and pointed towards the direction of a physiologic pro-oxidative state of the mucosal microenvironment. This finding was of particular interest because T lymphocytes are considerably sensitive to inhibition by pro-oxidative substances. This is due to the fact that they do not express the cystin/glutamate transporter complex in their plasma membranes. Thus, therefore, they cannot take up cystin which represents the precursor for the synthesis of Glutathion (GSH), one of the most potent intracellular reducing systems. Regular GHS-levels are required for cell-cycle progression, transcriptional activity and likely also for the stability of intracellular proteins (particularly those which exist as disulphide linked dimers). Given the above finding of a pro-oxidative state it was important to investigate the possibility whether GSH-levels in LPT would be significantly lower than in PBT which was indeed the case. Thus, LPT contain only 10% - 20% of the GSH concentrations as PBT. Needless to say, the availability of reducing substances leads to an increase in intracellular GSH-concentrations in LPT with an alteration of their functional phenotype e.g. with regard to proliferation.

Physiologically, antigen encounter by T cells only leads to clonal T cell expansion and an immune response specifically directed at antigen when antigens are presented on appropriate MHC molecules and when appropriate additional stimuli are provided towards them (co-stimulation).

Given that T cells lack expression of the cystin-transporter (see above) they are dependent for their GSH-synthesis pathway on cells which can produce a secrete cysteine in their vicinity. This is an important activity and contribution of monocytes to T cell responses. Comparative analysis of monocytes from blood and monocytes from the mucosa clearly demonstrated that mucosal monocytes, unlike their blood counterparts cannot produce cystein as a cofactor for T cell activation. Importantly, cystein production by blood monocytes does not occur spontaneously but has to be induced through engagement of particular cell-surface-receptors including CD14 (LTS-receptor) and CD58 (LFA-3) which are exactly those that are not expressed but LP/MO.

It was now very important to elucidate the mechanism how cell-surface-receptors such as CD14 or CD58, respectively, can be down-regulated on monocytes. There exists one cytokine known to exert such an effect, namely interleukin 10 (IL-10). It was to our surprise to discover that IL-10 is produced in large quantities by mucosal epithelial cells, which are known to secrete their products largely in a basolateral direction, i.e. towards the lamina propria. There, IL-10 could potentially down-regulate the above mentioned cell surface receptors with the consequence that monocytes can no longer be induced to produce cystein.

Having worked up our way from the T cell as the most distal effector element in an interactive compartment we identified mucosal monocytes as central regulators of T cell reactivity. The latter, however, are controlled by mucosal factors such as pro-oxidative substances and epithelial cell derived interleukin 10.

Most probably, activities of epithelial cells require induction or stimulation by luminal components. The intestinal microflora through interaction with epithelial cell components may play an important role for the physiologic function of the latter, which dictate the functional programs of immunocompetent cells homing to the lamina propria. Therefore, it will be of interest for an understanding of homeostasis in the largest

immunological compartment of the human body to elucidate precisely the mechanisms through which - in a symbiotic fashion - commensal flora influences functional phenotypes of the body's cellular elements.

In conclusion, the presented studies of mucosal immunity highlight aspects of physiological and pathological immune regulation and point to the direction that therapies of mucosal inflammatory processes which target immunocompetent cells are, despite ameliorating acute symptoms are unlikely to affect the causal processes underlying unwanted immune responses. The latter rather result as a consequence of a disturbed microenvironment, which is characterised by epithelial, cells interacting with microbial components. Therefore, eventually, therapies which are directed at those elements will address pathologic processes at their basis.

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OLD HERBORN UNIVERSITY SEMINAR ON HOST MICROFLORA CROSSTALK

MINUTES AND OVERVIEW OF THE DISCUSSIONS

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Elaine Vaughan: Approaches to investigate the diversity and functionality of intestinal microbes.

Some advantages and disadvantages of various molecular methods for investigating the microflora diversity were discussed. The microbial diversity and community behaviour over time of GI-tract faecal samples has been studied using fingerprinting techniques, such as DGGE or TGGE (denaturing or temperature gradient gel electrophoresis). TGGE/DGGE of 16S rRNA PCR products are especially suited to study diversity in samples with largely unknown microbial content, like the GI-tract, without culturing. The intensity of a band in D-TGGE is a semi-quantitative measure for the relative abundance of this sequence in the population. Bands can be excised from the D/TGGE gels, and advantage of this technique over other fingerprinting methods, and sequenced, and the identity determined by comparison to the databases. D/TGGE can also be done on gut mucosa biopsies. Fluorescent in situ hybridisation (FISH) with image or flow cytometry (rapid) analysis are excellent methods to identify and quantify bacterial groups in microflora without culturing, but do require prior knowledge of the microflora for 16S ribosomal RNA fluorescent probe design.

The question arose as to how the retrieval of mucosal biopsies affects the microflora. (The best material for mucosa-associated microflora comes from patients with abdominal pain who are biopsied in the colon. The best biopsy material for mucosa-associated microflora comes from patients who do not suffer infectious bowel diseases. Very early in the disease of Chronic Infectious Bowel Disease, changes in the histologic appearance and inflammation can be seen. The question was also raised as to how soon after onset of the disease does the microflora change? In man this cannot be studied because the colon needs to be flushed prior to scopy and biopsy. The benefit of scopy however, is the histology. Histologic appearance is the same in all patients while the microflora differs between individuals. Bacteria adhere to specific places (glycocalyx) which differ between individuals. In the crypts there are bacteria, which are perhaps not susceptible to oxygen; the way to isolate them is to make microtome (delete?) sections of the mucosa from the lamina propria up to the villi until the crypts are reached.

Using high throughput genomics methods involving DNA microar-

rays/chips, the diversity as well as the functionality of the microflora may be studied. Besides sequencing genomes of specific microflora commensals, the construction of metagenomic libraries consisting of the microflora DNA allows access to the genetic and functional diversity of the microflora in the absence of culturing. Samples from quite a number of people are required to study the influence of factors such as age and diseases. Thanks to the computer, molecular analyses as described above can be considered these days.

Vanya E. Grant: Flow cytometry: Can it help to analyse complex biosystems?

1. Validation by hybridisation as only a variable percentage is culturable.
2. Why do it?
3. High dimensionally; data handling i.e. an explosion of computer intensive data are generated.
4. Where is the money?

The reason why hybridisation is often difficult is because bacteria in the gut must be "stressed". This is because of lack of nutrients etc. The majority of the crypts are loaded with microbes, which are as yet not determined, even with 10-12 probes. Crypts may form a site for translocation. Gram-staining easily misses crypt colonisation by bacteria.

Questions that can be answered: Percentage of bacteria with intact barrier function that can be identified with probes for prokaryotics is relatively low, because only sick and dead bacteria can pick up the probe. Treatment with "Dibac C4" opens up the barrier. Some fluorescent stains act on Gram-positive bacteria only. This makes differentiation between Gram-positive and Gram-negative fractions in mixtures easy in flow-cytometry.

The 'money' (benefit) is in:

1. well cut biopsies (these should be least artificially changed).
2. allows analysis of many intestinal/faecal samples.

Note: In case bacteria have been exposed to antibiotics such as penicillins, cephalosporins, aminoglycosides etc. they may have become "leaky".

Hilde Ulvatne: Defensins and defensin-like molecules: Antibacterial Mode of Action.

Antimicrobial peptides (AMP) can be classified by structure, and include:

1. α -helices,
2. ß -sheets stabilised by disulphide bridges,
3. extended structures, and
4. loop-structures.

AMP are present in every organism so far investigated, and are located in epithelia, leukocytes, and mucosal secretions. Any organism may possess AMP from more than one structural class of AMP and many AMP within the same group (e.g. frogs have many different AMP of the α-helical group). AMP may act in synergy with each other and with other antimicrobial effector molecules in the host. AMP have different antimicrobial spectra, and some are more efficient against Gram-positive than Gram-negative bacteria, while others again are more efficient against Gram-negative than Gram-positive bacteria. Most AMP are gene-encoded, but some are generated by proteolytic cleavage of a native protein (e.g. lactoferricin and LL-37).

In humans, defensins are produced throughout the GI-tract, including the mouth, epithelia in the oesophagus, the stomach, and the small intestine and in colon. In the human gut, there are at least 9 different defensins (HNP 1- 4 in the neutrophils, HD-5 and 6 in the Paneth cells, HBD-1 in the small intestine and colon, HBD-2 in the gastric mucosa and colon, and HBD-3 in colon)

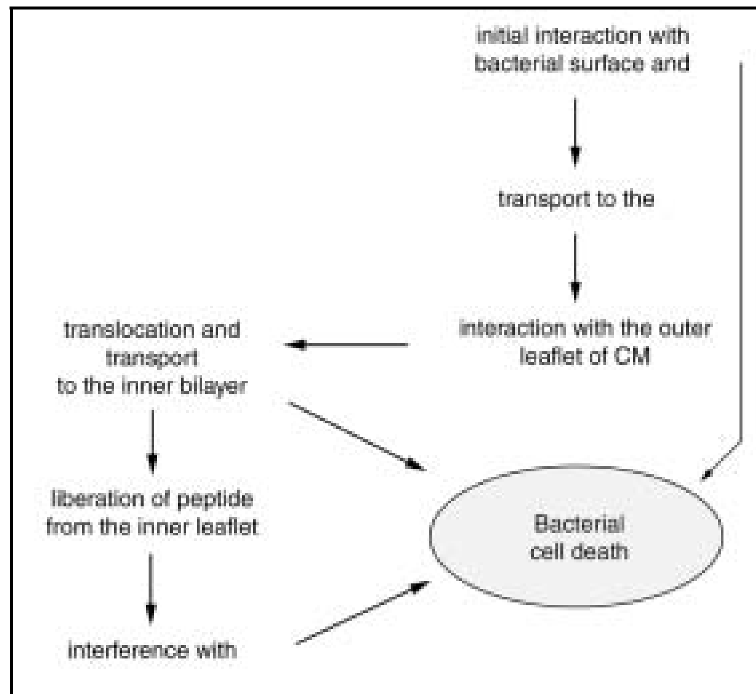

Figure 1: The interactions of antimicrobial peptides and the bacterial cell

The role of defensins in modulation of the intestinal microflora is difficult to elucidate, because of the high variability of bacteria in the intestine. There are also differences in the susceptibility pattern, and no clear-cut correlation between susceptibility and pathogenicity. Furthermore, bacterial proteases may destroy AMPs. Only little research has been done on development of resistance, but resistance mechanisms exist.

Lactoferrin (LF) is a multifunctional, iron-binding protein of 70 kDa. LF is present in secretions like milk and tears, in saliva, semen and colustrum, in addition to being a part of the secondary granules of polymorphonuclear leukocytes. Its strategic appearances on the mucosal surfaces and in leukocytes indicate a role in the primary defence of the organism. It has been shown to exert a broad antimicrobial activity, and the antimicrobial activity is not iron-dependent. Lactoferricin B, a 25 residue cationic antimicrobial peptide, is generated upon gastric pepsin cleavage of bovine lactoferrin.

The modes of action of AMP: Due to the amphipathic, cationic structure of most of these peptides, an effect on the cytoplasmic membrane of susceptible bacteria has traditionally been postulated as the main mode of action. Although an effect on the cytoplasmic membrane has been shown for most peptides, other mechanisms have also been reported, including interference with intracellular processes.

The mode(s) of action of AMP is illustrated in Figure 1. At several points, the interaction between peptide and bacteria may lead to the bacterial cell death, as indicated with arrows.

Lactoferrin B causes only minor leakage from liposomes, no general collapse of membrane integrity (measured by uptake of propidium iodide), minor effect on bacterial respiration

(measured by CTC), but causes concentration dependent depolarisation of the cytoplasmic membrane in *E. coli* (measured by JC-1). However, even though the membrane of *E. coli* gets depolarised, it does not necessarily cause death of these bacteria. This leads to the conclusion that 'membrane-effect' is not responsible for death of *E. coli*. The mode of action of lactoferrin B may be hypothesised as the following:

1. Attachment to bacterial surface.
2. Binding with the cytoplasmic membrane:
   * bacterial stress response?
   * induction of SOS-response?
   * inhibition of growth?
3. Intracellular action:
   * shut down of metabolism?
   * depolarisation?
   * bacterial action = MBC?

Preliminary results indicate that lactoferricin B affects the bacterial protein synthesis. At a concentration of 5 mg/ml suppression is observed, and 10 and 25 μg/ml further increase the effect. These *in vitro* findings do not necessarily reflect what may occur *in vivo* at these concentration levels. Through the bacterial response to defensin attack, bacteria may even be protected against the effect of other antibiotics, like aminoglycosides, which affect protein synthesis.

Lars-Göran Axelsson: Defensins and bacteria, a question of "live or let die?"

The human small intestine responds to bacterial challenge of the small bowel by secreting antimicrobial substances as in the mouse. Specific cells, Paneth cells, are strategically situated at the bottom of the small intestinal crypts and contain granula packed with bioactive substances. Paneth cell granules are degranulated upon stimulation and secrete e.g. lysozyme and phopholipase-2 into the lumen of the small intestinal crypts, together with the secretion of antibacterial defensins.

The importance of antimicrobial substances has been studied in patients having surgical by-pass treatment against morbid obesity. After Roux-en-y Gastric bypass (GBP), a standard surgical procedure for morbid obesity, food and oral-nasal-pharyngeal secretion pass directly into the small bowel without passing through the acid environment of the normal stomach. Postoperatively (3-6 weeks), complying patients underwent gastroscopy with jejunal biopsies using a sterile forceps. Postoperatively Gram-negative cocci, anaerobic bacteria and yeasts were found in a lower number than found in control individuals. The Paneth cell, histochemically, specific phloxine-tartrazine stain showed a striking reduction of stained granula in the postoperative biopsies, a result also obtained with the specific antibody to lysozyme. However, immunohistochemical staining of human defensin-5 showed an upregulation of the antibacterial peptide in granula in the entire lower crypt wall as well in the crypt lumen.

This indicates that the high exposure of the small intestine to environmental bacterial flora after GBS differentially regulates the secretions of antibacterial substances and thus controls bacterial colonisation and prevents deleterious bacterial overgrowth.

Mice having a conventional intestinal microbiota are known to produce at least 17 enteric antimicrobial peptides, i.e. defensins.

Germfree mice can be used to compare innate and classical immune responses to microbes. In the germfree mice, the basic activity of defensins can be studied and by mono-association with a single bacterial strain specific responses can be detected. HPLC fractionation of small intestinal crude extracts has shown that the sterile intestine

of germfree mice contains at least three antibacterial components. A mono-association with *Aeromonas hydrophila* (Bo-3N) produced two additional components. A pre-treatment with cortisone abolished the two first peaks with antibacterial activity, which implies a gene control by NFkB/IkB.

These results points to the role defensins can have in the new-born in controlling the establishment of a functional intestinal bacterial flora and later in protecting against unwanted colonisation of pathogens and deleterious microbes.

Conditions in humans where Paneth cell hyperplasia occurs, e.g. in the stomach, shows that there are genetic or mediator driven control of Paneth cells and its secretions. Lately there has been much interest in the pharmacological regulation or the administration of synthesised defensins to substitute for antibiotics to which microbes has acquired resistance against. Recently several methods to manufacture different antimicrobial peptides have been published.

In the near future there is the important and much promising possibility to induce and regulate or use defensins as new pharmacological entities in the control of infections caused by resistant or multi-resistant microbes.

Defensins might be important in the:

1. Regulation of the process of acquiring an functional healthy intestinal flora in the new-born:
   * are there qualitative differences between individuals,
   * if so, can the defensin production and secretion be up-, respectively down-regulated on demand in certain individuals under pathological conditions.
2. Maintaining a healthy intestinal flora under different environmental conditions:
   * maintaining the balance between commensals.
3. Protection against pathogens:
   * eradicate before critical infectious number of organisms is reached,
   * maintain commensal flora.
4. Treatment of disease:
   * maintain healthy flora vs. allergies,
   * maintain healthy flora vs. arthritis.
5. Treatment of cancer:
   * synthetic drugs.

Agnes Wold: Mucosal immunology.

Hygiene hypothesis by *Strachan* (1989): Microbial exposure reduces the risk of allergy development.

Children with elder siblings have fewer allergies. This could be due to a lower degree of contamination in the first child than in the siblings. Indeed, when "day care" is started early (<2 months) it seems to protect to allergy development. Also growing up on a "life-stock farm" reduces the risk for allergy.

Swedish infants develop IgA later than Pakistan children and children who develop allergy later on have a lower IgA in their saliva compared to infants who become not allergic.

When oral tolerance is induced in conventional mice (and in GF controls that do not become so easily tolerant following the same treatment) and their serum is injected into conventional nude (athymic) mice the latter become tolerant but not following injection of serum from the GF.

Strachan, D.P.: Hay fever, hygiene and household size. BMJ 289, 1259-1260 (1989).

Simon Murch: A link between mucosal regulatory lymphocytes and childhood food allergy.

A hypothesis linking parasites with development of allergy is presented. Low hygienic circumstances involving a high burden of pathogens/adjuvans disbalance the Th1/Th2 ratio. Viruses, bacteria and protozoa may stimulate DC1 cells and stimulate Th1 forming. Helminths and allergies on the other hand stimulate DC2 cells which stimulate Th2 formation.

Multiple Food Allergy:

* Breast-feeding has no preventive effect on allergy.
* Allergies are less severe in patients with enteropathies.
* May be found in subjects with "normal" bowel function (their villi however may be reduced)
* Transient IgA deficiency.
* IgG sub-clones deficiency like IgG2 and IgG4.
* Lymphocyte subset abnormality (low CD8, Low NK cells and CD19 cells).

Ovalbumin sensitisation can induce "allergic bowel dysmobility" in children.

Eotaxin attracts eosinophils, while IL-10 and TGF-β do the opposite as they induce development of suppressor cells.

"Para-cellular leakage" next to Peyer's patches attracts cells to infiltrate the lamina propria. In Gambian children this is seen at the age of one year and they may be dead by their second year. It seems that T-cells are involved but it is uncertain whether these are Suppresser cells. It is at the moment that "flora repair" will be of help to stop the process.

Stig Bengmark: Synbiotic treatment in clinical praxis.

Allergy is also important in human adults. People who grow fat and produce high amounts of volatile fatty acids, TNF and PAI-1. These patients may show Prostate hyperplasia, hypertension, diabetes, arteriosclerosis, hyperinsulinimia, hyperuraemia and obviously obesity. Their risk for development of cancer is increased.

Cows milk that comes from cows that are fed on hay (not fresh grass) may carry:

* trophic hormones,
* bovine growth factor (IGF-1), and
* xeno-estrogens.

According to Swidsinsky, intestinal mucus is normally free of bacteria. In patients with inflammatory bowel disease (IBD) on the other hand, the bacterial concentration in the mucus may be high. This may either be the cause or the result of IBD.

Elisabeth Norin: Phenotypic expressions in the small intestine.

In groups of 5 to 7 mice and rats the MACs were studied under several conditions:

* Fasting overnight.
* Vincristine injection.
* Similac diet.

In the rats, samples were taken at standard (same) places of the intestines. Significant differences were seen in the mitotic index of the crypt cells in the germfree animals. In their caecum a difference in mitotic index (MI) was seen between males and females. The MI was highest in rats following feeding of *Lactobacillus rhamnosis* GG and *Clostridium difficile* (in particular in toxin producing strains on the 3rd day).

In mice, similar results were obtained (young male animals had a higher mitotic index than females). Rats show higher mitotic indexes than mice.

Speculation: The effect of treatment on the MI is immunological, mediated by γδ T cells.

Barbara H. Iglewski: Quorum sensing in *Pseudomonas aeruginosa.*

Quorum sensing is bacterial communication co-ordinated activity of cells involved in the crosstalk. In this respect it is important to know whether bacteria of different species can "talk" with each other.

Cell-Cell signalling:

* Gram-positive bacteria: Post trans-stationally modified peptides are transported out of the cell to act on receptors on the cell membrane (outside the cell). Classic is two components systemic kinase.
* Gram-negative bacteria: Quorum sensing occurs by small molecular cyclates WSL.

AI-1 is an auto-inducer. These small molecules are diffusing (or pumped) out of the cell and may then diffuse back in to bind specific regulatory receptors. AI-2 are structures like antibodies are diffusing (or pumped) out to bind to specific receptors on the cell surface. This results in synthesis of two component kinases. Among AI-molecules much homology exists between different bacteria.

In mice, when the following bacteria are co-cultured in agar beads and put in the mouse lung, *Pseudomonas aeruginosa* produces $30\text{-}C^{12}\text{-}HS\alpha$ which can activate has-R in *E. coli* containing HasR+LacB-Gfp.

The question rises whether bacteria can talk with host cells. Polarised lung tissue cells show their normal cillilar movement and other activities. When *P. aeruginosa* is added, the cells will produce AIs.

What type of bacterial behaviour is regulated by crosstalk (=quorum sensing)?

1. Virulence (toxins, exo-enzymes).
2. Invasion (swarming, chemotaxis, proteasis).
3. Antibiotic production (self-defence).
4. Siderophores (β-cepacia) to acquire iron.
5. Antibiotic sensitivity (efflux pumps) regulate siderophores which may act as two-edged sword.
6. Evade host defence (alter membrane proteins).
7. Plasmid transfer (transfers genetic info).

Pseudomonas gene chip has 5769 genes. *In vitro* 433 genes of which 259 genes upregulate and 179 down regulate. Efflux pump transport level in PAO1 effect of nutrients shows that three genes are involved at a very low level of quorum sensing.

Lessons for future experiments:

1. There is a need for three repeats per experimental variable.
2. High quality RNA is essential (free DNA and intact mRNA).
3. Gene 2 experiment in variable responses involving:
   a. growth stage,
   b. media,
   c. +/- O_2.
4. Limitations:
   a. can not see regulation short lived messenger RNA,
   b. can not see regulation if gene is poorly expressed,
   c. genes with multiple regulators pose challenges.
5. Transcript analysis is a good complement to other approaches (to mutagenesis, proteomics, gene transmission etc.

Alexander Parlesak: Developing an *in vitro* model on the investigation of the crosstalk among bacteria, enterocytes and leukocytes near the intestinal mucosa.

Model: It was clearly to be avoided to get a Graft versus Host effect between the monocyte layer underneath a filter, which separated them from the enterocytes. In this model crosstalk was

studied between bacteria and cells of host origin. The costs of testing one compound was high (€ 20,000.-).

Selective stimulation of Caco-2 occurred only in the presence of leukocytes on the basal side caused production of β-actin, IL-8, TNFα, IL-1β and TNF-γ.

Bacteria tested were human isolates:

1. *E. coli* K12 (two concentrations were tested).
2. *Lactobacillus johnsonii* (two concentrations were tested).
3. *Lactobacillus sakei* (two concentrations were tested).
4. LPS (endotoxin).
5. No treatment.

Both *E. coli* K12 and *Lactobacillus sakei* stimulated expression of IL-8, while all bacteria stimulated expression of β-actin.

The process follows three subsequent steps:

Step 1: leukocytes stimulate the bacteria,

Step 2: stimulate leukocytes stimulate Caco-2 cells (enterocytes),

Step 3: enterocytes affect bacteria.